NATIONAL INSTITUTES OF HEALTH

Review of INTRAMURAL RESEARCH 1961

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service

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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health, Bethesda 14, Maryland

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FOREWORD

The National Institutes of Health, one of the five Bureaus of the United States Public Health Service, has been assigned the mission to conduct and to support research, research training, and other related activities. This mission is discharged partially through an extramural support program which, administered from Bethesda, reaches into virtually every institution in the United States engaged in biomedical research, and is rapidly expanding throughout the world. And it is partially discharged through a direct operation, the intramural program, housed in laboratories in Bethesda, and representing something of a microcosm of the total effort. This publication focuses exclusively on the intramural enterprise, describing it in a series of reviews designed to illustrate the compass and flavor of the local research activities of each Institute. Thus, it is not a comprehensive presentation of the program of either the United States Public Health Service or of the National Institutes of Health.

Glimpses of the extent to which the intramural research effort has participated in the broad forward thrust of medical research are provided in the eight Annual Summary Reports comprising this compendium. The reports are protected from editorial interference, and prepared by the originators in accord with only the most general guidelines. This procedure, considered and deliberate, has been adopted to allow the reader to savor the diversity of outlook and attitude which prevails among a group of brilliant scientists loosely knit and almost imperceptibly harnessed for the attainment of common categorical goals. In such a presentation, it is possible to overlook the thread of mission that ties each of these operating research organizations into the broad program of the National Institutes of Health. It is appropriate, therefore, to pursue this aspect briefly.

The mission of the National Institutes of Health as a whole and of its components is to develop the facilities, resources, and attitudes most effective in acquiring new knowledge concerning disease processes, and relieving suffering, bringing about cure and rehabilitation, and assuring the prevention, whenever possible, of disease. Broadly considered, this mission involves providing the wherewithal and cultivating suitable soil for a systematic study of man and his milieu with the ultimate objective of contributing to improved health. From this overall point of view, the NIH does not differentiate between what is done intramurally and extramurally. However, within these conceptions are contained more specific objectives only some of which can be sought for within an intramural research program, while the others may be searched out most expeditiously by support of work in other institutions through the extramural program.

This second Annual Review of NIH Intramural Research provides evidence of the magnitude of the effort—both in breadth and depth—and of the type of achievements that have placed this installation in the forefront of research in the medical sciences.

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NATIONAL CANCER INSTITUTE

INTRODUCTION

In this report I have commented on a particular area in order to illustrate some of the directions and problems of current research upon cancer at the National Cancer Institute. The very simplicity of the name of the Institute's categorical mission, "Cancer," disguises the biggest problem—that like the term "Infection," it includes a great diversity of biological, biochemical, and disease phenomena. In recent years it has become ever more apparent that generalizations about cancer in man and animals are unwise and that research at this time must focus upon fragments. The success enjoyed by Institute scientists in choriocarcinoma emphasizes the specificity of cancer research.

As one reads through the annual reports, one becomes aware of the extent of interest in the leukemias. In almost every laboratory and branch some aspect of the leukemias is being studied, and it is fair to say that research in these diseases constitutes a major NCI commitment. The following remarks will survey some of the Institute's programs on the causation, development, and management of the leukemias.

THE LEUKEMIAS

Causation

In animals and man the leukemias arise "spontaneously," that is, by some unknown stimulus, or follow exposure to several types of irradiation. In mice it has been shown that most types of spontaneous leukemias are due to viruses. New research findings have increased understanding of some of these phenomena during the past year.

One of the major barriers to the demonstration of the viral causation of spontaneous animal tumors has been the relatively low concentration of virus in the tumor-bearing animal. A whole series of developments, to which NCI scientists have made major contributions, now permits the isolation and identification of these small amounts of virus. Work on the quantitative relations between amount of virus and production of Rous sarcoma in fowl led to greatly improved methods of extraction and concentration of this virus. At the same time a much more highly susceptible host for murine tumors—the new-born mouse—was developed in work on the milk agent, the Gross leukemia, and polyoma viruses. It became possible to grow polyoma virus in quantity on tissue culture of mouse kidney and mouse embryo cells. Concurrently, the refinements in the techniques of electron microscopy, especialy by one NCI scientist, has increased the ability to identify oncogenic virus particles. All of these techniques, beginning about 1957, were focused on the problem of finding new oncogenic viruses, with considerable success in the mouse leukemias.

In 1958 the Moloney leukemia virus (MLV) was isolated from Sarcoma 37. Its history and characteristics have appeared in previous reports, and here only a few aspects will be considered. It should be emphasized that MLV has provided an excellent model to help understand the pathogenesis of virus-induced leukemia and to predict new relationships.

The virus has been found budding from the plasma membrane of malignant lymphoblasts and the intracellular membranes of megakaryocytes. In the latter cells, a large number of virus particles can be seen in the cytoplasm. They have also been demonstrated in platelets. The largest amount of virus, however, has been found free in the blood, and this provides a technique for isolating large amounts of pure virus. The amounts to be harvested are limited by the size of the animal, and so far the hamster is the largest susceptible species. Attempts are under way to transmit the disease to monkeys and cattle, but these possibilities will not be fully examined until much larger doses of virus can be given to these larger animals. MLV is excreted in the milk and vertical transmission of the disease to suckling mice has been shown. Lateral transfer of virus has not been found.

The pathologists have made good use of MLV leukemia in mice and rats to observe early microscopic lesions. The first changes occur in thymus epithelium. This is followed by splenic hyperplasia and later by lymphoid leukemic involvement of marrow and lymph nodes. If the virus is inoculated into thymectomized mice, reticulum cell sarcoma results rather than leukemia. If inoculated into splenectomized mice myeloid (chloro-) leukemia rather than lymphoid leukemia appears. However, prior to the appearance of microscopic evidence of leukemia in the rat, virus particles can be shown in megakaryocytes.

MLV is now growing well in tissue cultures of a cell line derived from mouse spleen. The cell line, probably a reticular cell, is in continuous culture, and attempts are under way to increase the size of such cultures to produce large amounts of pure virus.

The new techniques have resulted in the isolation of new leukemia viruses. These are the C–60 virus isolated from a Swiss mouse with the Schoolman-Schwartz disease; the R–NCI–2 virus from similar mice; and the Breyere-Moloney agent from Balb/c mice.

The lessons of the murine leukemia viruses seem to hold some promise for clues to the solution of the clinical problem. Studies in both the animal and patient systems are proceeding, and based on murine leukemia it would seem highly likely that a "virus" or some chemical or event capable of modifying the DNA-RNA mechanisms would soon be identified in association with one of the human leukemias. In preparing for what at this moment seems inevitable, it is profitable to ask what should be provided for the scientist to facilitate his examination of the various possibilities.

Electron microscopy represents at this time the most rapid and direct end-point in relating biological phenomena of tumors to the presence of a virus. But even this requires several months and a scientist can make relatively few observations, as shown by the fact that in 6 months only 16 of 100 samples of human leukemic blood could be examined. The problem is to insure the training of more men in this field. This is particularly true in Pathology and the clinic, for if a human tumor is found to be related to a virus or infectious nucleic acid then rapid examination must be available for diagnosis.

To find a more rapid way of proving the presence of a virus other direct techniques are needed. The most promising at the moment is that of viral interference developed in the Laboratory of Viral Oncology. Based on the observation that Moloney virus will interfere with the action of influenza virus in fowl embryos, an assay is being developed which can be read in 5 days. However, the test is non-specific and a number of controls to rule out contaminating viruses are needed. Another solution would be the rapid growth of the virus in tissue culture, but considerable research is needed on the cell lines themselves, as well as on the virus in culture, before any assay will be possible.

Since the incubation period for the oncogenic viruses in animals is in large part dependent on size of infective dose, considerable planning is under way to provide facilities in which large amounts of viruses can be made available. An outside contract has been established which will not only ensure production of Moloney and Rauscher viruses in mice and rats but will also develop tissue culture as a source of large quantities of these viruses. Such large amounts of pure virus will speed up studies on assay systems, and will also permit examination of the chemical characteristics of the viruses, their transformation into infectious nucleic acids, the possibility of infecting large mammals such as primates, dogs, cattle, and horses, and the manufacture of vaccines.

Large amounts of the murine viruses will also permit the infection of large numbers of animals for viral chemotherapy studies. Even with the amounts now available some of the chemotherapy studies in transplanted tumors have been replaced by studies of the chemotherapy of Moloney and Rauscher virus leukemias. Preliminary results indicate that the whole cell transmitted disease can be cured by means of drugs, but death ensues due to recrudescence of (Moloney) virus induced leukemia. Active collaboration with the Virus Research Resources Branch will provide chemicals with potential anti-viral activity.

Pathogenesis and Bodily Economy

In the animal given Moloney virus, particles are first demonstrated in the grossly normal megakaryocyte and later in the malignant lymphoblast.

Then follows histologic change in the thymus and spleen, and finally, the production of excess lymphoblasts which infiltrate many organs. Even if the same procession of events occurs in patients after the initiating leukemogenic stimulus, at the present time most observations in leukemic patients are related to the late situation of leukocyte over-production with consequent replacement of normal tissues. The greatest impact in acute lymphocytic leukemia is upon the bone marrow and the central nervous system. As a result of bone marrow infiltration there is interference with red cell production, and a sharp reduction in production of platelets and granulocytes, giving rise respectively to anemia, hemorrhage, and infection. If remission in the bone marrow phase of the disease can be induced, there often occurs luxuriant growth of leukemic cells in the meninges with the production of hydrocephalus. Each of these major manifestations of acute lymphocytic leukemia has been studied in the clinic with the purpose in mind of controlling these life-threatening processes.

The anemias of the leukemias and related diseases have been examined by physicians of the Metabolism Section. The mechanism is not one of simple failure of red cell synthesis due to bone marrow replacement. The widest spectrum of mechanisms was found in chronic myelocytic leukemia where one can demonstrate either an increase or decrease in rates of red cell production, and a shortened red cell life span. Later in the acute myeloblastic stage there is markedly shortened red cell survival and an absence of red cell synthesis. It is difficult to generalize about the mechanism of anemia in a particular patient. As is true of so many of the physiologic deficits of disease, when precise methods of examination become perfected, the specific "anemia" can be shown to be the resultant of several processes, weighted differently and even oppositely, in several patients with the same disease. While this heterogeneity of mechanisms within single disease entity is discouraging for those who wish to generalize, their recognition sometimes permits specific corrective measures to be undertaken for a given patient.

In addition to studies of the direct effects of leukocyte over-production upon the bodily economy, there are nucleic acid abnormalities in some of the leukemias which may or may not be related to the excess white cells. Studies on nucleic acid biochemistry are being pursued intensively in a number of our laboratories. Most of these are necessarily in simple artificial systems; mention will be made here only of a few early attempts at studies in patients.

In the Metabolism Section the main interest has been in pseudouridine (5-ribosyl-uracil) and uracil. Pseudouridine was found in some leukemic patients, and is of interest because it is not catabolized. When labelled pseudouridine was used as a measure of pyrimidine production, it was calculated that patients synthesized between 1.1 and 1.6 grams of pyrimidine nucleoside per day. In the Laboratory of Physiology, pseudouridine has also been under study in patients and its excretion has been shown to be partially diet dependent. Increased amounts of pseudouridine were found in acute lymphocytic leukemia but not in patients with acute myeloblastic type.

The large number of closely related pyrimidines and their congeners in urine has held up progress in the study of their relationships to disease and metabolism. When data processing equipment was put in the Laboratory of Physiology it became possible to resolve some of these problems, as well as to make available to Institute scientists the possibility of fairly direct solution of other computational and data retrieval problems. The scientists of the Energy Metabolism Section have taken the leadership in assisting other scientists to program their problems and to develop mathematical models for the further exploration of their data. In addition to making feasible the analysis of urinary pyrimidines, programs have also been developed for the study of the sequences of bases in the nucleic acids; for the prediction of toxicity and therapeutic effect of drugs in the L1210 screen; for retrieval and correlation of autopsy data; and for the analysis of metabolic data. One can expect the rapid application of these types of programming to a variety of laboratory and clinical studies.

Management of the Leukemias

The ability of our physicians to influence favorably the course of patients with the acute leukemias has improved considerably in the past year. These improvements have resulted in better con-

trol of hemorrhage and infection, and of the disease itself by improved chemotherapy.

The hemorrhagic complications of the acute leukemias have been reviewed by physicians of the Medicine Branch and have been shown to be due almost exclusively to lack of platelets. Studies on platelet replacement have continued at a rapid pace, and it is now possible to use pooled platelets. This has permitted control of platelet level, and therefore hemorrhage, in adults as well as children, and at the present time 85% of patients with such difficulties can be satisfactorily managed. This has become a regular ward procedure, and although it has been done by the Institute, it is such a standard part of operations it will soon be transferred to the Blood Bank. In addition, research on preservation of platelets for future use should be undertaken in conjunction with the Blood Bank, since only when it becomes possible to store platelets in advance of need will full use of this procedure be made.

The techniques learned in the gentle handling of platelets have led to successful studies of the replacement of granulocytes in the treatment of Pseudomonas septicemia. This is a common complication of acute leukemia and in a previous study was shown to be related to the absence of granulocytes. Physicians of the Medicine Branch have been able to give doses of 10–30 billion granulocytes, obtained from patients with chronic granulocytic leukemia, to patients with Pseudomonas septicemia who were also receiving antibacterial chemotherapy. In 22 patients with this infection treated in former years with chemotherapy alone, 21 were dead within 4 days and the 22nd survived about 10 days. Six of the last ten patients given granulocytes have been cured of their Pseudomonas septicemia. Further work is proceeding in getting more granulocytes and obtaining them from normal donors. Future work must face up to the problems of granulocyte preservation; of the dangers of possible homotransplantation of the white cells, and the remote possibility of transfer of a "virus" of chronic granulocytic leukemia.

In last year's report, attention was drawn to the usefulness of the L1210 mouse leukemia, as studied in the Laboratory of Chemical Pharmacology, to predict activity of new drugs for the acute leukemias. Using this model, two new agents of importance have been added—methyl-glyoxal-bis-guanylhydrazone (CH_3–GAG) and the terephthalanilides. In addition, the model has predicted a better way of using methotrexate but has failed in picking up an agent vincristine (leurocristine) which is quite active in acute lymphoblastic leukemia.

CH_3–GAG has been used in 13 patients with acute granulocytic leukemia. Eleven have shown objective improvement and 9 of the patients have achieved complete remission. Since 6–MP, the only drug useful for this disease, induces less than 20% complete remission, the new drug offers additional benefit to the adult with acute leukemia. The drug has a low therapeutic index and is not absorbed after oral administration. A variety of analogues is being synthesized for additional studies. Its major toxic effect seems to be on the epithelium of the upper digestive tract. The terephthalanilides, with high activity in L1210, have proven to give unexpected oculomotor paralyses in man at doses below predicted therapeutic range. However, the L1210 model has recently shown that these drugs when administered with a small amount of methotrexate, give rise to a synergistic effect. This observation is being extended to other anti-leukemia agents, and the methorexate-terephthalanilide combination is under clinical trial. In addition, there are many analogues of the latter drug available for further screening for synergism.

The L1210 model indicated sometime ago that methotrexate given every four days was more efficient than the same total amount of drug given by daily dosage. This has now been extended to patients with acute lymphocytic leukemia and 15 of the first 18 patients (83%) treated intravenously are in complete remission. Since the daily dosage regimen of oral methotrexate gave only 29% complete remissions, the comparison is encouraging. A direct comparison in patients will now be made.

Vincristine (leurocristine) is a drug developed by Eli Lilly and Company as an analogue of the periwinkle alkaloid, Vincaleukoblastin (Velban). It has marked activity in P–1534 leukemia, a lymphocytic leukemia closely resembling L1210. The activity in L1210 is only 20% of that of methotrexate. In 11 patients with acute lymphocytic leukemia, 7 have entered complete remission. Interestingly, this was accomplished much more

rapidly than the antimetabolines and without bone marrow toxicity. It causes alopecia and some changes in peripheral nerve function. Other alkaloids of this series have been made available and will be studied. Also, there is under way a comparative study of the P–1534 and L1210 leukemias in order to study whether both are needed in the leukemia screen.

With the above advances, namely, the ability to control hemorrhage and Pseudomonas infection, new drugs with a high remission rate in both forms of acute leukemia, a much more effective way of using methotrexate, and the possibility of a synergist for the antifolics, the survival rates and durations will be markedly increased. It is too soon to quantify the impact of these advances on survival times, but one would expect that it would at least double in patients treated with all these techniques. How soon does one deliberately try to apply these new methods across the country? Part of this will be accomplished when the total survival figures are available and when the results are published, but there is already sufficient proof to suggest that a more directed approach would result in a more prolonged life for those with acute leukemia. How this might be done by the joint efforts of the scientists of CCNSC, the intramural group and the cooperative groups is the subject of present planning.

BIOCHEMICAL AND PHYSIOLOGICAL STUDIES OF CLINICAL CANCER

While the clinical branches of the National Cancer Institute are chiefly concerned with the diagnosis and treatment of cancer in man, a significant effort is being made within the National Cancer Institute in the study of the biochemistry and physiology of man as modified by the presence of a tumor. They are studies intended to elucidate either mechanisms of disease, mechanisms by which the tumor produces metabolic changes or studies in man of the biochemical and physiological problems associated with the treatment of cancer. These studies should be viewed as an integral part of the larger program. A review at this time of the activities in these fields of the National Cancer Institute would serve to indicate the broad approach that the clinical investigators have developed for the study of disease. In many instances parallel studies are being carried out in experimental animals. These will not be reviewed here since it is intended that this review be confined to studies in man.

Nucleic Acids

The nucleic acids, both DNA and RNA, are directive both qualitatively and quantitatively of macromolecular synthesis and thus are central to many metabolic processes. For this reason a number of studies of nucleic acid metabolism have been undertaken.

Column chromatographic methods for isolating the pyrimidine pseudouridine (5 ribosyl-uracil) and uracil from urine have been developed. Elevated excretion of these compounds was found in patients with gout, leukemia, and psoriasis reflecting increased catabolism of nucleic acids in leukemia and psoriasis, but in gout the explanation for this increased excretion is not at hand, since it is not correlated with uric acid excretion. The excretion diminished following administration of azauridine because of suppression of uridine synthesis. Biosynthetically labeled pseudouridine containing C^{14} or tritium was prepared in rats. In both man and the rats, pseudouridine is not catabolized; therefore, excretion rates reflects synthesis rates in the steady state. Ring labeled orotic acid was administered to patients and the excretion of C^{14} as respiratory $C^{14}O_2$ (greater than 50%) uracil (1–2 %) and pseudouridine (2–3 %) determined. From the cumulative excretion of isotope as pseudouridine and its excretion rates, estimates of the pyrimidine production rate in three patients ranged between 1.1 and 1.6 grams of pyrimidine nucleoside per day. These are the first estimates to be made of total pyrimidine synthesis in man. With C^{14} labeled uracil, evidence was obtained indicating the virtual absence of re-utilization of this material, indicating that at nearly physiological levels, few cells of the body are likely to be able to use this compound as a substitute for endogenously produced pyrimidine.

An unusual group of nucleotide-peptide compounds have been studied, certain of which were tentatively identified as 5′ uridylic acid, adenylic acid, and 6-methyladenylic acid. The principle

amino acids were glutamic acid and asparatic acid. Studies with P^{32} indicate a rapid turnover of these compounds.

Elevation of serum uric acid has been found to be characteristic of psoriasis. Patients with this disease have been found to over incorporate C^{14} labeled glycine into uric acid as is also observed in primary gout, but to a lesser degree. This appears to be secondary to an increased turnover rate of the hyperplastic psoriatic epidermis, since the degree of hyperuricemia and pseudouridine excretion are well correlated with the percentage of skin involved with psoriasis.

Studies of the ribonucleic acid content of white blood cells derived from patients with chronic myelogenous leukemia and chronic lymphatic leukemia indicate that guanine and adenine exists in excess. There are also unusual adenylic acid like nucleotides present. These studies suggest that the white blood cell RNA differs quantitatively from the RNA of more differentiated tissues. In chronic myelogenous leukemia and chronic lymphatic leukemia, preliminary evidence indicates that acid soluble nucleotide containing components of RNA (phenol prepared) are relatively abundant as compared with those found in the liver.

Adenylic, guanylic, and inosinic pyrophosphorylase levels have been determined in leukocytes of patients with leukemia and in normal white blood cells. No consistent pattern of enzyme abnormality has been observed. Patients who were clinically resistent to 6-mercaptopurine have not shown the loss of inosinic pyrophosphorylase activity that has been observed in various bacteria and in transplantable mouse tumor cells that develop resistance to 6-mercaptopurine.

Azauridine, a pyrimidine analogue inhibits both *in vivo* and *in vitro* the enzyme, orotodylic decarboxylase. This is one of the steps in pyrimidine synthesis. Administration of this compound to man results in excretion of orotic acid and orotidine. Since these two compounds occur in the metabolic process immediately prior to the inhibited enzyme their appearance in the urine might be anticipated. Azauridine also induces a uricosuria and a lowering of the serum uric acid. This is thought to be a direct effect on the kidney and represent inhibition of the tubular reabsorption of uric acid. The enzyme, orotodylic acid decarboxylase, in the white blood cells of patients with chronic myelogenous leukemia is depressed both *in vivo* after administration of the drug, and *in vitro*. Following therapy with this drug, partial remissions have been observed including a decrease in the white count and a decrease in organ size. In some patients after several weeks, the white blood count increases and the spleen enlarges in spite of continued azauridine administration. Associated with this clinical evidence of resistance is a decrease in the degree of enzymatic inhibition.

Amino Acids

The urinary excretion of amino acids, as alpha amino nitrogen, in children with acute leukemia was found to be normal.

Plasma Proteins

Distinctive serum and urine proteins associated with multiple myeloma and certain lymphocytic neoplasms, characterized by immunochemical and physicochemical techniques have been found to fit into one of four classes of globulin: 6.6S gamma globulin, Beta 2_A globulin, 18S macroglobulin or Bence Jones protein. By studies of electrophoretic, chromatographic, ultra-centrifugal properties and antigenic determinants, the range of molecules formed in malignant plasma cells was defined, and a comparison of these proteins with those in normal serum undertaken. The proteins formed in malignant plasma cells closely resembled normal globulins and, in fact, could not be shown to be abnormal either in man or the mouse.

The genetic properties of malignant plasma cells were assessed by measurement of the Gm groups a, b, and x present on myeloma proteins and macroglobulins. Normal gamma globulins have these genetic traits which reflect the genetic constitution of normal plasma cells. The 6.6S gamma myeloma proteins also were found to carry the Gm genetic traits indicating that the genetic properties of malignant plasma cell (in which myeloma proteins are formed) are similar to the genetic properties of normal plasma cells.

Ultrafiltration using nitrogen under high pressure and a collodion membrane for the concentration of dilute solutions containing proteins of

dilute solutions containing proteins of cerebrospinal fluid, sweat, and urine have been carried out. These concentrated solutions were then studied by paper electrophoresis. Abnormal gamma globulins, Beta 2_A and Beta 2_M globulins were present in the nasal secretions in patients with multiple myeloma. The cerebro-spinal fluid in central nervous system leukemia shows minor changes in the albumin and Beta globulin components. There was no protein found in the sweat in patients with multiple myeloma. This method of concentration of proteins also proved satisfactory for studying protein excretion in the urine.

Twenty patients with lymphomatous disease were studied with Iodine 131 labeled albumin to determine the rate of synthesis of albumin, total body albumin, and if the gastrointestinal tract was a site of protein loss in these patients. These included three patients with marked diarrhea and three with large chylous effusions. Ten of these twenty patients, and an additional ten, were also studied using iodinated polyvinylpyrollidon (PVP). All but five of these patients had a serum albumin concentration under 3.5 grams percent. The three patients with chylous effusions had a prolonged albumin survival and an increased total albumin pool. The remaining 17 patients had markedly reduced body albumin pools with a normal or a slightly short albumin survival, but decreased albumin synthesis. In no instance in a patient in this group was gastrointestinal protein loss demonstrated.

The preparation of chromium 51 labeled albumin and the demonstration that it was superior to iodinated PVP represents a major advance in the means for detecting gastrointestinal protein loss.

Biochemistry of the Skin

Protein fractions have been extracted from exfoliated scales of psoriasis. Both soluble and insoluble fractions have been found which can then be further fractionated into four sub-fractions. Following the administration of glycine C^{14}, glycine was isolated from each of these eight fractions and its radioactivity determined. The time of appearance of isotope in each of these fractions differed, indicating that each fraction was synthesized at a different rate within the epidermis or at a different time following administration of the isotope.

Split thickness skin grafts cultured in a medium containing tritiated thymidine showed evidence of synthesis of DNA. This important observation indicates that under the conditions used, the skin continues to be capable of synthesis and opens the way to many further studies of the biochemistry of skin.

Incubation of the skin of subjects with albinism and vitiligo with tyrosine indicated the presence of tyrosinase in the skin of the albino, but not in the depigmented skin of persons with vitiligo. Thus the pigment synthesizing defect in albinism is still to be determined.

Endocrine Studies

Patients with multiple basal cell carcinoma have been given parathormone and their urinary phosphate excretion was measured. Two patients with the syndrome of basal cell carcinoma, bifid ribs and cysts of the mandible have failed to respond to this hormone with phosphate diuresis as did patients with ordinary basal cell carcinoma and five normal subjects.

Seven of 95 patients with trophoblastic disease were hyperthyroid as evidenced by elevated PBI and/or I^{131} uptake. In several of these patients, the hyperthyroidism or apparent hyperthyroidism subsided after treatment with methotrexate which led to substantial tumor regression.

Study by the Ouchterlony diffusion technique and immunoelectrophoresis indicates that the chorionic gonadotropic hormone in pregnancy is associated with a gamma globulin, whereas in choriocarcinoma it is found with the beta globulin fraction. Antisera produced to both gonadotrophins were shown to be equally potent indicating that from the immunological standpoint these materials are equivalent.

Section of the pituitary stalk in patients with advanced breast cancer shows that thyroid function may persist after interruption of the hypothalmic hypophyseal portal system indicating that the thyroid, in some instances, functions independently of the hypothalamus.

In collaboration with Dr. B. Hökfelt of the Karolinska Hospital, it has been shown that 17 alpha-hydroxy pregnenolone is a precursor of cor-

tisol in man as it is in the rat and the guinea pig. This is a previously unidentified pathway of cortisol synthesis in the adrenal gland. Testosterone was synthesized from progesterone in slices from the ovarian cyst wall taken from a patient with a virilizing syndrome. Measurement of aldosterone excretion and secretion rates indicate that the diuresis that occurs in patients with refractory edema can occur without change in aldosterone excretion rates.

Patients with adrenal cancer treated with o,p'DDD absorb only 20% of an oral dose. When the body fat stores become saturated an approximately constant blood level can be maintained.

Studies on patients with adrenal cortical carcinoma have shown that in those patients with Cushing's syndrome, an increased excretion of tetrahydro S substances has been found in each patient; whereas the increased excretion of dehydro-epiandiosterone is not constant. In patients with adrenal cortical carcinoma isotope dilution studies demonstrated that the increased etiocholanalone: androsterone ratio resulted from the metabolism of C^{21} precursors in contradistinction to findings in the normal subject.

Hematopoiesis

The effects of various doses and schedules of endotoxin on fever, WBC changes and antibody production was studied in normal prisoner volunteers. Differing dose-response relationships obtained for fever index, mature granulocyte increment and immature granulocyte increment. The variability of response to a given dose was slight. In patients with neoplastic disorders, the variability of response to a given dose was greater and appeared to be related to involvement of the reticuloendothelial system. With repeated daily endotoxin administration tolerance to the febrile effect occurred within 7 days whereas the granulocyte increment persisted.

Studies of hematopoiesis in patients undergoing total body radiation indicates that the increase in the half time for the plasma clearance of intravenously administered radioiron precedes any change in hemoglobin, reticulocyte, or platelet counts, and furthermore, that the clearance half time returns to normal before the reticulocyte count and hemoglobin rises. Post-radiation, white blood cell mobilization in response to endotoxin is progressively depressed.

A new assay system for erythropoietin, using the polycythemic mouse has been developed. It exhibits markedly increased sensitivity and requires smaller volumes of starting materials than previously available procedures. Erythropoietin has been demonstrated in an extract of cerebellar hemangioblastoma tumor tissue, cerebellar cyst fluid and the plasma of a patient with polycythemia, but not in the renal cyst, pancreatic cyst fluid, or an extract of a kidney tumor from this patient. Erythropoietin has also been demonstrated in an extract of the tumor from a patient with pheochromocytoma who was polycythemic, and from the plasma of two of three additional patients with pheochromocytoma. These findings suggest that the polycythemia associated with pheochromocytoma and cerebellar hemangioblastoma is a result of production by these tumors of an erythropoietic substance. In five of ten patients with renal cystic disease erythropoietin has been demonstrated in the renal cyst fluid. Three of the five patients were polycythemic. In two of three cases, resection of the renal cyst produced a reversal of the polycythemia. These studies suggest that the kidney is a major site of erythropoietin synthesis in man. Elevated erythropoietin titers have been demonstrated in the serum of only one of four patients with polycythemia secondary to von Hippel Lindau's disease and in none of the six patients with polycythemia vera. A group of patients with leukemia, multiple myeloma, chronic infection and aplastic anemia have been found to have elevated erythropoietin levels when the peripheral hemoglobin concentration was less than 8 grams percent even when there was severe renal impairment. This indicates that the anemia of renal disease is not due to the absence of erythropoietin. There was a direct correlation of the erythropoietin titer with the severity of the anemia. With the exception of the patients with aplasia of the bone marrow, there was little or no correlation between the erythropoietin level and the reticulocyte count. Endogenous erythropoietin production was decreased by transfusing 3 anemic subjects with three units of packed cells.

The plasma erythropoietin activity decreased with half times of 9½ to 19 hours.

The molecular weight of the biologically active portion of erythropoietin was found to be 25-30,000 using varying doses of radiation from the van de Graff accelerator for inactivation and assuming a spherical molecular shape.

Diisoproplyfluorophosphate P^{32} (DFP^{32}) was evaluated as a label for the measurement of red cell life span in the dog and man, and found to be satisfactory; in fact, probably the method of choice for most situations requiring this measurement.

Studies of erythropoiesis including determination of total red cell volume, rate of production of red cells and red cell life span were carried out in patients with multiple myeloma, macroglobulinemia, Hodgkin's disease and chronic myelocytic leukemia. In multiple myeloma, the predominant pattern of erythropoiesis was that of erythropoietic insufficiency with a moderate shortening of red cell survival. The red cell life span was finite but short. Variations in these patterns were found depending upon the stage of disease. Androgen therapy in large doses produced an increase in the rate of production of red cells and an increase in the total red cell volume in four of five patients studied. In chronic myelocytic leukemia a spectrum of patterns of erythropoiesis emerged. This varied from a normal total red cell volume, normal rate of production of red cells, and normal red cell life span through increased to decreased rate of production of red cells; a finite shortening of red cell life span in the order of 75 to 80 days, and later markedly decreased erythrocyte survival in the acute myeloblastic stage with a virtual absence of red cell synthesis. In macroglobulinemia, several distinct patterns were found. At one extreme, two patients with erythroid aplasia were observed, and at the other extreme, marked shortening of the red cell life span. In two patients, shortening of red cell life span could be related to clinical activity of the disease and the concentration of serum macroglobulins. A preliminary review of studies in nine patients with Hodgin's disease indicates that shortening of red cell life span is the predominant abnormality except in the late stage where there is bone marrow failure.

Folic Acid Antagonists

Dichloromethotrexate (DCM) containing radioactive chlorine was given to patients both intravenously and orally to study the mechanisms involved in the metabolism of this compound in an attempt to explain the differences in therapeutic efficacy in the mouse as compared to man. Following oral administration the urinary output of isotope was less than when given intravenously. This is thought to be due either to a failure to absorb a part of the dose through the gastrointestinal tract or alternatively that following absorption from the gastrointestinal tract the material is transported directly to the liver where it is excreted into the bile. The urinary excretion pattern shows an increasing percentage of the isotope being excreted as a metabolite, dyhydroxy-dichloromethotrexate. This metabolic pattern is similar to that observed in the mouse. This differs from studies of the same compound in the dog in which animal this material is not metabolized, and in the rat in which the material is converted to the matabolite at a higher rate. These studies have been made possible by significant advances in the development of methods for a separation of folic acid-like compounds on DEAE columns and by high voltage electrophoresis.

Metabolic Balance Technique

A common metabolic technique in the experimental animal is paired feeding in which the experimental animal receives a diet, or is allowed to select a diet *ad libitum* and a second animal is fed an identical diet. This permits an evaluation of the effect of any procedure by comparison with a normal animal receiving an identical diet. A somewhat modified version of this technique consists in the preparing of identical trays of food for each meal and allowing the patient to select food *ad libitum*. Then by analyzing the initial diet and comparison with the residue left, the total intake is calculated. In this manner the effect of applied variables on the selection of food both in terms of quantity, chemical composition and balance can be evaluated. This technique has been successfully applied and data concerning the anabolic and catabolic effects of tri-iodo thyronine.

cortisone, testosterone, and growth hormone have been readily demonstrated. Little or no immediate quantitative or qualitative effect on food intake was observed in conjunction with profound anabolic and catabolic stimuli.

Calcium Metabolism

Calcium metabolism was studied in patients with multiple myeloma with lytic bone lesions and in patients with breast cancer metastatic to the skeleton. With radioactive calcium[47] and metabolic balance techniques estimates of the rate of bone formation, bone destruction intestinal absorption and endogenous calcium secretion have been carried out. All patients studied have been in negative calcium balance particularly as a result of a markedly increased intestinal excretion rather than excessive urinary loss or inadequate absorption from the gastrointestinal tract. External counting over bone revealed that the osteoblastic metastases of breast carcinoma pick up labeled calcium in greater quantities than does normal bone, while the lytic lesions of multiple myeloma show little deviation from normal.

Serum Lactic Dehydrogenase

A method for the determination of serum lactic dehydrogenase has been set up and values have been established for the normal. In various disease states when disease is clinically static the values obtained are stable indicating that technically the procedure is satisfactory. Following total body radiation of patients with chronic leukemia, within approximately one hour, the serum lactic dehydrogenase level falls reaching a minimum at 24 to 48 hours. This represents one of the earliest measurable changes following radiation. In patients with solid tumors, particularly cervical epidermoid carcinoma and adenocarcinoma, the serum lactic dehydrogenese levels are variable from case to case and at the moment cannot be correlated with any other measurement of disease state. Three patients with rhabdomyosarcoma had consistent and elevated levels. Following extensive surgical procedures, the rise in serum lactic dehydrogenase was not comparable to that seen following a myocardial infarct; although in the surgical procedure considerably more tissue may be traumatized. In Hand-Schuller-Christian disease, elevated serum lactic dehydrogenase levels have been found.

Mitosis Inhibiting Activity in the Serum

Serum from patients with cancer and other diseases and from normal controls has been extracted with ether and saline and the fractions injected into partially hepatectomized rats. In the normal, two fractions are found. One that inhibits hepatic cell regeneration, and the second, that stimulates hepatic cell regeneration. The serum from patients with cancer lack this inhibiting fraction. This deficit can be replaced with the inhibiting fraction. Administration of the inhibiting fraction delays the time of appearance and slows the rate of growth of the L–1210 mouse leukemia.

Carcinoid

The excretion of 5-hydroxy indolacetic acid and total indole excretion were decreased in patients given cytoxan and 5-fluorouracil; however, when these patients were calorically restricted to the same extent as occurred during drug therapy, the same effect was obtained, thus no effect of the drug per se could be demonstrated. This is in contrast to similar studies in mice with a mast cell tumor.

Immunology

Studies of the effect of malignant disease on immune processes were undertaken in patients with multiple myeloma and with macroglobulinemia. The serum levels of the gamma globulins and related immunoglobulins were low in most of the 40 myeloma sera and 16 macroglobulinemia sera tested, indicating that serum antibody levels might be low in these malignant diseases. This was supported by the finding of low isohemagglutinin levels in multiple myeloma (35 of 40 patients) and macroglobulinemia (all of nine patients). Antibody response to antigen administration, now being tested, appears to be deficient in these two diseases.

The molecular basis of immunity has been under investigation by both physiochemical and immunochemical techniques. Previous studies have shown

that antibodies in man may be among the 6.6S gamma globulins or the 18S macroglobulins, and studies elsewhere have indicated that Beta 2_A globulins may carry antibody activity. These three groups of proteins have distinctive properties but they also share some properties in common. This possibility that antibody-forming cells have some specific common property was investigated through structural studies of these three classes of globulins. Enzymatic fragmentation of gamma and Beta 2_A globulins provided evidence that these proteins are made up of three subunits, and that two of the subunits in each class (gamma or Beta 2_A) were simliar, whereas the third subunit was markedly different for gamma and Beta 2_A globulins. Such studies narrow the molecular components in which distinctive globulin properties are sought, and help to put immunologic observations on a physiochemical basis.

METABOLISM SERVICE

Amino Acid Transport

In vitro studies using C^{14} and S^{35} labeled amino acids in rat renal cortex slices indicate that this tissue accumulates amino acids in intracellular water from two to five times the concentration in extracellular fluid. This fulfills the major criterion of an active transport system. Using Michaelis-Menten enzyme kinetic methods, an affinity constant was calculated for each amino acid studied. Compartmental analysis of the data has resulted in a formulation of a three compartment transport model from which influx and efflux rate constants have been calculated. Phlorizin was found to stimulate cellular accumulation of amino acids although it is a potent inhibitor of the transport of sugar. This was shown to result from specific inhibition of the efflux of amino acids without demonstrable influx effect. Lysine, ornithine, arginine, and cystine transport studies were undertaken in search of a common carrier mechanism since all four are excreted in excess in human cystinuria. Arginine and ornithine inhibited the transport of lysine C^{14} due to competitive inhibition.

Rats fed either a 30% galactose diet or maleic acid develop amino-aciduria which results from defective renal tubular absorption. *In vitro* studies with maleic acid suggest that inhibits both influx and efflux. These studies are part of a program defining the mechanisms of amino acid transport and determining the relationship between transport and subsequent protein synthesis.

Plasma Proteins

The study of plasma proteins on the Metabolism Service has been concerned with: (1) The physical-chemical and immunochemical properties of proteins produced in normal and malignant plasma cells, and, (2) Metabolic behavior in terms of rate of synthesis and degradation.

Proteins in normal and malignant plasma cells

The distinctive serum and urine proteins associated with multiple myeloma and certain lymphocytic neoplasms were characterized by immunochemical and physicochemical techniques and found to fit into one of four classes of globulin: 6:6S gamma-globulin, Beta 2_A globulin, 18S macroglobulin or Bence Jones protein. Parallel studies of serum and urine proteins associated with 20 transplantable plasma cell tumors in mice established the existence gamma and Beta 2_A myeloma proteins and Bence Jones proteins in this species which are very similar to the analogous proteins of man. By detailed studies of electrophoretic chromatographic and ultracentrifugal properties and antigenic determinants, the range of molecules formed in malignant plasma cells was defined, and a comparison of these proteins with those in normal serum could be undertaken. The proteins formed in malnignant plasma cells closely resembled normal globulins and, in fact, could not be shown to be abnormal either in man or the mouse.

Biosynthetic studies with plasma cell tumor slices and C^{14} labelled amino acids showed clearly that Beta 2_A myeloma proteins and Bence Jones proteins as well as gamma myeloma proteins are synthesized in plasma cell tumors. Thus, the properties of these proteins reflect the properties of the malignant plasma cell.

The genetic properties of malignant plasma cells were assessed by measurement of the Gm groups a, b and x present on myeloma proteins and macroglobulins. Normal gamma globulins have these genetic traits which reflect the genetic constitution of normal plasma cells. The 6.6S gamma

myeloma proteins also were found to carry the Gm genetic traits indicating that the genetic properties of malignant plasma cells (in which myeloma proteins are formed) are similar to the genetic properties of normal plasma cells.

Metabolic Behavior

These studies are principally concerned with measurement of the turnover of plasma proteins. Transplantable plasma cell tumors in mice accelerate the rate of turnover of gamma globulins and gamma myeloma proteins without altering albumin metabolism. Plasma cell tumors, however, differ in their metabolic effects for a plasma cell tumor producing Beta 2_A globulins did not accelerate gamma globulin turnover. The gamma globulins formed in malignant plasma cells were found to behave metabolically in the same way as proteins produced in normal plasma cells. Gamma myeloma proteins were found to be synthesized at the same rate per gram of tissue origin as albumin.

Twenty patients with lymphomatous disease were studied with Iodine 131 labeled albumin. These included three patients with marked diarrhea and three with large chylous effusions. Ten of these twenty patients, and an additional ten, were also studied using Iodinated polyvinylpyrollidon (PVP). All but five of these patients had a serum albumin concentration under 3.5 grams percent. The three patients with chylous effusions had a prolonged albumin survival and an increased total albumin pool. The remaining 17 patients had markedly reduced body albumin pools with a normal or a slightly short albumin survival, but decreased albumin synthesis. In no instance in a patient in this group was gastrointestinal protein loss demonstrated. In four patients with Whipple's disease marked gastrointestinal protein loss was demonstrated. Iodinated albumin turnovers on two of these patients showed a shortened albumin survival. These studies indicate that deficient protein synthesis is also a factor in hypoproteinemia seen in patients with Whipple's disease. When treated with antibiotics and steroids there was almost a complete reversal of this gastrointestinal protein loss. Twenty-seven patients with idiopathic hypoproteinemia with serum albumin concentration under 2.5 grams percent were studied. None of these patients had renal or hepatic disease. Twenty-five patients had idiopathic hypocatabolic-hypoproteinemia. The synthetic rate for albumin was normal or increased in all patients but gastrointestinal protein loss resulted in a shortened albumin survival. The features that these twenty-five patients shared in common was as follows: they were all below the age of twenty-eight at onset, had generalized edema, reduced serum albumin, total globulin, gamma globulin, and iron binding protein. There was a normal or low serum cholesterol. The majority of the patients had only mild gastrointestinal symptoms. Within this group of 25 patients, four clinical syndromes could be recognized. The first consisted of three patients with transient disease, which disappeared spontaneously in six weeks to three months. The second group included four patients with cardiac lesions, three with constrictive pericarditis and an interatrial septal defect in one. Surgical correction of the cardiac lesions produced a reversal of the protein abnormalities. Three patients had chronic pulmonary disease, eosinophilia and a familial history of allergy. In all three marked amelioration of the gastrointestinal protein loss followed therapy with adrenocortical steroids. In 15 patients gastrointestinal protein loss was thought to be due to dilated lymphatic channels in the small intestine. Microscopic examination of the small bowel in these patients showed edema of the wall and a dark brown pigmentation. The lymphatics were dilated and contained lipomacrophages. The mesenteric lymphatics were greatly thickened and fragmented. Surgical, steroid, and dietary treatment of these patients was ineffectual.

Twenty patients with hypogammaglobulinemia were studied. Fifteen patients had serum albumin concentrations under 3.5 grams percent and in six there was less than 2 grams percent. Three of these six patients had decreased albumin synthesis while in the remaining three, gastrointestinal protein loss was the major factor in producing hypoalbuminemia. This protein loss was reversed on therapy with cortisone or tetracycline, and a gluten free diet.

Two patients with failure to produce albumin were studied. In both, the iodinated gamma globulin survival was normal, indicating that the catabolism of albumin and gamma globulin are independent. All the abnormalities in these pa-

tients could be corrected by albumin infusion with the exception of a prolonged albumin survival.

The demonstration that chromium 51 labeled albumin was superior to iodinated PVP represents a major advance in the means for detecting gastrointestinal protein loss.

Immunologic Studies

Studies of the effect of malignant disease on immune processes were undertaken in patients with multiple myeloma and with macroglobulinemia, and two transplantable plasma cell tumors in mice. The serum levels of the gamma globulins and related immunoglobulins were low in most of the 40 myeloma sera and 16 macroglobulinemia sera tested by immunoelectrophoretic and chromatographic techniques, indicating that serum antibody levels might be low in these malignant diseases. This view was supported by the finding of low isohemagglutinin levels in multiple myeloma (35 of 40 patients) and macroglobulinemia (all of nine patients). Antibody response to antigen administration is now being tested and appears to be deficient in these two diseases.

When challenged with specific antigens mice bearing transplantable plasma cell tumors had impaired antibody production. The two tumors differed, however, in their quantitative effects, indicating the need for further investigation on the mechanisms of immunity. For this purpose and to establish a laboratory model for detailed investigation of tumor effects on immunity, investigations of the characteristics of antibody production and the properties of antibodies were undertaken in imbred mice. Studies to date clearly indicate that mice, like man, can produce both 6.6S gamma globulin and 18S macroglobulin antibodies.

The molecular basis of immunity has been under investigation by both physiochemical and immunochemical techniques. Previous studies have shown that antibodies in man may be among the 6.6S gamma globulins or the 18S macroglobulins, and studies elsewhere have indicated that Beta 2_A globulins may carry antibody activity. These three groups of proteins have distinctive properties but they also share some properties in common. This possibility that antibody-forming cells have some specific common property was investigated through structural studies of these three classes of globulins. Enzymatic fragmentation of gamma and Beta 2_A globulins provided evidence that these proteins are made up of three subunits, and that two of the subunits in each class (gamma or Beta 2_A) were similar, whereas the third subunit was markedly different for gamma and Beta 2_A globulins. Such studies narrow the molecular components in which distinctive globulin properties are sought, and help to put immunologic observations on a physiochemical basis. In addition, the functional and genetic properties of malignant plasma cells can be compared with normal plasma cells by means of the proteins (gamma globulins) produced in these cells. Related studies with myeloma proteins are described in section on Plasma Proteins.

Nucleic Acids

Column chromatographic methods for isolating pseudouridine and uracil from urine were developed. Elevated excretion of these compounds was found in patients with gout, leukemia, and psoriasis. The excretion diminished following administration of azauridine. Biosynthetically labeled uridine containing C^{14} and tritium was prepared. In both man and the rat, pseudouridine is not metabolized. Ring labeled orotic acid was administered to patients and the excretion of C^{14}, as respiratory $C^{14}O_2$, uracil and pseudouridine followed. From these data, estimates of the pyrimidine production rate in three patients ranged between 1.1 and 1.6 grams of pyrimidine nucleoside per day. These are the first estimates to be made of pyrimidine synthesis rate in man. With C^{14} labeled uracil evidence was obtained indicating the virtual absence of re-utilization of this material.

An unusual group of nucleotide-peptide compounds have been studied, certain of which were tentatively identified as 5′ adenylic acid, 5′ uridylic acid, adenosine diphosphate and 6-methyladenylic acid. The principle amino acids were glutamic acid and aspartic acid. Studies with P^{32} indicate a rapid turnover of these compounds.

Calcium Metabolism

Calcium metabolism was studied in patients with multiple myeloma with lytic bone lesions or

in patients with breast cancer metastatic to the skeleton. With radioactive calcium 47 and metabolic balance technique estimates of the rate of bone formation, bone destruction, intestinal absorption and endogenous calcium secretion have been carried out. All patients studied have been in negative calcium balance particularly as a result of a markedly increased intestinal excretion rather than excessive urinary loss or inadequate absorption from the gastrointestinal tract. External counting over bone revealed that the osteoblastic metastases pick up labeled calcium in greater quantities than does normal bone, while the lytic lesions of multiple myeloma show little deviation from normal.

Porphyrin Metabolism

Studies of the biochemical lesions of porphyria in patients and in experimental animals have been carried out. In patients with acute intermittent porphyria an increase in carbohydrate and/or protein intake decreases the rate of excretion of porphobilinogen. Orthostatic hypotension and the "restless leg" syndrome have been demonstrated as manifestations of acute porphyria. An elevation of the protein bound iodine was found; this may be related to a diminished rate of degradation of the thyroid hormone.

The synthesis of delta-aminolevulinic acid, the porphobilinogen was studied in the liver *in vitro*. There appears to be diminished ability to oxidize the alpha carbon atom of glycine to CO_2 in experimental porphyria. In experimental porphyria the following studies of the rate of utilization or oxidation were normal: rate of utilization of porphobilinogen and the rate of oxidation of the four carbon atom of delta-aminolevulinic acid, the oxidation of alpha-ketoglutarate, the one and six carbon atoms of glucose, the two carbon atom of pyruvate and the two carbon atom of acetate.

Erythropoiesis

The studies of erythropoiesis can be divided into the following areas: (1) The studies on site production of erythropoietin, (2) Studies on means of measuring red cell survival, (3) Clinical studies of erythropoiesis.

Erythropoietin Production

Paraboitic rats in which one partner of the pair was either nephrectomized or the ureter ligated and exposing one ainmal to room air, and the other to a mixture of 9% oxygen indicates that the kidney is the major but not the sole site of erythropoietin production. A new assay system for erythropoietin, using the polycythemic mouse of markedly increased sensitivity and requiring smaller volumes of starting materials has been developed. This assay system utilizes the polycythemic mouse.

Erythropoietin has been demonstrated in an extract of cerebellar hemangioblastoma tumor tissue and cerebellar cyst fluid, the plasma of a patient with polycythemia and von Hippel Lindau's diseases but not in the renal cyst, pancreatic cyst fluid, or an extract of a kidney tumor from this patient. Erythropoietin has also been demonstrated in an extract from the plasma of two or three additional patients with pheochromocytoma. These findings suggest that the polycythemia associated with pheochromocytoma and cerebellar hemangioblastoma is a result of production by these tumors of an erythropoietin. Renal cyst fluid and/or plasma has been obtained from ten patients with renal cystic disease and in five of ten patients with renal cystic disease erythropoietin has been demonstrated in the cyst fluid. Three of the five patients were polycythemic. In two of three cases, resection of the renal cyst produced a reversal of erythropoietin production in man. Elevated erythropoientin titers have been demonstrated in the serum of only one of four patients with polycythemia secondary to von Hippel Lindau's disease and in none of the six patients with polycythemia vera. A group of patients with leukemia, multiple myeloma, chronic infection and aplastic anemia have had elevated erythropoietin levels when the peripheral hemoglobin concentration was less than 8 grams even when there was severe renal impairment. This indicates that the anemia of renal disease is not due to the absence of erythropoietin. There was a direct correlation of the erythropoietin titer with the severity of the anemia. With the exception of the patients with aplasia of the bone marrow, there was little or no correlation between the erythropoietin level and the reticulocyte count.

Endogenous erythropoietin production was decreased by transfusing 3 anemic subjects with three units of packed cells. The erythropoietin activity decreased with a half time of 9½ to 19 hours.

The molecular weight of the biologically active portion of erythropoietin was found to be 25–30,000 using varying doses of radiation from the van de Graff accelerator and assuming a spherical molecular shape. Using the polycythemic mouse it was determined that the control of erythropoiesis is determined by the total red cell volume rather than the peripheral hematocrit. (Waldmann)

Red Cell Life Span Methodology

Diisopropylfluorophosphate P^{32} (DFP^{32}) was evaluated for the measurement of red cell life span in the dog and man, and found to be very satisfactory. A new method for producing a cohort of labelled cells utilizing both cold DFP and radioactive DFP^{32} was developed. This method involves the administration of a large (blocking) dose of cold DFP followed in several days by a dose of labeled DFP^{32}. The DFP^{32} labeling only those cells which were synthesized in the time interval following the administration of the cold DFP. In the dog, the red cells produced in the first six days in response to an acute hemorrhage (15% blood volume) have a shortened survival, but the cells produced by the ninth day have a normal survival. (Berlin)

Clinical Studies of Erythropoiesis

In man, studies of erythropoiesis including measurement of total red cell volume, rate of production of red cells and red cell life span were carried out in patients with multiple myeloma, macroglobulinemia, Hodgkin's disease and chronic myelocytic leukemia. In multiple myeloma, the predominant pattern of erythropoiesis was that of erythropoietic insufficiency with a moderate shortening of red cell survival. The red cell survival was finite but short. Variations in these patterns were found depending upon the stages of disease. Androgen therapy in large does produced an increase in the rate of production of red cells and an increase in the total red cell volume in four of five patients studied. In chronic myelocytic leukemia a spectrum of patterns of erythropoiesis emerged. This varied from a normal total red cell volume, normal rate of production of red cells, and normal red cell life span through increased to decreased rate of production of red cells; a finite shortening of red cell life span in the order of 75 to 80 days, and later markedly decreased erythrocyte survival in the acute myeloblastic stage with a virtual absence of red cell synthesis.

In macroglobulinemia, several distinct patterns were found. At one extreme, two patients with erythroid aplasia were observed, and at the other extreme marked shortening of the red cell life span. In two patients, shortening of red cell life span could be related to clinical activity of the disease and the concentration of serum macroglobulins. A preliminary review of studies in nine patients with Hodgkin's disease indicates that shortening of red cell life span is the predominant abnormality except in the late stage where there is bone marrow failure.

LABORATORY OF BIOCHEMISTRY

During the past year the Laboratory of Biochemistry was divided into five sections:

Cytochemistry, Head, Dr. Dean Burk
Nucleic Acids, Head, Dr. Walter C. Schneider
Nutrition and Carcinogenesis, Head, Dr. Harold P. Morris
Protein Chemistry, Head, Dr. Elbert A. Peterson
Tumor-Host Relations, Head, Dr. Robert E. Greenfield

Cytochemistry Section

Uridine and certain 5-fluorinated derivatives constitute useful tools for the study of the role of inorganic phosphorous in regulating cell metabolism. The glucose requirements for anti-tumor action of these substances were examined to determine the possible chemotherapeutic application. Triphosphopyridine nucleotide was shown for the first time to effect glycolysis by both living cancer cells and subcellular fractions thereof.

Using glycolytic assay procedures a naturally occurring cytocidal complement-dependent antibody system has been shown to be lacking in the blood of germ-free pigs but was present in natu-

rally contaminated animals maintained on the same diet. With cells of the reticuloendothelial system the role of endotoxins in increasing nonspecific (phagocytic) and specific (antibody) resistance to infection was investigated. It has been shown that endotoxins of gram negative bacteria exert profound effects on the metabolism of mammalian cells by their insulin-like effect on the hexokinase reaction. Endotoxin and insulin appeared to act at the level of mitochondrial bound hexokinase, both stimulating mitochondrial glycollysis. Mitochondrial suspensions prepared from mouse melanomas, ascites tumors and brain displayed highly significant glycolytic activities which were sensitive to insulin: anti-insulin hormonal regulation. The degree of sensitivity to hormonal regulation corresponded approximately to that obtaining in white cells of the tissue from which the mitochondria were obtained. Data obtained from a series of rat hepatomas indicated that in these tumors, as in malignancies of other tissues, with the development or progression of the malignant state there was an increase in capacity to glycolyze which was associated with decreasing sensitivity to restraint by anti-insulin hormones.

Effects of vincaleukoblastine (VLB) on glycolysis and respiration of ascites tumor cells in vitro have been demonstrated with several lines of the L1210 mouse leukemia and in the Ehrlich carcinoma. Protection against the effects of VLB on aerobic glycolysis of thioguanine resistant cells by monosodiumglutamate and L-arginine hydrochloride were observed. In line with reports of glutamic acid and arginine protection against VLB with leukemic cells in tissue culture and Ehrlich ascites cells in vivo, it was concluded that this action of VLB involved gross metabolic parameters, glycolysis and respiration, basic to the production of cell energy.

Procedures employing trypsin and DNAase were developed for preparing suspensions of large number of single viable and metabolically intact cells from a variety of solid tumor sources which made possible the measurements of metabolic or other parameters in the absence of contamination with host or necrotic tissues. Normal and myeloblastic leukocytes were found to contain myeloperoxidase and a cytochrome b-like pigment but to be relatively low in cytochrome c or oxidase. The lethal effects of intraperitoneally injected thermophilic chlorella cells into mice seemed to result from the induced carbohydrate depletion of the animal as well as the possible toxins liberated by the plants.

Nucleic Acids Section

Studies of the synthesis and metabolism of nucleic acids have centered around precursors of deoxyribonucleic acid (DNA). Basic to much of this work has been a microbiological assay which used *L-acidophilus* R–26 for the determination of deoxyribosidic compounds. Ribonucleotides were found to be potent inhibitors of the assay when deoxyribonucleotides were being measured but this inhibition could be avoided by hydrolyzing the nucleotides to the non-inhibitory nucleosides with snake venom. This was the basis for an improved assay procedure.

The investigation of the occurrence of large quantities of deoxycytidine diphosphate chlorine in the Novikoff hepatoma was continued. Enzymatic studies of the synthesis of this compound and of its utilization for the formation of lecithin showed that the synthesis of deoxycytidine diphosphate choline was about the same in the tumor as in normal liver but that the ability to form lecithin was only one third as great in the hepatoma as in normal liver. These results indicated that the high levels of deoxycytidine diphosphate choline in the Novikoff hepatoma were due to the decreased utilization of this compound for lecithin formation, perhaps indicating a mechanism by which the tumor can provide a larger pool of precursors for DNA synthesis. The intracellular localization of these enzymatic activities was also studied. Preliminary findings indicated that the synthesis of lecithin occurred in the microsome fraction while the formation of deoxycytidine diphosphate choline was catalyzed by the soluble fraction of normal rat liver.

Studies on the occurrence of deoxycytidine, deoxyuridine and 5-methyl deoxycytidine in the urine of rats were continued. Improved procedures for the determination of the four deoxynucleosides without prior isolation and purification were developed which made use of an *E. coli* mutant 70 V–464, *L-acidophilus* R–26, and chromatography on XE–64 resins. On the

same experimental regime females excreted one fifth the amount of deoxyribosyl compounds of males, an unexplained observation. In both sexes, excretion in the urine was elevated on the day following partial hepatectomy. The increase was not seen in pair fed controls or after 20% partial hepatectomy. The data obtained agreed with other work indicating that the synthesis of DNA during liver regeneration was almost complete within 24 hours.

Four seperate kinase systems responsible for the phosphorylation of thymidine monophosphate (TMP), deoxycytidine monophosphate, deoxyguanosine monophosphate (dGMP) and deoxyadenosine monophosphate, respectively, and required for total DNA synthesis were studied simultaneously in normal mouse liver cells and in mouse ascites hepatoma cells. The limiting kinase activity in both hepatoma and liver cells was found to be TMP kinase. However, when phosphorylation was studied, only 10–15% of the TMP was converted to the di- and triphosphate by normal cells while sixty percent conversion was obtained with the ascites hepatoma cells. A similar but more exaggerated situation was observed with dGMP in that nearly complete conversion to the di- and triphosphates occurred in the hepatoma cells. The results suggested that liver cells possessed a mechanism for controlling di- and triphosphate synthesis which was absent from the hepatoma cells. The hepatoma cells also appeared to have fewer degradative processes than normal liver cells.

As part of a continuing study of the structure and sequences of nucleic acids, chromatographic techniques were used to effect separation of nucleotides resulting from enzymatic degradation of nucleic acids. Members of a homologous series isolated from digests of synthetic polyadenylic acid have been obtained in sufficient purity and yield to permit a study of the relationship between nucleotide chain length and secondary structure. The hypochromic effect appeared with the dimer and remained constant with increasing chain length after the trimer. At a chain length of 7 and above, evidence of polymer-like secondary structure has been obtained from spectral shifts and melting curves. Sephadex and ion exchange Sephadex have potential use for the fractionation of oligonucleotides. Variation in the concentration of salt and eluting medium introduced an additional parameter which has produced advantageous subfractionation.

Partial and complete enzymatic digestion coupled with chromatographic techniques have been used to characterize enzymatic activity, to follow the purification of specific enzymes and to compare RNA samples derived from different sources. Examination in this manner of three different strains of tobacco mosaic virus (TMV) have clearly distinguished the RNA of the HR strain from that of the TMV- and M-strain in some of their nucleotide sequences. Similar differences have been reported for the protein component of the TMV virus obtained from these strains.

Since progress in obtaining the nucleotide sequences of a nucleic acid depends on specific cleavage along the chain, the search for nucleases with hydrolytic activities directed toward a specific nucleotide linkage was continued. A method for the preparation of ribonuclease T1 (specific for guanylic linkages) has been developed. Quantitative isolation and characterization of the mono-, di-, tri-, and tetranucleotides found in partial and complete RNA digests produced by the action of this enzyme indicated a strict specificity for guanylic linkages, since only 3′-guanylic-terminated oligonucleotides were found. Ribonuclease T_2, reportedly exhibiting specificity for adenylic linkages, was purified. Results indicated a preferential splitting of adenylic linkages but did not support a strict specificity.

Purification and characterization of the acid and alkaline ribonuclease activities of spleen uncovered at least two enzymes which fractionated very closely with but could be removed from the ribonuclease activities, an acid phosphatase which readily dephosphorylated adenylic acid, and a phosphodiesterase without ribonuclease activity, which hydrolyzes cyclic adenylic acid to yield 2′-adenylic acid. Although at complete digestion both RNases had hydrolyzed yeast RNA through the cyclic mononucleotides to the 3′-nucleotides, distinct preferential hydrolysis was observed at intermediate stages, the acid RNase digest containing little or no cytidylic acid, whereas the alkaline RNase digests showed very small amounts of adenylic acid.

The ribonuclease activities of homogenates and active nuclease preparations from normal rat liver, Novikoff hepatoma and hepatoma 3683 (Morris) were examined over a pH range of 4.8 to 9.0. The addition of the sulfhydryl reagent, parachloromercuribenzoate (CMB) increased the alkaline ribonuclease activity of the homogenates 2–3 fold by releasing the enzyme from a previously recognized inhibitor. Hepatoma 3683 showed less RNase activity than normal liver or Novikoff hepatoma both before and after the addition of CMB. The acid ribonuclease activities, however, were inhibited by CMB and this inhibition could be reversed by excess cysteine or glutathione. Further evidence in support of the sulfhydryl dependence of the acid RNase was found in a definite correlation between the rate of inhibition of activity and the rate of formation of the mercaptide as titrated spectrophotometrically.

Nutrition and Carcinogenesis Section

Transplantable tumors whose enzymatic complement showed a minimum deviation from that of the normal tissue have been induced in a number of ways. The prototype of the minimal deviation tumor, the transplantable malignant liver neoplasm (Morris 5123), was studied in 25 different laboratories throughout the country. Through the cooperative effort of this group of interested biochemists a large number of unusual enzymatic characteristics have been uncovered in this tumor as well as in the newer neoplasms which have been developed. The Morris 5123 tumor was developed in an inbred strain of Buffalo rats available only at NIH and a large operation has been required to make these tumors available to other investigators. Four sublines of this tumor have been established and it has already appeared that differences in their enzymatic complements have developed. Studies on the "minimum deviation" tumors so far completed suggested that extreme caution must be used in the interpretation that there is a "convergence of enzyme patterns to a single type" in the development of liver cancer. Chemical induction of tumors in inbred strains of rats was especially important since it permitted the development and transplantation of slow growing tumors which otherwise might not have survived in non-inbred rats and would have been permanently lost.

Contrary to the general belief that only negligible amounts of catalase existed in tumor tissue, the newer liver neoplasms that have been developed had high levels of catalase. A transplantable hepatoma induced by feeding a simple aromatic amine, 2,4,6-trimethylaniline grew very slowly and exhibited a high catalase activity. The recently developed Reuber tumor induced by ingestion of 2-fluorenyldiacetamide which grew much more slowly than two neoplasms induced by this chemical in this laboratory many years ago, also possessed catalase activity. The more recently developed and slow-growing neoplasms in general seemed to have a high catalase content whereas catalase activity was absent from almost all transplantable liver tumors previously studied.

The metabolism in various susceptible and resistant species of the carcinogen N-2-fluorenylacetamide (2-FAA) labelled with carbon-14 has been investigated using biochemical, pharmacological, histochemical and tracer techniques. Physical-chemical studies on the carcinogenic metabolites have been performed and the binding of the carcinogen to proteins of the liver and their separation by chromotography has been initiated (J. H. Weisburger, E. K. Weisburger and H. P. Morris). Substitution studies on the hydrogens of the omega carbon atom of 2-FAA series indicated that substitution of the hydrogens by the halogen fluorine, in the 2 and 7 position of the fluorene molecule increased carcinogenic activity. In fact, two transplantable tumors from the same liver have been induced by ingestion of this chemical. These neoplasms had strikingly different growth rates and the preliminary indications were that the more rapidly growing tumor had a carbohydrate enzyme pattern similar to that of the Novikoff hepatoma.

Previous studies of the mechanisms of carcinogenesis indicated that hydroxylation and conjugation were metabolic processes concerned with the detoxification and excretion of the administered carcinogen. The blocking of the usual sites of hydroxylation of simple aromatic amines yielded information which suggested that interference with this process increased carcinogenesis, in some instances at least, and induced transplant-

able tumors having valuable metabolic characteristics.

A compound related to 2-FAA, the 2,7 derivative, N,N,2-7, fluorenyl-bis-acetamide, (2,7-FAA), has been shown to be a very active carcinogen when compared with 2-FAA. The results of one series of experiments with rats have been published as J.N.C.I. monograph No. 5. Some of the most important findings included the induction of tumors of the small intestines and of the glandular portion of the stomach, salivary gland tumors, and neurogenic tumors. This appeared to be the first instance of induction of gastric carcinoma by an orally adminstered carcinogen. Extensive atrophy of many organs was found in those rats that ingested 2,7-FAA.

The rate of tumor induction and growth on adequate diets of natural foodstuffs and on diets of similar composition made from pure or chemically defined ingredients was compared at isocaloric intakes. The results indicated that the intake of the simulated diet was definitely poorer than that of the natural diet with resultant poorer growth. When, however, equal but limited amounts of each diet were fed daily, such that all were consumed, the growth rates were equivalent. Partial underfeeding was employed during the carcinogenic period.

Fischer strain rats receiving 2-FAA plus added DL-tryptophan and propylthiouracil developed liver, ear duct and bladder tumors. Bladder tumors could also be induced in Fischer strain rats by 2-FAA in the absence of added tryptophan. Previous studies indicated that rats ingesting the 2-FAA carcinogen had an increased requirement for pyridoine and that low levels of dietary pyridoxine resulted in an increased induction period although the incidence of liver tumors was essentially unchanged. Continuation of these studies with diets containing 2-FAA and high in pyridoxine permitted the development of mammary tumors earlier than those on the low pyridoxine diet without significantly changing the incidence of liver tumors. The difference in liver tumor incidence between male and female rats on this regimen disappeared if the mammary tumors of females were removed surgically although studies during the precancerous period indicated that histological changes in the liver occurred sooner and more extensively in male rats than female rats. Testosterone was able to accelerate the early liver histological changes of castrated male rats over that of intact males.

Biochemical studies of experimental cancer have shown that the metabolism of 2-FAA by the rhesus monkey resembled that of the guinea pig and not of the rat. The similiarity of the metabolic pattern to that of the guinea pig suggested that the rhesus monkey was relatively resistant to the carcinogenic action of 2-FAA. No tumors were found after six months of continuous feeding.

Examination of the glutamic-oxalic transaminase activity (GOT) of the Morris hepatoma 5123 showed an activity several-fold greater in serum and liver than that of normal animals. The Reuber tumor induced by N-2-fluorenyldiacetamide has a low GOT activity. Passage of the Morris hepatoma for several transfers in tissue culture and then reinoculation into rats has resulted in approximately a two-fold increase in growth rate. The GOT activity, however, seemed to be at the same level as in the slower growing tumor lines not passed through tissue culture. The Dunninghepatoma however, which grew as rapidly as the tissue culture grown 5123 tumor, showed a low GOT activity. Examination of the subcellular distribution of GOT activity in normal liver and in hepatoma indicated a different distribution of activity in neoplastic tissue, with more activity in the supernatant fraction of the hepatoma.

The enzyme responsible for the hydrolysis of D or L peptides seemed to be localized for the most part in the microsome fraction of rat kidney. The sum of the various fractions, however, far exceeded that of the homogenate. This discrepancy was found to be due to the presence of a powerful inhibitor or series of inhibitors in the soluble fraction of the homogenate. The inhibitor system has been shown to be sensitive to the sulfhydryl reagents or simple aerobic incubation. Cysteine was a powerful reversible inhibitor. Cobalt, and to a lesser extent other cations, overcame the action of the inhibitor and further activated the hydrolysis of glycyl-D-amino acids.

Protein Chemistry Section

Since the effectiveness of chromatography in its various applications to macromolecules depends

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upon appropriate adjustment to the properties of the substances under study, various types of adsorbents have been developed for those macromolecules which resist resolution by the existing cellulose exchange adsorbents. A new exchanger, ECG-cellulose, was made by attaching glycine molecules to cellulose through the amino groups and it possessed both positive and negative charges. Because of their proximity to each other, these charges were less effective in binding polyelectrolytes and permitted desorption with low salt concentrations in a more physiologic pH range. Polyglucosamine, obtained by controlled hydrolysis of deacetylated chitin, was investigated as another source of charged groups for attachment to cellulose. Appropriately sized glucosamine polymers, separated on cation exchange cellulose was used to prepare a series of adsorbents having widely separated clusters of uniform charges. These adsorbents should avoid the excessively tight binding encountered with some large molecular weight nucleic acids and nucleoproteins.

Polyamino acids are being used as model proteins to investigate the effect of increasing size on binding to anionic and cationic adsorbents. Homologous polyamino acids ranging in size from n-2, to n-15-20 have been resolved from polymeric mixtures of lysine, glutamic acid, aspartic acid, and mixed polymers of tyrosine and glutamic acid. These studies have demonstrated that chromatographic analysis of polymer mixtures can be used to characterize the molecular weight distribution in the mixture and thus to study the kinetics of polymerization and of acid or enzymatic hydrolysis. Large scale isolation of individual members of a given series will permit use of a given chain length or chain sequence in physical-chemical and biological studies.

Examination of serum proteins has continued with emphasis on the chromatographic purification of factor VIII (antihemophilic factor). Starting with Blomback FIO fraction, high purification has been achieved with recoveries of 85–100%. Factor VIII could also be adsorbed on DEAE-cellulose directly from diluted fresh plasma and recovered in high yield by elution with sodium chloride. Factor V and prothrombin, emerging before factor VIII, were also recovered in good yield. These factors are required for research and clinical applications, and their extraction directly from diluted plasma should increase their availability for experimental use.

Close chromatographic examination of bovine plasma albumin provided evidence for various types of heterogeneity of the albumin molecule. Besides mercaptalbumin (1 mole SH per mole), the major component of serum, three other monomeric albumin components have been observed which can be differentiated by their SH content. In addition, dimers and higher polymers have been detected and concentrated. Two different reactions are involved in the ethanol-induced dimerization of albumin, of which only one requires the participation of free-SH groups. Preliminary data with human plasma albumin suggest a similar degree of heterogeneity.

Of the fifteen β-hydroxy amino acid derivatives tested for inhibition in three microbiological screening systems, the N-chloroacetyl derivative of hydroxymethylserine, α-methylserine, and hydroxyleucine produced growth inhibition. The behavior of the three hydroxyamino acid derivatives was quite different from that of the amino acid, in which the zwitter-ion characteristics were present. The size and charge of the acyl blocking group plays some role in this inhibitory action.

The cytochemical organization of normal and malignant cells was studied primarily in liver cells and in plasma cell neoplasms. Microsome fractions prepared from the latter elicited good antibodies which were usually highly specific against the corresponding circulating myeloma protein. Particular attention has been devoted to those neoplasms which produced high concentrations of urinary proteins in tumor-bearing mice, analogous to the Bence-Jones protein in multiple myeloma. The Potter plasma cell neoplasm (RPC–20) produced urinary protein primarily with a sedimentation rate of 2.9S but with some 4.9S material present. The larger component could be converted to the smaller material by mercaptoethanol treatment. A strong antibody to the native urinary protein has been produced in rabbits by injecting rat microsomes. It failed to react with the normal serum components of mice or with any other mouse myeloma protein examined. The antigen was located in the endoplasmic reticulum; none appeared in the ribonucleoprotein particles.

Beta-glucuronidase activity was used as a biochemical marker for several strains of C3H mouse cell tissue cultures in the study of spontaneous or directed transformation of these cells in vitro. Four new C3H liver cultures which had been initiated have produced one culture line with a very high activity, and three other lines with a low activity of β-glucuronidase.

Fractionation of the ribonucleoprotein particles of rat liver by chromatographic and centrifugal techniques has resulted in two ribonucleoprotein peaks differing significantly in sedimentation coefficients and the ratio of protein to nucleic acid. Stepwise elution procedures producing extremely sharp ribonucleoprotein peaks made feasible the rapid isolation of ribosomes on a large scale. In the presence of high concentrations of magnesium, tight binding to the adsorbent occurred, apparently as a result of aggregation of the ribosomes. In the absence of magnesium the ribosomes dissociated into large and small subunits. Attempts to isolate the large subunit were frustrated by the tendency to reassociate under the conditions employed. The possibility of chromatographic isolation and fractionation of mitochondria was investigated since it had recently become possible to adsorb mitochondria on ECTHAM-cellulose and remove them with a nonionic detergent.

Tumor-Host Relations

A method for the isolation of single cell suspensions of various tissues of the rat was described in last year's report and involved intravenous injection with trypsin and dispersion of the cells with solution of 10% sucrose and 20% polyvinylpyrrolidone (PVP). Under these conditions viable cells were isolated in high yield, relatively free of other cell types, and did not release proteins or enzymes into the solution. More recently, the samples of PVP were highly toxic and caused cell disruption. The molecular weight distribution of PVP of around 25,000 was found to be satisfactory for the separation of cells but those with the molecular weight of less than 10,000 or around 60,000 were not. The toxic component in PVP could be removed by Dowex-1 or by boiling in hydrogen peroxide solution. Removal of the toxic substance again enabled cell preparation to be prepared which exhibited constant respiratory rates over periods of 3–4 hours. Isolated hepatic cells have shown respiratory rates of 30 to 60 microliters per hour and were still capable of anaerobically reducing tetrazolium salts intracellularly to formozan after four hours incubation. Hepatic cells maintained at 37° under culture conditions showed a "tree-like" organization attached to the bottom of the culture vessel. This organization did not occur at lower temperatures or at unphysiologic pH levels.

Tumor cells isolated from lymphosarcoma R2788 and hepatoma 3683 grew well on reinoculation into compatible hosts. Metastases to the lungs were noted with intraperitoneally injected cell suspensions but not with undispersed cells.

Close correlation between the activity of the enzyme lactic acid dehydrogenase (LDH) in the plasma and the growth of a variety of different types of mouse neoplasms has been found by Riley in animals bearing tumors which have been transplanted for many generations. However, animals with spontaneous tumors or transplanted tumors of more recent origin were not associated with markedly elevated plasma LDH activity. The plasma LDH was lost when the Moloney leukemia virus was passed through rats and then back into mice although the oncogenic properties were maintained. Diverse findings suggested that the transmissable agent was probably a virus of mouse origin being carried along by a number of transplanted tumors.

A study of the in vivo environment of the cells has been continued utilizing "tissue isolated tumors" (P. M. Gullino) with an examination of the in vivo production of connective tissue problems by rat and mice tumors. It has been observed that the ratio between total proteins and those of connective tissue was constant for each tumor over many transplant generations. Epithelial cells of Novikoff hepatoma grown in millipore chambers placed into the peritoneal cavity of the host were unable to form collagen but did produce an amount of connective tissue proteins characteristic of Novikoff hepatoma when grown "tissue isolated." Thus it was demonstrated that the connective tissue proteins must have been formed by the host. Transplantation of the same cell population into different strains of rats led to tumors with an amount of connective tissue protein which varied from one strain to another, whereas that

within a host strain was constant. It was possible to separate two lines of tumor producing cells from one hepatoma which differed two-fold in the connective tissue protein content. The results were interpreted as indicating that hepatoma cells "stimulated" production of connective tissue protein by the host and that the amount formed depended on the cell type of the hepatoma.

The interaction between tumor cells and host cells and the potentialities for growth differentiation of the free cells of the reticulo-endothelial system (lymphocytes, macrophages, polymorphs and mast cells) were examined in diffusion chambers implanted intraperitoneally into the rat. Pretreatment of the chambers by placing them empty into mice before adding the lymphocytes provided better growth and survival than if the cells were put directly into freshly prepared chambers. Lymphocytes exhibited better growth and morphological integrity if they were grown in chambers together with connective tissue. Thoracic duct lymphocytes have also been studied with these techniques.

Tumor cells from the rat and mouse have been separated from host cells by serial transplantation of the tumor cells from chamber to chamber. The connective tissue cells remained fixed to the millipore filter and the tumor cells could be scraped off and transplanted. Connective tissue was diluted out after three such transplantations. Novikoff hepatoma cells (low connective tissue formers) prepared in single cell suspensions by the trypsin-PVP-sucrose techniques were cleanly separated from all stroma cells by serial transplantation in the chambers. However, hepatoma 5123 and an ethionine-induced hepatoma, both of which produced dramatic connective tissue response, were much more resistant to the separation of connective tissue from tumor cells because of the limited growth potential of the tumor and the very intimate association between tumor islets and the connective tissue.

Normal C3H mouse connective tissue could be grown for longer periods of time in diffusion chambers implanted into C3H mice than in tissue culture. Thus fibroblasts which were removed from the chambers after 12 months growth and cultivated in vitro, became malignant after 3 months (or a total of 15 months of isolation from the mouse). Whereas the fibroblasts that were allowed to remain in the chambers were still normal after 18 months. After 23 months, however, the fibroblasts grown only in the chambers also produced tumors upon injection into C3H mice. It was of interest that transplantation of the tissue from chamber to chamber was not an inducing factor in this transformation. Tissue kept in the same chamber for 378 days produced tumors as rapidly and with the same incidence as tissues that were transplanted 11 times during the same interval.

Studies on the kinetics of catalase synthesis and destruction have indicated that the lowering of liver catalase by tumors is more nearly duplicated by a protein-free diet than by starvation. Hypophysectomized animals showed a level of liver catalase similar to that seen in starvation. Hypophysectomized animals bearing thyroid tumors exhibited a market decrease in liver catalase indicating that the effects of hypophysectomy and tumor growth were additive and that hypophysectomy did not block the effect of the tumor on liver catalase (M. Rechcigl, Jr.). Utilizing the specific inhibitors, 3-amino-1,2,4-triazole (AT) and allyliospropylacetamide (AIA), identical rates of catalase destruction in rat liver and kidney have been demonstrated although the rate of catalase synthesis in the liver was four times that of the kidney. Incorporation of Fe^{59} into liver catalase was almost completely blocked by AIA. Isolation studies however indicated that a small fraction of catalase was resistant to AIA. This small catalase pool turned over rapidly but so far has not been shown to differ from the main catalase fraction.

The finding that certain of the induced rat hepatomas had elevated levels of catalase activity was unexpected as all the tumors previously studied had very low activity. Furthermore, it had been postulated that the tumor itself produced a substance "toxohormone", which was capable of lowering catalase activity of the liver. Studies of the catalase in the Morris 5123 hepatoma have indicated that this tumor had a smaller rate of catalase synthesis and a greater rate of catalase destruction than the normal liver.

Catalase may well be a biochemical marker with which to follow the transformation and differentiation of tumor tissue. An ethionine induced rat hepatoma, arising in OM/N rats, has given rise to

two lines of hepatomas: one possessing extremely high catalase activity, and the other with low catalase activity. The low catalase line grow more rapidly than the high catalase line. These differences have persisted over ten transplant generations.

During the period of most rapid tumor growth and despite the onset of anemia, rats bearing lymphosarcoma R2788 gained appreciably more weight than normal controls with almost identical food consumption. Rats bearing hepatoma 3683, on the other hand, consumed less food and grew at equal or slower rates than the controls. Both types of tumors, however, exerted similar effects on the host with regards to the development of anemia, elevation of plasma aldolase and depression of liver catalase, etc. Carcass and tumor analysis showed that the lymphosarcoma bearing animals had sufficient water retention to explain their increased weight gain but an equally high degree of hydration was observed with the hepatoma bearing rats whose body weight gains were lower than those of the normal controls. Onset of anorexia and negative nitrogen balance was not prevented by an increase in the sodium chloride intake nor was there any apparent gain in weight, although the fluid consumption of both tumor groups had increased. In fact, a decreased survival time was observed among tumor bearing animals ingesting saline. In free choice experiments, neither tumor bearing rats nor control animals showed a preference for fluid containing sodium chloride.

LABORATORY OF BIOLOGY

The research program of the Laboratory of Biology presents a rather broad biological approach to the problem of cancer. Primary interest is in the etiology of cancer with pathogenesis and therapy through the biological approach also receiving some consideration. The host is given equal consideration to that of the etiologic agent and the developing neoplasm.

In a summary it is impossible to list all findings given in the individual reports, but the reviewer has attempted to select those which to him appear to illustrate best the scope of the program of the laboratory. Findings are grouped under six headings (carcinogenesis, immunology, virology, genetics, drug resistance, and pathogenesis), but in most cases the boundary lines are not clear and many projects fall within the scope of more than one discipline. Furthermore, the work of no investigator falls in one category alone. Many of the reported findings are the result of collaborative work between members of the laboratory, with scientists in other laboratories of the Institute, and with outside investigators.

Carcinogenesis

Carcinogenesis is considered in its broad biological sense by members of the laboratory. Work is directed not only toward further study of the effect of extrinsic chemical and physical carcinogens, but also toward further analysis of intrinsic factors involved and the nature of the carcinogenic process. Carcinogenesis is the central theme of the research program of the laboratory.

In continuation of his work on the role of the thymus in leukemogenesis, Dr. Law in collaboration with Dr. Bradley discovered that in thymectomized, irradiated C_{57}BL/Ka mice subcutaneous, isologous thymic grafts develop into lymphomas, whereas those transplanted under the kidney capsule do not. The grafts under the kidney capsule reconstitute and continue to grow but fail to show any of the morphologic changes characteristic of thymic tissue transforming to neoplasia. The mechanism of this inhibition is being studied further.

Dr. Potter has continued the study of induction of plasma cell tumors that at last provides a consistent experimental system for studying the pathogenesis of multiple myeloma. Mineral oil and mineral oil adjuvants, complete Freund's adjuvant and the adjuvant-*staphylococcus* mixture all injected interperitoneally are effective in inducing plasma cell tumors on BALB/c mice. Three injections of the oil alone results in the induction of this neoplasm in 60 percent of the mice. Such high frequency permits study of other aspects of the induction of this neoplasm including susceptibility of other strains, influence of genetic factors, role of antigen incorporated into the oil, and the effect of route of administration. This work has practical significance in that mineral oil and mineral oil adjuvants have been injected clinically into man. However, Drakeol-6VR, the oil approved

for this purpose, has thus far resulted in but few plasma cell neoplasms in mice.

Dr. Deringer has added further evidence that urethan, once thought to be limited to induction of lung tumors, is a multipotential carcinogen. A high percentage of hairless strain HR mice painted with urethan in ethylene glycol developed epidermoid carcinomas compared with only very few of those painted with ethylene glycol alone. Hepatomas and hemangioendotheliomas had been shown in this laboratory to be induced by urethan, and in other laboratories urethan was shown to augment the effect of a number of other agents in inducing leukemia.

Dr. Heston reported what may be a basic observation on the role of the hypophysis on induction of neoplasms. $(C_3H \times Y)F_1$ male mice hypophysectomized at one month of age were kept under observation until they were 16 months old—much longer than any hypophysectomized mice had previously, to his knowledge, been observed. At that time not one of these males showed any neoplasm, although normally 60 percent of them would have been expected to have hepatomas. This emphasizes the need for further studies of the necessity of normal growth processes in carcinogenesis.

There has been continued interest in the nature of the carcinogenic process or the neoplastic change in the cell, and one approach has been through dose-response studies. Earlier Heston and Schneiderman published a straight line pulmonary tumor response to graded doses of dibenzanthracene indicating a single event action which, if it were a gene change, should be considered as a dominant mutation. Left unexplained, however, was the fact that the extension of the line to zero dose fell below the point observed. Response data for these low doses have now been obtained and analyzed in collaboration with Mr. Mantel and Miss Gurian. They were found to be in accord with the earlier interpretation if it is assumed that each mouse has its own threshold for the carcinogen and its own characteristic sensitivity. The dose-response studies were extended to a soluble carcinogen, urethan, that also gave a straight line response. In addition, the study provided an adequate comparison of the carcinogenic activity of the two carcinogens, urethan, generally considered to be a potent carcinogen, was only one percent as active as the dibenzanthracene.

Immunology

Work of the laboratory in this area generally concerns the field presently called "immunogenetics", which covers an area of overlap between the two classical disciplines.

Dr. Barrett has continued to work with the Barrett-Deringer phenomenon of enhancement few years ago. The original observation was that the tumor grew better in the backcross generation after having been transplanted from the parent strain of origin into the F_1 that differed genetically from the parent strain. The effect does not hold for all tumors, however, for he reports failure of altered transplantability in a recently tested C_3H tumor.

Dr. Barrett reports further on the observation made in collaboration with Dr. Breyere of tolerance induced in female mice by parity. The effect has now been observed in three genetic systems with three transplanted tumors, and with skin homografts in two combinations. Tolerance can be seen when the materials are the same or different at the H–2 locus. In one of the tumor-host combinations the tumors in the tolerant female displayed an interesting growth pattern. After reaching a certain size the tumors became umbilicated in the center without necrosis until they appeared as doughnuts with no tumor grossly or microscopically visible at the inoculation site. This observation suggests a "local immunity".

Other observations reported by Barrett are that, in contradiction to the idea often held that transplantation antigens mature late and are not present in newborn mice, he was able to immunize suitable mice against the implantation of a tumor by prior inoculation of either embryos or placentas. He points out that this was really a repeat of an old and apparently forgotten experiment.

In collaboration with Miss Uphoff, Dr. Barrett has carried out experiments relative to the often held concept that transplantation immunity, once established, cannot be abrogated by irradiation. They used preimmunized mice, lethally irradi-

ated, and protected with bone marrow. Their results thus far are in contradiction to this generally held concept.

Miss Uphoff has continued her studies of the immunogenetic aspects of bone marrow protection against x-irradiation. Much of this work is in the area of irradiation protection, but certain aspects are directly concerned with neoplasia. She reports further observations of the histocompatibility patterns of lymphomas arising in irradiation induced chimeras of AKR mice receiving bone marrow from other strains. The lymphomas arising in these mice are primarily of donor tissue origin. Some were of host origin but they may have arisen from leukemic cells that were not destroyed by the radiation. Some appeared to be of both donor and host origin.

Part of Dr. Potter's work on plasma cell tumors falls in the field of immunology. Since normal plasma cells are the source of humoral antibody the question arises as to whether neoplastic plasma cells can make antibody. He has transplanted plasma cell tumors from hosts which have in their circulation good titres of anti-ovalbumin antibody, but to date no precipitating antibody production has been adoptively transferred through the transplants.

Virology

Members of the Laboratory of Biology continue to show interest in the viral etiology of cancer. The mammary tumor virus that has been studied for many years in this laboratory continues to demand considerable attention. Dr. Andervont now has four sublines of strain RIII from which the mammary tumor virus has disappeared or in which it has become inactive with the normal route of transmission. He is now reporting on experiments designed to determine why the virus disappears from certain RIII mice but not from C_3H mice. Some of the results indicate that the viruses differ either quantitatively or qualitatively. The RIII virus consistently gives a lower tumor incidence in C_3H mice than does the C_3H virus in C_3H mice. Other results indicate that the mouse strains likewise differ. It appears that the C_3H virus may also occasionally disappear when placed in RIII mice.

Doctors Deringer and Heston continue to study the development of mammary tumors in lines of mice deprived of the mammary tumor virus. Dr. Deringer has developed her agent-free lines by transfer of fertilized ova. She reports that her DBA/2eB line without the virus has very few tumors in virgin females, but the incidence can be increased by breeding and even more increased by force-breeding. However, in each case the incidence is lower than in the comparable C_3HeB group. She has also started an RIIIeB strain without the virus. Dr. Heston has developed his agent-free lines by foster nursing following Cesarean birth. In a newly established Af line no mammary tumors have been observed to date. In these agent-free lines the effect of the hormonal stimulation and of the genes becomes more apparent in the etiology of mammary tumors, for greater differences in tumor incidence caused by these factors can be observed.

Dr. Law in collaboration with Dr. Moloney has been studying congenital transfer of the Moloney and the Gross leukemia viruses. Transfer is through the maternal line with the milk providing an efficient means of transfer. They have been unable to demonstrate transfer through the paternal line. This is in sharp contrast with the usual leukemias of the high leukemic strains such as AKR and C_{58}, which are transsmitted through the male as readily as through the female. A reappraisal of the question of whether or not viruses can be considered to be involved in the etiology of the usual leukemias of these high leukemic strains is, therefore, indicated.

Relative to their discovery of histocompatibility tolerance induced by parity, Drs. Barrett and Breyere in collaboration with Dr. Moloney, have found that this tolerance may decrease the latent period of the Moloney virus.

In continuation of their studies of an attenuated strain of the polyoma virus grown in a milk medium, Drs. Law and Rabson have observed readaptation in a serum medium resulting in a return of the oncogenic properties, although in the milk medium the virus has remained attenuated. Some success has been achieved in immunizing C_3Hf/Bi mice against the potent oncogenic strain of polyoma virus with the attenuated strain.

Mr. Melroy has continued to operate his laboratory for testing the stocks of mice in Biology and

Viral Oncology for polyoma virus. Sixty-one percent of the tests have been for the Laboratory of Biology and 39 percent for Viral Oncology. Fortunately most of our colonies of inbred mice are free of the virus.

Dr. Heston and Mr. Vlahakis, in collaborative work with Dr. Moloney reported that in seven transplanted tumors carried for years tests for oncogenic viruses have for the most part been negative. This suggests that despite the fact that the Moloney virus was isolated from a well known transplantable tumor, oncogenic agents cannot be demonstrated by present techniques in most of our transplantable tumors.

Genetics

Probably the most basic of all research in the laboratory is that in genetics. The geneticist is concerned with the information passed on to the individual at the time of conception and the manner in which this information directs developmental processes and acts in conjunction with other factors to determine whether or not a neoplasm will occur. He is interested in the genetic information of the cell characterizing the neoplasm. All work of the laboratory is related in some way to these problems.

We must continually be characterizing new strains of mice. Dr. Law is characterizing strain C_3Hf/Fg in which he has observed a leukemia incidence of 80 percent in the females and 50 percent in the males. This is principally lymphocytic leukemia. Dr. Deringer has further characterized strain BL in describing amyloid in all animals of the strain at 15 months of age. Dr. Andervont has reported the occurrence of tumors in his colony of wild mice. It is significant that of a total of 225 mice, 43.5 percent had developed spontaneous neoplasms. Lung tumors occurred most frequently, with reticulumcell neoplasms next. Of particular interest in respect to the virus etiology of cancer was the fact that while the colony is known to harbor the mammary tumor virus, only 6 percent of the breeding females and none of the virgin females developed mammary tumors.

Dr. Law has made a genetic analysis of the difference between strain $C_{57}BL/KaB$, none of which develop thymic or parotid neoplasms when injected with a thymotropic polyoma virus strain, and strain C_3Hf/Bi, 100 percent of which develop such neoplasms when injected with the virus. Results of hybridization studies suggest that the resistance to the oncogenic effects of this virus is due to a single dominant gene.

Doctors Heston and Deringer are approaching the problem of the genetics of cancer in a more definitive manner by identifying effects of specific genes on the occurrence of specific neoplasms. To date 8 different genes on 6 different chromosomes have been shown either to increase or inhibit the occurrence of lung tumors. The lethal yellow gene is most interesting because it can be analyzed in an isogenic system. Heston showed that this gene not only increased lung tumors, but also hepatomas and mammary tumors. These observations have now been confirmed by Deringer, using different strains. There is a correlation between the effect of the gene on lung tumors and its effect on normal growth, and elimination of the effect of the gene on normal growth by food restriction, or injection of goldthioglucose likewise eliminated its effect on occurrence of the tumors.

Dr. Potter is laying some ground work for somatic cell genetics in his plasma cell tumors. Using immunoelectrophoresis and double diffusion precipitin methods and straight agar gel electrophoresis he has identified over 20 plasma cell types producing different proteins. It may be possible to use these different proteins as genetic markers in studying gene action in the origin and nature of the neoplasm.

Transformation of leukemic cells by DNA preparations was reported by Dr. Bradley and Dr. Law. The character chosen was 8-azaguanine-resistance in the P–388 lymphocytic neoplasm. P–388 cells incubated *in vitro* with DNA prepared from cells with a high level of resistance, regularly developed clonal, resistant colonies at a frequency nearly 40 times that observed in control cultures. Further studies by Bradley concerning antagonism by DNA extracts from other sources and substitution of adenine for DNA extracts points up the danger of interpreting this type of experiment without the further investigation of the true nature of the change.

Drug Resistance

Dr Anderson has continued her work on biochemical differences in the metabolism of azaserine-sensitive and -resistant lines of the plasma cell neoplasm 70429. Duazomycin that inhibited growth of sensitive cells to 3 percent of that of controls compared with inhibition by azaserine to 16 percent of controls, was tested against the azaserine-resistant tumor line to look for cross-resistance to duazomycin. Marked cross-resistance was observed. While collaborating with Dr. Wallace Brockman in further exploration of the biochemical effects of the three inhibitors, azaserine, duazomycin, and DON, in the sensitive and resistant lines of 70429, she exposed growing tumor cells to radioactive formate *in vivo* following pre-exposure to a single dose of each inhibitor and determined incorporation of the tracer in the nucleic acids. Each of the three compounds inhibited the incorporation of the tracer.

Dr. Anderson has studied uracil anabolism in fluorouracil- and fluorouridine-resistant lines of mast-cell neoplasm P815, and has observed the following pathway:

$$\text{uracil} \overset{1}{=} \text{uridine} \overset{2}{-} 5'\text{-uridylic acid}$$

Fluorouracil is apparently anabolized by the same path in mammalian cells. Step 2 is catalyzed by uridine kinase, and marked decrease in activity of this enzyme is associated with resistance.

In collaborative studies with Drs. Brockman and Davidson, Dr. Law has observed deletion of enzyme (pyrophosphorylase) activity in anti-purine-resistant cell lines. All of these observations will probably tie in with somatic cell genetics.

LABORATORY OF CHEMICAL PHARMACOLOGY

Research in the Laboratory of Chemical Pharmacology continues to be progressively re-oriented toward what is currently designated as "Molecular Biology." The emphasis is on the understanding of biological problems in terms of the interactions of the biological systems with chemical agents. The latter include compounds of low-molecular weight and of uniform composition, and polymers of high -molecular weight. The macromolecules under study are those isolated from living systems, and synthetic ones prepared to serve as models of the former. In the context antigens and antibodies are considered to be merely macromolecules with specialized biological properties in consequence of their physico-chemical characteristics.

The several lines of work are not rigidly walled-off from each other as unconnected academic enterprises. They rather are interconnected in many ways despite the fact that different academic disciplines are the different tools employed. For example, increasing attention is being given to *Viruses:* Dr. Mora's group is studying the transformation *in vitro* of normal cells to malignant as accomplished by viruses which elicit cancer in mammalian species; Dr. Landy's group, in investigating the question of the presence in cancer tissue of antigens not found in the normal tissues, has as one working hypothesis that such antigens may not be endogenous in the tumor cells but rather may reflect the presence of viruses; and Dr. Goldin's group, in laying the basis for chemotherapy of virus-induced tumors in laboratory animals, has found it necessary to add an additional target, viz., anti-viral chemotherapy.

Another example is the follow-up on the pioneering work of Dr. Shear with bacterial polysaccharides which include hemorrhagic-necrosis in tumors of animals and patients. Drs. Oroszlan and Mora have made progress in the further separation of the active fraction, from other components, in the tumor-necrotizing polysaccharide material previously prepared by Perrault and Shear; a 4-fold increase in potency was obtained, and this more-concentrated agent makes possible more sophisticated investigation of its chemical properties and of the mechanisms of its biological properties. Work in the Immunology Section led to the discovery of a component in normal blood serum and in liver cells which inactivates such endotoxic polysaccharides; in an extension of these findings it was found in the Macromolecular Chemistry Section that the inactivation with an extract from liver cells was a conjugation of the anionic polysaccharide with a cationic protein and that this inactivation could be reversed with either a more strongly acidic or basic polymer. Dr. Landy, in collaboration with Dr. Edgar Ribi of the Rocky Mountain Laboratory of the NIH in Hamilton.

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Montana, has made a series of important contributions to the currently controversial subject of the chemical features of endotoxins which are responsible for their biologic properties. Dr. O'Malley has been investigating the mechanisms through which such bacterial polysaccharides damage solid tumor tissue; he has been throwing useful new light on the responses of the blood vesels in the tumors and on the appearance of a hitherto unknown substance in the blood of mice which had received a single injection of the active polysaccharide from *S. marcescens.*

The basic science aspects of research in chemotherapy, conducted by Dr. Goldin and his associates, have continued to be fruitful both in the intramural Biochemical Pharmacology Section and in the contract operation with Microbiological Associates, Inc. Dr. Goldin dovetails his chemotherapy studies with clinical objectives and works in intimate relationship with the clinical investigators of the NCI.

The results obtained in the three Sections of this Laboratory may be summarized as follows:

Immunology Section

Under the leadership of Dr. Landy, various lines of work were prosecuted with gratifying success. Collaborative investigations were also carried out with men in other institutions both here and abroad.

The Tumor Antigen Problem

In the face of all the negative results over half a century, newer work carried out in various parts of the world has re-opened this question. Bjorklund (Stockholm) had provided us with a supply of anti-serum obtained from the horse after prolonged immunization with pooled human carcinoma tissues. This was absorbed here, exhaustively, with pooled normal human tissues to remove antibodies against normal tissues. This treatment removed anti-human reactivity without appreciable loss of cytotoxicity for Hela cells or of capacity to agglutinate erythrocytes coated with Hela cell extracts.

Thereupon, horses of our own were immunized, in weekly injections for more than a year, with suspensions of fresh Hela cells; for control, immunization was also carried out with pooled suspensions of normal human cells (kidney, liver, and spleen). Both anti-sera were found toxic for Hela cells. While absorption with normal human tissue materials removed this potency from the antiserum to normal human tissue it left it essentially unaltered in the anti-Hela serum. These antisera, when tested against tanned erythrocytes coated with extracts of Hela cells, yielded analogous results.

Role of the Antigen in Immonologic Tolerance

Immounological tolerance is elicited more readily in neonatal animals than in adults. The greater the retention of antigen in neonatal chicks, the greater the degree of immonologic tolerance (previous work of Dr. Hirata). In the newer work S^{35}-labelled Bovine Serum Albumin (BSA) was injected into neonatal and older chicks. The uptake by spleen was greater in the neonatal. He found that the antigen, incorporated in liver, disappeared at similar rates in the two age groups whereas the rate of disappearance from spleen differed for the two groups, indicating that the material retained in the spleen may be qualitatively different in neonatal chicks than in older ones.

Endotoxin

Various aspects of endotoxins, and of their biological effects, have been investigated.

It is generally believed that immunity in the classic sense plays no part in *tolerance to endotoxin* because of the non-specificity of its elicitation. However, earlier work of Dr. Landy and his associates showed that natural antibodies to Gram-negative bacteria are widely distributed in mammalian species, and that administration of any endotoxin leads to a prompt elevation of these specific antibodies. Hence the previous concept of non-specificity was open to question. Intravenous injection in rabbits of colloidal RES blockading agents, in doses which promptly abolish tolerance, were found to cause an equally prompt and precipitous fall in circulating antibodies to endotoxins. Serum from endotoxin-tolerant or normal rabbits, after incubation *in vitro* with such "blockading" agents, showed a decrease in antibody content. In the past it had been believed that these colloidal materials exerted their effects primarily on phagocytic cells of

the RES. These newer findings suggest that they may operate through reducing the level of circulating antibodies in the blood and thus interfere with the clearance of endotoxin. This line of work provides data for an immunological basis for tolerance to endotoxin.

Work in the Immunology Section on endogenous agents in mammals capable of inactivating endotoxin was brought to an appropriate stopping point. Of the organs of the perfused rabbit, only the liver yielded extracts which consistently displayed high endotoxin-inactivating potency. This work, conducted in collaboration with Dr. V. Waravdekar of the AFIP, showed that the intracellular agent obtained from liver resembled in its properties the one (EDC) in blood except that it was not demonstrably affected by divalent cation.

In collaboration with Dr. E. Ribi of the Montana Laboratory of the NIH, excellent progress was made in removing from an endotoxin (*S. enteriditis*) associated materials (lipoidal and nitrogenous) which do not contribute to endotoxic properties: lipoidal content was brought down from about 6 percent to below detectable levels, and the nitrogen content from about 6 to approximately 0.5 percent. The final product, a high-molecular weight polysaccharide, contained only small amounts of fatty acids and phosphorus. It is noteworthy that the analytical values for this highly potent purified endotoxin were virtually the same as for the low-molecular weight haptene which is devoid of endotoxic properties. Since differences in chemical composition were not found between endotoxin and haptene which could account for the striking difference in their biological behavior, it is surmised that physico-chemical features of these complexes may turn out to play the critical role.

It was visualized that host reactivity could be dependent on the macromolecular properties of endotoxin. A critically large size of the complex might be essential, and the loss of potency which we found to occur within minutes after treatment with dilute acetic acid might result from a simple depolymerization into particles of the size of haptene.

This line of work on *correlation between structure and biological properties of endotoxin* did not support a widely-publicized theory that the lipid content was responsible for the host responses. When differences in chemical composition were ruled out, experiments were designed to test the physico-chemical hypothesis.

The endotoxin preparation was boiled with 0.1 N acetic acid, which rapidly and progressively reduced endotoxic activity. Samples of the hydrolytic products were removed at intervals and examined in gel diffusion (Ouchterlong) tests and in the analytical ultracentrifuge, and also assayed for biological potency. The starting endotoxin behaved as a single entity in the ultracentrifuge. As hydrolysis progresses, a second, much slower moving boundary appeared and, as the fast moving endotoxin boundary decreased, the area under the slow moving boundary, subsequently shown to be haptene, increased correspondingly. The gel diffusion tests likewise provided evidence of a progressive increase in the concentration of haptene during the exposure of endotoxin to acid.

The findings obtained with these parameters were strikingly consistent and revealed that the rate at which host reactive properties were abolished paralleled the rate of dissociation of the complex into haptenic units, particles whose size was of the order of 1/100 that of the original endotoxin.

Potent endotoxin can exist in aggregates ranging all the way from 190 Svedberg units down to about 10 S; within this spectrum there has not yet been established a correlation between size and potency. Likewise, the size of the aggregate in the original endotoxin does not appear to affect the rate of destruction of potency nor the dissociation of endotoxin into uniform particles of the size of haptene. These findings give no indication of the formation of particles of *intermediate* size during the reduction of endotoxin to haptene. Rather, it would seem that cleavage of endotoxin by acid yields haptene directly, i.e., dissociation of an aggregate into its primary component particles. These findings suggest that the major requirement for endotoxin to elicit the spectrum of host reactions may well be a macromolecular complex of critical size.

Further work, in collaboration with Dr. Mark Woods, was carried out on *the effects of endotoxin on the metabolism of normal mammalian cells.* The earlier findings on tumor cells were extended to include an array of normal mammalian cells.

Similar stimulation of glycolytic activity by endotoxin was demonstrated on peritoneal exudate and bone marrow of the rabbit, granulocytes and lymphocytes of man, and peritoneal macrophages, kidney and spleen cells of the mouse. Special attention was focused on the peritoneal macrophages of mice inasmuch as these are the phagocytic cells directly involved in the resistance of this species to peritoneally induced experimental infections; endotoxin is known to increase resistance to these infections.

Direct exposure *in vitro* of various cells to high concentrations (50 μg or more/ml) of the most potent endotoxins resulted in enhanced glycolysis, but no evident injury to the cells. This was not always the case *in vivo*. In mice given endotoxin, biphasic responses were clearly apparent in peritoneal macrophages and spleen cells. The level of aerobic glycolysis displayed by these cells after harvest continued at a constant rate. The cells removed from the host did not pass through the progressive changes in activity which are found at various times *in vivo*. With small doses of endotoxin only stimulation occurred, but with higher doses there was an initial period of depression followed by a period of stimulation, which with large doses lasted for as long as 8 days. The stimulation by endotoxin of glycolytic activity of macrophages and spleen cells *in vitro* was well correlated with the enhanced glycolytic activity of these cells harvested from mice that had been injected with a small dose of endotoxin. In parallel metabolic and infection studies, the glycolytic behavior *in vitro* of mouse peritoneal macrophages was correlated with their phagocytic activity *in vivo*.

Natural Antibodies

The intriguing nature of natural antibodies was illuminated by several lines of study, in which various workers collaborated. One study dealt with *the increase in natural antibodies after administration of endotoxin*. The annual report for last year described the bactericidal method which was then employed to explore the humoral changes in mice and rabbits given endotoxin. It was found that following the administration of endotoxin to mice, the bactericidal activity of their serum for *S. typhosa*, *E. coli*, and *Sh. dysenteriae* was consistently raised to an activity 1½ to 4 times that found in normal mouse serum. Just as had been reported previously in our studies on the increase in resistance brought about by endotoxins, many factors were found to influence the onset, magnitude and duration of these changes. Among these factors were the properties of the endotoxin itself, the dose, the strain of mice, the interval between injection of endotoxin and withdrawal of serum samples and the antigenic relationship of the endotoxin to the challenge culture. Three endotoxins from different sources and with individual immunological specificities were effective but varied considerably as regards the quantity required to increase the levels of antibody against unrelated *Enterobacteriacea*. It was found repeatedly that, with one hour, the intravenous administration of endotoxin produced a substantial increase in antibody levels against immunologically unrelated Gram-negative species. The magnitude of this elevation and the duration of the enhanced antibody levels appeared to be related to the dose of endotoxin. For example, the augmented levels following 10–50 μg persisted for 72 to 96 hours, whereas those for 1 and 0.1 μg lasted about 24 and 6 hours, respectively. It is significant that, following subsisdence of the endotoxin effect, there was a return to the original levels of antibody. It was found that, within one hour after a large dose of endotoxin (50 μg), bactericidal activity for the homologous strain fell, presumably as a result of complexing of endotoxin with homologous antibody in the host. During this period bactericidal activity for heterologous strains was increased markedly, indicating that the response involved specific antibodies. Further evidence for specificity was provided by absorption and inhibition tests with endotoxins of differing antigenic specificity.

Thus, following endotoxin administration there occurred a general release of substances active against *Enterobacteriaceae* which show the specific activity characteristic of antibodies. These alterations in the level of antibody were produced with endotoxin in amounts known to modify resistance of mice to experimental infection. It is of interest that the magnitude and duration of this serum effect was dose dependent in a manner analogous to the changes in "properdin" levels and resistance such as had been reported from this Section previously.

The capacity to opsonize organisms for phagocytosis is a distinctive property of specific antibody. Since these antibodies effectively prepare bacteria for the lethal action of complement, it is a reasonable assumption that they can also function as opsonins. Although no direct correlation has been established, these changes are likely to be involved in determining the outcome of infection since they affect (a) the very cells which are concerned in host defense against intraperitoneal challege and (b) a humoral factor which is known to increase the phagocytic efficiency of these cells.

Exploratory experiments were performed to determine whether the administration of endotoxin affected the levels in serum of natural antibodies other than those directed against Gram-negative bacteria. Microgram amounts of endotoxin produced, in rabbits, a rapid increase in the level of natural antibodies against various *Enterobacteriae*, in a manner even more pronounced than that observed in mice. There also occurred, however, analogous increases in levels of natural antibodies to foreign erythrocytes and to mouse tumor cells. These findings suggest that the effect of endotoxin may well involve a variety of situations where the host is naturally and continually subjected to antigenic stimulation.

There has developed an increasing awareness that the characteristic reactivity of mammals to endotoxin may be a consequence of the continued presence of Gram-negative bacteria in the intestinal tract from shortly after birth. To determine whether the intestinal flora contribute to endotoxin hyperreactivity, *germ-free* and conventionally reared mice of similar genetic stock, maintained on the same sterilized diet, were compared as regards their reactions to bacterial endotoxin. No significant difference was found as regards the following: elevation in the levels of antibody to Gram-negative bacteria; stimulation of metabolic activity of spleen cells and of peritoneal macrophages; susceptibility to infection with *S. typhosa;* induction by endotoxin of increased resistance to infection; and LD_{50} of endotoxin. Thus the presence or absence of a bacterial flora does not appear to control the susceptibility of mice to endotoxin.

A study was made of the origin, occurrence, and properties of *natural antibodies to Gram-negative bacteria in the* normal serum of *several animal species.* Antibody was measured by a procedure described in the preceding annual report, based on the bactericidal reaction carried out under conditions in which activity was a function of the amount of antibody contributed by the test serum.

Antibodies to representative test strains of *Salmonella*, *Shigella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Serratia*, and *Aerobacter* were demonstrated in normal human serum. These antibodies were also found to be widely distributed in the sera of various experimental animals. Absorption with homologous bacteria and inhibition of bactericidal activity with purified homologous somatic antigen affirmed the specific nature of these antibodies. Absorption with graded amounts of bacterial suspensions showed that a large excess of bacteria led to nonspecific removal of antibodies. Analogous findings were also made with immune antibody. These observations help to explain how earlier workers arrived at the concept of nonspecificity of natural antibodies reactive with *Enterobacteriaceae.*

As determined by quantitative absorption tests no difference could be found in the avidity of natural and immune antibody. Natural antibodies of various species were found to be significantly less heat stable than immune antibodies. It has been suggested that natural antibody is more heat labile than immune because it is present in low concentration. However, no differences were detected between the heat liability of diluted and undiluted immune serum added to saline or to absorbed normal serum.

The time of appearance of antibodies to *S. typhosa* and *E. coli* varied in young animals of different species. Mice developed these antibodies at the earliest age, with guinea pigs, rats, and rabbits following in that order.

These findings attest to the multiplicity of natural antibodies in serum. The ubiquity of Gram-negative bacteria makes it inevitable that animals may be exposed to them in ways ranging from frank or overt infection, commensal status, or the simple process of continued ingestion of dead bacteria. It seems unlikely, however, that the smaller animals, in their comparatively short life span, could be exposed to the entire range of Gram-negative species. It is more likely that

this category of antigenic determinants is more widely distributed than the *Enterobacteriaceae* and can be derived from non-bacterial sources.

Animals reared in a bacteria-free environment lack the antigenic contribution normally derived from living intestinal flora. The level of gamma globulin in such animals is known to be reduced, but is nonetheless present in significant amounts. If *living* bacteria are important stimulator of antibody production to Gram-negative species, germ-free animals should have significantly reduced levels.

Accordingly, *serum from adult germ-free mice*, rats, chickens, rabbits and swine were *examined for antibody* to *E. coli* and *S. typhosa*. Animals of comparable age and genetic stock, maintained on the same sterilized diet, but exposed naturally to bacterial contamination, served as controls. Periodic checks indicated that these conventional controls consistently harbor various bacterial species including Gram-negative serotypes. It was found that, as measured by the bactericidal test, the levels of antibody in germ-free mice was similar to that found in control mice. On the other hand, no detectable antibody against *E. coli* or *S. typhosa* was found in sera of germ-free rats, chickens, rabbits, and swine. In conventional controls, however, these antibodies were present in substantial concentrations. It is known that immature animals of different species vary considerably in their capacity for absorption of intact protein from the intestine. If natural antibodies do in fact arise as a consequence of antigens absorbed from the digestive system, this species difference may have special significance.

Experiments were conducted to obtain information on the stimulation of antibody production by antigenic material in the food, particularly killed bacteria. Adult germ-free rats, without demonstrable antibody to *E. coli* and *S. typhosa*, were fed the usual autoclaved diet in which heat-killed *S. typhosa* had been incorporated to the extent of 0.1% on a dry weight basis. Another group of Germ-free rats was given a single injection i.p. of 100 μg typhoid endotoxin. Serum specimens for antibody measurement were collected at various intervals. Little or none of these antibodies was found in the serum of these rats during 30 days of ingestion of the killed typhoid bacilli. In the rats that received a single injection of endotoxin, antibody to *S. typhosa* was detectable 1 day later and reached high levels within 4 days.

Immunological distinctions between normal and neoplastic tissues may represent the most subtle of the various ways in which they differ. Greater understanding of such differences would enhance the likelihood of development of more effective measures of control. Information on the nature of the host's agencies, both humoral and cellular, which participate in either natural or induced immunity, and the manner in which they participate, is essential for this understanding.

Macromolecular Chemistry Section

Fundamental studies, under the leadership of Dr. P. T. Mora, were carried out on macromolecular interactions in biologic processes, e.g., enzyme-substrate and antigen-antibody interactions, viral infection of cells, etc. Such interactions were profoundly altered, or prevented altogether, by polyelectrolytes both anionic and cationic.

Synthesis and Structure

To the previously accomplished synthesis of anionic polysaccharides, there has been added the *synthesis of cationic polysaccharides*. Basic derivatives of the synthetic polyglucoses were prepared with a very high degree of substitution of cationic groups. It is possible to introduce up to 1.6 catonic groups per anhydroglucose unit and thus synthesize, without degradation of the polysaccharide, a very strongly cationic derivative. The best method was the reaction of 1-diethylamino-2, 3-epoxypropane with polyglucose in aqueous sodium carbonate, when the ether derivative formed with hydroxyls of the polysaccharide. The derivative is a tertiary amine. It was then easy to convert this tertiary amine derivative to the corresponding quaternary ammonium derivative which is even a stronger base.

Work has been carried out aimed at the understanding of the *structures of synthetic polysaccharides, polypeptides, and proteins*. The details of the molecular structure are being studied by an able group of polysaccharide chemists under

Prof. G. G. S. Dutton at the Chemistry Department, University of British Columbia, Vancouver, supported by NIH Grant No. RG7652C1. We are helping these studies by providing samples of polysaccharides (polyglucose, polyxylose, polygalactose and polyarabinose up to this time) and technical information from our earlier synthetic work and from our macromolecular characterization studies. The knowledge of the fine structure will help in correlating structure and immunilogical properties, since these synthetic polysaccharides, when the molecular weight is above about 200,000 possess antigenic properties.

Certain structural problems of proteins were approached by Dr. S. Shifrin who was interested in checking the claim that the α helix configuration of the polypeptide chains, and interaction of tyrosin and tryptophane residues, causes the observed fluorescence of proteins. For models he chose synthetic polypeptides which, from optical rotatory dispersion studies, are said to be 100% in the α helix form in dimethylformamide solution (Sela). When he compared absorption and fluorescent properties of the aromatic polyamino acids poly-L-tryptophane, poly-DL-tryptophane, poly-L-tyrosine and poly-L-phenylalanine with the corresponding N-acetyl derivatives, he could not find any spectroscopic effect of adjacent aromatic residues apart from the fluorescence quenching expected from concentration quenching. This indicates that side group interactions such as interactions of tryptophane residues cannot be used to explain protein fluorescence. In general a more careful reinvestigation of the α helix in protein and polypeptide structure is in order, since the optical rotatory dispersion was used until now somewhat indiscriminately to infer helical structure. Actually, similar optical rotatory dispersion can result from changes in the solvent structure around the protein, and more careful studies with fluorescence and other independent methods would be helpful to provide fundamental information on the secondary structure of polypeptides and proteins.

Furthermore, fluorescence is an extremely sensitive method of studying molecular interactions, for example enzyme co-enzyme and substrate interaction. It is with this in mind that Dr. Shifrin is constructing a sensitive spectrophotofluorometer, and we expect that such an instrument would be of great use for many diverse studies on macromolecular interaction in this Laboratory.

In another approach to protein and enzyme structure he tried to develop a specific and sensitive fluorescent reagent for combination with the sulfhydryl groups of proteins and thus develop information on the position of these groups in the protein chain. The sulfhydryl groups in proteins are important because they are very reactive and, if they are tied up, the enzyme activity is usually lost; thus they probably relate to the active center of the enzymes. Also they form S-S cross links and stabilize the tertiary structure of proteins. Dr. Shifrin condensed the highly fluorescent N, N-dimethylamino-naphthaline-5-sulfonyl chloride with the hydrazide of isomaleimide, the latter chemical being a specific reagent which in turn condenses with sulfhydryl groups. However, because the condensation products of the two organic molecules turned out to be unstable, this approach was discontinued.

Enzyme Inhibtion With Polyelectrolytes

In continuing the lead of our earlier work on the general phenomena of *reversible inhibition of enzymes* with oppositely charged polyelectrolytes, the inhibition of the cationic protein ribonuclease with the anionic polyelectrolyte polyglucose sulfate was chosen for detailed examination of pH and salt dependence. The inhibition of RNase was much more effective at lower pH than at higher, when measured at pH values 6, 7 and 8, and also in lower salt concentration at any of these pH values. This is in line with an explanation that electrostatic forces hold together the enzyme and the inhibitor polyelectrolyte in a complex, and that such forces are the most important causes of this type of enzyme inhibition: at lower pH the RNase is more cationic, and the charges of the macromolecules are less shielded in low ionic media.

Advice and samples of polyglucose sulfates and of polyglucose carboxyl derivatives have been provided to scores of biochemists who are now regularly using these polyanions to inhibit various catonic enzymes (RNase, DNase, trypsin, cytochrom C-oxidase, etc.), following our original publications.

Inactivation of S. marcescens Polysaccharide

In following up earlier leads, obtained in other parts of this Laboratory, on detoxification of endotoxic polysaccharides with a system in normal blood and a subcellular fraction from liver, a component was leached out of normal liver cells by Dr. Oroszlan of higher potency inactivating the tumor-necrotizing capability of *S. marcescens* polysaccharide. In the fractionation studies he noticed that inactivating potency increased as the cationic property of the protein fraction increased. It became evident that the endotoxic polysaccharide, which is an anionic macromolecule, can be inactivated with various kinds of cationic macromolecules including our synthetic polyglucose amines. Furthermore, such inactivation can be reversed by adding a more strongly anionic polymer than the bacterial polysaccharide (for example by adding polyglucose sulfate); the bacterial endotoxin then again displays tumor-necrotizing activity. This indicated that the mechanism of inactivation of the tumor necrotizing potency by these proteins from liver cells was through interaction of oppositely charged macromolecules rather than through an enzymatic process. Dr. Orozlan has also been studying the macromolecular properties of the endotoxic polysaccharide preparation from *S. marcescens*, with the purpose of separating the most active component from the relatively inert components still present.

Bacteriophage

The concept of inhibition of various kinds of biological activity by means of complexing the active polymers with oppositely charged polyelectrolytes was extended to the demonstration of *reversible blocking of an antibody.* In the antiserum against T2 bacteriophage the antibody activity can be blocked with a strongly charged polyelectrolyte (for example, with the anionic polyglucose sulfate) but the antibody recovers its activity if there is added to this system an oppositely charged polyelectrolyte to that employed first (for example, the cationic spermidine). Highest reactivation occurs when the second polyelectrolyte is added in stoichiometrically equivalent amount to the first polyelectrolyte, just sufficient to neutralize the charges. Thus it was possible to inactivate *and* to reactivate the T2 bacteriophage antiserum in a predictable and controlled way. Our explanation for the inactivation of the antibody was that the strong polyelectrolyte added first (anionic polyelectrolytes are generally more effective, but cationic polyelectrolytes also show this effect) complexed with the amphoteric antibody protein; in the complex the antibody was unable to combine effectively with the virus. When a second strong polyelectrolyte, oppositely charged to the first polyelectrolyte, was added, a preferential complex formed between these two, and the liberated antibody was now free to combine with and neutralize the virus. Analogous research on better defined systems (using isolated antibody and antigen) would undoubtedly lead to better understanding of the forces which bring about the antibody-antigen interaction. Also, our results suggest new methods for the fractionation of the antibody from the antiserum with the help of polyelectrolytes. Finally, it gave some leads on how to influence (block and reactivate in a controlled way) antiserum activity against a virus *in vitro.*

Earlier work in this Section had shown that incubation of T2 phage with anionic polyelectrolytes abolished the ability of the phage to reproduce in *E. coli* cells without destroying phage structure. Dr. Rizvi gained further insight into this process. He developed new methods to inactivate high concentrations of T2 bacteriophage (10^{12}phage/ml) with polyglucose sulfate (1 mg/ml) without any concomittant release of T2 DNA from inside the protein coat. He achieved this by using properly selected buffers with the polyglucose sulfate; thus the phage was not exposed to local extremes of pH. Dr. Rizvi currently is extending the polyanion inhibition of the T2 bacteriophage into the whole T series of bacteriophages from T1 to T7. on a large scale, with special attention to the T4 O_1 mutant which has a protein coat permeable to low molecular compounds and can be prepared without the internal cations putrescine or spermidine. The cations putrescine and spermidine, found recently by B. Ames of NIAMD to be present inside of the protein coat of certain of the bacteriophages, have an important role in neutralizing the anionic charges of the DNA.

Our polyanion treatment undoubtedly causes some increase to the permeability of the protein coat. Dr. Rizvi observed that the percent in-

activation of bacteriophage viability after the polyanion treatment is the same as the percent loss of the internal low molecular weight cations putrescine and spermidine, released by this treatment through the protein coat from inside the phage. Polyglucose sulfate apparently competes with the DNA and preferentially complexes with these cations. The loss of the cations must disturb the original structure of the DNA in the head of the bacteriophage; with the permeability change of the protein coat, this appears to be the main cause of the loss of viral infectivity.

Virus Inactivation With Polyelectrolytes

The cationic polyglucose derivatives prepared in this Laboratory were examined by Prof. A. Di Marco in Milan, Italy, for their effect on influenza virus (strain A2/W29). In very low concentrations these polymers inhibited the hemagglutinating action of the virus; they also reduced somewhat the mortality of mice when infected intranasally with this virus previously incubated with the cationic polyglucose amines. Polyglucose amines themselves showed hemoagglutinant action at higher concentration: this action decreased when such solutions were kept incubated at 37° C with chorio-allantoic membrane. Apparently this hemoagglutinant action of the polyglucose amines were exerted at sites different from those which the influenza virus uses to agglutinate red cells, because when erythrocytes were incubated first with influenza virus and the virus was eluted, then these erythrocytes still agglutinated with the polyglucose amines.

Cationic polyelectrolytes also inhibit the bacteriophages. Our work showed that this inhibition is strongest in about 0.9% saline, and decreases both above and below this salt concentration. This indicates that other than purely electrostatic factors must participate in such inactivation.

Viability of the mouse infectious encephalomyelitis virus was found by Drs. K. K. Takemato and H. Liehaber of NIAID to be reduced by a direct interaction with polyglucose sulfate. They also found that this anionic polymer affected plaque morphology in a fashion similar to that of a sulfated polysaccharide present in small concentration in the agar usually employed in the overlay during the assay of these viruses.

The charged polysaccharide derivatives synthesized by J. W. Wood continued to be in great demand by biochemists working on enzyme and on virus inhibition studies, both in this country and abroad.

Biochemical Pharmacology Section

Dr. Goldin and his group have continued to investigate a considerable number of facets in the field of basic science chemotherapy. Their objectives have been: chemotherapy studies of antitumor and antiviral agents and the mechanism of their actions; synthesis of new agents; study of host-tumor relationships with respect to drug action; and establishment of new therapeutic principles and procedures in the management of tumor growth in animals.

Many aspects of the program were the product of collaborative work with investigators in other parts of the NCI and in institutions elsewhere. Close working relationships were maintained with clinical colleagues active in chemotherapy investigations in patients.

Immunity

It had been noted in previous work in this Section that, after treatment of advanced leukemia 1210 in F_1 hybrids of C×DBA with halogenated derivatives of amethopterin, an appreciable number of mice survived. Many of them were found to be immune to a second inoculation of the sensitive subline of L–1210, and also to antifolic-resistant sublines. It was than found that this host immunity could aid in the chemotherapy of the amethopterin-resistant sublines.

These observations were followed up in a number of ways. Immunization of hybrid mice with normal spleen, x-irradiated normal spleen, and x-irradiated leukemic spleen, followed by challenge with L–1210 or skin grafts, suggested that L–1210, which originated in DBA mice, possesses some antigenicity for the C×DBA hybrid.

Since leukemia L1210 is a transplantable tumor with a long laboratory history, it is not unexpected that it possesses some slight incompatability to the isologous host. Without therapy this incompatability is masked by the rapid growth of the tumor and early death of the animal. Therapy with highly effective drugs such as the halogenated

derivatives of amethopterin, by holding the tumor growth in check, apparently permits sufficient time for the host to respond to the weak antigenic stimulus. The immune response in turn is added to the direct antitumor action of the agent, resulting in extensive survival times and cures.

It is of considerable interest that therapy of leukemia L1210 in DBA/2 mice is less successful than in C×DBA F_1 hybrids. In DBA mice, halogenated derivatives of amethopterin prolonged survival time but failed to achieve "cures". It had been considered previously that this was attributable to lower drug tolerance of the DBA mice. Evidence has now been obtained that the DBA mice are less responsive to immunization than the hybrid mice. Investigations are in progress on the nature of this unusual observation and its possible relationship to the greater resistance to therapy of the leukemia in DBA mice, the strain of origin.

Increases in survival time were obtained in mice inoculated intracerebrally or subcutaneously with FR–3, an antifolic-resistant subline of L1210, on treatment with 3′-bromo-5′-chloromethopterin (BCM) if the mice were first treated with BCM for intracerebral inoculation of sensitive L1210.

The series of experiments on the effect of surgical adjuvant therapy reported last year has been extended and completed. It had been demonstrated that a combination of surgery and chemotherapy (6-mercaptopurine) was more effective than surgery alone or chemotherapy alone in increasing the lifespan of mice bearing advanced adenocarcinoma Ca-755. Complete remission of tumor occurred in a high percentage of cases following surgery and adjuvant therapy. Studies have been conducted showing that host immunity contributes to the therapeutic response to 6–MP. Survivors of adenocarcinoma 755 resulting from successful surgery plus treatment with 6–MP showed immunity to reinoculation. This immunity was shown to occur relatively early during the course of therapy. Also, mice inoculated intraperitoneally with x-irradiated carcinoma 755 were highly refractory to a challenge with viable carcinoma 755.

Without surgery, in the presence of a large tumor mass, the moderate immune response to carcinoma 755 apparently does not augment therapy sufficiently to permit extensive tumor remission. However, when a large part of the tumor is surgically enucleated, the combination of chemotherapy and immune response is sufficient to result in extensive regression and a high percentage of survivors.

It had prevously been found in this Section that several strains of L1210 elicited a typical homograft response in a variety of lines of mice and that the homograft response was readily suppressed by treatment with folic acid antagonists. Extension of these findings showed that variation in the size of the inoculum can alter the homograft response. With progressive dilution of the inoculum in out-of-strain mice, the proportion of tumor takes terminating in death of the animals increased markedly. This phenomenon was designated as "the dilution effect".

Antifolic therapy of the resistant subline resulted in progressive growth of the leukemia and death over the entire range of inoculum concentrations, thereby removing the *dilution effect.* Simultaneous administration of the metabolite, citrovorum factor, prevented the abrogation of the homograft response by amethopterin, and in essence, restored the *dilution effect.*

Concomitant injection of normal tissue (spleen and blood) or x-irradiated leukemic tissue prevented the lethality observed with low concentrations of leukemic inocula, thereby also removing the *dilution effect.*

Suppression of host immunity, rather than the *dilution effect* would appear to account for the observation that therapy of a resistant tumor abrogates the homograft response. However, the *dilution effect* would appear to be an important phenomenon in relation to screening and drug evaluation with transplantable tumors.

The growing interest in the extent to which *antitumor agents suppress host immune reactions* had led to the development of a model system for the evaluation of such antihost effects of chemotherapeutic agents. Previously, we had reported that therapy of a resistant tumor abrogated the homograft reaction. In that system, however, therapy of the tumorous animal complicated the evaluation of the anti-host effects of the compounds.

The current system measures the extent of growth of leukemia L1210 in homologous mice following pretreatment with the candidate drug.

The system avoids any direct effect of the drug on the tumor, thereby permitting a critical evaluation of the effect of candidate compounds on the immune response of the host. To date, sarcolysin, triethylene melamine, Cytoxan, amethopterin, and 6-mercaptopurine have been tested in this system, employing leukemia L1210 in C57B1/6 mice. The alkylating agents were quite effective, with sarcolysin producing the most extensive suppression of host immunity. 6-Mercaptopurine was less effective, and amethopterin was relatively ineffective in this system. Homograft suppression, as evidence by an increased incidence of takes, was apparent only at toxic levels with these agents, and then was not extensive.

In other experiments the system described above was modified to include both skin and tumor homografts. To date, a good correlation has been observed between survival of skin and tumor homografts in control animals, in which both are rejected, and in animals whose immunological response has been impaired by drug treatment. BALB/c animals pretreated with sarcolysin and subsequently implanted with leukemia, died of progressive tumor growth. In comparable groups of pretreated animals, the median survival time of DBA skin grafts was approximately twice the median survival time of the tumorous animals. In contrast, on pretreatment with amethopterin, survival of skin graft and tumor approximated that of the respective controls.

Continuous daily post treatment with an optimal dose of amethopterin abrogated the homograft response to the tumor, and the animals all succumbed. In comparably treated animals the skin homograft reaction occurred, but graft survival was increased approximately 50 percent over the median survival time of tumor animals.

Virus Leukemia

Experiments have been carried out *in vivo* with the Maloney virus and with the Rauscher virus.

Mice bearing transplants of whole-cell leukemia generated by the Maloney virus survived longer on treatment with alkylating agents such as Cytoxan, TEM, and sarcolysin than with antifolics, purine- and pyrimidine-antagonists, or antibiotics. The several lines of transplantable virus leukemia that have been established showed a wide range of drug sensitivity, e.g., to Cytoxan. These observations indicate that a population of mice in which the Maloney virus had induced lymphocytic leukemia would probably show the broad range of sensitivity to drugs characteristically seen in clinical leukemia.

One of the whole-cell lines was quite sensitive to therapy with several alkylating agents and x-irradiation. Following apparently successful therapy of this transplanted whole-cell line, animals subsequently succumbed, frequently after 100 days. Pathologic examination of some of these leukemic long-term survivors, by Dr. Thelma Dunn, revealed a widely disseminated lymphocytic neoplasm.

Since the transplant inoculum contained both leukemic tisue and the inducing virus, it was of fundamental importance to determine whether the animals were succumbing to slow-growing variants in the transplanted cell population, or to a primary leukemia, induced by the virus. Data obtained from transplantability studies with tissue obtained from these long term survivors strongly suggest that the agents (TEM, Cytoxan, sarcolysin and x-irradiation) eradicated the whole-cell disease, and that the virus in the initial leukemic implant subsequently induced a second lymphocytic neoplasm in these animals.

The system described above, in which therapy induced a remission, which was followed by subsequent reappearance of leukemia, appears to approximate closely the clinical experience observed with acute lymphocytic leukemia in children. Thus, this system provides an experimental tool for investigating maintenance of remission and "induced" resistance. In addition, it raises the question as to a possible role of virus as an incitor of human leukemia.

Cytoxan, 5-fluorouracil, and TEM, in preliminary experiments, produced a decrease in spleen size in mice bearing the Rauscher virus.

CNS Tumors

Cytoxan was found to be much less effective in the treatment of intracerebral L1210 than of the subcutaneous. This finding was obtained both on intracranial injection of the drug as well as with subcutaneous.

The relative effectiveness of a series of drugs against L1210 inoculated I.C. or S.C. was ascertained with a single S.C. injection of the maxi-

mum tolerated dose of the drug 24 hours after implantation of the leukemia. All the drugs tested were less effective against I.C. than S.C. leukemia. Cytoxan was the most effective against both sites.

The duration of effective drug levels was determined by giving a single dose of the drug at various intervals prior to inoculation of leukemia I.C. or S.C. Chlorambucil was ineffective. The others (Cytoxan, TEM, and L-Sarcolysin) were more effective against the S.C. than I.C. leukemia. The effective dose level, however, is maintained for only a brief period—inhibition was obtained when the interval between the drug and tumor administration was one-half an hour, but preinjection of the drug one hour prior to inoculation of tumor was without effect.

Sequestration of tumor cells in the brain, and less efficient entry of drug into the brain site, have important bearing on refractoriness to complete remission in chemotherapy. Detailed pathologic studies of these mice showed features similar to those in the brain and meninges of patients who died of acute leukemia.

Systemic chemotherapy of I.C. leukemia with Cytoxan apparently destroyed all, or most, of the leukemic cells in the spleen, bone marrow and dura, but had little, if any, effect on the infiltrate of leukemic cells in the arachnoid space. Progressive growth of leukemic cells in the arachnoidal and perivascular space occurred while animals were receiving daily subcutaneous doses of Cytoxan.

Evaluation of Antitumor Agents

Assay procedures which had been developed in this Section were employed in the evaluation of new compounds of clinical interest. The tumors used in the assays were L1210 and resistant variants, Sarcomas 37 and 180, carcinoma 755, and the Ehrlich ascites tumor. These studies were carried out primarily in the Drug Development and Evaluation Program (Contract No. SA–43–ph–2371). This line of work is fully presented in a separate report (NCI 216), but a few of the major points may be mentioned here.

During the past year 67 compounds were examined for ability to increase the lifespan of mice with systemic L1210. This brought the total to over 300 compounds. Summary data on 235 compounds were included in a comprehensive "Manual" prepared in collaboration with Dr. Howard E. Skipper of Birmingham and Dr. Leon Schmidt of Cincinnati. The most active compound was 2-chloro-4′-di-2-imidazolin-2-ylterephthol-anilide (NSC 38,280). Of the alkylating agents, none was more effective than Cytoxan. In general, alkylating agents of the ethylene-imine type were more effective than nitrogen mustards, methane sulfonates, or epoxides. No purine or pyrimidine derivative was more effective than 6–MP. Of the antibiotic materials, Duazomycin A exhibited ⅓ to ½ the activity of MTX, but showed no significant advantage over the structurally-related azaserine.

For a series of compounds, variations in the treatment schedules were investigated. The relative advantages of drug administration daily, every 2 days, and every 4 days were compared with the effectiveness of a single treatment. Each drug displayed its own characteristics in this regard.

Combination therapy with two drugs was found to be more effective than with either alone in the case of the following pairs: NSC 38,280 plus MTX; Cytoxan plus MTX; and Cytoxan plus 6–MP. In contrast, combination of MTX plus 6-aminonicotinamide was no more effective than MTX alone.

Of the 17 additional compounds evaluated against Ehrlich ascites tumor, none produced a greater survival time than previously obtained in this Laboratory with N-methyl-formamide. As regards the newer data on increased time of survival of mice bearing the three solid tumors, alanine nitrogen mustard continued to be the most effective for S37 and S180; Ca755 has been particularly sensitive to many purine derivatives and to Cytoxan.

A comprehensive study of the relationship between inhibition of the local tumor and increase in survival time elicited by the treatment of mice with S37 or Ca-755 was conducted. No necessary correlation between inhibition of the growth of the local tumor and increased survival time was found. For example, when mice bearing early Ca-755 were treated, daily to death, with Cytoxan or 6-MP, both compounds provided a 100 percent increase in median survival time. With Cytoxan, the increase in survival time was accompanied by

only a moderate inhibition of the local tumor. The equivalent increase in survival time produced by 6-MP was accompanied by a marked inhibition of the local tumor. Doses of Cytoxan which caused marked tumor inhibition resulted in decreased survival times because of their toxicity for the host. A-139 produced a high degree of tumor inhibition in mice with early Ca-755, but failed to increase survival time. N-Methylformamide increased the survival time of mice with early S-37 at dosage levels which are not markedly inhibitory to the local tumor. 6-Thioguanine produced a high degree of tumor inhibition of S-37 but failed to increase the survival time of the mice. In general, these studies showed that drug efficacy in increasing survival time may be accompanied by a greater or lesser degree of tumor inhibition.

Collaborative work has been in progress with Prof. Orrie Friedman of Brandeis Univ., who is synthesizing new compounds related to Cytoxan and other types of mustards with carrier groups. Cytoxyl alcohol, a possible metabolic product of Cytoxan, was markedly less active against L1210. Arrangements were made with Dr. G. M. Timmis of the Chester Beatty Institute in London to investigate here the antitumor effects of selected compounds synthesized there.

Folic Acid Antagonists: Mechanisms

Prefolic A, both naturally occurring and synthetic specimens, was found to be active as a metabolite in the L1210 system. (Drs. Keresztesy and Donaldson, NIAMD had isolated prefolic A from liver, then synthesized 5-methyltetrahydrofolate, and showed them to be identical). It was found to be as effective as citrovorum factor in reducing the toxicity of amethopterin in mice and in reducing its antileukemic effect. The N5-ethyl and N5-propyl derivatives, prepared by Dr. Keresztesy, were tested for activity against early L1210; neither compound produced prolongation of survival.

Our previous observations on the metabolite activity of dihydrofolic acid, in which it was shown that this compound had biological activity in the presence of amethopterin similar to fully reduced derivatives of folic acid are now being extended to an *in vivo* assay, in collaboration with Dr. P. Condit, Oklahoma City. This system measures directly the ability of animals, treated with amethopterin, to convert folate or dihydrofolate to tetrahydrofolate derivatives. Preliminary results have shown that amethopterin at low doses will completely inhibit the conversion of folate to tetrahydrofolate, but that a dose as high as 500 mg/kg of amethopterin will not inhibit the conversion of dihydrofolate to tetrahydrofolate. This is not in agreement with *in vitro* studies of the reduction of folic acid in which the reduction of folate and dihydrofolate are equally sensitive to inhibition by amethopterin. It raises the question as to whether the enzyme systems which catalyse this reduction *in vivo* are the same for folate and dihydrofolate.

With Prof. M. Friedkin (Tufts Univ.) we have shown that in some antifolate resistant sublines of leukemia L1210 there is a considerable increase in the level of the enzyme dihydrofolate reductase. This led to the suggestion that it might be possible to utilize this property of the resistant varients to achieve a lethal synthesis of tetrahydrofolate analogs by introducing a substrate which would be preferentially reduced by the tumor cells with high reductase levels. In preliminary work, several simple derivatives of folic acid have not shown significant activity. A series of antifolic analogs is being synthesized to test for lethal synthesis in leukemic variants with high content of dihydrofolate reductase.

We have observed that the toxicity and antileukemic effect of amethopterin, in mice, produced by daily treatment can be reversed by dihydrofolic, but not by folic acid. With Dr. Condit, we have shown that conversion of folic acid to citrovorum factor in mouse liver is inhibited by small doses of amethopterin, while the conversion of dihydrofolic acid to citrovorum factor is not. To explain this behavior, Drs. Schrecker and Mead have carried out *in vitro* studies on the kinetics of inhibition of dihydrofolate reductase. The enzyme was obtained from a highly resistant subline of leukemia L1210 with 80-fold increase in dihydrofolate reductase activity. It was found that this enzyme catalyzed the reduction of both folic and dihydrofolic acid at pH 5, but that only dihydrofolic acid was reduced at pH 7. At pH 5, the reduction of both folic and dihydrofolic acid was inhibited irreversibly by amethopterin and 3,′ 5′-dichloroamethopterin. On a molar basis, both antagonists were equally effective inhibitors.

This was in agreement with previous findings obtained by Zakrewski, Werkheiser and Nichol, who used an enzyme isolated from chicken liver. At pH 7, on the other hand, both amethopterin and dichloroamethopterin were found to be non-competitive inhibitors of dihydrofolate reduction, i.e. dihydrofolic acid was able to reverse partly the inhibitory effects of the antagonists. At this pH, dichloroamethopterin was a more effective inhibitor than amethopterin. These findings agree with previous findings of Huennekens, Bertino and Friedkin. An amount of inhibitor that completely represses the reduction of folic and of dihydrofolic acid at pH 5 was found insufficient to suppress the reduction of dihydrofolic acid at pH 7. These findings may help explain the reversal of amethopterin-induced toxicity and antileukemic effect by dihydrofolic acid, but not by folic acid.

Drug Resistance

The development of resistance to a drug to which the tumor had been initially responsive is a phenomenon of such importance that considerable attention was devoted to experimental work on this subject. Many variants of L1210 were developed in which each subline was resistant to a particular agent. Examination of the responses to other agents revealed no generalization regarding cross resistance, for each variant was a special story in itself. The following findings exemplify the results in this line of work.

Seventeen compounds were tested for their activity in increasing the survival time of mice with the 3′, 5′-dichloroamethopterin resistant variant, L1210/M663R. This subline of L1210 was cross resistant to MTX, but did not display cross resistance to Cytoxan, NSC 38,280, 6-MP, 6-MP-riboside, tetramin, methylglyoxalbisguanylhydrazone, 1–aminocyclopentane carboxylic acid, thio-TEPA, TEM, 2-amino-1,3,4-thiadiazole, 5-fluorouracil, 5-fluorodeoxyuridine, streptovitacin A, actinomycin D, 4-aminopyrazolo(3,4-d)pyrimidine, or 6-aminonicotinamide.

The L1210/C95 variant of leukemia L1210 arose from successive treatment with MTX, 6-MP, and Cytoxan. This subline is markedly resistant to 6-MP and MTX and displays a high degree of resistance to Cytoxan. The L1210/C95 variant displayed no resistance to methylglyoxalbisguanylhydrazone or NSC 38,280.

6-Mercaptopurine (6-MP) and 6-mercaptopurine riboside (6-MP-R) were compared for their effectiveness against the L1210/AgR subline of leukemia L1210. L1210/AgR is resistant to 8-azaguanine and has displayed cross-resistance to 6–MP. The studies showed that L1210/AgR was equally resistant to 6–MP and6–MP–R.

In collaboration with Dr. D. Hutchison and J. Biedler of the Sloan-Kettering Institute, a number of resistant L1210 sublines were investigated as regards certain genetic and biochemical factors, viz., the chromosome complement of the leukemic cells and their specific dihydrofolate reductase activity were determined at several transplant generations.

Cells of the sensitive parent line possess a submetacentric chromosome. A mercaptopurine (MP) resistant subline which was not collaterally resistant to amethopterin, still possessed the submetacentric chromosome, and had the dihydrofolate reductase activity of the parent line. An amethopterin-resistant subline had lost the submetacentric chromosome, and its reductase activity was increased 11-fold. Another subline, treated serially with a combination of amethopterin, MP, and fluorouracil was resistant to these drugs singly and in combination. During the first 56 transplant generations, it still had the submetacentric chromosome, and its dihydrofolate reductase activity was that of the parent line. Between the 56th and the 63rd transplant generations, a mutation apparently occurred, leading to loss of the submetacentric chromosome and an 18-fold increase in dihydrofolate reductase activity. These findings suggest a possible correlation between loss of the submetacentric chromosome and increased dihydrofolate reductase activity.

The biochemical basis of drug resistance to leukemia was investigated by Drs. Schrecker, Mead and collaborators with regard to: (a) correlation of dihydrofolate reductase activity with increasing degree of antifolate resistance in mouse leukemia; (b) correlation between inhibition of dihydrofolate reductase and of purine biosynthesis in leukemic tissues by folic acid antagonists: and (c) use of a computer to explore the use of dihydrofolate reductase as a tracer of amethopterin-resistant leukemic cells.

When L1210 was made resistant to amethopterin, the enzyme activity increased 11-fold. Made this variant resistant also to a second antifol (dichloroamethopterin) resulted in a 40-fold increase. When the latter compound was used alone, a variant developed with partial resistance to this antifol, but there was no concomitant rise in this reductase.

A subline of L1210 leukemia had previously been developed by serial transplantation and treatment with amethopterin for 10 generations, followed by similar treatment with dichloroamethopterin for 10 generations. This subline was biologically fully resistant to the two drugs. Immediately following establishment of this subline (FR–8), its dihydrofolic reductase activity was increased 80-fold. After 11 transplant generations in the absence of treatment, dihydrofolic reductase activity was still 60-fold that of the parent line, but fell to a 25-fold level at generation 14. At the 29th and 33rd transplant generation in the absence of treatment, dihydrofolate reductase activity was the same as in the sensitive parent line, although the tumor was still resistant to amethopterin and dichloroamethopterin. Treatment with 75 mg/kg/day of dichloroamethopterin during one generation after 13 or 29 untreated transplant generations restored the maximal (80-fold) dihydrofolic reductase activity. This would be consistent with a mechanism of enzyme or with elimination of sensitive cells produced by partial reversion of the resistant subline to sensitivity. Purine biosynthesis *in vivo* was measured concurrently in several transplant generations by means of determining the incorporation of radioactive formate into the adenine of leukemic spleen and tumor in the absence and the presence of amethopterin or dichloroamethopterin. Dose-response curves were obtained. It was found that, as the dihydrofolate reductase activity was increased in the leukemic tissues, increased amounts of drug were required to produce the same inhibition. When dihydrofolate reductase activity was increased 80-fold, no inhibition of purine biosynthesis was observed at all, even with very high doses. This would suggest that the cells contained enzyme in excess of the amount that could be inhibited by the maximal drug concentration capable of being established inside the cells.

Mathematical models were employed in an attempt to relate the interplay between such factors as life span of a leukemic animal, how early drug treatment is initiated, the survival of sensitive and resistant leukemic cells in treated animals, the incidence of mutation from the sensitive state to the resistant state, and the dihydrofolate reductase levels in mixed populations of sensitive and resistant cells. A computer was used to generate theoretical mixed populations of resistant and sensitive leukemic cells during three simulated passages in drug-treated animals. This was done so as to mimic the actual enzyme data collected during the development of an amethopterin-resistant subline. It was concluded that dihydrofolate reductase can be used as a meaningful biochemical marker in studies of emerging antifolate-resistant leukemic cells.

A model experiment was conducted by S. Humphreys and collaborators to determine whether resistant leukemic cells with high levels of dihydrofolate reductase might serve as biochemical markers, reflecting the efficacy of therapy. This was considered feasible since the high levels of dihydrofolate reductase could readily be measured in tissues showing any extensive degree of invasion by leukemic cells. In the model experiment, the high dihydrofolate resistant leukema was inoculated intracerebrally; treatment was with Cytoxan.

A close correlation was found of biochemical, biological, and pathological observations. Cytoxan was effective in increasing survival time. The systemic disease was held in check by treatment, as shown by the low level of dihydrofolate reductase activity in the spleen and the reduced transplantability of spleen suspension. Following therapy, pathologic examination of the spleen, liver, bone marrow, and other extracranial tissues revealed little evidence of leukemia. There was much more difficulty in holding the disease in check in the brain. This, again, was reflected in the progressive increase in dihydrofolate reductase in the brain, the success of retransplant, and the infiltration of the brain seen on pathologic examination.

Enzymatic markers of this type may be useful in studies of fate and distribution of drugs, and of the blood-brain barrier. They may be employed in investigations of tumor invasiveness, and the origin of resistance and the efficacy of therapy.

High dihydrofolate reductase in antifolic resistant leukemia can serve as a marker which, in conjunction with the biological studies and the pathology, may provide a means for detailed investigation of chemotherapeutic agents.

Other Topics

In collaboration with Prof. N. O. Kaplan of Brandeis Univ., an extensive series of pyridine derivatives was studied with regard to ability to function as metabolites like nicotinamide, or as antimetabolities like 3-acetylpyridine. The large amount of data has been summarized in a comprehensive review which provides a basis for correlation of the foreging activities with ability to stimulate DPN synthesis *in vivo* and to form analogs of DPN by exchange with its nicotinamide moiety.

Dr. Chirigos has been investigating the transport of amino acids, notably tyrosine, in S37 ascites cells. The uptake of L-tyrosine was rapid: an intracellular concentration nearly 7 times that in the external medium was accomplished in less than one-half hour. Little sterospecificity was observed: D-tyrosine, DL-tyrosine and CH_3-tryrosine were concentrated nearly as well as the α-form. Tyramine and p-hydroxyphenylacetic acid were not concentrated. Heating at 60° C in buffer, and treatment with a variety of metabolic inhibitors such as sodium azide and 2,4-dinotrophenol inhibited tyrosine transport.

During a study of the influence of Sarcolysin (phenylalanine mustard) on tumor growth; it was observed the amino acid analogue p-fluorophenylalanine emitted appreciable fluorescence in the ultra-violet. The fluorescence characteristics of the positional isomers of other halogenated phenylalanines were therefore investigated by Dr. Chirigos. It was concluded that fluorine, uniquely among the halogens, enhances the fluorescence of aromatic rings.

Much labor, space, animals, and expense may be conserved in the continuous maintenance of stocks of transplantable tumors by preserving them in the frozen state and then thawing them only when required for new experiments. The viability, and the resistance to chemical agents, of variants of L1210 were found to be satisfactorily preserved by the storage of frozen tumor cell suspensions. Three sublines of L1210 (M46R, AgR, and M663R), after freezing and thawing, yielded comparable reactions to antitumor agents as they did when maintained in continuous passage in animals. A variety of other tumors, notably sarcomas and carcinomas, which had been carried in live passage in other parts of this Laboratory have now also been preserved in the frozen state.

DERMATOLOGY BRANCH

The research activities of the Dermatology Branch concern two major areas: (1) Study of normal and abnormal growth and differentiation of the epidermis and related epithelial tissue: (2) Study of the lymphomatous disease, Mycosis fungoides.

Epidermal Growth and Differentiation

The aim of this program is to identify and characterize the varying and various biologic behavior patterns of epidermis and related epithelial tissues under normal and pathological circumstances, and to determine the nature of influences that naturally exist, or that can be experimentally exerted, which predictably can alter or determine a specific pattern of response of the epithelial cell.

It has been demonstrated over the past 40 years that embryonic epithelium responds in markedly different patterns to environments of different connective tissue stroma. Thus, the normal eventual form and function of an epithelial cell is the result of the stromal environment in which it is found.

Recent work in our Branch, involving autotransplantation of epithelial cells into various anatomical sites in the human, has indicated the same is true for post-embryonic epithelia.

It is readily apparent that the cutaneous epithelial cell possesses a wide potential range of speed of reduplication and pathways of differentiation, depending upon its anatomical location. For example, the rate of reduplication of epithelial cells of the hair root is known to be considerably more rapid than those of the epidermis, although the precise rate of each has not heretofore been determined; and epithelial cells of the epidermis differentiate and produce fibrous proteins which

are different from those produced by the hair root cells.

The direction of a portion of the investigations of this Branch to date has been to quantitate the rate of reduplication of the various epithelial tissues of man and biochemically to define the products of differentiation. Through these studies it has been found that the germinative cells of the hair root double their populations every twenty-four hours; the time required for the epidermis to reduplicate itself has been found to be twenty-eight days, whereas the epidermis in the disease psoriasis requires but three days. Several insoluble proteins have been identified as products of the normal and abnormal epidermis and their peptide content studied.

The studies of the epidermis in psoriasis have indicated that the lesion is one of benign epidermal hyperplasia (increased rate of reduplication, increased mitotic index, excessive production of protein). The effects of mitotic "arrestors," antimetabolites, alkylating agents, and radiation on the lesion have been studied. These agents have been found to inhibit the rate of cellular reduplication, but in the doses employed, showed no inhibition of differentiation (in this instance, keratinization). On the contrary, keratinization is accelerated under these circumstances.

A consistent pattern of mitotic activity has been elucidated in human hair roots. Mitotic activity has been found to be correlated with certain geometric data, regardless of the size of hair roots. Thus, the mitotic index of any given segment in the hair root can be formulated by the equation, $X=113-0.58\ Y$, where Y is the distance (in microns) between the segment and the proximal tip of the connective tissue hair papilla. No such relationship has been found for normal epidermis nor the hyperplastic epidermis of psoriasis, in both of which mitotic activity is confined to the region of basal cells. The pattern of mitosis in basal cell tumors is being studied.

In an attempt to determine what factors promote keratinization of the epidermal cell, split thickness specimens of skin have been cultured *in vitro* under different conditions. Results of this work suggest that a high pH of the culture medium and/or high CO_2 tension of the ambient atmosphere promotes the keratinization process.

Studies of basal cell carcinoma indicate that this neoplastic lesion can be characterized by an inability for its constituent epidermal cells to keratinize. Although chemotherapeutic drugs inhibit the growth of this lesion, no evidence has been found that these compounds promote normal keratinization of the epidermal cell in psoriasis. Inhibition of growth basal cell tumors by methotrexate, appears to be proportional to the mitotic activity of the tumor. Attempts have been made to stimulate mitotic activity within tumors, with both ultraviolet light and X-ray, prior to administration of methotrexate. Initial observations suggest that prior-irradiated tumors show a greater damage response from the drug than non-irradiated tumors.

Mycosis Fungoides

Approximately fifty patients with this rare lymphomatous disease have been studied and treated during the past eight years. Its histopathogenesis, from its onset in the skin to its involvement of internal organs, has been carefully investigated. Treatment with X-ray, high energy electrons, and several chemotherapeutic drugs has been given. Although remissions of the disease have been attained, no curative responses to these measures have been observed. Perhaps significant, however, is the fact that prolonged remission has occurred in four of twelve patients in response to therapy with the drug Cytoxan, one patient remaining virtually free of lesions now for over one year in response to continuous therapy.

A seemingly pertinent observation in this disease has been the occurrence of a spontaneous "cure" in one patient following an allergic drug eruption. Whereas spontaneous remission of this disease has been unknown, this patient has been free of disease for over four years. Transfusions of blood from this patient to another patient with the disease has been without effect. Attempts to provoke allergic skin eruptions in several patients with the disease have been unsuccessful.

ENDOCRINOLOGY BRANCH

The studies of this Branch have been directed towards increasing our knowledge of the processes

of growth differentiation and control of certain normal tissues and their neoplastic derivatives. These tissues include the endocrine glands and those organs whose maintenance or growth is dependent upon specific humoral agents.

In the area of trophoblastic disease, follow-up studies of 30 patients who were successfully treated for metastatic disease revealed that relapse has not occurred after a remission of over one year. In methotrexate-resistant cases, Actinomycin D has proved a valuable therapeutic adjunct resulting in a 50 percent remission rate in this group. Methotrexate has been used in women with persistent evidence of disease following a hydatidiform mole and has caused a complete remission in each of 8 cases. Since these patients are prospective victims of metastatic trophoblastic disease, this response may be regarded as an example of specific cancer prophylaxis by chemical means. The endocrinological effects of trophoblastic disease have been examined and a singular coincidence of hyperthyroidism noted. The hyperthyroidism has subsided with treatment of the trophoblastic disease.

Eight strains of human choriocarcinoma are being carried by serial transplantation in the hamster cheek-pouch. These strains are sensitive to many therapeutic agents some of which had been ineffective in the patient. The implications of this with respect to the usefulness of heterologous tumor transplants are being examined. Several classes of agents have been screened for therapeutic usefulness in this system and several alkaloids to Vincaleukoblastine proposed for clinical trial.

Immunological techniques have been used in an attempt to distinguish between tumor gonadotropin and normal gonadotropin. No differences were found notwithstanding the different distribution of these hormones in plasma proteins.

Among the humoral controls of tissue growth, the steroids play an important role. Methods have been devised for the analysis of several steroids whose precursors are important in adrogen synthesis. The precursors of several androgens in adrenal cancer have been investigated and were shown to arise via pathways not significant in the normal steroid synthesis.

Adrenal androgen biosynthesis in gonadal dysgenesis was shown to be decreased suggesting that the genetic factors important in gonadal dysgenesis also influence the development of biosynthetic pathways in the adrenal gland.

The analysis of the prostatic response to ACTH has been continued and it was shown that thyroxine and growth hormone enhanced the effect of ACTH. Prolactin did not augment the effect of androgens on the growth of the ventral prostate.

The pharmacological alterations of adrenal secretion by derivatives of compounds related the insecticide, DDT are being examined in the dog in the hope of finding a less toxic agent than o,p′ DDD. Complete chemical adrenalectomy in the dog could be obtained by prolonged administration of o,p′ DDD.

Twenty-six patients with metastatic adrenal cancer have been treated with o,p′ DDD. Hormonal remission was achieved in 18 and tumor regression in 10. Comprehensive analysis of urinary steroids in these patients has uncovered defects of steroid synthesis in adrenal cancer and resulted in the finding that the increased excretion of tetrahydro substance S in patients with Cushing's syndrome is highly suggestive of cancer (Hertz and Lipsett)

The pituitary hypothalamic level, a series of patients subjected to hypophyseal stalk section for metastatic breast of these hormones in plasma proteins.

Among the humoral controls of tissue growth, the steroids play an important role. Methods have been devised for the analysis cancer has been examined. The therapeutic effectiveness of stalk section was less than that of hypophysectomy. Persistence of the normal thyroid-pituitary relationship can occur after stalk section. In the rat, it has been shown that the formation and release of thyrotropin from the pituitary *in vivo* is greatly enhanced by hypothalamic tissue. The secretion of melanocyte stimulating hormone in the frog was demonstrated to be under hypothalamic control. Studies of the various factors affecting this melanocyte response have been described to provide a basis for the development of a bioassay method. These studies have been initiated to obtain some insight about the complex relationship between the central nervous system and the endocrine system.

Other growth factors are being examined. Growth hormone was ineffective in primordial

dwarfism suggesting that this entity is due to an end-organ resistance.

The adjustment of the normal subject to a variety of anabolic and catabolic agents has been determined using newly devised paired-tray technique. This has the potential of answering several questions that cannot be handled by the classical balance technique.

MEDICINE BRANCH

Chemotherapy Service

A number of new agents have been studied clinically as regards their ability to produce tumor regression. These include cytoxan, guanylhydrazone, vincaleukoblastine, leurocristine, dichloromethotrexate, the terephthalanilides, and sarcolysin. A guanylhydrazone derivative, methyl-glyoxalbis-guanylhydrazone, has produced complete remissions in approximately 50 percent of adults with acute myelogenous leukemia. This is the first major therapeautic advance to occur in this disease which previously was slightly responsive to only one drug, 6-mercaptopurine. Guanylhydrazone has also produced tumor regressions in patients with lymphosarcoma and mycosis fungoides. The toxicity of guanylhydrazone has been considerable and relates primarily to the gastrointestinal tract, the skin, and the bone marrow. In collaboration with the Cancer Chemotherapy National Service Center and the Midwest Research Institute a number of related quanylhydrazone congeners are being prepared in an effort to determine the active chemical sites, and hopefully to improve therapeutic index. Guanylhydrazone C^{14} has been synthesized and initial pharmacology studies in rodents are under way. The mechanism of action of guanylhydrazone is independent of that for the other known cancer chemotherapeutic agents in that cross resistence does not occur.

The periwinkle alkaloid, vincristin, has also been shown to be capable of inducing remissions in children with acute leukemia and tumor regression in a large proportion of patients with Hodgkin's disease and lymphosarcoma. There is preliminary evidence that this compound is active in patients with carcinoma of the breast and perhaps certain other patients with solid tumors. Dose schedule studies with this compound in man reveal that the tolerated dose per unit time is independent of the schedule. While vinblastine, a related alkaloid, also produces tumor regression in patients with Hodgkin's Disease and lymphosarcoma, it was inactive in patients with acute leukemia. Thus, major differences not only in toxicity but in the therapeutic effects exist between these chemically very closely related Periwinkle alkaloids. (Carbone and Karon) Vincristin produces marked metaphase arrest. In the bone marrow the proportion of cells in metaphase rises from a control of 10/1000 to in excess of 70/1000 at 12 to 24 hours after intravenous administration.

A comparative study of cytoxan, uracil mustard and nitrogen mustard in patients with various solid tumors including lymphoma has revealed no significant difference in either the antitumor activity of these agents or in the overall toxicity. Sarcolysin, a phenyalanine mustard, is the first drug in our hands to produce significant antitumor effects in patients with multiple myeloma. Though complete regressions have not occurred, substantial decrease in abnormal protein production, a decrease in myeloma cells of the marrow, bone healing, and improvement in anemia have occurred in a substantial inumber of patients. A comparative study of urethane versus placebo in patients with multiple myeloma has revealed that urethane is inactive and that spontaneous improvement of any sort is rare in patients with multiple myeloma. Other clinical studies include comparative dose-response studies of 6-mercaptopurine and 6-mercaptopurine riboside in patients with solid tumors; a comparative study of fluorouracil, fluorodeoxyuridine and methotrexate in patients with carcinoma of the breast and colon; studies of azauridine, both in terms of its clinical antileukemic effect and its ability to inhibit the enzyme orotidylic decarboxylase in patients with chronic myelogenous leukemia; and studies of the effects of cytoxan, fluorouracil, vinblastine and duazomycin A in patients with carcinoid syndrome. This latter study was undertaken when it was observed that certain of these compounds are highly active in a serotonin producing mast cell rodent tumor. In association with tumor regression qualitative and marked quantitative changes in tryptophane metabolism occurred.

In association with these clinical trials there is continued experimentation with and improvement of experimental design as well as extension of our knowledge of the natural history of advanced neoplasms in man. The results of these quantitative antitumor studies have been carefully compared to a number of the screens, particularly the rodent tumor screen, used in selecting antitumor agents for tumor trial. In this regard the L1210 mouse leukemia system which in general would appear to predict fairly well for human acute leukemia failed to disclose significant activity for vincristin, a compound which was found to have considerable activity in human acute leukemia. The rat lymphomas and leukemias which had been extensively used in the evaluation of alkalating agents would appear to correlate poorly with human tumors in terms of ranking therapeutic agents. (Frei and Rall)

The use of plasmapheresis to obtain platelets from normal donors for the prevention and control of bleeding in thrombocytopenic recipients has been attended with considerable success. Eighty-five percent of recipients will have a rise in their platelet count and will not demonstrate diminishing response with successive transfusions. In addition to becoming an established therapeutic procedure it allows for the application of *in vitro* techniques for measuring platelet function and their collation with the *in vivo* observations and the study of the efficacy of the various methods of platelet preservation. More recently the same general techniques have been applied in the acquisition of white cells from donors with chronic myelogenous leukemia. One hundred transfusions of such cells have been given to granulocytopenic individuals, mainly patients with acute leukemia. A significant rise in granulocytes in the recipcient occurs in the majority of patients. Many of the patients had severe infections at the time of transfusion, for example, pseudomonas septicemia. In the majority of these, prompt control of the infections was achieved. The implications of the observation that we can now control both hemorrhage and infection in patients with acute leukemia are major. From 90 percent of the patients with acute leukemia and in excess of 50 percent of patients with malignant disease generally die of these complications.

The technique for chromosome study of clinical material has been simplified and applied in 40 patients with acute leukemia and 10 patients with chronic leukemia, 4 patients with multiple myeloma and a number of other disease entities which allow for the acquisition of relative homogenous cellular preparations. Abnormalities both in number and quality of chromosomes are relatively frequent but as yet, with the exception of the Philadelphia chromosome in chronic myelogenous leukemia, no consistent pattern has evolved. Detailed clinical correlative studies are under way to look for relationships to the chromosome abnormalities.

Clinical Pharmacology and Experimental Therapeutics Service

Major emphasis continues to be given to the problem of drug distribution. Considerable progress has been made concerning our understanding of factors which influence the entry and exit of drugs in the central nervous system. Exit from the central nervous system may result from:

1. Passive diffusion for lipid soluble compounds.
2. Bulk flow. This obtains for large lipid insoluble compounds. Evidence concerning the importance of this latter mechanism was obtained when it was observed that Acetazolamide, a compound which decreases cerebral-spinal fluid production, and therefore bulk flow, will decrease elimination of large lipid insoluble compounds.
3. Sterospecific active transport. *In vitro* studies of the animal choroid plexus have delineated this mechanism. Two related problems are being pursued. One concerns the absolute rate of production of cerebral-spinal fluid and the other concerns dynamic studies of the entry and egress of magnesium from the cerebral-spinal fluid. This latter study was prompted by the observation that the concentration of magnesium in the cerebral-spinal fluid is greater than that in the blood. The broad implications of these observations for a drug development program are apparent. Clinical application of these concepts and techniques have been made, particularly as they may be applied in the treatment of that important clinical entity, meningeal leukemia. The tolerated dose and the magnitude of entry and egress of methotrexate from the central nervous

system has been studied. Since methotrexate does not pass the blood brain barrier in any quantity it must be delivered by lumbar puncture. The diffusion throughout the subarachnoid space from this site has been studied in monkeys using I^{131} labeled rose bengal. Certain mechanical maneuvers at the time of lumbar tap may assure adequate distribution.

The Medicine Branch has a major commitment to the folic acid antagonist area which extends from synthesis up through clinical trial. Studies with radioactive chlorine labeled dichloromethotrexate (DCM) have revealed a considerable variation in organ distribution depending upon the specie. By the use of ion exchange column techniques and UV spectroanaylses the metabolites of methotrexate and dichloromethotrexate have been defined. In contrast to methotrexate, dichloromethotrexate has a major metabolite, 4,7-dihydroxy folic acid. The magnitude of conversion to this metabolite also varies from specie to specie and this probably is the major explanation for the lesser toxicity of DCM.

The mechanism of resistance to thiopurines in tissue culture and in many animal tumor systems relates to the selection of cells incapable of converting 6–MP to the active nucleotide. These studies have been extended to man and preliminary evidence would suggest that deletion of the enzyme involved does not occur in human leukemia cells resistant to 6-mercaptopurine.

Studies of carcinogenic materials in newborn animals including newborn monkeys have indicated that squamous cell papillomas can be produced regularly when methylcholanthrene in olive oil is injected intradermally. In addition to carcinogenesis, the reproducibility of this system is such that it may be used in chemotherapy studies.

LABORATORY OF PATHOLOGY

The Laboratory of Pathology has a more complex organization than many other laboratories of the National Cancer Institute. This is because the Pathologic Anatomy Branch performs functions related to patient care and clinical research in the Clinical Center; the Histopathology Laboratory prepares microscopic sections on a service basis for the entire Institute; and another group in the Cancer Induction and Pathogenesis Section is devoted primarily to non-clinical research. There is a close association among all the pathologists in the Laboratory of Pathology and frequent collaboration. Opportunity is given to members of the Pathologic Anatomy Branch to do research, and pathologists not directly responsible for the autopsy or surgical biopsy service may still observe autopsies and avail themselves of human material.

The work in this laboratory is not restricted to a single project, or to a group of closely related projects but each pathologist follows his particular line of interest and training. It is therefore convenient to divide this summary into a number of sections.

Collaborative Research

It is recognized that many research projects at the National Cancer Institute require the collaboration of a pathologist, especially in the final evaluation of the effect of an experimental procedure on laboratory animals. The Laboratory of Pathology has always tried to make this assistance available. The pathologist may take an active part in planning an experiment and in following it through; he may take the responsibility for all autopsies and histologic diagnoses in an experiment; he may review only the histologic sections in a given experiment; or he may serve as a consultant to review selected material with no responsibility for the entire experiment or its publication. Finally, he may make use of material accumulated by other investigators for independent studies concerning pathologic alterations. It is emphasized that full collaboration of the pathologist at the time the experiment is planned is the most satisfactory arrangement for it insures the best and most economical selection of material for pathologic studies. Examples of collaborative research now in progress are: a) Dr. Stewart and Dr. Snell with Dr. Morris of the Laboratory of Biochemistry in studies of the effect of new carcinogenic agents in rats. As a result of these studies a monograph on lesions produced by N,N′–2, 7-fluorenylenebisacetamide has been published. b) Dr. MacCardle with Dr. Potter of the Laboratory of Biology on the cytology of induced plasma cell tumors. These studies have disclosed a cytologic correlation between the neoplastic cells in partic-

ular transplantation lines, and indicate that different globulins are produced in the protein-secreting plasma-cell tumors at different stages of maturation of the plasma cell. c) In observations made by Dr. MacCardle with Dr. Frederic Bartter, a new clinical syndrome has been found in which hyperplasia of the juxta-glomerular apparatus accompanies hyperaldosteronism and hyperkalemia with a normal blood pressure. d) Dr. O'Gara with Dr. Kelly of the Medicine Branch, NCI, on the effects of injecting chemical carcinogenic agents into newborn animals. This work revealed that extremely small doses were effective. e) Dr. Dunn with Dr. Moloney of the Laboratory of Viral Oncology in a study of morphologic changes produced by his virus. This study showed that a number of changes preceded the development of lymphocytic leukemia. f) Dr. Dunn with Dr. Andervont, Laboratory of Biology, in a review of tumors appearing in wild mice. In this study, the pathologist did no more than make a diagnosis of the tumors, but the results showed that morphologically these tumors closely resembled tumors observed in inbred mice. g) Dr. Swarm with Dr. Morris and Dr. Dyer on carcinogenesis in rats with congenital hyperbilirubinemia. Also with other members of the NIH staff where his specialized knowledge of radioisotopes and autoradiographic techniques was required. h) Dr. Banfield in a number of investigations where electron microscopy was required. Examples are studies of Whipple's disease, psoriasis, mouse thymic agent, acanthosis nigricans, and the Arthus reaction. i) Dr. Malmgren with other members of the NIH staff where the flourescent antibody technique is required. j) Dr. Sidransky with Dr. Morris on the pathologic changes produced by N–2-fluorenylacetamide in male and female rats.

Laboratory animals

This is a continuing activity in the Laboratory of Pathology. Precise knowledge regarding the normal anatomy of laboratory animals is often lacking, particularly as regards variations in inbred strains and the alterations appearing when animals reach the age when cancer can be expected. The investigator using laboratory animals must know his basic material for he relies upon it much as a chemist relies upon the substrates in a reaction. Dr. Stewart and Dr. Snell are accumulating data on aged rats from five inbred strains. For the second year, Dr. Snell has given a lecture as part of a course at the Armed Forces Institute of Pathology on the use of Laboratory Animals in Research. This lecture describes spontaneous lesions in old rats and the incidence of cancer in the various strains. Dr. Snell is also collecting data on the Mastomys, a species of rodent recently introduced to the Laboratory. Dr. Banfield, Dr. Dunham, Dr. Herrold, Dr. Chu, and Dr. Swarm are collecting data on the hamster, a species now popular in cancer research, and about which our knowledge is still limited. Dr. Dunn continues to collect information on the endocrine system of the mouse. Dr. Swarm has under observation a breed of rats with an inborn error in the matabolism of bilirubin. Basic data are required before many of these animals can be used with greatest profit.

Transplantable Tumors

The first studies on transplantable tumors in animals were made by pathologists and this interest has continued. The interpretation of the effects of certain tumors on the host has given important information regarding the function of normal organs, especially the endocrine organs. Work on transplantable adrenal cortical carcinomas is being continued by Dr. Snell and by Dr. Mulay, each of whom is carrying a tumor of this type. Dr. Mulay has found a difference in the secretion of the tumor and material obtained from the normal adrenal gland. Dr. Mulay has also found that hepatogenesis is modified by the hormonal state of the rat and that changes in adrenocortical chemistry precede neoplastic changes.

Dr. Banfield, assisted by Mrs. Darlene Brindley, is studying a transplantable reticulum cell sarcoma in hamsters which is transmitted from one animal to another in the same cage. It is the first instance of such natural transmission that has been found recorded. The possibility that a cell transfer and implantation occurs when other hamsters eat the cancerous tissue is being investigated, and also the possibility of infection by a transmissible agent is being explored. A second tumor of this type has now been found. Dr. Swarm is continuing his studies of a transplantable teratoma of the testis. This tumor produces

cartilage, and the behavior of the neoplastic cartilage, and cartilage from normal animals after subcutaneous transplantation is being compared.

Virus Research

Members of the Laboratory of Pathology have been interested in exploring the morphologic alterations produced by oncogenic viruses both in tissue culture and in the experimental animal. Dr. Clyde Dawe is carrying on fundamental studies in observing the alterations produced by the polyoma virus on whole organ tissue cultures of salivary glands of mice. It is disclosed for the first time that the action of the mesenchyme profoundly affects the reaction of epithelial tissue to the virus. These studies also prove that tissue from the adult is as susceptible as tissue obtained from the newborn. This finding is contrary to *in vivo* observations for the effect of the virus has been largely restricted to newborn animals. Dr. Dawe has also shown that if adult salivary gland tissue is exposed to the virus for two hours and then transferred subcutaneously in a mouse, a salivary gland type of tumor will develop at the site of implantation. This observation opens up many possibilities for future research and continuing work with this system offers a hope that the mechanism by which the virus produces its oncogenic effect may yet be disclosed.

Dr. Rabson has found that a strain of polyoma virus which lost its oncogenic property when grown in cells in a milk medium regained its oncogenic potency when grown in serum. This finding has fundamental significance for it proves that an alteration may be induced in the virus as well as in the host. The milk-medium strain with a low oncogenicity forms a small plaque when compared with the strain having greater oncogenic potency. Now that the actuality of oncogenic viruses is generally accepted it becomes urgent that the mechanisms by which they operate be determined. The oncogenic outcome is the result of an interaction between the virus and the host, and both elements of this interaction require intensive study. Other observations made by Dr. Rabson show that the polyoma effect first appears in the nucleus and terminally in the cytoplasm. The effect of polyoma virus in Mastomys is being investigated for it is known that the effects of this virus vary in different inbred strains of mice and in different species of animals. If viruses play a part in human cancer, in order to prove this, we must understand the variable manifestations which they produce in non-human species. Dr. Rabson's attemps to recover a virus from human cancer have so far been unsuccessful.

Dr. Dunn has worked in collaboration with Dr. Moloney, Dr. Rauscher, Dr. Manaker, and Dr. Bryan of the Laboratory of Viral Oncology in pathologic studies of animals infected with leukemia viruses and with an agent obtained from a human gastric cancer. With the Moloney virus the most interesting finding is that in intact BALB/c mice and rats, the first neoplastic lymphocytic cells are found in the thymus, but this finding is preceded by hyperplasia in the blood-forming organs and atrophy of the thymus. When the Moloney virus is given to thymectomized mice, the incidence of lymphocytic neoplasms is reduced almost to zero, but occasional cases of granulocytic leukemia (extremely rare in BALB/c mice) and frequent cases of reticuloendothelial dysplasia, often resembling Hodgkin's disease in man, appear. This is of considerable interest in the controversy regarding the relationship among different types of malignant lymphomas in man. The findings in mice suggest that the same stimulus may produce a lymphocytic, a granulocytic, or a reticulum cell neoplasm, depending upon the reaction of the host. It is not necessary to postulate a separate virus for each of these diseases. The action of the Moloney virus also shows that a derangement of the reticular tissues precedes the appearance of the neoplasm, and the action of the virus is indirect. A virus also obtained by Dr. Moloney from an undifferentiated plasma cell neoplasm and the leukemia virus obtained by Dr. Manaker seem to affect the host in much the same way as the first Moloney virus, but more work on these is required. Pathologic studies on mice with the Rauscher virus are only at the beginning, but it appears that a lymphocytic neoplasm begins in the thymus, as with the Moloney virus. The neoplastic condition, however, is preceded by an extreme erythrocytopoiesis, with enormous enlargement of the spleen which frequently leads to hemorrhage and death before the neoplasm develops. With the Moloney and the Rauscher virus we appear to have two distinct agents each

leading to the same form of lymphocytic neoplasm, but preceded by very different conditions in the host. The agent recovered by Dr. Ray Bryan from the human gastric cancer has produced a significant number of renal tumors (a very rare condition) in BALB/c mice. These renal tumors are always accompanied by cysts in the liver and occasionally by carcinomas of the acinar cells of the pancreas of a type never before seen in mice. The evocation of rare and previously unknown lesions in the mouse, and the association of three apparently unrelated conditions has some similarity to the action of the polyoma virus.

Dr. Robert Love, before leaving the National Cancer Institute, was engaged in cytochemical studies of the nucleic acids in normal and neoplastic cells infected with riboviruses and deoxyriboviruses. Using his toluidine blue molybdate staining procedure he has shown that the earliest alteration in P388D_1 cells infected with polyoma virus is enlargement of the nucleolinus which is subsequently extruded and then forms numerous particles in the nucleoplasm.

Chemical Carcinogenesis

This continues to be a fruitful field for study by the pathologist, since pathogenesis is always of major interest. A notable achievement was work performed by Dr. Mearl Stanton which provides (1) a new method for the induction in rats of lung tumors that histologically resembled human lung tumors, and (2) the demonstration that two factors acting concurrently will induce lung tumors in rats, while neither was effective alone. Multiple infarcts were produced in rat lungs by the intravenous injection of a fluorocarbon, and when this treatment was combined with injections of methylcholanthrene, bronchiogenic tumors resulted. This observation is highly significant for human lung cancer because exposure to a carcinogen may be relatively harmless in a healthy lung and dangerous in a diseased lung.

Several carcinogenic studies have been stimulated by observations on the geographic distribution of cancer in man and on studies of special types of human cancer. Dr. O'Gara is trying to induce cancer by exposing animals to material obtained from utensils used by African natives with esophageal cancer. Doctors Herrold and Chu are studying the development of uterine cervical cancer in mice and hamsters. Dr. Dunham is devising techniques to compare the possible carcinogenic effect of adsorbates obtained from the drinking water of a city with a high bladder cancer rate (New Orleans) with water from a city with a low rate. Dr. Herrold and Dr. Dunham have produced lung cancer in Syrian hamsters by the intratracheal instillation of benzo(a)pyrene. Thorium wire and urethane in the bronchus were ineffective. They have also tested the hamster cheek pouch for carcinogenic response to different substances, and found this site generally unresponsive. Dr. Swarm continues to study the late effects of the injection of thorium dioxide in man and in mice, in rats and in rabbits. Similar cancers of the liver are developed in all four species.

Geographic Pathology

The project comparing uterine cancer in various ethnic groups in New York City, Israel, and Washington, D.C., has been completed and a manuscript is in preparation. A similar survey of cancer of the uterus in Negro women in New York City and in Washington, D.C., is being analyzed. A project is in progress to survey bladder cancer in New Orleans and to look for possible factors to account for the frequency in that city. Dr. Herrold is continuing her collection and review of lung cancer among Veterans. All cases are examined histologically and Kreyberg's classification has proved satisfactory. Any relationship of smoking to the histologic type will be considered when the findings are tabulated. Dr. O'Gara visited the Transkei Region of South Africa where a high incidence of cancer of the esophagus is reported among the natives. Since his return he is testing various possibilities on laboratory animals for etiologic factors in human esophageal cancer.

Plasma Cell Neoplasms in Mice

Dr. Malmgren has employed the fluorescent antibody technique to demonstrate that antisera to globulins in plasmacytomas is localized within the tumor cells. Dr. MacCardle has determined that a corelation exists between the cytology of the neoplastic plasma cell and the type of protein it

produces. Dr. Kobayashi found that all transplantable plasma cell neoplasms that he studied would produce osteolytic lesions if introduced intravenously. Osteolysis therefore appears to be a special property of the neoplastic plasma cell and is not restricted to only a few transplant lines. Kidney lesions, however, are restricted to those transplant lines in BALB/c mice in which a Bence-Jones protein has been demonstrated.

PATHOLOGIC ANATOMY BRANCH

In addition to duties connected with the surgical biopsy, exfoliative cytology and autopsy service of the Clinical Center, the Pathologic Anatomy Branch pursues a number of research studies relating to human pathology. These are described in detail in the reports from various members of the Pathologic Anatomy Branch. Publication of the pathologic studies is often incorporated in studies by clinicians in which the biopsy or autopsy findings add significant information. The pathologist may also make case reports where the findings are primarily of pathologic interest. In addition to this, several studies have been carried out where the autopsies of numerous cases are reviewed and the information is synthesized in order to afford a unified concept. An example of this is a study by Dr. Louis B. Thomas on the skeletal and nervous system lesions in acute leukemia. This human study is now being correlated with a study of the central nervous system lesions in mice given the L-1210 lukemia and treated with cytoxan. In a similar fashion the Cytology Section examines material from patients for diagnostic purposes, and is also engaged in research activities related to technical improvements of methods to recover cancer cells from the blood and other fluids and also in estimating the prognostic significance of circulating cancer cells. Dr. Chu by employing her skill in the cytodiagnosis of human vaginal smears has followed the painted uterine cervices of hamsters with a carcinogen. Carcinoma resulted and a comparison of the histologic appearance and the cells in vaginal smears showed that a good correlation existed.

Dr. John H. Edgcomb has been an exchange fellow in Moscow since June 23, 1961. He expects to return at the end of December but will soon go to Ghana to head a research laboratory to be established there. While in Moscow he has studied the histochemistry of transplantable melanomas and participated generally in the activities of the Institute of Experimental and Clinical Pathology. His reports of his life in Russia and observations of cancer research have been valuable and interesting.

Dr. Thomas has investigated the effects of schistosoma from Egypt and from the Gold Coast on the bladder epithelium of hamsters. No bladder cancers have been found. This negative evidence is of interest in considering the problem of the high bladder cancer rate in Egypt and the frequency of schistosomiasis.

LABORATORY OF PHYSIOLOGY

Because of the diversified training and endeavors of this laboratory, the annual report presents a number of avenues of research. Yet, in spite of this, the ultimate goal sought is to throw light on the cancer problem. In general, all the research is directed towards a study of tumor-host relationship *in vivo*. Wherever supplementary data or exploratory data from *in vitro* experiments can assist the studies *in vivo*, such experiments are encouraged.

Dr. Pratt is oriented toward the physiological and biochemical systems that exist in the tumor-bearing animals. The large number of parameters resulting in such a study made data processing and computer utilization a necessary adjunct. As a result, a digital computer (IBM–1620) was obtained. The instrument has two main uses: (1) to act as a data processor for monitoring and directing long-term experiments, and (2) to afford a powerful computational facility for developing an applied mathematical methodology for research projects. He has been (a) formulating mathematical models for the interpretation and evaluation of cancer chemotherapy screening data, (b) with Dr. Thomas of the Pathological Anatomy Branch, he is setting up a system for storing and retrieving information of pathology diagnosis, (c) with Dr. Sober, he is developing chemical, mathematical and computer programming techniques to describe the arrangement of mononucleotides within polynucleotides, and (d) continuing his study on the

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total metabolism (energy, carbon, nitrogen and water) in normal and tumor-bearing rates.

Dr. Shack is continuing his elegant techniques in attempts to characterize and compare nucleic acids and related compounds, as well as enzymes and enzyme inhibitors of normal and malignant tissues. He has been (a) making intensive studies on the action of bivalent ions (Ca, Mg, Sr) in the activation of deoxyribonuclease, and (b) developing methods which appear to offer promise of separating nuclei acids into their component fractions for further identification. He has demonstrated that although Ba, Ca and Mg are poor activators of ribonuclease by themselves, they seem to increase the activity of Mg ions when added to the latter. This strongly suggests that two metal ions are concerned with the formation of a nucleic acid-protein complex. As a result, this system may be a useful model to study the factors involved in the formation and stabilization of other types of nucleoproteins. One of the major problems involved in characterizing nucleic acids is accounting for the partial denaturation which occurs during isolation. By employing electrophoretic techniques, Dr. Shack has been able to demonstrate the extent of the denaturation and which appears to be related to the proportions of the various purine and pyrimidine bases. It now makes it possible to separate nucleic acids of different composition with exact information on controlled denaturation. Separation of nucleic acid of different composition following controlled denaturation is now possible.

Dr. Rabinovitz continues his studies on action of inhibitors which produce a "biochemical lesion" in protein synthesis at the protein assembly site, namely the ribosome. In studying the effect of the antibotic, puromycin, on the Ehrlich ascites tumor cell, he has produced what he calls a dissociative lesion where the partially assembled proteins are prematurely released from the ribosome. Employing the lysine analog, S–(β-aminoethyl) cysteine in the rabbit reticulocyte, there occurs what he calls an accumulative lesion whereby the assembled proteins either cannot be released as a final product or can be released with difficulty and therefore appear as excess precursors on the ribosome. These results suggest there may be a qualitative difference in the behavior of ribosomes of mammalian cells during protein synthesis.

Dr. Winitz is continuing his studies with his water-soluble diet. He is attempting to determine the minimal protein requirement in terms of amino acid intake. It has been suggested that the free amino acid levels in the blood of a given animal species in the fasting state might serve as a precise indicator of the dietary essential amino acid requirements of that species. Hence, time-consuming and expensive nitrogen balance studies could be eliminated. Indeed, Dr. Winitz has demonstrated that diets prepared according to the proportions of the amino acids in the plasma induced growth comparable to the best available known synthetic diet and compared favorably with Purina chow. Studies to elucidate the implications of this diet in studying nutritional deficiencies and the impact of a growing tumor on the host are underway.

Dr. Wollman is continuing his studies on the early events leading to the formation of thyroid hormone in normal thyroid tissue. He has made extensive studies on the iodide-concentrating mechanism and the site of formation (including kinetics of formation) of protein-bound iodine. More recently he has turned his attention to examining the mechanism of the secretion of thyroid hormone. In collaboration with Dr. S. S. Spicer, he has identified intracellular colloid droplets in thyroid which have the properties of lysosomes. These droplets contain colloid derived from the lumen of the follicle as well as hydrolytic enzymes. It appears therefore that the droplet is an intracellular organelle in which thyroxine is released from peptide linkage in thyroglobulin prior to secretion of the thyroxine into the blood. Dr. Wollman has also developed a variety of functional and non-functional thyroid tumors in the Fischer rat. It was not possible to distinguish between the functional and non-functional follicles on the basis of shape, size or staining properties. Some of the functional tumors can compete effectively with the thyroid for a hormone precursor. He was able to divide these tumors into two classes: (a) tumors which suppressed severely the ability of the thyroid to clear radioiodine from the blood, and (b) tumors which had almost no effect on thyroid clearance.

Dr. Elkind continues his elegant research on the kinetics of the growth and survival properties of surviving cells (as a result of exposure to vari-

ous doses of X-irradiation) after the termination of the division delay period. He has demonstrated that the post-delay growth is not synchronous (as commonly presumed) but exponential with a doubling time very close to that of unirradiated cells. Thus the so-called "mitotic block," presumed to be the explanation of the irradiation-induced delay, is not a true block but rather a delay. Dr. Elkind is attempting to formulate a mathematical model of the growth and radiation responses of cells and tissues. If successful, this model should contribute much to our understanding of radiation effects. He plans to study changes in nucleic acids and proteins in cells after irradiation.

Dr. Reid is continuing his study on urinary excretion patterns in human leukemia with special reference to nucleic acid congeners. During the year, Dr. Reid had a total of 13 patients under investigation: 3 normals on a free diet and on the control diet used for pathology studies; 3 untreated cases of acute myeloblastic leukemia; 4 untreated cases of acute lymphoblastic leukemia; and 3 of the acute lymphoblastic cases in remission after therapy. Because of the wide variety and number of components present, a good deal of time has been spent in the reduction and analysis of the profile data and upon the systematic investigation of the composition of the mixed peaks obtained in the chromatographic procedure. The introduction of data processing machines into the Laboratory has been highly successful and information of a concrete nature should be available very soon. Preliminary work has unequivocally demonstrated the presence of pseudouridine. Its excretion has been shown to be diet dependent. There is no relation between pseudouridine excretion and myeloblastic leukemia. There is a definite relationship between pseudourdine excretion and lymphoblastic leukemia.

Dr. Millar and Dr. White have continued to study tumor-host relationships using the Walker 256 tumor. We have demonstrated clearly the great demand for Na ions by tumor-bearing animals (far in excess of that supplied in the normal diet). In collaboration with Dr. J. O. Davis (Laboratory of Kidney and Electrolyte Metabolism, NHI), a study was made of the corticosteroid output of the adrenal gland. Aldosterone output was found to be markedly increased over normal.

These investigators have also made some preliminary studies of the isolated protein of Walker tumor as a source of nitrogen for growth. Two tentative (need verification) observations have been made: (a) animals ingesting a 6 per cent casein diet show a 20 per cent higher increment in growth when supplemented daily with 300 mg. of tumor protein than with 300 mg. of casein, suggesting a higher growth efficiency of tumor protein, and (b) animals bearing tumors and ingesting tumor protein (20%) grow statistically larger and have larger tumors than do a similar group of animals ingesting casein protein (20%).

In addition, Dr. Millar in collaboration with Dr. Winitz and Dr. Pratt are studying the gross body composition in normal and tumor-bearing rats on a completely water-soluble, chemically-defined diet. It is now possible to compartmentalize each component that contributes to body growth and ultimately demonstrate the impact of the tumor on the host in terms of nitrogen, water, and electrolytes.

Dr. Willie Smith has been studying spontaneous and induced recovery from radiation damage. She finds that age is an important factor in spontaneous recovery and that its effect may vary with particular components of recovery, thus granulocyte recovery and recovery from gastrointestinal damage seem to improve with increasing age of the mouse while recovery of the immune mechanism occurs somewhat more rapidly in young animals. A single injection of endotoxin before or immediately after irradiation leads to an early recovery of bone marrow cellularity as well as peripheral granulocyte, erythrocyte and platelet counts. There is also an increased survival. Toxic manifestations of endotoxin are undesirable and other products which may be less toxic are being sought. Studies of a similar nature are being conducted with colchicine and colchicine derivatives.

Dr. Draper is studying radiation immunology to contribute to our understanding of both radiation effects and the nature of the immune process. He has demonstrated the reduction of the deleterious effects of a given dose of radiation by fractionation (over-all exposure time is increased and the average dose thereby lowered) and has extended this observation by using hemolysin formation in rabbits as an indicator system. These

procedures have also provided experimental data which indicate that tissue or cellular sites of antibody synthesis are not equally radiosensitive. This suggests the possibility of using radiation as agent for the selection of certain sites of antibody production. It is also possible that the manifestation of immune reactions *in vivo* may depend on the participation of non-specific processes. The mast cell has been shown to play a role in the tissue reaction to the combination of antigen and antibody in tissue. Thus it becomes necessary to know the effects of radiation in this and other nonspecific events when measuring the effects of immune reactions in tissue.

Dr. Maxwell continues his long-term studies on the effect of ionizing radiation on amino acids. His objective is to determine the mechanism of the chemical reactions induced by ionizing radiation in aqueous solutions of amino acids. These simple systems of biological interest result in information which may be applied to more complex biological systems, which is not easily available by direct investigation. From the simple system of aqueous amino acid, ionizing radiation has produced 13 products which have been isolated and identified. Effects of temperature, pH and concentration of glycine are being studied. Only about one-half of the glycine is accounted for by these products. C^{14}-glycine will be used to search for additional products.

Dr. Riesz is also engaged in studying the effect of irradiation of *in vitro* systems. He is bombarding aqueuos solutions of acetone with cobalt gamma rays and studying the reaction products. He has isolated hydrogen gas, hydrogen peroxide, hydroxyacetone and 2,5-hexanedione. In dilute solutions the yields can be quantitatively explained in terms of the reactions of hydrogen atoms and hydroxyl radicals. In concentrated solution, the concept of hydrogen atom precursors is required to explain the observed results.

RADIATION BRANCH

The year 1961 has seen the separation from the Radiation Branch of the experimental radiobiological research components.

In respect to service functions, the Radiation Branch is responsible for meeting the medical radiation therapy needs of the Clinical Center. As a measure of the size of this operation some 434 courses of patient irradiation comprising 1,798 irradiations were administered and 202 medical consultation requests were processed in 1961.

Non-clinical irradiation services are also performed as a continuing responsibility of the Radiation Branch to meet the requirements of the NIH and, on request, to other agencies of the Federal Government. The scope of this operation is very broad; 2,651 high and medium energy exposures being given to a variety of biological materials, each exposure generally involving the simultaneous irradiation of multiple units, and 2,100 animals have been irradiated for variously prolonged periods of time in a "low-level" irradiation facility. Thirty-six percent of these irradiations were performed for the operations of the Radiation Branch and 16% of the high-energy irradiations were performed for other government agencies. The remaining 40% were performed for other NIH laboratories. These irradiations are performed, largely, by the physics staff.

The clinical research activities of the Radiation Branch can be grouped conveniently in two categories, the one having to do with the definitive relief of human cancer and the other with the human patho-physiology of ionizing radiations. In the former belong the studies of the significance of the relationship of dose and time and related considerations in the cancerocidal effect of irradiation and the chondrosarcoma and the childhood cancer studies.

The dose time studies have been extended to include studies of tumor and patient responses at extreme ends of the dose and time scales. The cases studied have been those of carcinoma of the upper food and air passages. Single irradiations with tumor doses of the order of 2500 roentgens are being applied in order to have a basic experience and to define some responses as a background for other clinical studies where, as in high-pressure oxygen radiotherapy, prolonged or multiple treatments may not be possible. These responses are being compared with those at the other extreme of the dose time scale, that is, those cases receiving doses of the order of 9000 r over 100 days. Some of these cases are still being followed and many interesting observations have resulted. Among the more interesting conclusions

that can be drawn from the observations is that dose-*per se*, is critical at either extremity. This material will be worked up in detail at an appropriate time as several years are required to accumulate data of these sorts. In respect to chondrosarcoma studies, a method has been elaborated for the determination of the "volume of distribution" of sulfur and this can now be studied before and after; for example, amputation, resection, or external beam or radioisotope radiation therapy. Extensive experience has now been gained with the clinical, radiotherapeutic, and other aspects of the so-called undifferentiated solid cancers of childhood, especially those of the head. A very substantial experience has also been accumulated in respect to the indications for and the responses to local irradiation of the infiltrative local lesions of acute childhood leukemia.

The detailed hematological responses and the recovery of radiation-injured hemopoietic functions are being studies in patients receiving therapeutic total body irradiation. This total body irradiation is being delivered at a faster rate than has been reported and the responses which have been observed suggest that there are real rate differences in the degrees of response to a given dose. A detailed study of the quantitative relationship of the prolongation of the time in which radio iron is removed from the plasma (a measure of erythropoiesis) to the dose of total body irradiation and the quantitative relationship of changes in the white blood cell mobilization response to bacterial toxin to the dose of total body irradiation is being undertaken. Renal function studies are showing for the first time, by the use of certain refined tests of renal function, the changes which may occur as a result of therapeutic irradiation of the abdomen and renal regions for metastatic abdominal cancer and which changes are not ordinarily reflected in common tests of renal function. An observation of great interest is that changes are demonstrable after what would ordinarily be considered relatively small doses of radiation. Calcium metabolic radiation effect studies have so far largely been limited to calcium balance studies and some tracer studies with radioactive Calcium [47]. To a limited degree, we have been able to demonstrate local changes in calcium deposition in destructive neoplastic bone lesions following radiation therapy. (J. R. Andrews)

A quantitative mammalian cell radiobiology system has been devised. It is unique in that it normally is an anoxic one in respect to the oxygen effect. The full exploitation of the system has been handicapped by lack of adequate numbers of animals, but, nevertheless, a substantial amount of quantitative information on the influence of cell population number, ionization density (LET), oxygenation, dose-fractionation, and chemical modification has been obtained. Most of this information has a direct applicability to problems in clinical radiotherapy research and it points the way to directions along which such research should move.

SURGERY BRANCH

Local Wound Chemotherapy

The effectiveness of local wound chemotherapy was studied in a number of systems. Using urine 5-hydroxyindoleacetic acid content and time of onset of palpable tumor of the P–815–MI ascitic mast cell tumor as measures of tumor growth, previous animal studies with formaldehyde suggested that when the drug was applied to the "wound" prior to inoculation with a tumor cell suspension, an increased growth of tumor was observed. Further studies this past year have shown that a true "pretreatment" effect was not seen when an open wound was used, i.e., when an incision was made simulating clinical surgical procedures. However, when an air bubble "wound" was created the tumor appeared earlier, and an increase growth rate was observed.

In other studies of local chemotherapy a 90–95% effectiveness of proflavine hemisulfate in preventing the "take" of S–91 melanoma or mast cell tumor in open axillary wounds was shown. This effectiveness was diminished to 48% if the wounds were made hemorrhagic previous to instillation of the drug. This would seem to be important in any clinical application of local chemotherapy. The bloody and serous drainage could "neutralize" any local drug.

Clinical application of local chemotherapy has been under study for some time. At first, the operative wounds were sprayed with saline for cytologic studies. Later, a group of patients had

their wounds treated with 0.5% formaldehyde. Followup studies of a group of head and neck and cervix cancers showed very little effect of the formaldehyde in changing the incidence of local recurrences. However, as followup information becomes more complete, it would appear that local wound treatment may be of value. In a study of 119 uterine cervix cancers that received radical surgical excision, there were 54 that received no wound treatment, 27 that had 0.5% formaldehyde, and 38 that had formaldehyde plus the use of picris acid-treated sutures. The numbers of advanced cases were the same in each of the 3 groups, and the proportion with positive wound washings was the same. The gross local recurrence experience for the 3 groups was 21 of 54 (39%) for those with no treatment, 4 in 27 (15%) for the formaldehyde group, and 10 of 38 (27%) for the combined treatment. At 30 months after surgery, when almost all the local recurrences had manifested themeselves, the cumulative percent with local recurrence, calculated by life table methods, was 49% for the no treatment group, 28% for picric acid group, and 17% for the formaldehyde treated patients. Since the numbers involved in each group are small, the significance is questioned. However, when the date is examined from another point of view, it takes on added significance. If the wound treatment results are correlated with wound washings, the cumulative percent with local recurrence at 30 months in 11 patients with positive wound washings was 56% for those with no wound treatment, and 57% for those positive cases treated. The 23 patients with negative wound washings and no treatment had 64% incidence of local recurrence, while the 40 treated negative cases had only a 15% incidence of local recurrence. This could be interpreted as a quantitative factor in wound contamination, that is, if the wound has a heavy contamination with tumor cells, enough to give positive wound washings, the locally applied drug is not effective. The 58% cumulative local recurrence experience is the same for the entire group of positive washing cases. However, if the operative wound has only minimal contamination so as to make it difficult to wash and identify cancer cells from a wound, the local drug treatment is able to kill these individual cells.

It has been difficult to understand why patients with positive wound washings did not develop a significantly greater proportion of local recurrent cancer than those with negative washings. In the 119 cervix cancers under study, wound washing studies were performed in 96. Twenty-three of these were positive for cancer cells. The local recurrence experience for this group was 9/23 (39%) for the positive washings, and 17/63 (27%) for negative washings. This difference is not what one would expect, if the true picture of wound contamination were seen in the study of wound washings. (Smith)

In addition to the re-evaluation of wound washing results, wound drainage has been under study for the past 1½ years. A review of 40 cases in whom both wound washing and wound drainage fluid was examined, revealed additional cases that had cancer cell contamination of their wounds. There were 8 with positive wound washings and an additional 4 in whom wound washings were negative, but wound drainage collected as late as 68 hours after surgery contained cancer cells. This now means that calculation of local recurrence rates in relation to wound contamination must be based upon 12 of the 40, and not 8 as in previous studies. This may account for some of the "false" negatives.

In addition to evaluation of local recurrence rates based upon the above data, studies of circulating cancer cells shows a high incidence of cells in local blood draining a tumor. The role of circulating cancer cells in relation to the true degree of wound contamination remains to be worked out.

A satisfactory method has been developed for sampling blood from patients at regular intervals. Tumor cell embolization is greater between 6 a.m. and 4 p.m. Increased embolization during certain phases of operating room events was demonstrated. However, increased tumor cell "showers" during surgery is not uniform. It was noted that in 20 uterine cancer patients, a circulating tumor cell was found in 1 or more speciments from 5 patients. Life table method analysis suggests that the true prevalence of circulating tumor cells in peripheral blood in these patients was about 30%. The yield of "positives" for cancer of head and neck seems to be lower than uterine cancer.

The circulating tumor cell studies have also made possible observations on the occurrence of megakaryocytes in blood. The range is variable, but in general is greater in blood from the inferior

vena cava than in antecubital or jugular vein blood. As with tumor cells, there are greater numbers of megakaryocytes found during the 6 a.m. to 4 p.m. interval. The individual peak occurrence of circulating tumor cells and megakaryocytes do not coincide. The numbers of these cells do not correspond to the extent of tumor present in the patient. Preliminary observations suggest that circulating megakaryocytes are extremely sparse in normal individuals.

Tumor Growth and Metastases

A biologic test system to measure the rate of mitosis in the rapidly regenerating liver following partial hepatectomy in the rat has been developed. The rate of cell growth has been measured by means of dry weight, mitotic counts, and measurements of DNA synthesis by tritiated thymidine. Data accumulated to date indicates that serum from normal individuals contains two fractions; one capable of inhibiting cell growth, and the other capable of stimulating growth of the regenerating liver. Serum from patients with cancer contains a stimulating fraction, but does not contain an inhibiting fraction in the same concentration as does normal serum. The addition of the inhibiting fraction of normal serum produces inhibition of liver regeneration, indicating that the deficit in cancer serum can be replaced. The periodic injection of inhibiting substance into animals with a transplanted tumor delays the time of appearance of the tumor, and slows its growth rate.

It has been consistently found with five animal tumor systems that removal of a primary tumor decreased the number of lung metastases. The metastases which did develop in lungs after primary tumor removal grew to a larger size than those which were found in comparable primary tumor-bearing animals. Complete amputation of the primary tumor sharply reduced the number of metastatic foci, while incomplete removal of the primary tumor did not significantly alter the number of foci. When only part of the cancer was removed, results were similar to the unamputated group. This suggested that surgical trauma *per se* does not result in enhanced formation of metastases.

The effect of pregnancy on spread of tumor has been studied. Mated mice, whether pregnant or not, have larger primary tumors when sacrificed 39 days after tumor inoculation. No clear effect of mating on the occurrence of lung metastases was noted, but growth of such metastases was found to be inhibited in pregnant mice.

Blood of newborn mice, from mothers with S-91 melanoma, revealed cells which were characteristic of malignancy. Control blood from normal fetuses did not contain such cells. It was noted that maternal pelvic organ metastases were not present in 2 of the 8 positive groups. Metastases were not evident in any of the offspring. Thirty-one of the placentas were recovered. Serial sections of placentas, fetuses, and newborn lungs failed to disclose any metastatic foci.

Growth hormone increased primary tumor growth, carcass weight, and number of large pulmonary metastases in 2 animal tumor systems. Adrenocorticotropic hormone (ACTH) simultaneously decreased tumor growth, carcass weight, and number of large metastases. When growth hormone and ACTH were administered together, only the effect of growth hormone was observed. The two drugs seemed to have an antagonistic action. Neither altered tumor take, latent period, or growth rate of the primary or secondary tumor was not transferred when the primary tumor was transplanted to untreated animals.

Virus and Surgical Treatment of Cervical Cancer

During the past year the data obtained from the cervix virus study cases have been collected. Of special interest is the survival data in those patients in whom virus was injected into the cervix prior to excisional surgery. It is apparent that those patients who had virus placed in their cervix, and were then subjected to pelvic excisional surgery, the life survival curve was increased for the first 24–36 months after surgery. The cumulative percent surviving at 12 months of those who had Coxsackie virus plus definitive surgery was 93%. Adenovirus and surgery gave an 88% survival at 12 months, while for surgery alone there was 68% survival at 12 months. Statistical analysis shows that at 6 months the 35 patients with surgical excision and no virus had 74%, while 22 virus-treated patients had 91% survival.

Head and Neck Cancer

A study of 89 patients with invasive head and neck cancer revealed over 20% showing clinical evidence of distant metastases during the patient's life. At autopsy the incidence of distant metastases during the patient's life. At autopsy the incidence of distant metastases was 50%. This is far higher than generally recognized, and demonstrates the true "cancer" characteristics of these neoplasms. The overall degree of metastasizability of head and neck cancers is less than other cancers, i.e., in a cooperative study of 600 laryngeal cancers only 151 (25%) had lymph node metastases at the time of admission, none were below the clavicle. The overall five year survival rate was 65%. Therapy for early stages gave good results, whether by radiation or surgery (91% for Stage 1, 75% for Stage 2, 45% for Stage 3, and 22% for Stage 4). Radiation therapy was not of value in advanced stages. There were no survivors in Stage 4 cancers treated by radiation, while surgical therapy resulted in a 34% five year salvage. Even bulky tumors when treated by surgical excision, resulted in 60% salvage. The important prognostic factor was the development of a lymph node metastasis. When a lymph node metastasis was observed on admission, survival rate was less than half that seen for similar primary tumors without node metastases. Only 30% of the 151 with node metastases survived five years.

Cancer of Uterine Cervix

A true contrast of cancer behavior is seen in the cervix study. The overall salvage of these cases seen at the Clinical Center was 37%. This study consisted of 206 cervix cancers. Twenty-two of these had their cancer arise in a cervical stump. There were 46 that were inoperable on admission because of extent of disease, medical status, etc. An additional 41 patients were explored and found inoperable. There were 37 radical hysterectomies, 19 anterior exenterations, 63 total pelvic exenterations. Eighty-seven percent of the operable group had advanced disease (Stage C or higher). There were 7 operative deaths in the 119 cases (5.6%). Six of these occurred in the total pelvic exenteration group. The causes of these operative deaths are interesting. One died on the operating table of cardiac arrest. There was one massive pulmonary embolus on the seventh postoperative day. One patient died on the eighth day after re-exploration for intestinal obstruction. The remaining 4 deaths were associated with septicemia, occurring as late as one month after exenteration and resulting from multiple complications associated with pre-existing pelvic inflammatory disease, fistula formation, etc. Five of the 6 deaths in the exenteration group occurred in post-radiation patients.

The cumulative percent surviving 5 years (life table method) for the hysterectomy group was 57%. The same survival was noted in the anterior exenteration group. Five year survival following total exenteration dropped to 17%. Survival was related to extent of disease, and not on the operative procedure performed. The acceptance of total pelvic exenteration has depended upon the development of a satisfactory means of urinary diversion. The first exenterations done at this hospital were given a "wet colostomy." One-third of these few cases died free of cancer in 24–36 months from renal failure secondary to infection. Another ⅓ rapidly died of their cancer before their renal failure became severe enough to cause death. This remaining group was either converted to an ileal loop, or died of their cancer. The loop method of urinary diversion is now utilized. We have had no incidence of renal failure, and no deaths attributed to this type of urinary diversion.

LABORATORY OF VIRAL ONCOLOGY

The work of two of the three sections of the Laboratory of Viral Oncology, namely the Virus Oncology and Cellular Biology Sections, has been in the area of viruses in relation to cancer, although the additional areas of (1) carcinogenesis associated with nonviral agents, (2) ultrastructure (electron microscopic) of various types of cancer and normal cells, (3) cancer immunology, and (4) radiation have also been embraced. The work of the Tissue Culture Section includes studies with tissue cultures of the phenomenon of malignant transformation *in vitro*, the malignant cell, and the normal cell from which it arose. Much work has been done also on the development of tissue culture methodology and on the applications of newer methods to the studies mentioned.

I. Viruses in Relation to Cancer

a. Laboratory Animal Systems

1. Murine Leukemia Viruses

a. Moloney virus: Dr. Moloney has continued his studies with the virus he discovered in 1958, and first reported upon in 1959. His studies on animal specificity have been extended to include (1) a total of 8 strains and 5 hybrid groups of inbred laboratory mice, (2) wild mice (in collaboration with Drs. Andervont and Dunn), (3) two strains of rats, (4) hamsters, (5) guinea pigs, (6) rabbits, and (7) sub-human primates (6 rhesus and 1 cynomologus monkeys). All types of mice and rats tested, including wild mice, are susceptible to the Moloney virus. Hamsters also developed neoplasms of the reticular system, diagnosed tentatively by Dr. Thelma Dunn, as reticulum cell sarcoma. The first hamster tumor appeared 12 months after inoculation of the virus neonatally, and at 14 months the incidence among different groups of hamsters varies from 12.5 to 33%. These virus-induced neoplasms are readily transplantable in hamsters and studies are now under way to determine whether the virus is recoverable from the original and transplanted neoplasms. No lesions have as yet developed in other species. The studies in monkeys were started only recently and have been in progress for only a few months.

Further additional information of importance has been obtained by Dr. Moloney on the stability of his virus and on its chemical, physical and biological properties. Of special interest is the fact that it is inactivated by heating at 56° C for 30 minutes, that is, at a temperature slightly lower than that used for pasteurization. Significant contributions have been made by him also to a number of collaborative studies to be reported upon by others. One joint study, in collaboration with Dr. Dalton, has led to some of the most important developments of the year. Here, the contributions of each of these investigators were interdependent and of equal import in the developments to be described.

Pursuing the finding reported last year of an abundance of virus particles in megakaryocytes of the bone marrow of animals infected with the Moloney virus, electron micrographic studies were made of the blood platelets, which derive from megakaryocytes. Platelets were found to contain characteristic virus particles, but what was more significant was the presence of free virus particles in spaces between the platelets in the specimens examined under the electron microscope. This led to fractionation of larger volumes of blood and separation by ultracentrifugation of the "microsome" fraction, which would be expected to include any virus. Duplicate pellets of sedimented material were used for electron-microscopic studies by Dr. Dalton and for biological tests in mice and rats by Dr. Maloney. Both procedures confirmed the presence of large amounts of leukemia virus in the fractions derived from blood. The biological studies indicated a yield of virus about 10 times higher than previously had been obtained from leukemic-tissue sources (on a gram equivalent basis), and the electron micrographs indicated that the virus preparation derived from blood was in a fairly high state of purity with respect to formed elements, there being little else but virus particles and some debris, probably of platelets.

These results led to the following further important developments: (1) Dr. Moloney was able to modify and refine his differential centrifugation procedures so that apparently very high purification of the virus was achieved, as judged by electron microscopy. This achievement is comparable to that reported earlier by Beard and his associates for the virus of fowl myeloblastosis, which also is present in high concentration in the blood of infected fowls, and which can be separated from the blood in practically pure form by the simple procedure of differential centrifugation. This development makes it possible, and work has already been initiated to produce relatively large quantities of highly purified virus for chemical, physical and biological studies, as well as for large scale use in producing leukemic animals for chemotherapy research and screening, and for studies on vaccine production.

(2) The consistency of the association between biological activity and the presence of virus particles in electron micrographs has added a valuable tool not only for studying the pathogenesis of the disease in animals (e.g., prior to the appearance of lesions), but also has opened a door to the human problem by providing a fairly simple and practical procedure for the potential detection and isolation

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of human leukemia viruses, if such exist, comparable to this murine virus.

Using improved lead staining procedures in his electron micrographic studies, Dr. Dalton has found the mature Moloney virus particle to measure 97.5 millimicrons in diameter. The particle is bound by a double limiting membrane, the inner measurement of which is 8 millimicrons in diameter. A central, more electron dense nucleoid measures 65 millimicrons in diameter. Similar studies and determinations of particle size were made under identical conditions on four additional murine leukemia agents. Thin section studies of two separate radiation induced leukemias of mice showed virus particles similar to those of viral leukemia in one but not in the other. Characteristic particles were also found in the megakaryocytes of bone marrow of leukemic mice of two different strains which develop the disease spontaneously. In all cases in which the particles were observed, they were found budding from the plasma membrane of malignant lymphoblasts or from intracellular membranes of megakaryocytes.

Dr. Merwin has used the transplantable S 37 mouse tumor known to carry the Moloney virus (originally isolated from S 37) to test her hypothesis that viruses in tumor tissue placed inside Algire diffusion chambers (which retain cells but allow diffusion of virus), and then placed intraperitoneally in test mice, might be protected from host resistance and therefore have a better chance to adapt to a new host. Since virus would continue to be produced by the living tumor cells within the chamber it might be possible to detect virus in tumors which, at a given time, is too low in concentration to be detected by other methods. Dr. Merwin's experiments are still in progress but a fairly high incidence (about 20%) of leukemia and Hodgkins-like lesions have already developed in the BALB/c hosts. These tumors come up much earlier, and are to be distinguished from the sarcomas and plasma cell tumors which sometimes result from the implantation of the blank chambers alone. Similar studies with other virus-associated tumors of mice are under way. Dr. Merwin now plans to use this technique to test human tumor for their ability to induce characteristic lesions in mice.

Dr. Manaker has previously reported the successful propagation of the Moloney virus in primary tissue cultures of cells derived from mouse spleen. The cells which grew out under the conditions employed, and which supported growth of the virus, were amoeboid cells, probably of the reticular or macrophage type. Dr. Manaker has now succeeded in establishing a continuous line of these cells in tissue culture and has found them to be suitable for propagating the Moloney virus. This makes possible the large scale *in vitro* production of the Moloney virus in certified cell lines, without danger of infection with extraneous viruses as might occur when primary cultures prepared from many mouse spleens are used.

b. Manaker (C–60) virus: Dr. Manaker has isolated a new strain of mouse leukemia virus which differs in certain electron micrographic and biological properties from other murine leukemia viruses previously described. The isolate was picked up in tissue culture studies in which attempts were being made to propagate the elusive Schoolman-Schwartz agent isolated from a Swiss mouse leukemia. Whereas the latter agent induces a solitary mesenteric tumor nodule as the characteristic lesion, the new strain isolated by Dr. Manaker produces a generalized leukemia. Another difference is that active virus could not be extracted from the mesenteric tumor nodule by Schoolman and Schwartz and their associates, but only from the brains of mice bearing the induced tumor. Dr. Manaker's virus on the other hand, can be readily extracted from all involved tissues of the generalized leukemia. Since the Schoolman-Schwartz agent is in the background of the picture, Dr. Manaker concludes that his virus is a derivative, or strain of that agent. The possibility remains, however, that the two are not related and that Dr. Manaker has discovered a new virus in the growing family group of murine leukemia viruses. Extensive serological and immunological studies will be required to answer this question.

The Manaker virus reaches a high concentration in leukemic tissues, comparable to the Moloney virus. Dr. Manaker has made extensive studies on the character of his virus and has now established it as a new laboratory model available for the study of neoplasia.

c. Rauscher virus: Dr. Rauscher has also discovered a new murine leukemia virus (designated as R–NCI–2) which differs dramatically from any hitherto described. Again, the Schoolman-

Schwartz agent was in the background. Experience with that agent had shown that most experimental attempts to pass it by means of cell-free filtrates fail, and only occasionally was success achieved. When successful, the tumors appeared within 3 or 4 weeks, or not at all.

In a large scale attempt to get at the factors associated with the elusiveness of the Schoolman-Schwartz agent, Dr. Rauscher inoculated a total of 508 test mice. None of them developed lesions within the 3 to 4 week period. However, after 11 weeks one of this number developed a solid subcutaneous tumor at the site of needle penetration of the skin. This has not previously occurred with the Schoolman-Schwartz agent. The solid tumor was carried by transplantation through 9 passages in BALB/c mice and attempts were then made to isolate a virus. A virus was isolated which, when inoculated into suckling mice, causes a tremendous enlargement of the spleen, up to 60 times the size of normal spleens, within a period of about 15 days. Death results within about 35 days. Sections of such spleens show large numbers of nucleated red cells as well as undifferentiated cells of the white cell series. Peripheral blood counts show 28,000 to 250,000 nucleated cells per cc. Anemia is not present. Depending on the conditions of the experiment (dose, age at inoculation, etc.), some mice do not develop the initial spleen enlargement, and others which do develop large spleens survive beyond the 35-day period. In such animals a second syndrome appears which includes all of the manifestations of generalized leukemia, including thymic and peripheral lymph node enlargement. This second syndrome is followed by a second mortality peak. Virus isolated from animals which develop the early syndrome only and die early, or virus from animals which develop only the later syndrome (typical leukemia) and die after several months, is in both instances still capable of inducing both types of response. It appears therefore that both diseases are produced by one virus.

Dr. Thelma Dunn is studying the pathogenesis of this agent and has determined the second syndrome as a true leukemia, probably originating in the thymus. It has been tentatively classified by Dr. Dunn as a stem-cell leukemia, and is distinct from any other murine viral leukemia heretofore reported.

The early enlargement of the spleen grossly resembles the Friend virus disease. However, the early syndrome produced by the Rauscher virus differs from the Friend disease in the following important respects: (1) the high incidence of true leukemia (80–100%) in mice which survive early death due to erythrolastosis; (2) the early and continuing presence of greatly elevated levels of nucleated cells in the peripheral blood; (3) ED50 end titers up to 10^{-6} and 10^{-7} within 70 to 90 days; (4) the relative ease with which fragments of spleen, thymus and lymph nodes give rise to solid tumors at localized sites on transplantation, (5) the high susceptibility of mice of many different strains (the Friend virus grows in only 2 strains); (6) the early severe viremia of infected mice; and (7) clear differences in histopathology of the two diseases.

The virus also differs antigenically from both the Friend and the Moloney viruses, since antisera against neither will neutralize it.

The Rauscher virus will infect adult, as well as suckling mice and thus far, 9 different strains or crosses of inbred mice have been found to be susceptible to it.

The rapidity of appearance of palpable lesions (enlarged spleens), that is, within 5 to 15 days, makes bioassay of this virus much more practical, and less costly than for any other murine leukemia virus (which require several to many months). Chemotherapeutic studies on tumor viruses are also more practical with this agent, and collaborative studies with Drs. Chirigos and Boldin have already been initiated.

Another new and somewhat strange member has been added to the family of murine leukemia viruses.

d. Breyere-Moloney virus: The isolation of a new virus which produced a generalized leukemia in BALB/c mice was reported last year by Doctors Breyere and Moloney. The biological activity of this virus has been enhanced during the past year by serial selective passages and further studies have been carried out on animal susceptibility. Both C3H mice and Osborne-Mendel rats have been shown to be susceptible to this agent.

2. Polyoma Virus

Dr. Sarah Stewart is continuing her studies on the polyoma virus which she isolated in collaboration with Dr. Bernice Eddy several years ago.

Since this virus is the only known agent which has the combined properties of producing tumors, causing cell lysis, and producing hemagglutination of red blood cells, Dr. Stewart has been attempting to determine whether all of the properties are possessed by a single virus particle, or whether more than one type of particle may be involved.

In collaborative studies, Dr. Robert Cramer of the Pasteur Institute, Paris, has separated Dr. Stewart's virus preparations into various electrophoretic fractions and return them to Dr. Stewart for further study. Although earlier work on this project previously reported showed that the virus could be separated from inhibitors of the hemagglutinating property by zone-electrophoresis, the continued studies have produced no evidence to date that more than one type of viral particle is involved in the three reactions, but the data are not yet conclusive.

In other collaborative studies with Dr. M. Ida of the M. D. Anderson Hospital, Houston, the ability of the polyoma virus to cross placental barriers in mice and hamsters is being investigated. Offspring of mothers which had been inoculated at various stages of gestation were taken by caesarian section and raised by foster nursing on non-infected mothers. At the end of 12 months, surviving test mice were divided into 2 equal lots and one lot was treated with 150 r of total body radiation for 4 successive times at 3-day intervals. The other lot was not given any additional treatment. None of the offspring of infected mothers, neither mice nor hamsters, developed tumors of the polyoma virus spectrum, indicating that the polyoma virus does not pass the placental barrier, at least in sufficient amounts to induce neoplasia. Antibody titers for the detection of subclinical infection with the polyoma virus could not be studied simultaneously in this experiment due to the lack of isolation facilities and the presence of other infected animals in the same room.

Dr. Stewart's assistant, Mr. John Landon, is studying the influence of fluorocarbon on lung tumor induction in hamsters by polyoma virus. Fluorocarbon was introduced intravenously into 18-day-old hamsters and two days later they were inoculated intratracheally with polyoma virus. Control animals received only fluorocarbon intravenously or only polyoma virus intratracheally. Several of the animals which received polyoma virus only developed lung tumors, but many died from liver hemangiomas. The animals which were inoculated with both fluorocarbon and polyoma virus had many more lung tumors which attained a larger size. Many of them were squamous cell carcinomas. No lesions were produced by fluorocarbon alone.

Mr. M. B. Melroy continues to perform the valuable service of running an extensive program for testing for the presence of polyoma virus antibody in mice used for experimentation in tumor-virus research. This service is under the general supervision of Dr. Fink.

3. Rous Sarcoma Virus

Doctors Rauscher and Fink are continuing their studies using the Rous sarcoma virus model in attempts to develop more sensitive methods for the detection of tumor virus and, perhaps, making possible the detection of virus in tumors not now known to be virus induced, including human tumors. That the use of Freund's adjuvant in association with virus inoculation enhances the response to Rous sarcoma virus was reported last year, and this technique was introduced into the screening program for the detection of human viruses by the use of newborn mice (see section on human studies (B)). The studies with Rous sarcoma virus are now nearing completion and it has been established that: (1) with test chickens pretreated with Freund's adjuvant before inoculation of the virus, large granulomas appear within 4 to 5 days. Inoculation into the granulomas of very small doses of Rous sarcoma virus (e.g. 0.01 ED50) which ordinarily do not produce tumors or result in recoverable virus from inoculated hosts, caused the granulomatous lesions to grow rapidly, become tumorous, and invade surrounding tissues. Virus was recoverable from such lesions in relatively high concentration (10^2 to 10^4 ED50 per gram). The technique therefore permitted propagation and recovery of virus in fairly high yields from subliminal doses, which under ordinary conditions would not have led to tumor formation or recoverable virus.

When the adjuvant was injected at a later date into chickens which had previously received, but failed to respond to subliminal doses of the virus

by developing tumors, relatively high titers of antibody against the virus appeared. Thus, it was possible with this technique to establish that chickens were infected with subclinical doses of Rous sarcoma virus, although tumors did not appear within the usual latent period (35 days). Whether tumors would appear after prolonged periods (e.g. many months) remains to be determined. This is an important question since it involves the possibility that Freund's adjuvant may, in time, activate latent infections with subclinical doses of virus.

As reported last year, the Rous sarcoma virus could be propagated serially in post-hatched Japanese quail, but not on the chorioallantoic membrane of the quail eggs. These studies, in collaboration with Dr. James A. Reyniers, have been continued and it has now been found that after ten passages in post-hatched quail, a "quail adapted" strain of Rous sarcoma virus has been established which can be propagated on the chorioallantoic membrance of embryonated quail eggs. During twelve passages in quail eggs there was a gradual increase in the ability to grow in quail eggs, with a concomitant decrease, and finally complete loss, of the ability to grow also on the chorioallantoic membrane of embryonated chicken eggs. Thus a new, quail-egg adapted, strain of the Rous sarcoma virus has been established which will permit more extensive and more economical studies on host-virus interaction in miniature fowl hosts.

4. OTHER VIRUSES IMPORTANT TO TUMOR VIRUS RESEARCH

a. Indigenous viruses

(1) Ziegel Agents: Dr. Ziegel reported last year the discovery of a virus-like agent which he found budding from acinar cells of the pancreas in apparently normal chickens. The particles had all of the characteristics, including their formation by budding from plasma membranes, of the known tumor viruses in both fowls and mice. It appeared therefore that Dr. Zeigel's agent might be a latent tumor virus, possibly the lymphomatosis virus which is known to be present, at least as a subclinical infection, in most strains of chickens. Dr. Ziegel has carried his electromicroscopic studies further and, in collaboration with Dr. Rauscher who is making parallel biological studies, has found his agent budding from pancreatic cell in chickens from flocks known to be infected with lymphomatosis virus (from antibody studies), whereas he has yet to observe this phenomenon in chickens of a strain obtained from Dr. B. R. Burmester (U.S.D.A., East Lansing, Michigan) which has a very low incidence of the disease. By studying the offspring of hens showing serological evidence of infection with lymphomatosis virus, Dr. Zeigel has obtained additional evidence that his agent may be identical with the lymphomatosis virus. If this is the case, then Dr. Zeigel has discovered a very important, probably the most essential, site of replication of this virus in host tissues and has opened the door to early pathogenesis studies on this important disease of fowls. In addition to the possibility of controlling this very important economical problem through greater knowledge of pathogenesis of the etiological agent, the understanding of this disease in chickens will contribute greatly also to the understanding of viral neoplasia in general. With the recent introduction of *in vitro* methods for quantitative assay of the lymphomatosis virus by Rubin (based upon interference with the Rous sarcoma virus) this fowl tumor virus model has become one of the most important laboratory models for studying the natural history and pathogenesis of tumor viruses.

(2) Kilham ("K") Virus: Dr. Ziegel is also carrying out electronmicrographic studies in collaboration with Dr. Dalton on the "K" virus of Kilham, which is indigenous in certain strains of mice. He has found that the ultrastructure of this agent is similar to and suggests at least a morphological relationship to the polyoma virus.

(3) Manaker Hepatitis Virus: Dr. Manaker has completed his studies on characterization of the indigenous hepatitis virus of BALB/c mice which he isolated in 1959 and reported upon last year. The general characteristics of this virus (designated MHV–59) indicate membership in the "hepatoencephalitis group" of viruses. Serological tests suggest a close relationship to one member of this group. The virus was found to produce widely disseminated inapparent infection in experimental mice. Precise quantitative methods for *in vitro* assays of the virus, and serum neutralization methods for the detection of antibody developed during the course of these studies

should aid in the detection of this contaminating virus and lead to its elimination from defined colonies of experimental mice.

B. Viruses in Human Neoplasias

1. HUMAN CANCEROUS TISSUES

a. Specimens from surgical operations: Cancerous tissues of various types removed at surgical operation are obtained through the cooperation of the U. S. P. H. S. Hospitals at Baltimore, Md., and Staten Island, N.Y. The specimens are frozen immediately upon removal and stored in low temperature chests (−50° C. or below) until used. For study, the specimens are homogenized and extracted in citrate buffer and the "microsome" fraction separated and concentrated by differential ultracentrifugation. Aliquots of this fraction, which would be expected to contain any viruses present, are used for a variety of procedures designed to demonstrate the presence of viruses and to propagate them in the laboratory. These procedures include: (1) Direct examination of the primary fraction under an electron microscope (2) inoculations into various human and other cell lines in tissue culture; (3) inoculations into newborn mice; (4) inoculations onto chorioallantoic membranes of embryonated chicken and Japanese quail eggs; (5) tests for ability to interfere with known viruses when inoculated into mice and embryonated eggs; (6) extraction of nucleic acid fractions and testing them in animal and tissue culture systems; (7) similar secondary tests including all of the above (1–6) on fluids and cells from tissue cultures which received the primary inoculations.

The following investigators are participating in these joint studies; Doctors Dalton, Dunn, Fink, Manaker, Moloney, Rauscher, S. Stewart, and Zeigel, with the assistance of Miss Calnan, Mr. Kvedar, and Miss Valentine.

Ten experiments initiated in calendar year 1960 have reached the critical observation period during the current year. They involved the following human tumor specimens: 4 carcinomas of the stomach, 3 carcinomas of the breast, 1 carcinoma of the colon, 1 carcinoma of the lung, and 1 sarcoma of the pelvis.

In one of these experiments, involving a carcinoma of the stomach, 21.5 percent of the mice injected with the microsome fraction when newborn have developed neoplasms, whereas none of the control mice in the same experiment have developed such lesions by the 13th month of the observation period. Of 11 tumorous animals out of a total of 51 inoculated in the test groups, 9 have developed bilateral kidney carcinomas, a type of cancer which has never been observed to occur spontaneously in the strain of mouse employed, i.e., BALB/c. Significant also is the fact that such tumors have been observed to occur only very rarely among laboratory mice of all strains. In addition to the kidney tumors, several of the mice also had primary tumors of the pancreas and liver. The mice have been tested and shown to be free of polyoma virus infection. (Kidney *carcinomas* have not been observed with the polyoma virus.)

The studies involving newborn mice are under the direction of Dr. Fink. In one of the two test groups which received the microsome fraction of the gastric carcinoma, Freund's adjuvant was added to the inoculums by Dr. Fink. The tumors appeared several weeks earlier in this group than in the group receiving straight microsome fraction (see also studies on animal systems, under *Rous sarcoma*). (A(3)). No tumors have occurred with adjuvant alone, with buffer alone, or among the uninoculated controls.

These results confirm those of 5 other laboratories which have reported the induction of tumors in mice injected neonatally with extracts or fractions of human tumors. They differ from the results of others, however, in that the predoninant type of tumor induced was not one which sometimes occurs spontaneously (with low frequency) in the strain of test mice employed.

No significant results were obtained in the other 9 experiments of this series. Although tumors of the types that BALB/c mice develop spontaneously with low frequency as they grow old (e.g. lung tumors and leukemia) are now appearing among the older mice (14 to 16 months) in several of the experiments, such tumors occur with equal frequency among both test and control mice.

The positive results reported from other laboratories have been interpreted either as: (1) the induction of tumors in mice by the replication and direct action of a human viral agent, or (2) the enhancement, or activation of a latent mouse tumor virus by some factor in human tumors, prob-

ably viral in nature. In either event, the possibility exists that newborn mice may serve as a valuable biological indicator for the detection of human tumor viruses. The present series of experiments was for the purpose of further investigating this possibility.

Even a single positive experiment among the 10 which have been carried out justifies a more extensive investigation of this potential test system. Such studies have already been initiated under a contract with a private industry.

No other evidence of a virus associated with the human specimens has been obtained with the other methods employed in this series of experiments.

b. Specimens derived at autopsy: A single large experiment involving a total of 300 BALB/c mice (50 in each of 3 test and 3 control groups) was carried out in an attempt to repeat the studies of Schwartz et al. in which it was reported that microsome fractions of brain removed at autopsy from human leukemia subjects were capable of inducing leukemia in test mice. The strain of mouse used by Schwartz and associates was Swiss albino.

The methods employed in the present study were the same as described by Schwartz (Blood 15: 758–760, 1960), except that a different strain of mice (BALB/c) was used.

No leukemias were induced in BALB/c mice within a period of a few weeks as reported by Schwartz et al. for Swiss mice, and no significant tumor induction has occurred among mice of this experiment within a 12 month observation period.

2. Human Leukemic Blood and Bone Marrow

As a result of the significant findings with a murine leukemia virus model and the development of practical methods for the examination of blood and bone marrow described in another section of this report (*Murine leukemias*, Moloney virus (II(A)), Doctors Dalton and Moloney carried out exploratory studies on human leukemic specimens using the techniques worked out with the animal system. Briefly, the method consisted in the fractionation of citrated blood, or of bone marrow homogenates in citrate buffer, by differential centrifugation and concentration of the "microsome" fraction by ultracentrifugation. Replicate pellets of the concentrated materials were then fixed for thin-section electron microscopy, used for inoculation into newborn mice, or stored at low temperature for future use.

The experiments have been in progress for only 5 months, and none of the test mice have as yet developed neoplasms.

Out of a total of 14 human leukemia cases studied thus far, 11 specimens have revealed virus-like particles under the electron microscope. In some of these cases a careful search had to be made in order to find an occasional characteristic particle, but in several of the specimens many particles having a morphology similar to murine and fowl leukemia virus particles, could be seen within a single field under the electron microscope. Other replicate pellet suspensions from such cases were turned over to the other members of the laboratory who are collaborating on this problem, for attempts to propagate the agent in the laboratory. In particular, efforts to propagate a virus in various types of human cell lines in tissue culture are being made by Doctors Manaker and Stewart.

It should be emphasized that the finding of viral particles does not necessarily indicate that they are etiologically related to the human neoplastic disease, since they could represent extraneous, or contaminating "passenger" viruses. For determining this possibility it will be necessary to propagate each such virus isolate in relatively large quantities in the laboratory so that extensive serological, immunological and biological studies can be carried out for identifying or characterizing it. Looking toward this end a viral diagnostic laboratory, as well as other "service" laboratories, have been set up on the Bethesda campus, and off-campus facilities for producing and testing large quantities of virus have been activated through contracts with private industry. In addition to virus production for characterization studies, exploratory studies on vaccine production will also be undertaken in the off-campus facilities.

The suggestive preliminary findings have also stimulated a closer collaboration between the laboratory and clinical branches of the N.C.I., and arrangements have been made through Dr. Emil Frei for the obtaining of an abundance of clinical specimens. Dr. George Porter is acting as liaison between the laboratory and clinical groups, and is

participating in both the laboratory and clinical research.

Specimens are also being received from field cases of leukemia, particularly from such "cluster" areas as Niles, Ill., through the cooperation of the Epidemiology Branch of the Field Studies Division. Doctors L. Zelkowitz and Donald Armstrong of this Branch have been collaborating with Dr. Moloney, Dr. Stewart and Mr. Culler in the procurement of field specimens.

3. Other Human Lesions

Another type of human disease being investigated by Dr. Sarah Stewart with respect to a possible viral etiology is the Histiocytosis X group of diseases. The gross pathological manifestations of the proliferative stages of these diseases are sometimes confused with early neoplastic lesions, particularly of the bone. Dr. Stewart received a biopsy specimen from a case of eosinophilic granuloma in a 20-month child, with the provisional diagnosis (before frozen section histological diagnosis) of osteosarcoma. Dr. Stewart inoculated tissue cultures with the biopsy material and was successful in propagating a filterable agent which appears to be a virus. Further efforts to characterize it are in progress. The agent produces cytopathogenic changes in cells in tissue culture, and subcutaneous granulomatous lesions and hydrocephalus when innoculated into newborn hamsters. There is no proof as yet that the agent which has been isolated is related etiologically to the human disease, but through collaboration with Dr. Robert Andrews and other members of the staff of the Radiation Branch of NCI, other cases of the histiocytosis X group of diseases are being admitted to the Clinical Center for further attempts to isolate similar agents and determine their relationship to etiology.

II. Tissue Culture

A. General

The outstanding contributions of members of the Tissue Culture Section to the field of tissue culture, both as a method of approach and as a new branch of cellular biology, are well known. The work of this section continues to be of the same high quality and step-wise progress continues to be made on several difficult problems of importance to cancer research that have been embraced during recent years.

The use of short-term tissue cultures in various branches of biomedical research and routine laboratory testing, particularly in virology, is now so extensive and so commonplace that, to many, the term "tissue culture" has come to represent a method rather than a field of research. The ultimate goals of achieving truly *long-term* maintenance of mammalian cells *in vitro*, while preserving their normal states and physiological properties so that life processes may be investigated at the intracellular level, requires continuing basic research on cell biology and on factors associated with cell homeostasis. It is such studies, directed toward such goals, with which the research of the Tissue Culture Section is primarily concerned. Perhaps the time has come when this field of endeavour should be given some other label, e.g., *In Vitro Cell Biology*.

B. In vitro Cell Biology Studies

1. Nutritional and Metabolic. Doctors Evans, Sanford, Westfall, and Bryant and their associates are continuing their studies on the nutritional requirements of cells of various types and species of origin. A completely chemically defined, protein free, culture medium which sustains the growth of several lines of cells was reported last year. Several additional cell lines have been adapted to the completely defined medium during the past year, of particular importance being two sub-lines of Gey's HeLa strain of cervical adenocarcinoma which is so widely used in cancer and virus research. Further extensive studies have been made on the activity of the vitamins and reducing agents in the medium. These studies led to the first clear-cut demonstration that biotin is required for survival of mammalian tissue cells *in vitro*. Also, the addition of serum protein to an otherwise chemically defined, but controlled vitamin and amino acid deficient. medium was found to prevent development and manifestation of certain vitamin and amino acid deficiencies. It appears therefore that serum protein contributes appreciable amounts of vitamins and amino acids to cells. By supplying appropriate quantities of such required components, a further improvement has been made in the completely chemically defined, protein free, medium but some lines of cells still cannot be adapted to it. Much work still needs to be done in the development of this important problem. Of particular impor-

tance, and now being emphasized, is the adaptation of stable lines of cells which support virus propagation, for eventual use in the mass production of vaccines for human use. So long as animal products such as serum have to be used, the danger always exists of bringing in contaminating viruses which may be present in serum. Protein free media which support the growth of cells and the propagation of viruses have the same significance to virology, therefore, that sterile media in general have to bacteriology. Rhesus monkey kidney cells have already been adapted to chemically defined, protein free media, and an encouraging start has already been made (through 3 culture generations) with "green monkey" kidney cells.

Metabolic studies by Doctors Westfall, Evans, Sanford, and Bryant and their associates are being continued with respect both to what cells take out of culture media and what they put back that was not there before, or was there in different quantity. These basic studies underlie a number of fundamental problems, such as (a) the further improvement of media (e.g., it was partly upon the basis of such studies that successful media were devised); (b) identification of metabolic pathways and intracellular mechanisms that may be involved in neoplastic transformation *in vitro;* (c) designing of counter measures which may aid in combating the neoplastic process; (d) understanding of normal life processes at the biochemical level.

The appearance of extremely high amounts of the enzyme arginase in a clone derived from a cell strain which showed only barely detectable amounts of this enzyme was reported last year. Another clone showed a moderate increase in this enzyme. These findings have been found to be characteristic of the respective clones in further investigations, and sufficiently stable under constant tissue culture conditions to serve as chemical markers for a new type of study involving the possibility of transfer of genetic material between mammalian cells, i.e. "hybridization", *in vitro.* Such studies are now in progress in this and other laboratories who have been supplied with the characteristic clones.

Other results of interest were the findings in collaboration with Dr. Kuff (Lab. of Biochemistry) of a higher B-glucuronidase activity in freshly explanted mouse liver cells than in original liver from which the explants were derived. Another interesting aspect of this study was that the enzyme activity was considerably influenced by the medium used for cultivation of the cells.

These alterations in enzyme activity illustrate the fact that "metabolic transformations" occur in tissue culture as well as certain other types of transformations that have long been known to occur i.e., malignant transformation, and chromosomal changes.

2. Malignant Transformation in Vitro. Doctors Sanford, Evans, Westfall, and Earle and their associates are continuing their studies of many years duration on the malignant transformation of cells *in vitro.* Every long-term strain of C_3H mouse cells established from normal tissue and maintained in the laboratory has in the past, when adequately tested by injection into mice of the strain of origin, demonstrated a capacity to give rise to malignant neoplasms. Such lines had at some time in their life been grown in media containing foreign proteins such as those of embryo extract and serum. It therefore seemed of importance to determine whether cell lines established and maintained in chemically defined, protein free, media would also show such transformation. Parallel lines of cells of the same origin were therefore set up in protein-free media and in media containing sera. The cells grown in media containing serum showed ability to give rise to sarcomas after 124 days *in vitro.* On the other hand the cells in protein-free media have not yet shown such capacity, after growth for 21 months *in vitro.* It remains to be determined whether they will eventually undergo malignant transformation or whether they can be continued indefinitely in protein-free media without undergoing malignant change. Additional studies for this purpose are in progress.

C. Instrumentation and Methods

An important technical development during the past year has been the working out of a successful method by Doctors Evans and Bryant for freezing and storage of certain cell lines maintained in protein-free, chemically defined media. This is of importance to a large national program now engaged in efforts to characterize and standardize various lines of cells for use in basic studies on

cancer, growth of specific viruses, etc. The procedure requires only the addition of glycerine, a chemically defined ingredient. Success is critically related to speed of freezing and thawing, the concentration of glycerine as well as to the rate at which the glycerine is removed from the cells after thawing. Storage is at −180° C, in a liquid nitrogen refrigerator.

Another significant development in the technical area has been the demonstration by Mr. McQuilkin and Dr. Earle of the value and potentialities of the time-lapse cinemicrographic analysis of cell populations *in vitro*. The basic apparatus has been under test for several years and methods have now been worked out for the measurement of various cellular events, including cell growth, migration, adhesiveness to glass substrate, timing of events in mitotic cycle, etc. A tremendous amount of time has been required to transform the basic data on film to measured parameters which can be tabulated and further analysed. Dr. Earle and Mr. McQuilkin are now looking into the possible utilization of modern electronic scanning devices which may be capable to transforming film data directly to coded information which can be handled by electronic computers. Such a possibility would make feasible the exploration and study of intracellular life processes not now approachable with visual and manual measurement and correlative procedures.

Studies on the instrumentation and methods for growth of mammalian cells in large batch lots are being continued by Doctors Earle and Bryant. These include suspension cultures of various sizes as well as continuous-flow type of apparatus which permit exchange of culture fluids without disturbance of cells during continuous operation.

III. Other Areas of Investigation

(*A*) *Carcinogenesis*

1. Diffusion-chamber Induction of Plasma-Cell Tumors: Dr. Merwin reported last year that plasma cell tumors had been induced in mice in which blank diffusion chambers (i.e., chambers containing no living tumor or other tissue) had been placed in their peritoneal cavities. This was a follow-up study of an earlier observation that such tumors arose in mice bearing intraperitoneal chambers containing various types of tumor and other tissues.

Additional studies have been carried out to test: (1) blank chambers of different sizes; (2) the separate plexiglas disks, membranes, and other parts of which the chambers are composed; and (3) borings of different sizes of the plexiglas component of the chambers.

It was found that: (1) Large chambers induce more plasma-cell tumors than smaller ones; (2) Discs of the three chamber materials (i.e., plexiglas, millipore filters, and the cement used in assembly) induced plasma cell tumors whereas small pieces of plexiglas in several forms do not; (3) plexiglas borings of relatively larger size induced many plasma cell tumors however. A correlation was found between the induction of plasma-cell tumors and the development of fibrosis around the foreign materials. The factors associated with the induction of plasma-cell tumors by these materials appear to be similar to those reported by Oppenheimer and others for the induction of sarcoma in rats by cellophane and other plastics.

When a certain type of tumer tissue (a sarcoma of a BALB/c mouse) was placed inside the chambers the incidence of plasma cell tumors was increased considerably over that obtained with blank chambers alone. Another type of tumor tissue (isologous mammary tumor) placed within the chambers increased the incidence of sarcomas of the type occasionally induced by blank chambers.

2. Ultrastructure of Plasma-Cell Tumors: In collaboration with Doctors Merwin and Potter (Lab. of Biology), Dr. Dalton has carried out extensive studies on the ultrastructure of plasma-cell tumors of mice as well as upon the human counterpart of such tumors, namely multiple myeloma. Comparative studies were also made on normal plasma cells and on 6 fibrosarcomas of mice.

Normal plasma cells of the mouse were characterized by a wide range, from cell to cell, in size of the cisternae of the ergastoplasm. This wide range was not noted in neoplastic plasma cells. Neoplastic plasma cells of the mouse regularly contained doughnut shaped (Type A) virus-like particles in the cisternae of the ergastoplasm. The particles were found to originate by budding

from the endoplasmic reticulum. They were not observed in normal plasma cells nor in multiple myeloma cells in man. The particles resemble the immature form of certain known viruses but they are not considered by Dr. Dalton to represent etiological agents responsible for the induction of plasma cell tumors of mice since extensive efforts by Dr. Merwin to transmit the neoplasm by measures that have been successful with other murine tumor viruses failed.

The malignant cells of multiple myeloma were observed to be similar to the cells of plasma cell tumors of mice in ultrastructure, except that they did not contain the characteristic "A" type particles.

No correlation could be established between variations in detail of fine structure of cells of plasma cell tumors of the mouse and the type of serum globulin produced.

3. Transmission of Hamster Tumors by Feeding of Tumor Cells: A previous report by Brindley and Banfield (J.N.C.I. 26: 949, 1961) showed that a reticulum-cell sarcoma of the hamster could be transmitted to other hamsters by feeding. Submucosal tumors appeared in the larynx and pharynx of recipients. Dr. Stewart has repeated these studies with three different transplantable tumors of hamsters which arose during the course of other investigations. Two of the three tumors were transmissible by the feeding of intact tumor cells. One of them, a leukemia, produced generalized leukemia within 8 to 12 weeks after being fed. The other, a lymphosarcoma produced laryngeal tumors of the same type 4–8 weeks after being fed. A myxosarcoma showed no evidence of transmissibility on feeding. Tumors have thus far failed to appear in hamsters inoculated with cell-free filtrates of these tumors or with fluids from tissue culture explants of them.

B. Immunology and Serology

Except for the polyoma virus, which differs in immunological and biological properties from other known tumor viruses of animals, the usual serological and immunological methods for diagnosis and quantitative assay of viruses of infectious diseases have not been readily applicable to the tumor viruses. Dr. Fink has spent a considerable amount of effort during the past year in attempts to develop methods applicable to tumor viruses, using the Rous sarcoma and Moloney leukemia viruses as laboratory models.

Little success has beeen achieved thus far with the Moloney virus, but substantial progress has been made with the Rous sarcoma virus model. Using the "high potency" preparations of Rous sarcoma virus now available, Dr. Fink has been able to prepare strong heterologous antisera against this virus in rabbits. The ability of the antisera to neutralize the virus persisted after absorption of the serum with sheep erythrocytes (to remove antibody against Forssman antigen) and with normal chicken tissues (to remove antibody against normal chicken antigens). This finding strongly supports the conclusions that the Rous sarcoma virus does not contain the Forssman antigen, or normal chicken antigens as essential components. The absorbed heterologous antisera also fixed complement specifically with Rous sarcoma virus, in a titer of 1: 80. This achievement now make possible the use of the complement fixation reaction for diagnosing and titrating the virus.

It has long been known that antisera induced in chickens fail, in general, to fix complement, due to some pecularity of the chicken. Dr. Fink also failed to demonstrate complement fixation with the Rous sarcoma virus when whole antisera of chickens were used. Such antisera were capable of neutralizing the biological activity of Rous sarcoma virus however. In the further investigation of chicken antisera, Dr. Fink has found that the gamma globulin fraction of chicken antiserum against the Rous sarcoma virus separated by ammonium sulfate precipitation, will fix complement while the whole antiserum will not. It is probable that this successful accomplishment with homologous sera will permit the development of complement fixation tests which can be used for detecting antibody in epidemology type studies in chickens.

In other studies on methodology, Doctors Zeigel, Fink and Kuff (Lab. of Biochemistry) are collaborating in attempts to apply the technique of conjugating antibody with ferritin so that it can be visualized under the electron microscope. Examinations of such ferritin labeled antibodies

under the electron microscope have revealed complexes not seen with ferritin control preparations, indicating that conjugation, as reported by others, may have been successfully achieved. Serological studies to confirm this are in progress.

C. Radiation

In continuation of studies on tissue growth by means of light microscopy through the transparent chambers developed by Algire, Dr. Merwin has determined the influence of proliferating normal endothelial cells on the growth of x-irradiated transplants of tumor tissue and of the healing of wounds in irradiated areas within transparent chambers. Previous studies had shown that irradiated tumor failed to grow, and that healing was delayed in irradiated areas.

It was found that scattered foci of endothelium still capable of growing were present within irradiated tumors. Such growing endothelium supported the growth of surrounding tumor at a normal rate, but there was no growth of capillaries into adjacent areas which had become anoxic due to inadequate vascular supply. The introduction of normal, unirradiated endothelium into irradiated areas of wounds hastened the healing process. Further studies are necessary to determine whether the introduction of normal endothelium would be effective in the treatment of wounds, particularly surgical wounds, in irradiated areas.

NATIONAL HEART INSTITUTE

INTRODUCTION

As in the past, this review of intramural research in the Heart Institute consists of the assembled reports of the leaders of its major research groups. It will be apparent to the reader that it describes work of considerable breadth, depth and variety. We hope that it will also be apparent that is describes work of considerable scientific merit.

Note should be made of certain organizational changes which are reflected in this report and others which will be involved only in the next. This year for the fiirst time the group in Experimental Therapeutics, Clinical Endocrinology and Cardiology report as separate branches. This in reality constitutes recognition of an autonomy under which these groups have operated for several years. In the coming year it is anticipated that the Laboratory of Chemistry of Natural Products, the head of which left the NIH this year, will be abolished. A segment of its functions will be continued by a section to be incorporated into another laboratory. The impending departure of the head of the Laboratory of Cellular Physiology and Metabolism will be a serious loss to the scientific leadership of the Institute. The solution to the problem it creates has not yet been determined. However, it is anticipated that the Sections on Enzymes and Metabolism will be established as separate laboratories in recognition of the stature and independence of these groups.

LABORATORY OF CELLULAR PHYSIOLOGY AND METABOLISM

Section on Cellular Physiology

The program of the Laboratory of Cellular Physiology and Metabolism, Section on Cellular Physiology, has continued in the direction of investigating the structure, synthesis, function and genetic control of protein molecules. Experimental results over the past year have reinforced earlier conclusions that the three-dimensional structure of proteins is derived directly from their primary amino acid sequence. Thus globular molecules, previously converted to random chains in solution by reduction of disulfied bridges, are regenerated to the native enzyme upon reoxidation of the SH groups in spite of the extremely improbably occurrence of correct pairing of the SH groups in disulfide linkage. With the enzyme ribonuclease, for example, 105 forms of the protein, containing 4 SS bridges, are possible when its 8 half-cystine residues are oxidized in a random fashion. Nevertheless, theoretically maximum yields of native enzyme can be obtained by a spontaneous reaction.

In addition to studies specifically directed at protein molecules themselves, a number of investigations, reported below, have been carried out on more biological aspects of biochemistry, including such processes as electron transport in metabolic systems, the biochemistry and cytology of cell transport, and the mechanisms involved in the transport of fat.

Optimum conditions have been worked out for the formation of native ribonuclease from the reduced molecule containing 8 SH groups. These conditions, involving the use of low protein concentrations at a pH of 8.0 lead reproducibly to full regeneration of activity. The kinetics of the reoxidation process have been examined in detail and an interesting lag period has been observed during which no activity appears in spite of considerable disulfide bridge formation and the assumption of complex internal structure. Preliminary experiments suggest that absence of activity is due to the formation of randomly paired SH groups which then, subsequently, rearrange to yield the proper set of 4 disulfide bridges. Further evidence for this rearrangement hypothesis was obtained from experiments in which forms of ribonuclease, deliberately pre-

pared with random disulfide bridging, were exposed to dilute solutions of various sulfhydryl compounds. Such compounds catalyzed a rearrangement and led to high yields of the native configuration (which must therefore be thermodynamically the most probable form).

The general hypothesis that the primary sequence alone determines tertiary structure has been examined using other proteins as models. By methods similar to those used with ribonuclease, it has been shown that egg white lysozyme can also be subjected to reduction-reoxidation with efficient regeneration of activity. Experiments with trypsin were initially unsuccessful because of the extreme solubility of the reduced form. Subsequently, the molecule has been attached to carboxymethylcellulose and the resulting insoluble derivative has been used to prepare columns. The trypsin in these columns could be reduced and reoxidized with approximately 5–10% regeneration of enzymatic activity. Further tests on other protein molecules are underway.

In a continuation of studies of the role of various side chain interactions in the reoxidation process, ribonuclease has been reduced and reoxidized following the attachment of polypeptide chains to the epsilon amino groups of lysine residues. These derivatives, containing as many as 70 added alanine residues, can still be successfully reoxidized after reduction. The amino groups themselves must, therefore, not be directly required as information for refolding but may, perhaps, contribute only to the total charge of the protein in solution. This conclusion is reasonable since the added polyalanine side chains also possess a single terminal positive charge. On removing positive charges by acylation, the capacity to refold is lost.

These studies with polypeptide derivates are of added interest since it has beeen further noted that 3 of the 11 free amino groups in ribonuclease are completely resistant to peptidylation and are probably hidden within the structure of the protein. The results suggest that such methods might be useful in studying the chemical topography of poteins in general, and it is planned to apply these techniques to several other test proteins.

The ready formation of native ribonuclease from the reduced, random chain has suggested the possibility that one might attack the total synthesis of ribonuclease with some chance of success. Before beginning such studies, certain questionable features of the sequence, as reported by investigators at the Rockefeller Institute and in this Laboratory, were reinvestigated. The sequence in question has been corrected and the results from both laboratories now agree in full. The primary structure of the protein now appears to be correct.

The plan for chemical synthesis of the ribonuclease chain involves the limited cleavage of the polypeptide chain into 2 to 5 fragments, using trypsin to cleave, specifically, the peptide bonds involving arginine. Trypsin is chosen since it is the most specific of the known proteolytic enzymes. To limit cleavage to arginyl bonds, it is necessary to block the epsilon amino groups of lysine since peptide bonds involving this amino acid are also cleaved by trypsin. The blocking group must be easily removable to permit the ultimate regeneration of native enzyme. The reagent chosen for this purpose was the ethylthiolester of trifluoroacetic acid, and it has been shown that, using this reagent, all the requirements listed above can be achieved. A critical step in the acylation and deacylation involves the "correction" of incorrect disulfide bridges that have been formed by disulfide interchange during the acylation reaction. The correct pairing of SH groups was achieved by complete reduction, followed by oxidation of the reduced chain under optimal conditions.

When samples of trifluoroacetyl RNase were reduced, the resulting material yielded 5 fragments upon trypsin digestion according to prediction. The problem of joining these fragments together involves the formation of peptide bonds between each of the fragments arranged in their proper order. Since ribonuclease contains a number of free carboxyl groups which would also react during the chemical formation of peptide bonds, these must also be blocked in a reversible way before the reconstruction experiments can be initiated. Studies are now underway to investigate the suitability of esterification of these groups with methanol.

Studies on the structure of egg white lysozyme have been continued, and the total elucidation of the sequence is nearing completion. The determination of the disulfide bridges will be under-

taken in the next few months. As an additional point of interest regarding this enzyme, the protein has been isolated from incubations of minced hen's oviduct to which tritiated leucine had been added. The radioactive protein was isolated in pure form and leucine containing peptides were isolated from enzymatic digests of the polypeptide chain. The specific radioactivity of the individual leucine residues differed from one another by as much as 250% and the specific activity was found to increase in the order in which these residues appear along the chain. Thus leucines near the amino terminal end were much less radioactive than those near the carboxyl terminal end. These observations were consistent with a model which depicts protein biosynthesis as a process of unidirectional growth of a polypeptide chain (along a template) beginning at the amino and terminating at the carboxyl end of the chain.

The previously postulated involvement of lipid complexes or lipid compounds of amino acids in protein synthesis has been further examined, both in minced oviduct tissue and in cells of *E. coli*. It has been found that one particular purified amino acid lipid complex involves a covalent bond sensitive to hydroxylamine between the amino acid and the lipid moiety. This material was shown to undergo rapid metabolic turnover at a rate consistent with involvement in protein biosynthesis. Some evidence was also obtained for similar complexes in the *E. coli* system. The characterization of these materials, as derived from bacterial cells, has been much more difficult and requires further work. The overall findings have been included in a model suggested as a consistent interpretation of available observations on protein synthesis.

Studies on the genetic control of the structure of a lysozyme isolated from bacteriophage particles have been continued. A "map" of a number of mutational sites that lead to modifications in the functional aspects of this enzyme has been constructed and the sequence of the enzyme isolated from the parent strain of phage is well on its way to completion. It is hoped, during the coming year, to complete this latter investigation and to compare the structures of "mutant" lysozymes with that isolated from the parental strain. The results should give a direct answer to the question of the colinearity of the genetic map and the primary sequence and should, furthermore, provide direct information on the nature of the genetic code relating DNA structure with protein structure.

Studies on the structure and function of muscle proteins involved in contraction are continuing. The major component of the contractile system of muscle, myosin, has been investigated with regard to its structure, and the relationships between certain aspects of structure and ATPase activity have been examined. Physical-biochemical studies have shown that the long myosin molecule is composed of three identical polypeptide chains associated together in the form of a three-stranded rope. This complex may be dissociated into single strands and re-formed by careful removal of the dissociating agent. ATPase activity was, however, not regenerated, and experiments to overcome this deficiency are in progress. Other experiments have involved the blocking of certain essential SH groups in myosin that are known to be required for enzymatic activity. By enzymatic digestion of the blocked protein, followed by peptide separations, fragments have been isolated, in pure form, containing the blocked group. The determination of their structures should furnish direct information on the chemistry of the active center of myosin-ATPase. Similar studies on a second essential component of the contractile system, actin, have been initiated.

A new project in the Laboratory is concerned with the biochemistry and cytology of cell transport. A convenient mutant of the slime mold, *Dicytiostelium discoideum*, which does not aggregate but which grows vigorously in the amoeboid form, has been analyzed extensively for its phospholipid components. Experiments are now underway to prepare cell walls from these organisms and to examine their structure by chemical and electron microscopic methods. It is further planned to study the metabolic events occurring in cells during the phagocytosis and uptake of various materials. The uptake and transport of fat, in particular, will be investigated in these cells. Concurrently, studies on the transport of fats are being pursued with the lactating mammary glands of guinea pigs as an active fat metabolizing organ. Attempts will be made to correlate the ability of the gland to assimilate triglycerides with the lipoprotein lipase activity of the tissue.

The nature of the reaction catalyzed by lipoprotein lipase as well as the chemistry of the enzyme itself are under continuing investigation. In particular, chemical studies are in progress on the inhibition of the enzyme by polyanions and polycations. It is hoped that the study of such inhibitors will throw some light on the mechanism of the hydrolysis catalyzed by this specific lipase.

Section on Metabolism

While it is clearly recognized that the pathogenesis of atherosclerosis is complex and multifacted, there is adequate evidence to show that elevated levels of blood lipids play some role in the development of this important disease. A great deal of research has been done on the factors controlling levels of blood lipids but much of it is purely descriptive and empirical. Before the problem of hyperlipidemia can be approached rationally we shall have to acquire deeper insights into the mechanisms controlling the production and degradation of the serum lipoproteins and their component parts. Investigators in this section continue to concentrate their efforts primarily on basic studies of lipid metabolism directed ultimately at elucidation of the complex metabolic pattern that determines steady state concentrations of serum lipids.

Pathway and Inhibitors of Cholesterol Biosynthesis

Previous work in this laboratory has established that reduction in the rate of hepatic synthesis of cholesterol can lead to a depression of serum cholesterol levels both in animals and in man. A variety of inhibitory agents has been explored in recent years. Two years ago we established the mechanism of action of triparanol (MER–29), showing that it blocked the last step in cholesterol biosynthesis—the reduction of desmosterol to cholesterol. The clinical effects of the drug have been disappointing. Cholesterol levels are depressed very considerably (on the average 30 to 35%) but desmosterol accumulation is so great that total serum sterols are only reduced by about 15%. Some investigators have held out the hope that desmosterol might prove to be less atherogenic than cholesterol but studies in this laboratory have now shown that desmosterol is laid down in normal aorta and in atheromata of rabbits at a rate indistinguishable from that at which cholesterol is laid down.

The studies on the mechanism of action of triparanol have opened several interesting lines of investigation. These studies provided important evidence that desmosterol is a normal intermediate in cholesterol biosynthesis. Clinical studies completed this year show that desmosterol is an excellent precursor for bile acid synthesis and for adrenal steroid synthesis. The possibility that the introduction of a double bond into the side chain occurs during the conversion of cholesterol to bile acids must now be considered.

Application of the powerful technique of thin-layer chromatography has made it possible to study more simply the reductive reaction blocked by triparanol. Preliminary results show that the drug inhibits reduction of lanosterol to dihydrolanosterol, suggesting that perhaps the same enzyme is involved in reduction of the side chain double bond whatever the structure of the sterol nucleus. This enzyme has been shown to require TPNH as cofactor and to depend upon a reduced sulhydryl group for its activity. An unidentified sterol component of high specific radioactivity has been isolated in the course of these studies and its chemical structure is currently under investigation.

The Heart Institute was fortunate in having Dr. George Popjak as a Visiting Scientist during a part of the past year. He collaborated in studies which have established the details of the mechanism of squalene formation. It was shown that two molecules of farnesyl pyrophosphate condense in an asymmetric fashion to yield squalene. One atom of hydrogen from one of these two molecules is replaced by hydrogen derived from TPNH. This unusual finding, which corrects previously reported studies done in other laboratories, may disclose a new type of condensation reaction in mammalian systems.

Metabolism of Cholesterol Esters

The fatty acids associated with the cholesterol ester fraction in the serum of animals and of man are highly unsaturated. This has suggested the possibility that the well-known effects of unsaturated fats in the diet on serum cholesterol levels may be due to changes in cholesterol ester metab-

olism. In order to explore this possibility more basic information about the metabolism of cholesterol esters is required. Studies initiated this year were designed to explore several aspects of the problem. The esterification of cholesterol in the intestinal wall during absorption has been studied and preliminary results fail to reveal any differences in the rate at which saturated and unsaturated fatty acids are esterified.

The greatest fraction of newly absorbed cholesterol is known to appear in the chylomicrons in the form of cholesterol esters. The fate of these cholesterol esters has been studied by intravenous injection of chylomicrons containing C^{14}-labeled esters, obtained by the collection of chyle from the thoracic duct of rats previously fed C^{14}-cholesterol. Radioactive free cholesterol was largely removed from these preparations by an exchange process. It was shown that more than 80% of the labeled ester cholesterol of the injected chylomicrons was taken up by the liver in 20 minutes. Over the next 24 hours radioactivity appeared slowly in the blood and other tissues in free form. Thus it appears that ingested cholesterol is esterified during its passage from the intestinal lumen into the chyle, is deposited in ester form in the liver very rapidly, and then undergoes hydrolysis in the liver to be released as free cholesterol once again.

The nature of the enzyme systems that hydrolyze and synthesize cholesterol esters in the liver is under investigation. The cholesterol esters present in the soluble fraction have been purified 30 to 40-fold. The nature of this enzyme and its range of substrate specificity is being explored.

Effects of Dietary Fat on Cholesterol Metabolism

This project, being carried out in collaboration with Dr. Sven Lindstedt of the Karolinska Institute in Stockholm, has been continued in order to clarify results previously obtained in the study of bile acid metabolism. Results analyzed to date show that there is a consistent but small effect of unsaturated fats on bile acid formation. When all other factors are held constant the substitution of unsaturated fats for saturated fats causes a slight increase in bile acid excretion. An important outcome of these studies is the demonstration that the pattern of fecal bile acids is considerably altered as a function of the kind of dietary fat offered. Consequently the determination of the fecal excretion of any single bile acid component will not give a true picture of the total fecal bile acid excretion. Most of the results reported by other laboratories have been based on measurements of only one or two bile acid components. While quantitative evaluation of our results is not yet complete it appears that the observed changes in bile acid excretion are not large enough to account for the effects of dietary fats on serum cholesterol levels.

Lipidoses and Hyperlipemia

A new clinical entity, characterized by abnormal storage of cholesterol esters in the tissues, has been discovered. Two siblings living on the island of Tangier have been studied in great detail. These patients appear to have a complete deficiency of α_1-lipoprotein or high-density lipoprotein (HDL). Although they lack any demonstrable HDL they are able to form chylomicrons. This finding forces revision of theories implicating HDL protein as an integral part of the chylomicrons. The β-lipoprotein level in these patients is not particularly abnormal and it appears therefore that the production of these two density classes of lipoprotein is separately and specifically controlled.

An extensive survey of the population on the island of Tangier is being carried out. A very high percentage of the population shows a marked reduction in HDL concentration. An analysis of the genetic relations involved suggests that the observed abnormality is not due to a single allelic abnormality and nongenetic factors have not been entirely ruled out. However, it seems most likely that this syndrome is a genetically determined derangement of lipoprotein metabolism.

Another patient, apparently unrelated to those with Tangier disease, has been discovered with marked hypercholesterolemia, hepatic enlargement, and striking elevation of cholesterol ester concentration in the liver. Again, this patient shows a marked decrease in HDL. Whereas patients with Tangier disease have very little immunochemically identifiable HDL, this patient, even though she has very little lipoprotein with the density of normal HDL, does have immunochemically demonstrable HDL-protein in her serum.

Extensive studies of the lipid metabolism in these interesting syndromes are underway. The results should be of great value in determining the normal function of high density lipoproteins.

It as been recognized for some time that the clinical syndrome of hyperlipemia is heterogeneous but no systematic classification has been possible. An extensive survey is underway in an attempt to differentiate hyperlipemias of different metabolic origins. The use of a more sensitive technique for measurement of lipolytic activity after injection of heparin has revealed that patients with fat-induced hyperlipemia have, as a group, a diminished response. Siblings and parents of patients with hyperlipemia also tend to have lower values than normal even though they may not exhibit hyperlipemia. The enzyme assay method has been made available to a number of other clinics in the hope that the application of a standardized technique to large groups of patients may clarify this syndrome further. At least one other type of hyperlipemia can now be clearly separated—carbohydrate-induced hyperlipemia. In contract to the patients just discussed, who develop hyperlipemia on ingestion of fat, the carbohydrate-induced lipemics develop hyperlipemia only on diets high in carbohydrate. These patients show normal levels of clearing activity after injection of heparin.

Xanthomatous tissue removed from a patient with xanthoma disseminatum (a cutaneous form of Hand-Schuller-Christian disease in which plasma lipids are normal) synthesized cholesterol from acetate at a rate five times that of normal skin while eruptive xanthomas from a patient with hyperlipidemia had an essentially normal rate of synthesis. This finding, although it requires confirmation in a few more patients, is the first biochemical proof of an earlier theory of Thannhauser that the lesions in the former disease were probably synthesizing most of the lipid stored within them.

Metabolism of Adipose Tissue and Hormonal Effects on Fat Mobilization.

This laboratory is continuing with a broad series of investigations on the biochemistry of adipose tissue. It is considered essential to obtain a deeper insight into the factors controlling the metabolism of adipose tissue since without this information it will not be possible to reach understanding of the complex hormonal influences on this tissue.

In previously reported studies the step by step mechanism of triglyceride synthesis in adipose tissue has been determined and during the past year some of the steps involved have been characterized in greater detail. Alpha-glycerophosphate has been shown to be an obligatory acceptor of fatty acids for triglyceride synthesis in this tissue. More sensitive techniques still fail to demonstrate the presence of any enzymes that can phosphorylate free glycerol. It has been shown that a number of glycolytic intermediates can give rise to α-glycerophosphate by well known reactions. Evidence has been obtained that adipose tissue has transaldolase activity but the physiologic importance of this reaction in adipose tissue is still uncertain. The tissue contains an active phosphatase but this enzyme has activity only at an acid pH. It will be interesting to determine whether changes in the activity of this enzyme may influence rates of triglyceride synthesis. It has also been shown that α-glycerophosphate can be formed by a transphosphorylation reaction and again further studies will be needed to determine the physiological importance of this reaction.

The last step in triglyceride synthesis in adipose tissue has been studied in further detail. A particulate enzyme system from chicken adipose tissue cataylzes the reaction of diglycerides with activated fatty acids to form triglycerides. Diglycerides containing at least one unsaturated fatty acid were more reactive than saturated diglycerides. However, the relative positions of the saturated and unsaturated fatty acids in mixed diglycerides did not appear to influence the rate of this reaction very significantly.

Because glycerol can not be activated and reutilized for triglyceride synthesis the rate of release of glycerol has been used as a measure of the rate of a lipolysis independent of changes in the rate of triglyceride synthesis. Using this technique it has been shown that there is continuing lipolysis in adipose tissue under all conditions. Even when triglyceride synthesis is strongly stimulated by the addition of glucose, glycerol continues to be released at rates not significantly different from those seen under control conditions. This confirms earlier conclusions based on measurements of 1-C^{14}-palmitic acid incorporation to the effect that glucose exerts its suppressive effect on fatty acid release primarily if not exclusively by stimulating triglyceride synthesis. Epinephrine, norepinephrine, glucagon and adrenocortico-

tropic hormone all increase the rate of glycerol release, indicating that they stimulate the rate of breakdown of triglycerides. These findings are in conflict with tentative conclusions previously drawn on the basis of measurements of incorporation of labeled fatty acids. In these earlier studies it was shown that the rate of incorporation of fatty acids into triglycerides was inhibited by these several hormones. More recent studies have shown that the pool of free fatty acids in the adipose tissue is heterogeneous. After incubation of the fat pad with labeled palmitic acid it has been shown that the free fatty acids in different fractions of the tissue can, under some circumstances, have widely different specific radioactivities. Which of these fractions, if any, represents the true precursor pool remains to be determined. Preliminary studies, carried out in collaboration with Dr. A. T. James in London, appear to rule out a direct incorporation of free fatty acids from the medium and suggest that the figures obtained for triglyceride turnover in earlier experiments will not require very much revision. Further quantitative studies along these lines are planned.

The nature of the lipolytic system in adipose tissue is being investigated since this appears to play a central role in controlling the mobilization of fatty acids. The properties of the enzyme system under investigation are quite different from those attributed to lipoprotein lipase. It has been shown that the activity of this enzyme system in tissues that have been exposed to epinephrine is increased, although the changes are not as large as the changes in the rates of release of fatty acids induced by epinephrine. The fact that the largest fraction of the enzyme becomes closely associated with the adipose tissue neutral fat and is concentrated at the top of the tube when homogenates are centrifuged holds out hope that purification may be accomplished with relative ease.

Metabolic Fate of Free Fatty Acids

Previous studies in this laboratory showed that when free fatty acids were mobilized from adipose tissue at a high rate under the influence of catechol amines there was a marked net deposition of triglyceride in the liver and, to a lesser extent, in the muscle. *In vitro* studies have now shown that the rate of triglyceride synthesis in liver slices increases as a function of the concentration of fatty acids in the medium. The rate of fatty acid oxidation and the rate of phospholipid synthesis were increased but to a lesser extent. *In vitro* studies of skeletal muscle have also shown that the available concentration of free fatty acids influences rates of triglyceride synthesis and rates of fatty acid oxidation in a similar fashion.

It is now possible to synthesize the results obtained over the past several years into the following hypothesis concerning the fate of free fatty acids:

> The rate of mobilization of free fatty acids from adipose tissue is increased in the fasting state and under conditions of stress. Catechol amines, released directly at nerve endings or carried to the adipose tissue by way of the blood stream, strongly stimulate fatty acid release. During active fat mobilization the serum levels of FFA are elevated. This causes an increase in the uptake of FFA in the liver and a large part of the fatty acid taken up is converted to triglycerides there. If the rate of fat mobilization is very high these triglycerides accumulate in the liver. A part of the fatty acid brought to the liver is incorporated into lipoproteins and secreted back into the serum compartment. It is suggested that the elevation of serum lipoproteins that is seen after injection of catechol amines or under stressful conditions is directly or indirectly attributable to the extra load of FFA delivered to the liver. The *in vitro* studies of skeletal muscle suggest that the rate of peripheral utilization of FFA is to some extent conditioned by the concentrations of FFA in the serum. It will be important to explore the possible hormonal control over this latter process.

Previous studies in this laboratory established the remarkably rapid rate of turnover of serum free fatty acids. Kinetic analysis of these results was complicated by the unusual pattern at later time intervals. These activities have now been clarified by the finding that even highly purified radioactive fatty acids contain sufficient impurity to affect significantly the late portions of the serum disappearance curves. Only by purifying the labeled substrates by means of gas-liquid chromatography is it possible to obtain materials sufficiently pure to give unambiguous results. Con-

flicting findings reported from several laboratories are probably resolved and explained on the basis of contaminant radioactive materials in the preparations used.

Using labeled fatty acids prepared by gas-liquid chromatography it has been shown that the unsaturated fatty acids (oleic and linoleic acids) disappear from the plasma FFA pool more rapidly than does palmitic acid. In view of the well recognized effects of dietary fats of different degrees of saturation on serum lipoprotein levels this finding may be of great significance. A number of isotopically labeled fatty acids have been synthesized and exhaustively purified to permit extension of these studies.

On Phospholipid Metabolism

It has been postulated that the transport of ions through membranes may be intimately linked to phospholipid metabolism. Preparatory to investigating this problem studies have been initiated to determine the properties of the system in red blood cells that synthesizes phospholipids. It has been shown that the ghosts (membranes) of human red blood cells can incorporate labeled fatty acids into phospholipids. Coenzyme A and ATP are both essential requirements but α-glycerophosphate does not stimulate incorporation. Since, in all other phospholipid-synthesizing systems, α-glycerophosphate has been shown to be an obligatory intermediate, these findings suggested that the incorporation observed did not represent net synthesis. It has been shown that C^{14}-palmitic acid is incorporated primarily into lecithin and that the palmitic acid is located selectively in the alpha position. In other words it appears that there is an active turnover of preformed phospholipid molecules in the membrane. The possible correlation of this with the function of the membrane is the subject of further study.

Metabolism of Ricinoleic Acid and Other Hydroxy Fatty Acids

Castor oil is one of the most widely used agents in medicine and yet little or nothing is known about its mechanism of action. Ricinoleic acid makes up over 80% of the total fatty acid content of castor oil and it is almost certain that the unusual effects of castor oil are related to this hydroxy fatty acid. It has been shown that the fatty acids of castor oil are absorbed both by the rat and by man. Rats maintained on daily intake of castor oil accumulate significant amounts of ricinoleic acid in their adipose tissue. Castor oil undergoes enzymatic hydrolysis at rates comparable to those seen for the more usual oils but the rate at which ricinoleic acid is activated to form the coenzyme A derivative is much lower than that seen for the common fatty acids. Thus, it is possible that free ricinoleic acid may accumulate and lead to purgation. Whether this is due to specific properties of ricinoleic acid or only to the fact that it accumulates in higher concentration remains to be determined.

In vitro studies with rat adipose tissue have shown that hydroxy fatty acids (ricinoleic acid, 9-hydroxystearic acid and 10-hydroxystearic acid) are incorporated into triglycerides but at rates significantly lower than the rate of incorporation of palmitic acid. At high concentrations in the medium these hydroxy acids inhibit the incorporation of palmitic acid.

In connection with clinical studies of the fate of ingested castor oil a technique for measurement of fecal fat had to be developed. Methods currently in use failed to measure hydroxy acids because these acids are so much more polar than the common fatty acids. A sample technique for quantitative collection and sampling of feces was developed and the method for extraction and determination of lipids was devised.

Protein Metabolism

The syndrome in which serum proteins "leak" into the gastrointestinal tract and, because of proteolysis, are lost to the body (protein-losing gastroenteropathy) has been studied in a wide variety of clinical conditions. The syndrome is seen in patients with a variety of gastrointestinal diseases recognizable by other clinical manifestations. In addition, however, it has been shown to occur in patients without clinically apparent gastrointestinal disease. Now it has been shown further that patients with congestive heart failure, particularly those with constrictive pericarditis, may exhibit the syndrome.

A new technique has been devised for demonstrating protein-losing gastroenteropathy. Albumin labeled with chromium[51] is injected intravenously and the amount of labeled chromium

appearing in the feces is measured. After the tagged albumin is lost into the intestine the chromium appears not to be reabsorbable and the recovery of it in the feces reflects the amount of protein leaking into the intestine.

Basic studies on the structure of proteins of biological interest have been carried out along two major lines. The structure of the fibrinogen molecule has been further investigated and it has been shown that trypsin destroys the ability of fibrinogen to form a clot, probably by splitting a single peptide bond. When digestion is allowed to proceed further a large number of peptide bonds is split and the molecule appears to be reduced to three fragments of approximately equal size and shape. The relation of these findings to clot formation is the subject of further investigation.

In the course of studies on serum albumin metabolism it was noted that the commonly used HABA dye method gives different color yields with the albumin of different species. These differences reflect the fact that the dye-albumin complex formed has different structures in different species. A systematic study of serum albumin from 10 species has been carried out. It has been shown that the albumins from closely related species form similar complexes while those of phylogenetically divergent species form distinct complexes. It is hoped that close analysis of the spectral characteristics of the dye-albumin complex may be useful in inferring structural characteristics of these different albumin molecules.

Section on Enzymes

The research program of the Section on Enzymes is concerned with studies of diverse biochemical reactions involved in the intermediary metabolism of one, two, and three carbon compounds and in the metabolism of lipids, amino acids, heterocyclic compounds and onium compounds. Led by the consideration that all living organisms make use of similar if not identical enzymatic reactions in the course of their metabolism, the biological material used for these studies consists mostly of specialized microorganisms that have been isolated on selected substrates and offer operational advantages over other experimental media for the specific studies undertaken. The various metabolic processes chosen for investigation represent model systems that present unique opportunities to obtain an insight into biochemical mechanisms of general, fundamental significance. The results of these investigations are summarized below.

One-Carbon Metabolism

Formate Activation. Experimental evidence for the occurrence of formyl-CoA as an enzymatically active one carbon derivative has been obtained through studies with cell-free extracts of *C. kluyveri*. Extending the studies of Lieberman and Barker who observed that extracts of this organism catalyze the formate-dependent decomposition of acetyl-phosphate, it has now been established that formyl-CoA is an intermediate in the overall process. Three coupled enzymatic steps are involved as follows:

$$\text{acetyl-P} + \text{CoA} \xrightleftharpoons{\text{phosphotransacetylase}} \text{acetyl-CoA} + \text{Pi} \quad (1)$$

$$\text{acetyl-CoA} + \text{formate} \xrightleftharpoons{\text{CoA-transferase}} \text{formyl-CoA} + \text{acetate} \quad (2)$$

$$\text{formyl-CoA} + \text{H}_2\text{O} \xrightarrow{\text{deacylase}} \text{formate} + \text{CoA} \quad (3)$$

$$\text{Sum: acetyl-P} + \text{H}_2\text{O} \longrightarrow \text{acetate} + \text{Pi} \quad (4)$$

Formyl-CoA is synthesized through enzymatic transfer of the CoA-moiety from acetyl-CoA to formate (reaction 2). The enzyme catalyzing this step is probably identical with the CoA-transferase previously shown to catalyze reactions between acetyl-CoA and fatty acids with three to eight carbon atoms. The deacylase catalyzing reaction 3 has little if any activity on other CoA derivatives but will catalyze the hydrolysis of formylpantetheine. Whereas these results point to a possible role of formyl-CoA in intermediary metabolism, the significance of this substance in one carbon metabolism remains to be established.

In the course of the above investigation it was discovered that formyl-thiolesters undergo rapid non-enzymatic reactions (at pH 7.0, in dilute solutions) with substances containing adjacent sulfhydryl, hydroxyl or amino groups or various

combinations thereof. Substances with an amino group adjacent to a sulfhydryl group undergo intermolecular thiolester transfer followed by intramolecular S→N migration to form the N-formyl derivative. Reaction of formyl-SR with bis-amino compounds results in formylation of one amino group followed by ring closure with the other to produce imidazoledines. Similar ring compounds are formed by interaction of formyl-SR with bis-hydroxy amines. These reactions may be helpful in explaining the mechanism of the enzymatic reactions of formyl thiolesters.

Another outgrowth of the above study was the observation that several times re-crystallized preparations of tetrahydrofolic acid (THF)-formylase isolated from *C. cylindrosporum* catalyze the formation of formyl hydroxamate when incubated with ATP, formate, and neutral hydroxylamine. It is anticipated that further studies of this formate activating activity may help to elucidate the mechanism of the THF-formylase enzyme; however, the possibility that a formate activating enzyme is a contaminant in the crystalline THF-formylase preparation has not been excluded.

Carbon Dioxide Activation. *Clostridium thermoaceticum* and several other strains of anaerobic bacteria have the capacity to synthesize acetate by a mechanism involving the condensation and reduction of two molecules of CO_2. In an effort to determine the nature of the CO_2-activation that is probably involved in this process, the enzymatic mechanism is being investigated with cell-free extracts of *C. thermoaceticum*. Treatment of such extracts with Sephadex, charcoal or Dowex-1, results in the loss of ability to fix $C^{14}O_2$ into acetate. This ability is restored by the addition of pyruvate, DPN^+, CoA and ATP and as yet other unidentified factors in boiled cell extracts of the organism.

Carboxylation of Acetyl-CoA. Malonyl-CoA is a critical intermediate in the biosynthesis of long chain fatty acids and of certain aromatic compounds. Malonyl-CoA is formed enzymatically by carboxylation of acetyl-CoA in a complicated reaction that involves interaction of CO_2, acetyl-CoA, ATP and a biotin-enzyme complex. It is assumed that a biotin-CO_2-enzyme complex is the reactive carboxylation intermediate. Extending previous studies in this and other laboratories showing that citrate stimulates fatty acid synthesis by animal enzyme preparations, it has now been established that the citrate effect is concerned solely with the conversion of acetyl-CoA to malonyl-CoA. With purified particulate enzyme preparations derived from rat adipose tissue, up to 20-fold stimulation of the carboxylation reaction by citrate is observed. Although other Krebs-cycle intermediates produce activation also, citrate is by far the most active substance tested. Experiments with $C^{14}O_2$ show that citrate activation is not associated with incorporation of isotopic carbon into the citrate molecule. Further experiments of the mechanism of the citrate effect are in progress.

Two-Carbon Metabolism

Fatty Acid Synthesis. The first step in the synthesis of fatty acids involves a condensation of malonyl-CoA with acetyl-CoA, accompanied by a release of the unesterified carboxyl group of malonyl CoA as CO_2 and the formation of an enzyme bound β-keto thiolester derivative. Reversibility of this reaction results in the exchange of CO_2 with the carboxyl group of malonyl-CoA:

$$\mathrm{ESH + HOOCCH_2COSCoA + CH_3COSCoA} \underset{\mathrm{E}}{\rightleftharpoons} \mathrm{CH_3COCH_2COSE + CO_2 + 2CoASH} \quad (5)$$

Two protein fractions derived from extracts of *C. kluyveri* are needed to catalyze the overall reaction. One of these is stable to boiling for 30 minutes, and is stable to 0. 1N acid or base for 30 minutes at 25°C. The other fraction contains an SH-enzyme that catalyzes a thiol transacylase reaction with free CoA or suitable analogues. This reaction was measured by the incorporation of P^{32}-CoASH into malonyl CoA according to the reaction:

$$\mathrm{Malonyl\text{-}CoA + P^{32}\text{-}CoASH} \rightleftharpoons \mathrm{malonyl\text{-}CoA\text{-}P^{32} + CoASH} \quad (6)$$

Acetyl-CoA, propionyl-CoA, and butyryl-CoA can substitute for malonyl-CoA in this ester interchange reaction whereas succinyl-CoA, methylmalonyl-CoA, hexanoyl-CoA and octanoyl-CoA cannot. It remains to be determined if the exchange reactions observed with the lower fatty acyl CoA derivatives are catalyzed by the same

enzyme that catalyzes the malonyl-CoA—P^{32}—CoASH exchange. Two CoASH analogues, pantetheine and phosphopantetheine, can replace CoASH in the exchange reactions; however, other mercaptans such as 2-mercaptoethanol and thioglycollate are not able to do so.

In confirmation of Lynen's experiments with yeast enzyme preparations, it has been established that enzyme bound labeled β-ketoacid is formed when either 2-C^{14}-malonyl-CoA and hexanoyl-CoA or 1-C^{14}-hexanoyl-CoA and malonyl-CoA are incubated with large amounts of exchange enzyme preparation from *C. kluyveri.*

Evidence that the malonyl-CoA-CO_2 exchange reaction represents an essential step in the synthesis of long chain fatty acids is obtained from the following observations: 1) two protein fractions that are required for the exchange reaction are required also in conjunction with other enzymes for fatty acid synthesis by enzyme preparations of *C. kluyveri;* 2) the exchange enzyme is purified coincidentally with the purification of the overall fatty acid synthetic activity of enzyme preparations derived from mammalian adipose tissue; 3) the exchange enzyme is present in all preparations thus far examined (three strains of bacteria, mammalian adipose tissue, and yeast) that catalyze the synthesis of long chain fatty acid; 4) reduced triphosphopyridine nucleotide, the electron donor in fatty acid synthesis, is a strong inhibitor of the exchange reaction. This inhibition is presumed to be caused by reduction of the β-keto acid intermediate thereby making it unavailable for the reverse of reaction (5).

In addition to the two protein fractions needed to catalyze the exchange reaction, a third protein fraction is needed to catalyze the synthesis of saturated fatty acids from malonyl-CoA, acetyl-CoA and TPNH. Following treatment of the purified fractions with charcoal, they must be supplemented with flavins (FMN or FAD or riboflavin) and with an unidentified cofactor present in boiled cell extracts in order to catalyze fatty acid synthesis.

In contrast to the mechanisms used for the synthesis of long chain fatty acids in *C. kluyveri,* it has been shown that the synthesis of butyrate by this organism does not involve malonyl-CoA as an intermediate. Instead butyrate is formed by the condensation of two-moles of acetyl-CoA with production of acetoacetyl-CoA which is reduced to butyryl-CoA.

The synthesis of long chain fatty acids from malmonyl-CoA and acetyl-CoA by rat adipose tissue is catalyzed by a particulate enzyme complex. During extensive purification of this particulate system, the β-keto-fatty-acyl-CoA reductase activity (but not enoyl-CoA hydrase or α-β-unsaturated fatty acyl-CoA reductase activity) is purified in parallel with the overall fatty acid synthetic system. Furthermore, loss of β-keto-fatty-acyl-CoA reductase activity during heat and acid denaturation parallels the loss of overall fatty acid synthetic activity. These observations suggest that the β-keto-fatty-acyl-CoA reductase is an integral part of the biosynthetic mechanism.

When 1-C^{14}-acetyl-CoA and malonyl-CoA are converted to palmitate in the presence of the particulate ezyme and a pool of butyryl-CoA, isotope is neither trapped in the butyryl-CoA nor does this substance cause dilution of the isotope incorporated into palmitate. These results eliminate from further consideration those theories of fatty acid synthesis that postulate an intermediary role of *free* saturated acyl-CoA compounds of intermediate chain length.

Incidental to the studies of fatty acid biosynthesis was the development of a highly sensitive method for the separation and identification of fatty acid acyl-CoA compounds. This method involves conversion of the thiolesters to their acetylated hydroxamate derivatives which are subjected to gas-liquid chromatography at elevated temperatures. When heated at the top of the column, acetylated hydroxamates undergo a Lossen rearrangement to form isocyanates, which separate readily upon continued column chromatography.

Ethylene Glycol Metabolism. Abeles has reported that the conversion of ethylene glycol to acetaldehyde by extracts of *Aerobacter aerogenes* involves an intramolecular rearrangement and a loss of water by a mechanism that is obligately dependent upon the presence of vitamin B_{12} coenzyme. This represents the simplest reaction thus far observed in which the B_{12}-coenzyme is required and it appears to offer the best opportunity to study the mechanism of B_{12} action at the

molecular level. In order to obtain a better experimental material for further studies of this curious reaction, an anaerobic bacterium was isolated from the soil that is capable of deriving most of its energy from the dissimilation of ethylene glycol. The new organism has been characterized as a new species belonging to the genus *Clostridium*. In addition to ethylene glycol which serves as the major energy source, this organism has nutritional requirements for 17 different amino acids and the three vitamins, biotin, pantethenic acid and riboflavin. Of numerous substrate analogues, only propylene glycol can replace ethylene glycol as an energy source. The fermentation of ethylene glycol leads to the formation of equal amounts of acetate and ethanol. This is consistent with a postulated mechanism in which the ethylene glycol is converted to acetaldehyde which undergoes a dismutation to form ethanol and acetate. An enzymatic analysis of this system to determine the role of vitamin B_{12} coenzyme is in progress.

Acetyl Cyanide Formation. Cell-free extracts of *C. kluyveri* catalyze the acetylation of amino acids in the presence of acetyl phosphate and high concentrations of hydrogen cyanide. The curious requirements for high concentrations of hydrogen cyanide prompted a more intensive study to determine its role in the acetylation reaction. It was found that an enzyme is present in extracts of *C. kluyveri* that catalyzes a transacetylation reaction between acetyl-CoA and HCN to form acetyl-cyanide. Acetyl-cyanide is a highly reactive substance which, in the absence of an acetyl acceptor, undergoes instantaneous hydrolysis; however, in the presence of amino acids or mercaptans it undergoes preferential aminolysis or acyl transfer to form the N-acetylated or thiolester derivatives. The HCN-transacetylase was partially purified and some of its properties were determined. Although the normal substrate for the enzyme has not yet been identified, it promises to be an interesting new type of acyl acceptor capable of forming active acyl derivatives at the energy-rich level. Of several substances tested, azide is the only compound other than HCN that can serve as an acetyl acceptor.

An outgrowth of the above study was the discovery that at high concentrations various thiolesters undergo non-enzymatic cyanolysis reactions with the formation of acetyl cyanide. The kinetics of this non-enzymatic reaction have been studied in great detail. The data obtained have been critical in establishing the role of acetyl cyanide as a product of the enzymatic reaction.

Metabolism of Heterocyclic Compounds

Riboflavin Degradation. It was previously observed that 3,4,dimethyl-6-carboxy-α-pyrone ($C_8H_8O_4$), urea, and oxamide, appear as products during the aerobic dissimilation of riboflavin by a pseudomonad isolated from soil enrichment cultures. 1-Ribityl-2, 3-diketo-1,2,3,4-tetrahydro-6, 4-dimethylquinoxaline and 3,4-dimethyl-2,3-quinoxalinediol were shown to be intermediates in the overall metabolism. Further studies with dried-cell suspensions of the bacterium have now established the stoichiometry of the main overall reaction which is described by the equation:

$$C_{17}H_2O_6N_4 + 7.5\ O_2 \longrightarrow 6CO_2 + NH_2{-}\overset{O}{\overset{\|}{C}}{-}\overset{O}{\overset{\|}{C}}{-}NH_2 + NH_2{-}\overset{O}{\overset{\|}{C}}{-}\overset{O}{\overset{\|}{C}}{-}NH_2 + C_8H_8O_4 + 2H_2O$$

With prolonged incubation, the α-pyrone ($C_8H_8O_4$) undergoes slow decomposition with the uptake of about 4 moles of oxygen, the production of 4 moles of CO_2 and the fixation of one mole of endogenous nitrogen which otherwise appears as ammonia. In addition to the above organism, three new strains of bacteria have been isolated that are capable of causing the degradation of riboflavin to lumichrome and as yet unidentified products. Enrichment cultures capable of catalyzing the anaerobic dissimilation of riboflavin have been obtained also and experiments are in progress to isolate the responsible organisms in pure culture.

Nicotinic Acid Fermentation. An organism capable of fermenting nicotinic acid with the formation of stoichiometric amounts of acetate, propionate, CO_2 and NH_3 was formerly isolated in this laboratory but it was unfortunately lost. Because this fermentation poses a number of extremely interesting and fundamental questions, another organism capable of fermenting nicotinic acid has been isolated from the soil and optimum conditions for its growth have been determined. Future studies will examine the postulated roles of methylmalonyl-CoA, malonyl-CoA, vitamin B_{12}

coenzyme and biotin in the dissimilation of nicotinic acid by this organism. The organism will be used also in combination with chemical degradation procedures to degrade nicotinic acid obtained biosynthetically from isotopic precursors. Such studies will give an insight into the pathway of nicotinic acid biosynthesis.

Phenazine-1-Carboxylic Acid Biosynthesis. Studies on the biosynthesis of phenazine-1-carboxylic acid by the bacterium *Pseudomonas aureofaciens* have been continued. It was demonstrated that all carbon atoms of this pigment are derivable from glycerol. The fact that lysine is also required, although lysine carbons are not incorporated into the pigment, suggests that the ring nitrogen atoms are obtained from lysine. The possibility that anthranilic acid is a precursor was eliminated. A pool of shikimic acid reduces the incorporation of glycerol carbon into pigment and is therefore tentatively assumed to be an intermediate.

Metabolism of Amino Acids

Glycine Reductions. Insight into the mechanism of anaerobic phosphorylation associated with the reductive deamination of glycine has been sought through studies with cell-free extracts of *Clostridium lentoputrescens*. With cell-free extracts prepared by means of the French pressure cell, DPNH serves as the ultimate electron donor for glycine reduction in a flavin mononucleotide and disulfide-dependent series of reactions. The DPNH-linked enzyme system is relatively unstable; aging results in extracts in which only 1,3-dimercaptopropanol or similar dimercaptans can serve as electron donors. The complexity of the overall reaction is indicated by the requirements for at least four protein fractions in addition to the various coenzymes. As yet no intermediates have been identified, but it has been established that ATP formation and ammonia release precede acetate formation. The glycine reductase portion of the reaction sequence requires relatively high ammonium ion concentrations for activity.

Lysine Degradation. Previous studies have shown that lysine degradation by *Clostridium sticklandii* and a newly isolated organism obtained from lysine enrichment cultures leads to the formation of one mole each of butyrate and acetate and to two moles of ammonia. It has now been established that in fermentations catalyzed by cell-free extracts of the new bacterium the acetate is derived from carbons 1 and 2 of lysine and that butyrate comes from the remainder of the molecule; carbon 6 of lysine becomes carbon 4 of butyrate. The overall reaction is sensitive to a number of metabolic inhibitors including those that inhibit electron transport phosphorylation at the flavin level. Treatment of cell-free extract with charcoal results in a loss of activity which can be partially restored by the addition of boiled cell extract or a mixture of DPN, CoA, vitamin B_{12} coenzyme, pyruvate and 1,3-dimercaptopropanol. This interesting enzymatic system promises to be a fruitful medium for further studies of electron transport phosphorylation and the mechanism of vitamin B_{12} coenzyme action.

γ-Aminobutyrate Fermentation. The conversion of γ-aminobutyrate to butyrate during fermentations by *Clostridium aminobutyricum* involves the intermediary formation of γ-hydroxybutyrate by a complicated series of reactions (see annual report, 1960). Continuing this study, evidence has been obtained to support the reaction mechanism postulated to explain the further conversion of γ-hydroxybutyrate to butyrate. Using the pantetheine analogues of the normal CoA intermediaries, the following enzymatic steps have been observed to occur in cell-free extracts: The dehydration of γ-hydroxybutyryl-pantetheine to crotonyl-pantetheine; the isomerization of vinylacetyl-pantethteine to crotonyl-pantetheine; the DPNH-linked reduction of crotonyl-pantetheine to butyryl-pantetheine; and the conversion of acetyl-CoA to acetate by phosphotransacetylase and acetokinase activities. Although dinitrophenol inhibits the overall reaction and crotonyl-CoA reductase, efforts to demonstrate electron transfer phosphorylation with either process have not been successful.

Metabolism of Onium Compounds

Choline Fermentation. Research has been continued on the intermediary metabolism of choline by a newly isolated anaerobic bacterium belonging to the genus *Clostridium*. Balance studies

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with growing cultures show that choline is converted to trimethylamine, acetate, and ethanol according to the overall equation:

$$2(CH_3)_3\overset{+}{N}CH_2CH_2OH + H_2O \rightarrow 2(CH_3)_3\overset{+}{N}H + CH_3COOH + CH_3CH_2OH$$

The stoichiometry of this fermentation is therefore similar to that previously observed for the choline fermentation of *Vibrio cholinicus*. However, the new organism is a better experimental medium to study the enzymatic mechanism since all of the enzymes needed for the overall fermentation are readily obtained in a soluble form and are therefore amenable to fractionation. Suspensions of aged dried cell preparations frequently catalyze the fermentation of choline only after a long lag period. After the lag period there is a substance in the extracellular solution which when added to a fresh suspension of these cells will promote fermentation without a lag period. Some properties of the "lag-reducing" activity have been determined. Isolation of the substance is in progress. Preliminary studies show that the fermentation by cell-free extracts is inhibited by iodoacetamide, and by compounds known to inhibit electron transport coupled phosphorylation; i.e., 2,4-dinitrophenol, 2,4-dinitrocresol, sodium amytal and quinoline-N-oxide, but not antimycin A. Following aging and treatment with charcoal and Dowex-1-acetate, the enzyme preparations are partially resolved of cofactors and can be reactivated by the addition of CoA, ADP, Fe^{++}, Mg^{++} or Mn^{++}, and by a heat labile protein fraction that is present in the extracellular solution of dried cell suspensions which have been previously incubated with choline. This factor is not identical with the "lag-reducing" factor present in the extracellular solution.

Fermentation of Sulfonium Compounds. In an attempt to determine if the high energy released during hydrolytic cleavage of the sulfonium bond can be used for biosynthetic purposes, an anaerobic bacterium capable of growing on dimethyl-β-propiothetin (DMPT) as a major source of carbon was isolated from the soil. It has been established that the fermentation of DMPT is described by the overall equation:

$$3(CH_3)_2\overset{+}{S}—CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3CH_2COOH + CH_3COOH + 3(CH_3)_2S + CO_2 + 3H^+$$

Washed cell suspensions also ferment α-alanine, β-alanine, acrylate and lactate to give propionate and acetate in a ratio of 2:1. Pyruvate is fermented to produce propionate and acetate in a ratio of 1:2. These results therefore suggest that this organism is related to *C. propionicum* which carries out similar fermentation of the 3 carbon compounds. Preliminary enzyme studies indicate that DMPT is activated to form the CoA derivative which undergoes cleavage to form acrylyl-CoA and dimethyl sulfide. The acrylyl-CoA is reduced to propionyl-CoA or alternatively is aminated to form β-alanyl-CoA. It is hoped that further studies with this organism will help to elucidate the mechanism of the propionate formation by anaerobic metabolism.

Transsulfuration

Studies on the mechanism of sulfur transfer between homocysteine and cysteine have been continued. A cystathionine cleavage enzyme isolated from an me-2 mutant of *Neurospora* has been shown to catalyze the decomposition of certain disulfides (L and meso isomers of cystine and homocystine). A similar reaction occurs also in animal tissues and may represent the major pathway for disposing of these compounds within the cell and may have relevance to cystinosis and cystinuria. The evidence obtained indicates that disulfide cleavage occurs by a β-disulfide elimination reaction. Strongest support for this conclusion is the successful trapping of an unstable alkyl hydrogen disulfide intermediate with iodoacetate, and identification of the reaction product as the mixed disulfide of cysteine and thioglycollic acid.

With regard to the cysteine ↔ homocysteine process itself, the work has served so far more to raise doubts about hitherto postulated pathways, rather than to supply an acceptable alternative. The reversible process has been thought, for many years, to be mediated by enzymes catalyzing irreversible reactions, 2 cleavages and 2 condensations; i.e., cysteine+homoserine (homocysteine+serine)→cystathionine→cysteine+α-keto-butyrate (homocysteine+pyruvate). Only one cleavage enzyme was found in *Neurospora*, which

catalyzes a heterogeneous reaction, decomposing cystathionine 10% by β-elimination and 90% by γ-elimination. Moreover, only the same unmodified enzyme can be found in mutants supposedly blocked in cleavage to homocysteine, and in condensation from cysteine.

LABORATORY OF CHEMISTRY OF NATURAL PRODUCTS

The work of the Laboratory may be summarized in four areas. These are (1) the development of gas phase chromatographic techniques for the qualitative and quantitative analysis of biologically important substances, (2) investigation of the isolation, structure, properties and biogenesis of plant alkaloids, (3) studies of the components of the kallikrein-kallidinogen-kallidin system, and of the chemistry of human polypeptide vasodilators, and (4) consultative and informal collaboration with various intramural research groups of the National Institutes of Health seeking the specific knowledge and equipment of the Laboratory for application to their particular problems.

Gas Phase Chromatographic Methodology

Research in gas phase chromatography has developed along several lines of specific interest. With liquid phases developed earlier in the Laboratory, new classes of compounds were studied for gas chromatographic behavior. These included the vitamins and provitamins D_2 and D_3, sugars (protected as acetyl derivatives), urinary 17-ketosteroids, epinephrine and indole metabolites. The sapogenins and steroidal amines, tomatidine, solasodine and many of their derivatives have been separated and identified by this method.

While the extreme sensitivity and high resolving power of gas phase chromatography are of considerable significance, chromatography of larger samples (over 0.5 mgm) has not been particularly successful. Progress has been made in increasing the amount of material that can be chromatographed, maintaining resolution comparable to that of the analytical columns. Several steroid and alkaloid separations have been successful using 7–25 mgm samples of 5-component mixtures.

A third research area in gas phase chromatography has been concerned with the development of new liquid phases to provide greater selectivity in dealing with compounds difficult to separate under normal conditions. Neopentyl glycol-succinate (NGS) and a fluorinated alkyl silicone polymer (QF–1) have proved to be promising new liquid phases for this purpose. Additional selectivity for the separation of closely related compounds has been achieved through the use of specific hydroxyl derivatives, either trifluoroacetyl esters or trimethylsilyl ethers of the alcohol function. The use of two-component phases and segmented packings has shown promise in exhibiting specificity towards various functional groups, making it possible to distinguish between steroids differing in type and degree of substitution.

Alkaloid Work

Minor alkaloids have been isolated from *Ormosia jamaicensis* and *Cassia excelsa*. *Astrocasine*, $C_{20}H_{24}N_2O$, has been isolated from *Astrocasia phyllantoides* (Euphorbiaceae). Structural studies in the major alkaloids of *Ormosia* and *Cassia* are continuing.

In spite of the chemical and pharmacological interest in alkaloids for hundreds of years, no satisfactory answers have been found to the questions of why a given plant makes alkaloids or in what manner. Studies are continuing in this Laboratory to determine the mechanism of alkaloid formation in plants. Feeding radioactive tyrosine and phenylalanine to various plants of the Amaryllidaceae has yielded radioactive alkaloids. No "scrambling" of the label was observed and it was concluded that the C_6–C_2 fragment of these amino acids is incorporated intact into the alkaloid. Prior to this discovery, an alternate theory, that condensation products of shikimic and prephenic acids produced the alkaloids was accepted by some investigators. Radioactive compounds, structurally more complex than the amino acids, have been prepared in the laboratory and fed to selected plants to study the individual steps by which the alkaloids are synthesized in the plant.

The Kallikrein-Kallidinogen-Kallidin System

The vasodilating substance kallikrein owes its action to the formation of the physiologically active polypeptide kallidin. The latter compound is formed when kallikrein acts on kallidinogen, a component of human plasma. Work directed to the isolation of kallikrein, kallidin and kallidinogen has been carried on as a joint study with the Laboratory of Cardiovascular Physiology.

The two kallidins, I and II, isolated from human plasma after reacting with human urinary kallikrien, as described in the 1960 report, have been tentatively identified. Kallidin I could not be distinguished from the nonapeptide, bradykinin (H. Arg. Pro. Pro. Gly. Phe. Ser. Pro. Phe. Arg. OH). Kallidin II was identified as the decapeptide H. Lys. Arg. Pro. Pro. Gly. Phe. Ser. Pro. Phe. Arg. OH by degradation and by synthesis (the latter by E. D. Nicolaides). Studies are being made to determine the physiological role(s) of these peptides.

Highly purified preparations of human urinary and pancreatic kallikreins have been obtained. Both were homogeneous in the ultracentrifuge. The former was also electrophoretically homogeneous, whereas the latter showed two bands.

Work is in progress to isolate pure human plasma kallidinogen, the protein on which the kallikreins act to form the kallidins, in order to determine unambiguously the nature of the peptide products resulting from the action of pure kallikreins and to do chemical studies on the kallidinogen molecule before and after enzymatic reaction.

Miscellaneous Informal Collaborative Research

In addition to conducting the research cited above, a significant amount of time and effort has been spent by several members of the Laboratory to help other scientists of the National Institutes of Health with specific problems. Our help has been sought primarily in three areas: (1) Large scale processing of microorganisms, plant materials, biological fluids, glands, and culture media. (2) Assistance in spectroscopic analysis and interpretation. (3) Advice in the construction and repair of gas phase chromatography equipment, in the preparation of satisfactory column packings and in preliminary studies to determine the feasibility of gas phase chromatography for the particular problem of the investigator. Consultative work of this type provides a stimulus for members of our Laboratory to broaden their research outlooks.

LABORATORY OF CHEMICAL PHARMACOLOGY

Interaction of Drugs With Physiological and Biochemical Function

The bridge between biochemistry and physiology is the biochemical-physiological interactions which explain control mechanisms. Since drugs change the quantity, but not the quality of function, they must act, directly or indirectly, on these control mechanisms. Thus drugs must perturb control mechanisms in the same way that these are affected by changes in environment. It is even useful to regard many diseases, at least symptomatically, as disturbances of regulatory mechanisms.

Function is regulated by changing the level of hormones at reactive sites. But such regulation can be achieved only if the hormone is in a stored form. The storage site or neurochemical transducer is acted on by the nerve impulse which is transmutted into free hormone; this in turn is transformed into a change in activity of the next nerve cell or of the end organ. Thus the body reacts to the stimulus or to a changing environment through actions on these neurochemical transducers. It follows that one of the basic problems in biology is the nature of these units which synthesize, store and release substances like norepinephrine (NE) and serotonin (5HT).

Amine Storage Site

Our studies now provide a reasonable picture of a unit which synthesizes, stores and releases NE or 5HT. We have shown that the amines in cells are stored in two pools. One, a mobile pool, is separated from monoamine oxidase (MAO) and from reactive sites by an active transport system which maintains the amine inside a lipoid membrane. Reserpine acts by inhibiting this transport system. The second, a reserve pool, appears to be a complex of amine in granules.

The nerve impulse causes amine in the mobile store to be discharged onto the active site. MAO, which surrounds the membrane, has the key role of controlling the content of stored amine in the face of continuous synthesis. The enzyme facilitates diffusion by inactivating the amine that diffuses through the membrane; in this way the steady-state level is maintained well below saturation of the transport system. The scheme answers such questions as: (1) How reserpine depletes the tissue of its amines though it does not act on isolated granules. (2) How a pool stored by active transport coexists with amine-containing granules. (3) How the stored amine is separated from reactive sites and MAO. (4) Selfregulation of brain content of amines. (5) Rise in brain levels after MAO blockade.

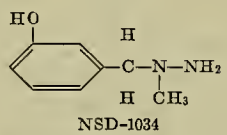
Control of Function by Drugs Acting on Storage Sites

Viewing the synthesis, storage, physiological release, metabolism and the reactive site as a single control unit, permits a broad picture of drug action. NE and 5HT control units are affected by drugs as follows: (1) Drugs may mimic amines: Neosynephrine mimics NE at peripheral sympathetic sites, amphetamine and LSD at central sites. We are searching for a drug that mimics 5HT centrally and has the same relation to 5HT that amphetamine has to NE. A new benzoquinolizine (P–2647) might be this drug; it exerts some central reserpine-like effects but has little effect on 5HT content; however, a more potent substance would be needed to be of clinical value.

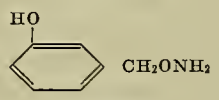
(2) Drugs that block the action of amines: Dibenamine blocks NE action peripherally, chlorpromazine in brain. With respect to 5HT, desmethylimipramine, the antidepressant used in endogenous depression, might possibly be a 5HT antagonist in brain.

(3) Drugs that block amine metabolism: A number of MAO inhibitors raise brain levels of both amines but the excitation in rabbits is associated with elevation of NE and not 5HT. The importance of NE in the action of these inhibitors was shown rather definitively by giving MO 911 (Abbott), a new non-hydrazine inhibitor, after reserpine. Reserpine sedation was soon reversed but this action was related to NE levels which rose later than those of 5HT.

(4) Drugs that block synthesis: (a) Decarboxylase inhibitors: These block formation of both 5HT and NE since Dopa and 5 HTP are decarboxylated by the same enzyme. However, decarboxylase can be blocked by 90% or more without affecting synthesis of NE. Apparently formation of this enzyme must be blocked virtually completely before the brain stem dopamine level is low enough to affect NE formation. (b) Dopamine hydroxylase inhibitors: Since α-methyl-m-tyramine is a substrate for the enzyme, we tested the isostere NSD–1034 in hopes it might inhibit but not be a substrate. This compound appears to be the first agent yet found that blocks NE formation *in vivo*. Doses of 40 mg/kg block formation of NE *in vivo* at the dopamine hydroxylase step without affecting 5HT synthesis. Dr. Udenfriend has tested this drug for us *in vitro* and has shown it to be the most active inhibitor yet developed. Another isostere, NSD–1024, seems even more potent. However, NSD–1034 lowers NE level only slightly in one hour. Thus blockade of NE synthesis probably will not elicit pharmacologic effects rapidly since stores must first be utilized.



NSD-1034



NSD-1024

(5) Blockade or activation of physiological release mechanism: Bretylium is a quaternary sympatholytic agent that lowers blood pressure by preventing the release of NE by nerve impulses. Our studies indicate that guanethidine, another sympatholytic agent, appears to activate the process whereby the nerve impulse releases NE specifically onto receptor sites; as a result NE is depleted peripherally. Pretreatment with bretylium prevents the depletion of heart NE by guanethidine but not reserpine. In addition guanethidine causes considerable sympathomimetic activity during the NE release phase, while reserpine does not. These results suggest that reserpine impairs storage so that amines spill over onto MAO, while guanethidine causes release directly onto active sites.

(6) Blockade of storage processes: (a) Reserpine and analogues: Reserpine inhibits the active transport processes which maintain NE and 5HT in their mobile pools. As a result, the stores are depleted. Synthesis remains unabated but release is no longer controlled by nerve impulses. The physiologic effects of reserpine will depend on the resultant amine level at active sites; this in turn will depend on the balance between synthesis and disappearance. Thus the level of NE at peripheral nerve endings is too low to produce an effect and a deficiency results. In contrast, uncontrolled release of 5HT in the gut results in a continuous level at receptors which control peristalsis. Evidence now indicates that reserpine does not elicit a deficiency of either amine at their active sites in brain because the blood-brain barrier prevents their rapid diffusion into the blood stream. (b) Alpha-methyl-m-tyrosine: This synthetic amino acid selectively releases catecholamines. It does not act *per se* but through its decarboxylated product α-methy-m-tyramine and the side chain hydroxylated derivative. Like reserpine, MMT is a "hit and run" drug, the action lasting long after MMT or its decarboxylated products have left the body. Of great potential importance in the development of new drugs is the observation that the sedative Rauwolfia alkaloids and the benzoquinolizines are derivatives of α-methyl-m-tyramine. (c) Competitive inhibitors of storage processes: We have shown that a number of drugs including desmethylimipramine (DMI) and cocaine, antagonize the uptake of low levels of NE by tissues and thereby prolong its action. Even though DMI inhibits at a concentration of 10^{-10} M it does not release measurable amounts of NE and the inference has been made that release and uptake of amines are different processes. By kinetic studies we have shown that these drugs compete with NE for the storage process. Since NE level is very high inside storage sites, only a small amount is released.

Role of 5HT and NE in Brain

The question of whether reserpine action is due to its effects on the storage of NE or of 5HT must be answered before reserpine can be used to disclose the role of brain 5HT. A number of agents that selectively block NE storage together with a mass of pharmacological and physicochemical data has definitely eliminated loss of NE stores as an important factor in central effects of reserpine.

If reserpine action were shown to be due to the effects of uncontrolled 5HT release, a great step would be taken in elucidating the role of the indole. Our studies show a quantitative relationship between central actions of reserpine, SU 9064 and Ro 4–1284. In these studies the central action is determined by five criteria. Each dose of drug is given to many mice and the change in brain 5HT is related to central activity expressed as the number of animals that display all 5 signs. The results show a close relationship between the degree of central activity and lowering of brain 5HT even when NE stores are first depleted by a selective NE releasing agent.

The close association of sedation to blockade of 5HT storage suggests a causal relationship and lends support to the view that the action of reserpine does reveal the role of 5HT in the brain.

Further insight into the role of the brain amines was provided by physiologic effects attained by their selective release after blockade of MAO. In mice the amines trapped in brain produce results which indicate that NE and 5HT integrate different neuronal systems. Thus, after release of NE by MMT, the animals behave as though given a super-active amphetamine and dash around, perfectly coordinated, at express speed for several hours. When both 5HT and NE are released, far less excitation results. When only 5HT is released (by first depleting NE with MMT, then giving a MAO inhibitor and reserpine) the animals show no excitation. From these results it appears that effects of 5HT and NE released onto their respective reactive sites produce opposite responses. Presumably they act on different neuronal systems.

Amines as Modulating Agents

Studies indicate that the NE present in sympathetic ganglia acts in a local feedback mechanism to modulate the effects of acetylcholine and to prevent the uncontrolled firing of ganglionic cells. These results may be a lead in looking for the cause of some kinds of hypertension. Further studies have shown that the effects of guanethidine on ganglionic transmission are erratic because the release of NE by guanethidine is erratic. NE is

not present in parasympathetic ganglia, but the presence of decarboxylase and MAO suggests that some amine is present.

Our results suggest that a main action or reserpine is on the limbic system which contains largely 5HT. The results are in accord with the view that 5HT in the limbic system is a modulator of acetylcholine action The electrophysiological effects of reserpine in the limbic system provide that first bioelectric phenomenon in brain that is associated with a change in brain 5HT.

Biochemical Behavior

Central Control of Energy Substrates

In past years we have studied how the brain controls biochemical processes through the hypothalamic-pituitary-endocrine and the autonomic systems. This past year, we have been concerned with how these systems interact, especially in mobilizing energy substrates.

(1) Role of brain and sympathetic nervous system in mobilizing energy substrates: The control by the nervous system of the release of free fatty acids (FFA) from adipose tissue was shown by stimulation of sympathetic fibres innervating omental adipose tissue *in situ*. Electrical stimulation increases (3-fold) the amount of FFA in effluent blood. The breakdown of triglycerides to FFA is exquisitely sensitive to free NE. After reserpine, free NE but not nerve stimulation mobilizes FFA; after dibenamine, neither nerve stimulation nor free NE mobilizes FFA.

In the absence of NE it appears that FFA and other energy substrates are not mobilized in physiological situations-requiring increased energy. For example, the effect of depot ACTH in mobilizing FFA from adipose tissue (and the resultant triglyceride deposition in liver) does not occur in animals depleted of NE stores; infusion of NE 2 hours before ACTH restores the NE stores in adipose tissue and the FFA are now released by ACTH. Similarly fat pads of animals which have been depleted of NE do not respond to ACTH or glucagon without addition of NE to the incubation mixture.

(2) Requirements for both corticoids and sympathetic nervous system in mobilization of FFA: Ethanol, morphine and many other drugs produce manifestations typical of pituitary-adrenal stimulation including a fall in adrenal ascrobic acid, a rise in plasma corticosterone, a rise in FFA, and deposition of liver triglycerides (TGL). These effects do not occur after hypophysectomy or adrenalectomy but still occur after adrenal demedullation. Similar results are produced by depot ACTH. If the rats are pretreated with dibenamine or Ecolid and then given ethanol, morphine or ACTH, the rise in corticosterone level still occurs but the mobilization of FFA and rise in liver TGL are prevented. This suggests that the release of FFA is dependent on both an intact sympathetic system and on the presence of corticoids.

The essential role of glucocorticoids in lipid metabolism was shown by the following experiments: a) NE infusion failed to raise FFA levels in adrenalectomized rats unless the animals were pretreated with cortisone; b) NE added to the fat pads of adrenalectomized rats does not mobilize FFA unless the animals have been pretreated with cortisone. Preliminary work indicates that the corticoids might be involved in the passage of FFA from adipose tissue, while NE is concerned in breakdown of TGL.

Central Control of Metabolism

Processes of conservation and restoration are set in motion by reserpine-like drugs. In doses that do not deplete adrenal catecholamines in rats, these drugs produce a sleep-like state with an increased parasympathetic output and the resultant economy of function. The evidence suggests that they activate processes that are superimposed on normal function and which might be similar to those elicited in sleep and hibernation.

A dramatic example of the action of reserpine in activating conservation processes is provided by its effects on energy production. Thus reserpine was found to produce a profound drop in the metabolic rate in normal and thyroidectomized animals. In thyrotoxic animals it rapidly reserves the high metabolic rate to below the normal level. This action is not exerted upon the thyroid gland but upon the tissues where it counteracts the effects of thyroxine. The action of reserpine is prevented by pretreatment of desmethylimipramine, a specific antagonist of the central actions of reserpine, showing that the reserpine effect on BMR is a central one.

Desmethylimipramine, a New Antidepressant

In studies of compounds that block the reserpine-induced syndrome, imipramine was found to counteract the central actions of reserpine and reserpine-like compounds in rats. Its delayed onset of action was explained by the slow accumulation of a metabolite, the monomethylanalogue (DMI). Unlike imipramine, DMI has no sedative action *per se;* it neither inhibits MAO nor affects the release of amines by reserpine. If given before reserpine (or other reserpine-like compounds) it prevents the complete reserpine syndrome including sedation, decreased reactivity to stimuli, increased parasympathetic output, decreased basal metabolism, etc. In higher dosage it reverses the reserpine syndrome, that is, it produces a hyperactivity which is not an increase in normal activity but appears to be the opposite of reserpine sedation, that is endogenous excitation.

The rats are not influenced by outside stimuli, but act as though controlled by an inner drive. They jump from great heights, relentlessly travel a treadmill till they drop and ignore the presence of other animals, as well as of food and water. These effects are elicited by reserpine up to 30 hours after a single dose of DMI, but if reserpine is given first and then DMI, the effects of the reserpine are no longer influenced.

Some experiments are in accord with the view that DMI competes with 5HT for reactive sites. Thus: 1) Rabbits given an MAO inhibitor followed by DMI die in hpyerpyrexia. 2) If rats are depleted of NE (with MMT) and then given DMI, reserpine action is still blocked. 3) DMI, in contrast to amphetamine, blocks the parasympathetic actions of reserpine. However, other experiments indicate that DMI might activate the action of NE in brain. Neither concept gives a satisfactory explanation for the fact that DMI has no antidepressant effect in normal animals.

The following suggest that the effects of DMI may be a link between biochemistry, physiology and a brain disease. 1) DMI does not produce excitation in normal subjects, but only in those having a primary depression. 2) Reserpine elicits an "endogenous" depression which is converted by DMI to an "endogenous" excitation. 3) Overdosage in subjects with endogenous depression can elicit hypomania. These results suggest that similar central pathways might be involved in endogenous and reserpine-induced depression.

Passage of Substances Across Membranes

Ca in Membrane Permeability

Ethylenediamine tetracetic acid (EDTA) increases intestinal absorption of lipid-insoluble compounds such as mannitol, inulin, decamethonium, and sulfanilic acid. The non-specificity of the effect was indicated by the increased passage of inulin-C^{14} from plasma to gut. These results suggest that binding of Ca changes the character of the boundary.

Penetration of Drugs Into Cells

Organic anions enter red cells while cations do not. Our studies show no indication of competitive inhibition, no saturation phenomena, and no effects by metabolic poisons, and suggest that anions pass into the red cells by a process of simple diffusion through positively charged pores.

Biliary Excretion of Drugs

The liver transports some quaternary ammonium compounds, e.g., procaine amide ethobromide, Darstine and benzomethazine, into bile. Strangely enough decamethonium and tetraethylammonium are not concentrated in bile.

Membranes Within the CNS

There is no barrier to substances leaving the cerebrospinal fluid (CSF). Various sugars such mannitol, sucrose, inulin and dextran (M. W. 180 to 40,000) injected into lateral ventricle or cisterna magna leave CSF into plasma at same rate, presumably via the arachnoid villi. The rate of passage appears to be a measure of bulk flow (or turnover) of CSF, since the exit of inulin is related to CSF hydrostatic pressure. In contrast, phenol red and PAH leave the CSF by a specialized transport system. Decamethonium and hexamethonium also appear to leave by a special mechanism, but the process differs from the one for acids.

Preliminary results suggest that highly polar substances can be trapped inside the blood-brain barrier. Thus the amines formed by decarboxylation of MMT remain in brain long after they

can be found in other tissues. In addition the guanethidine that can be forced into brain after intracisternal injection remains there for a considerable period of time. This trapping effect of the blood-brain barrier indicates that bulk flow between brain and subarachnoid space is slow. This finding can possibly be exploited in therapy.

Transport of Purines and Pyrimidines

Uracil is actively transported *in vitro*. Strong inhibitors have substituents at the 5 or 6 positions of the pyrimidine ring, while compounds with substituents at 1, 2, 3 or 4 positions were weak inhibitors. Hypoxanthine and compounds similar to hypoxanthine are the best inhibitors of uracil transport, although these compounds themselves are not transported.

Drug Metabolism

Antimetabolites Metabolized by Enzymes of Intermediary Metabolism

6-Methylaminopurine (methylated adenine or MAP) is one of several methylated derivatives of adenine and guanine found in trace amounts in soluble-RNA. An enzyme in rat liver converts adenine to MAP. Since inclusion of N-methylated purine in nucleic acid might upset genetic coding, studies on utilization were carried out. However, the results show that MAP is efficiently incorporated into RNA, mainly as adenine and guanine and only to a slight extent as MAP. In fact, in mammalian tissue and tumors it is a more efficient precursor of RNA than adenine. However, the first step in the metabolism of MAP does not appear to be demethylation. These results are in accord with the view that tissues have a small pool of MAP and that methylation may have an important role in the synthesis of nucleic acid, perhaps to transfer adenine from one intracellular pool to the other.

Substances Acted on by Non-specific Enzymes Not Involved in Intermediary Metabolism

a) Oxidation and reduction enzymes in microsomes: These important enzymes are involved in the metabolism of most drugs. The oxidative enzymes utilize O_2 from air and act through a reaction of TPNH oxidase, O_2 and TPNH to form an active hydroxyl donor. Data now indicate that the TPNH oxidase is a common denominator in both the oxidation and reduction of drugs. A common step to both kinds of reaction is: oxidase + TPNH > reduced oxidase + TPN, then in air: reduced oxidase + O_2 > "active hydroxyl donor". In the presence of an excess of FAD or anaerobically:

reduced oxidase + FAD = oxidase + FAD H_2

FAD H_2 + nitro compound > amino compound + FAD

TPNH oxidase has been solubilized. In addition nitro reductase has been solubilized. These might be important achievements, since their solubilization has been a barrier to elucidation of the mechanism of action.

b) Mechanisms of inhibition: Since drug combinations are often prescribed, it is important to know how one drug affects the metabolism of another. The kinetic analysis of microsome oxidation *in vitro* has suggested a number of mechanisms of drug inhibition—thus 1) Drug A (monomethyl-4-aminoantipyrine) competitively inhibits o-demethylation of drug B (p-nitroanisole) by combining with the enzyme. However, drugs A and B are metabolized by entirely different enzymes.

Substances like SKF 525–A, a potent and general inhibitor of drug enzymes, act non-competitively by unknown mechanisms.

c) Induced enzyme formation: There are three mechanisms by which drugs cause an increase in drug metabolism. 1) Our work shows that the effect of testosterone in increasing rates of drug metabolism in female rats is actually due to the anabolic effects of testosterone and can be produced by analogues having high anabolic activity and low sex activity. These substances do not increase synthesis of ascorbic acid and the inducing effects are additive to the general inducing action of drugs. 2) Hydrocarbons like 3, 4-benzpyrene and 3-methylcholanthrene have been shown to act through different mechanisms than phenobarbital and presumably other drugs. 3) The general non-specific action whereby many drugs induce an increased activity of their own and other drug metabolizing enzymes is still obscure. The action is not mediated through any known endocrine gland. It is of considerable interest that the repeated administration of MER 29 stimulates metabolism of acetanilide, hexobarbital, etc., and

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presumably its own metabolism. The clinical significance of this finding is unknown.

Metabolism of Imipramine (Tofranil)

The lack of correlation between effects and levels of imipramine, the antidepressant drug, made us suspect an active metabolite. Repeated doses in rats results in accumulation of a compound, identified by gas chromatography as the monomethyl analogue.

Deposition of Calcium

Studies of factors regulating fixation of Ca in the aorta and the relationship to cholesterol accumulation show: a) On incubation of aortas with serum containing Ca^{45}, there is no uptake for 24 hours and 10% of label is taken up in the next 48 hours. There is a parallel deposition of Ca and P measured chemically. The process is temperature dependent and is prevented by preheating at 80° C. for 10 minutes. Under nitrogen, Ca uptake is drastically reduced. X-ray crystallography and electron microscopy show typical hydroxyapatite formations which by histochemical methods was shown to involve an elastin component. b) Pre-incubation of aortas with various enzymes indicates that in contradiction to the postulated role of collagen, only elastin was important in calcification.

Plasma contains an inhibitor of calcification, probably a protein.

Deposition of Triglycerides (TGL)

TGL deposition in liver after the administration of ethanol is blocked by hypophysectomy. This is reflected by increased incorporation of C^{14} palmitate into TGL at expense of phospholipids. When CCl_4 is given, TGL deposition is only partly blocked by hypophysectomy. A main effect of CCl_4 is to impair hepatic release of TGL from liver to plasma. This is shown by fall in plasma TGL chemically determined, and by specific activities of TGL fractions in liver and plasma after administration of palmitate C^{14}.

Development of New Drugs

Reserpine Analogues

Reserpine is difficult and sometimes dangerous to use in prolonged therapy since it exerts a cumulative action owing to its high affinity for the transport system of amine storage sites. The reversibly acting SU 9064 (methyl ether of methyl reserpate) was developed in collaboration with Ciba Laboratories on the basis of physicochemical prognostications. It is now in clinical trial in the treatment of psychoses.

Antidepressants

We have shown that the action of imipramine is mediated through its metabolite, desmethylimipramine (DMI). Clinically this drug is effective and acts rapidly in primary depression. In collaboration with Geigy, Switzerland, we are looking for the structural characteristics of active compounds. We have found a number of monomethyl analogues of imipramine and chlorpromazine which reverse the characteristic reserpine-like syndrome. Trials in man will show whether DMI is the progenitor of more active antidepressants.

Dopamine Hydroxylase Blockers

Collaborative studies with Smith-Nephew (England) have yielded two isosteric analogues of NE (NSD–1034, NSD–1024) which block synthesis of NE at the last step. These drugs are potentially of clinical use in producing a selective deficiency of NE in certain parts of the body.

Benzoquinolizines and Other Compounds That Might Mimic 5HT Action

Structural relationships between reserpine, α-methyl-m-tyramine and other amine releasers have led us to believe that suitable modification might yield a compound which mimics the action of 5HT in brain. In studies of Pfizer's benzoquinolizines it appears that P–2647 might act like a weak reserpine, but releases only a small fraction of the brain amines. Attempts will be made to turn up more active derivatives. Analogues and isosteres of α-methyl-m-tyrosine that might act in a similar way are being studied in collaboration with Hoffmann La Roche.

Bretylium and Guanethidine-Like Drugs.

In collaboration with Burroughs-Wellcome, we are investigating structural requirements for activation or blockade of the system that releases NE at reactive sites. Small changes in structure convert one kind of compound to the other, but both are potentially useful in hypertension.

Development of New Methods of Analysis

The following procedures have been developed: 1) A simple sensitive extraction procedure to assay dopamine in biological material which provides a routine procedure since it eliminates the need for tedious column chromatography. 2) A colorimetric method for the determination of guanethidine in biological material. 3) Methods to determine α-methly-m-tyramine and α-methyl-β hydroxy-m-tyramine in tissue preparations. 4) A sensitive fluorometric method for agmatine, a good substrate for diamine oxidase. 5) A modification of the procedure of Shore and Olin to assay norepineprine in adipose tissue. Preliminary studies with paper chromatograms containing imipramine and metabolites indicate that at the temperature of liquid nitrogen these compounds have a pronounced phosphorescence. This property is the possible basis for fluorescence assay at low temperature. In drug action studies, classical methods of enzyme inhibition are often inappropriate since an enzyme may appear to be completely blocked *in vitro*, yet be functionally active *in vivo*. For this reason it has been necessary to develop methods of determining whether enzymes are blocked *in vivo* by measurement of rate at which it performs its function in the intact organism. Methods have been developed to assay monoamine oxidase, dopa-5HTP decarboxylase, and dopamine hydroxylase *in vivo*.

LABORATORY OF TECHNICAL DEVELOPMENT

Gas Chromatography

The work of this year consisted of efforts to develop methods for studying biochemical and physiological research problems. Primary emphasis continued to be on development of methods of analysis by gas chromatography including ultrasensitive methods of detection, methods for measurement of radioactivity in gas chromatographic effluents, and methods for applying these techniques toward the solution of specific biochemical and physiological research problems in which this laboratory collaborated or which this laboratory instituted or both.

The further development of the direct current electrical discharge detector for gas-liquid chromatography was continued. It had been shown elsewhere that the electrical conductivity of argon gas in the presence of a radioactive source and a high voltage was highly sensitive to the addition of small quantities of organic vapor. Because the presence of radioactivity is objectionable in many applications, a study was instituted to determine the conditions necessary to operate this device without radioactivity. By varying the voltage across the detector chamber, it was demonstrated that at low voltages the currents obtained are determined by the quantity of radioactivity present, while at higher voltages this dependence is lost. At higher voltages the fact that the current is independent of the presence of radioactivity makes operation of the device without radioactivity feasible. A detector was developed and its use was explored.

This device was found to operate only at very low concentrations of organic vapor. In its effective range this method is probably the most sensitive method for detection of organic vapors by ionization in argon. However, it is somewhat more sensitive than some of the other methods to atmospheric gas contaminants and to poisoning of electrode surfaces by adhesive anti-corona agents such as silicones.

The stable continuous discharge produced by a high direct current (D.C.) voltage in argon and helium can be used to replace a radioactive source of electrons. The plasma thus produced can be used as a conductance sensitive to organic vapors and measured with a second set of electrodes. As a result, the concentration over which the detector can be used is increased to higher levels, and the sensitivity to contamination is decreased. This detector is now in use in our laboratory.

The possibility of applying the continuous D.C. discharge to the detection of atmospheric gases analyzed by gas chromatography was explored. The device was found to be quite sensitive to oxygen, nitrogen, and carbon dioxide. The response

and the sensitivity to each of these gases was found to be dependent on the carrier gas used, its purity in the detector cell, and the nature as well as the concentration of contaminants present.

When argon is used as carrier gas, oxygen reduces its electrical conductivity. Detection of parts per million of oxygen is possible. Nitrogen in low concentration enhances the electrical conductivity. Higher concentration reduces it. The sensitivity for oxygen is approximately 10–20 times that for nitrogen. Addition of low concentrations of organic vapor increases the sensitivity to each of the atmospheric gases.

When helium is used as carrier gas, oxygen, nitrogen and carbon dioxide are measurable in the parts per billion range. They enhance the conductivity of pure helium. Small concentrations of each of these, however, overload the detector. The concentrations at which overloading occurs and the maximal conductivity obtainable varies with the different gases. It had been frequently reported that extensive purification of helium was necessary to obtain this effect. In the work described here commercial helium was used to obtain this highly sensitive detection of atmospheric gases.

Determination of Blood Gases

Experimentation with the D.C. discharge pointed the way to a series of methods for detecting atmospheric gases. Each of these methods is either more sensitive or easier to operate than the methods presently in use in analyses of blood gases. This higher sensitivity, for example, makes it possible to perform analyses on the oxygen contained in a microliter of blood.

The problem that has hindered the development of gas chromatography for blood gas analysis is that of removing the gases from the blood and delivering them to the flowing gas stream within a narrow time interval. It was found possible to remove the oxygen from oxyhemoglobin by heat. This permitted injection of blood into the inlet of a gas chromatography column system directly. The results obtained agreed quite closely with those obtained from blood oxygen using the conventional Van Slyke procedure.

The present effort is directed toward eliminating several experimental difficulties, such as those caused by coagulation of the blood in the introduction system. It is hoped to establish this as a working system of analysis and to extend the technique to a series of other determinations of physiological interest.

Radioassay: Scintillation Counting

A method has been developed whereby labelled compounds in the effluent of a gas stream are condensed quantitatively in the presence of a scintillator. The radioactivity is then assayed by scintillation counting.

Two approaches, using this method, have been explored. In one, the entire column effluent is collected in a single collection device which is monitored by scintillation counting throughout the procedure. This has been used with moderate success in studies of fatty acid synthesis. A major effort this year has involved extending this technique to the assay of sterols for a study of sterol synthesis. Unfortunately the sterols and the fatty acid esters differ sufficiently in volatility to require, at the moment at least, two different methods for condensing them. For fatty acid esters, the gaseous effluent is passed through a cigarette shaped glass cartridge filled with coated anthracene crystals. Upon emerging from the heated column outlet tube these are sufficiently volatile to pass promptly through the first layer of anthracene crystals. This layer is heated by the hot gas and the anthracene scintillates at less than optimal efficiency. By addition of a coolant air stream to the photomultiplier compartment, the successive layers of anthracene where the materials are condensed and retained are cooled sufficiently to scintillate well.

Sterols, however, condensed in the heated layer of anthracene when this system was used. An alternative system was devised whereby the column effluent was discharged into the cavity of a funnel containing anthracene crystals which were kept under suction. The top layer of anthracene was cooled by the air passing through it while the sterols were depositing on it. This system was developed and applied effectively in a study of cholesterol biosynthesis. Fatty acid esters, however, are too volatile to be retained in the funnel in the face of the torrent of gas necessary to bring the column effluent into the funnel. A successful method for retaining the shorter chain esters in this system has not yet been developed.

The second approach to radioassay by scintillation counting has involved collecting the effluent of the column in a series of anthracene-filled cartridges for subsequent scintillation counting. A careful study of the scintillation properties of anthracene, and of the mechanics of scintillation counting using a commercial liquid scintillation spectrometer, yielded a method for reproducible and quantitative recovery of labelled compounds. An automatic fraction collector for this purpose was developed.

The feasibility of collecting equal fractional aliquots for radioassay was demonstrated. This yielded a high sensitivity radioassay which, of the possible approaches, was least likely to allow operator bias to contribute to error in interpretation of the results.

Radioassay: Ionization Chamber

This project, initiated last year, first involved attempts at enhancing the response of ionization chambers for radioassay. It was predictable that the current yield per radioactive disintegration could be increased by using argon with a trace of organic vapor as ionization chamber gas. The combination of argon, high voltage, and a source of radioactivity has been used as a sensitive detector for organic vapors. It was shown feasible to substitute the radioactivity for the concentration of organic vapor as the variable. The system, however, remained sensitive to small changes in organic vapor concentration. This made the approach less attractive as a method for increasing sensitivity, since, in gas chromatographic applications, the organic vapor concentration could be expected to vary. A second approach was suggested by the work of Cacace, who used a large ionization chamber and thus achieved greater yield in ion current from each disintegration, while keeping the time constant of the system small by purging the chamber with an accessory gas flow. This approach was tried with a modification.

To avoid difficulties caused by differing volatilities of samples and by the operation of insulators at high temperature, it was decided to combust the sample to carbon dioxide and operate the ion chamber at room temperature.

A study of the behavior of this system was undertaken to determine its ultimate sensitivity, both for routine use in our laboratory and for comparison of this approach with that of the anthracene scintillation system. Conditions for complete combustion of organic vapors in the column effluent were determined. Changing the carrier gas used was found to have an effect on the result obtained, but the effect was not sufficiently large to prohibit the use of any of a number of gases should the need arise. Although the work is incomplete, it appears that the ultimate limit of sensitivity of this system will be determined at least partly by its sensitivity to change in the composition of the gas in the chamber, as well as by leakage currents and other more conventional difficulties with ion chamber measurement. The sensitivity, however, seems quite high. This work is currently being completed.

Analysis of Free Fatty Acids: Use of Carbon Dioxide as Carrier Gas

Several possible methods of analyzing free, long chain fatty acids were explored. These materials emerge from the gas chromatograph as asymmetrical peaks, making quantification difficult. The degree of asymmetry was found to depend on the concentration of fatty acids present in the column, higher concentrations yielding more asymmetrical peaks. Although the asymmetry is usually attributed to adsorptive binding on active sites of the solid support, it was also found when the inert material Teflon was used as solid support. Addition of phosphoric acid to the liquid phase was tried and found effective in reducing the asymmetry as had been reported elsewhere. The latter, however, remained when very small quantities of acids were analyzed. Carbon dioxide as carrier gas was also found to reduce the asymmetry of peaks, and, as did the addition of phosphoric acid, to reduce the retentivity of the liquid phase of the column.

These effects of carbon dioxide were not limited to the analysis of free fatty acids. Chromatography of sterols and fatty acid esters was similarly affected. No sacrifice of sensitivity is involved in the use of carbon dioxide since the hydrogen flame ionization detector is insensitive to it.

The great sensitivity of standard ionization chambers to radioactivity was exploited to extend the range of analysis of gas chromatographic

column effluents for C^{14}. A pyrolyzer was placed at the end of a fatty acid column and the $C^{14}O_2$-labeled products of combustion were run into the ionization chamber at room temperature. The ionization chamber was purged with gas at about 3 times its volume each minute. This simple system was found to effect the conversion of fatty acids completely to CO_2. It was found that samples with activity as low as 150 counts per minute could be detected.

The chromatographic apparatus and methods developed have been applied to several metabolic studies in collaboration with the Laboratory of Cellular Physiology and Metabolism.

Porphyrin

Porphyrin analysis by gas chromatography was considered for the study of the deposition of endogenous porphyrins in tissue and tumors. Several porphyrins were chromatographed at temperatures in excess of 400° centigrade but rapid destruction of those porphyrins containing oxygen and partial degradation of others indicated that the method had severe limitations. Methods of extracting porphyrins from tissue and determining their fluorescence were explored and developed to facilitate a study of the localization of endogenous porphyrins in rats bearing experimental Walker 256 carcinosarcomas. If localization in the tumor were sufficiently specific, it might provide a method for labeling or treating some new growths. In the course of studies it was noted that tetraphenylporphinesulfonate was concentrated to a greater extent than hematoporphyrin.

Blood Flow Measurement

Ultrasonic, nuclear magnetic resonance, and indicator dilution methods are being developed to facilitate hemodynamic measurements. These methods hold some promise of greater stability and sensitivity, especially for measurement of lower flow rates.

The ultrasonic blood flowmeter systems currently available cannot discriminate low flow rates from noise generated by the switching circuits that compare upstream and downstream sonic velocity to determine flow velocity. A refinement of this technique has been developed in which the upstream and downstream velocities are measured simultaneously without ambiguity by using a different carrier frequency for each channel. The signals are separated by means of filters. The difference in velocities gives a continuous indication of flow velocity. This procedure is free of errors introduced by comparing signals separated in time and obscured by switching noise.

The system, which has been constructed and tested, performs according to theoretical predictions. Construction of a transducer head suitable for application to blood vessels will allow testing *in vivo*.

The nuclear magnetic resonance flowmeter program is continuing with development of high stability, low noise electronic circuits and sensing heads. At the present time the system can be used to measure flows of the order of 5 to 300 cc/minute in a tube of 3 mm inside diameter. Flow in larger tubes can be measured with greater ease than small tubes. Several of the circuits developed in the design of these flowmeters have had novel features.

Work has been completed on a project for improving the use of ascorbic acid for the detection of cardiac shunts. A platinum electrode on the tip of an intracardiac catheter is used to measure the redox current which is related to the ascorbic acid concentration. The technique has been found valuable in detecting shunts during cardiac catheterization.

Ultrasonics

The chemical and physical interactions of ultrasound in various media have been studied to determine the extent to which chemical reactions can be modified or accelerated by this form of energy. The extent to which changes in velocity and attenuation can be used to measure the progress of chemical and enzymatic reactions has also been investigated.

Ultrasound at intensity levels sufficient to cause cavitation produces sonichemical oxidations by the action of hydroxyl free radicals. Tryptophan, for example, when irradiated produced formylkynurenine, kynurenine, and 3-hydroxy kynurenine. Irradiation of pthalic and benzoic acids produced fluorescent hydroxylated products. This system has been utilized in the development of a method for measuring these aromatic acids

by the fluorescence of the hydroxy-acids produced.

The attenuation and velocity of ultrasound was measured in a reaction cell to determine the time course of enzymatic reactions. It was possible to obtain a satisfactory record of the action of invertase on sucrose. Starch and alpha amylase have been shown only to give a satisfactory signal. The system is considered to have reasonable potentialities for the study of reaction where no good spectrophotometric methods are available.

Theoretical Analysis of Transport

The earlier work on integral equation methods in biological transport problems has been continued and expanded. Some of the fundamental notions in biological transport theory have been more closely examined. This has resulted in a compact set—theoretic definition of input-output systems. In this formulation a biological *event* is taken to have an undefined but intuitively understood meaning. Everything else is defined in terms of *event* and the primitives of set theory. This leads to the definition of a biological system as a set of ordered "stimulus-response" or "input-output" pairs in which each member of the pair is a set of biological events. This definition has the advantage that mathematical structure can be introduced into the analysis in a systematic way. For example, a linear biological system is represented as a linear operator defined on a linear vector space whose members correspond to states of the system. Such an operator representation provides a general and compact symbolic language divorced from computational details. For example, for states distributed discretely in time the operators, symbolized by single letters, are ordinary matrices, and for states distributed continuously are kernels of integral equations. Such a representation is also ideally suited for machine computation. The formulation also seems much more closely related to the intuitive ideas used by the experimentalist in studying transport problems than some other approaches. Its development makes possible a rather direct road to a variety of particular mathematical techniques.

Particular biological problems studied within this framework have been fatty acid transport, potassium washout from kidney slices, and countercurrent exchange.

Fluorescence and Phosphorescence

A series of isomers of several substituted acetanilides has been purified for the study of the relation of their phosphorescent spectra to structure. The compounds will be studied in the aqueous propylene glycol glass at 184°K and their spectra compared with those obtained in nonaqueous media at 184°K and 77°K.

Several materials (e.g. chlorpromazine, kynurenic acid and fluorescein) that have been shown to be phosphorescent on paper at liquid nitrogen temperature have been studied. At room temperature the feeble phosphorescence normally obtained can be enhanced by the use of infrared or electric fields. Subjecting them to such energy sources causes these phosphors to emit their phosphorescence more rapidly and makes it possible to obtain intensities that will allow sensitive measurement of such compounds. Various glasses, such as vinyl acetate, cellulose acetate butyrate and boroglycerine glasses have been shown to provide physical conditions that favor phosphorescence, but unless the stimulation technique can be used to enhance the emission the results to date indicate that samples of the order of milligrams will be needed for satisfactory measurement.

Freezing Point Determinations

A two-stage thermoelectric refrigeration system has been constructed. The apparatus will rapidly cool a sample holder positioned under a microscope so that samples can be observed while the temperature is controlled electrically. This system accommodates 8 samples of one micromilliliter each, under oil in holes in the sample holder. The current through the thermoelectric elements directs the flow of heat and rapidly changes the temperature. The apparatus, now operating, is being provided with a servo control system that will allow selection of the equilibrium temperature while the ice liquid equilibrium is observed in the microscope.

LABORATORY OF CARDIOVASCULAR PHYSIOLOGY

Electrolyte Heart Changes During Homeometric Autoregulation

The exhibition of homeometric autoregulation by the left ventricle has been amply confirmed in the isolated heart. It was therefore assumed that an increase in the amount of tension developed per unit of time by the myocardium must be followed by the development of some biochemical rearrangement which provides for an increased contractility. We had previously suggested the possibility that changes in intracellular potassium might play a role in this phenomenon. It has now been shown that a net efflux of potassium does occur when the total tension developed by the myocardium is increased either as a result of increasing aortic pressure or heart rate. The amount of the net efflux is small. However, it has been shown that this efflux is comparable to that observed when a comparable increase in contractility is produced by the administration of glycosides.

It will be difficult to establish a cause-effect relation between the hemodynamic and the biochemical changes observed. However, the magnitude of the intrinsic changes in contractility observed underscore the importance of continued efforts to characterize the biochemical changes.

The following premises are the basis of a working hypothesis:

A. Homeometricity is observed when myocardial TTI (the amount of tension developed by the heart per unit of time) is increased (established).

B. The O_2 consumption of the heart is determined by TTI; when TTI is increased the O_2 consumption of the heart is increased (established).

C. When the O_2 consumption is increased, CO_2 production is increased and intracellular pCO_2 and hydrogen ion concentration are increased; these are changes which influence intracellular potassium (assumed).

D. A net efflux of potassium occurs when TTI is increased (established).

E. When intracellular potassium is lowered myocardial contractility is increased (widely considered to be established).

One valuable piece of information growing out of this study is the recognition of the occurrence and the magnitude of Bowditch effect in the intact heart.

Isolated Papillary Muscle

The basic mechanics of heart muscle have been explored using the cat papillary muscle. By relating the force the muscle develops to its velocity of shortening, two helpful generalities have been clearly demonstrated. 1) Increasing muscle length increases the maximal force of the muscle but does not alter its intrinsic (maximal) velocity. 2) Inotropic interventions (heart rate or norepinephrine), at any one muscle length, increase intrinsic velocity of the muscle with a variable change in force. This increase in intrinsic velocity defines inotropism. The active compliances of the muscle have been shown to be constant in the face of these changes and thus do not contribute to the observed phenomena. Interrelations of work and power have also been studied. This has yielded information on the influence of afterload on performed work and work capacity. This type of analysis forms the background with which to compare phenomena observed in the intact heart.

Afferent Pathways Influencing Efferent Autonomic Discharge

Carotid Body Hypoxia.

As previously shown, carotid body hypoxia causes a marked bradycardia when respiration is controlled. This was reduced, but not abolished by vagotomy. The subsequent administration of hexamethonium completely abolished the response, thus establishing that the observed bradycardia is attributable to sympathetic withdrawal as well as increased vagal activity. Further, the contractility of the atrium is reduced by carotid body hypoxia and varying degrees of heart block are frequently observed. The latter responses are considered to be due to efferent vagal activity.

Ventricular function curves showed that carotid

body hypoxia causes a reduction, never an increase, of ventricular contractility, indicating a reduction of sympathetic discharge to the heart. The reduction in heart rate after vagotomy and the reduction in ventricular contractility are associated with a concomitant increase of total peripheral resistance. These findings show that hypoxic stimulation of the carotid bodies causes a dichotomous sympathetic response, that is, a reduction of sympathetic discharge to the heart and a simultaneous increase of sympathetic discharge to the peripheral vasculature. Such a dichotomy has not previously been demonstrated.

Central Nervous System Hypoxia

Since the response to total body hypoxia differs markedly from that observed with carotid body hypoxia, it was reasoned that other mechanisms might be involved. Accordingly, a preparation was developed in which differential cerebral hypoxia could be induced. A marked increase in ventricular contractility (as well as peripheral resistance and heart rate), such as is observed in total body hypoxia, occurred.

Cardiac Sympathetic Nerve Discharge During Carotid Body and Central Nervous System Hypoxia

An electroneurographic correlation with A and B above was obtained by means of separate studies in which nerve impulses were recorded from the lesser cardiac nerve in the cat. Whereas systemic hypoxia caused a marked and persistent increase in the sympathetic discharge to the heart even after denervation of the peripheral chemoreceptors, isolated carotid hypoxia lessened this impulse activity.

Vagal Afferent Pathways

The stimulation of certain afferent vagal fibers has been shown to produce a very marked increase in left ventricular contractility as demonstrated by the shift of the ventricular function curve. The possible role of these fibers, with special reference to the location and type of receptor they serve is the cause of considerable speculation in this laboratory at the present time. The presence of chemoreceptors on the inflow portion of the pulmonary vascular bed is being investigated.

Effect of Carotid Hypotension on Renal Blood Flow

A technique has been developed to meter and/or control renal blood flow without perceptible interference with renal innervation. Studies performed using this technique demonstrate the importance of reflex influences on renal function. The major findings in this study to date are:

(1) Carotid occlusion is accompanied by an increased renal vascular resistance; during occlusion renal blood flow may fall as renal arterial pressure increases.

(2) When the kidney is perfused at a constant flow, renal arterial pressure increases indicating that the response is due, at least in part, to extrinsic nervous influences and not simply to autoregulation.

(3) In many animals, a given change in renal perfusion pressure produced by carotid occlusion is accompanied by little or no change in renal blood flow, whereas the same change in pressure produced by decreasing renal inflow resistance produces a substantial change in renal blood flow.

(4) The fall in renal blood flow which may be observed during carotid occlusion can be changed to an increase in blood flow by dibenzyline, at a time when dibenzyline itself does not modify the resting blood flow.

(5) Conversely when arterial pressure is increased, as with stellate ganglion stimulation, renal blood flow rises and diuresis occurs.

Vagal Afferent Pathways Inducing Reflex Vasodilation and Hypotension During Myocardial Ischemia

The hypotension accompanying some instances of myocardial infarction has long been thought to be due almost exclusively to myocardial incompetence. In a dog hindlimb perfused at constant flow, it was demonstrated that acute occlusion of the circumflex branch of the left coronary artery reflexes induces a marked peripheral vasodilation. Cooling of the vagi abolished this response which in turn, reappeared when the vagi were rewarmed.

Further evidence of sympathetic inhibition under these circumstances was obtained by electroneurographic techniques in the cat; a persistently diminished sympathetic impulse rate was obtained, while the vagi were intact, when local myocardial ischemia was induced.

Dynamics of the Cardiac Cycle

The Ventricle. Hemodynamic Determinants of the Duration of Left Ventricular Systole, Ejection and Isovolumic Contraction

These studies were carried out in a newly developed right heart bypass preparation. The following observations were made:

1. The effect of increasing stroke volume is to prolong ventricular ejection with little or no effect on total duration of ventricular systole. There is, therefore, a progressive shortening of the isovolumic contraction period as stroke volume is augmented.

2. The effect of increasing mean aortic pressure is to reduce ventricular ejection time with little or no effect on the total duration of ventricular systole. There is therefore a prolongation of the isovolumic contraction period.

3. The effects of augmenting heart rate are to shorten both the duration of ventricular ejection and total systole, the former more than the latter so that duration of isovolumic contraction is decreased.

4. The independent augmentation of heart rate, aortic pressure and stroke volume each a) increase the mean rate of ventricular ejection, and b) increase the average rate of rise of ventricular pressure during isovolumic contraction.

The data are consonant with the view that increasing LVED fiber length, increasing heart rate and increasing mean aortic pressure each lead to a more vigorous ventricular contraction.

The Atrium

Study of the participation of the atria in circulatory hemodynamics and regulation has been continued with particular reference to the transport function of the atria. Evidence was obtained indicating that the duration of ventricular diastole, the vigor of atrial systole and the timing of atrial systole are the fundamental factors influencing the relationship between mean left atrial pressure and left ventricular end diastolic pressure and thus the relation between the central pressure head which must be overcome by blood returning from peripheral vessels and the hemodynamic stimulus to the ventricle's force of contraction. Independent variation either of mean aortic pressure or of stroke volume had little or no influence on this relation; thus, changes in heart rate and sympathetic and vagal tone, because of their influence on the vigor of atrial systole and on diastolic time appear to be those influences which most importantly affect this relation.

Studies on atrial fibrillation, induced either electrically or with aconitine, were initiated in the dog with surgically induced heart block. This was done so as to be able to ascribe observed changes to alterations in atrial activity with the ventricle beating at a constant and regular rate. The observations obtained thus far suggest that, as might be expected, the occurrence of atrial fibrillation is most important at the higher ventricular rates. Specifically above a ventricular rate of 120/min, inducing atrial fibrillation produces a rise in mean left atrial pressure for any given left ventricular end diastolic pressure. Further, it has been observed that, when atrial fibrillation is induced, mean aortic pressure and cardiac output decline even when left ventricular end diastolic pressure is maintained. These data appear to confirm our previous findings that a properly placed and vigorous atrial systole contributes to mitral valve closure as well as to ventricular filling. The atrium can be considered to perform in relation to the ventricle those functions that a booster pump supplies to a primary power pump in mechanical systems.

Electrophysiological Studies

Increased synchronicity of ventricular contraction may yield a more forceful contraction from any given ventricular end diastolic pressure and fiber length. Electrophysiological studies with special reference to timing have been initiated to investigate this possibility. These studies are aimed primarily at determining whether an intervention such as sympathetic stimulation produces a more synchronous ventricular systole.

The evidence obtained thus far indicates that, in the vagotomized animal and in the animal after ganglionic blockade, the effect of increasing heart rate is to prolong A–V conduction time whereas stellate stimulation and catechol amine infusions shorten A–V conduction times. Vagal stimulation prolongs A–V conduction time. Suggestive evidence was obtained indicating that the augmented heart rate in the animal after ganglionic blockade prolongs ventricular activation time (QRS duration) whereas a similar increase in rate produced by stimulation of the cardiac sympathetic nerves reduces ventricular activation time at any given heart rate. These data lend ECG support to those micro-electrode studies of similar effects of heart rate, sympathetic and parasympathetic influence on A–V conduction time.

The usual clinical ECG experience would suggest that increasing heart rate is associated with a shortening of the P–R interval. Our data indicate that the primary effect of increasing rate is to prolong the P–R interval and that the shortening which occurs clinically with increasing rate is, therefore, related to the associated increase of sympathetic tone. Finally these experiments suggest and hemodynamic studies of the relation of ventricular and atrial dynamics and the effects thereon of rate, vagal and sympathetic tone should include data concerning the time relationships between atrial and ventricular events and the activation time of the ventricular myocardium.

Myocardial Catechol Amines

Since the cardio-dynamic effects of cardiac sympathetic nerve stimulation are related to the release of myocardial catechol amines, experiments were undertaken to study the myocardial production and/or utilization of these amines. It was found that stimulation of the cardiac sympathetic efferent nerves was accompanied by a release of catechol amines into coronary blood. This release was a function of stimulation intensity and occurred even when stroke volume and coronary outflow were maintained constant. Comparison between the ethylenediamine and the trihydroxyindole methods for the analysis of plasma catechol amines showed both quantitative and qualitative differences; only the results with the latter method showed a consistent relationship to the hemodynamic changes observed during cardiac sympathetic stimulation. The acutely sympathectomized heart was found to extract catechol amines from coronary blood in the absence of nerve stimulation. The patterns of extraction observed indicated that the quantity of catechol amines in the myocardium modify the extent of extraction in the unstimualted state as well as the extent of release during stimulation. Continued stimulation of the cardiac sympathetics was accompanied by at least a partial depletion of the myocardial catechol amine stores. These stores appeared to be repleted by circulating catechol amines, since the response to any given intensity of stimulation was potentiated by a prior norepinephrine infusion. Dichloroisoproterenol prevented neither the myocardial extraction of catechol amines in the unstimulated state nor the release of catechol amines during sympathetic stimulation; it did lessen or abolish the cardiodynamic effects of sympathetic stimulation. Data were also obtained which indicated that a direct coronary vasoconstriction is one of the consequences of cardiac sympathetic nerve stimulation, the vasodilatation usually observed being due, at least in part, to overriding metabolic factors.

Having established the characteristics which appeared to modify the release and extraction of myocardial catechol amines during direct nerve stimulation and in the acutely sympathectomized heart, experiments were undertaken to determine whether, during carotid sinus hypotension when myocardial contractility is reflexly increased, the release of myocardial catechol amines also occurred. These experiments showed that carotid sinus hypotension can be accompanied by the release of catechol amines and that the release is prevented by bilateral stellectomy. This reflex release of catechol amines appears to be independent of changes in cardiac output, heart rate, coronary blood flow changes and changes in arterial blood pressure.

Experiments were also done to determine the effects of bretylium on the myocardial release and uptake of catechol amines. The initial response to the injection of bretylium tosylate was a release of myocardial catechol amines. This release appeared to occur from myocardial stores since it was observed after the injection of dichloroisoproterenol. After the initial myocardial release

of catechol amines, bretylium blocked the release of catechols associated with cardiac sympathetic nerve stimulation. The failure of nerve stimulation to release catechol amines after bretylium was not due to depletion resulting from the initial release, since tyramine still caused a release of catechols from the heart in the presence of bretylium. Although bretylium prevented the release of catechol amines associated with cardiac nerve stimulation it does not appear to interfere with the myocardial extraction of catechol amines even in the presence of dichloroisoproterenol. Thus the antihypertensive effect of bretylium appears to be related to blocking of sympathetic axon conduction.

Vasculature of Resting Musculo-cutaneous Areas During Exercise

By metering blood flow in resting areas as well as in those in which simulated exercise was induced, it was ascertained that the vasoconstriction that occurs in the resting areas is preceded by a substantial vasodilation. This is not blocked by atropine but is abolished either by local sympathectomy or TEAC indicating a brief initial diminution in sympathetic tone in the resting area with the onset of exercise. The cholinergic vasodilator system of Uvnas and Folkow is apparently not involved. The secondary vasoconstriction in the resting area was abolished by TEAC.

The possible role of the initial vasodilation in resting areas in facilitating the transition from rest to exercise is not at present understood.

The Kallikrein System

Studies on this hypotensive system have provided data to suggest that the inborn biochemical lesion in hereditary angioneurotic edema is due to a deficiency of a serum inhibitor of plasma kallikrein. Two biologically active polypeptides have been isolated from the incubation of human urinary kallikrein with acid-treated human plasma and the structure of these kallidins has been determined. Methods have been devised for the preparation of esentially homogeneous human urinary and human pancreatic kallikrein. Comparison of these proteinases with pure hog pancreatic kallikrein by electrophoresis on cyanagum gel has revealed that each hypotensive enzyme has its own characteristic mobility. Rabbit antibody to crude and partially purified human urinary and human pancreatic kallikreins has been shown to cross react, but antibody to human urinary kallikrein failed to precipitate in agar gel and to inhibit the vasodilator activity of hog pancreatic or dog urinary kallikreins.

The possibility was investigated that the vasodilation accompanying skeletal muscle exercise is due to activation of the kallikrein-kallidin system. These experiments revealed that the lymph from most dogs during exercise gave only a barely detectable level of kallikrein as measured on the uterine strip.

LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM

The research activities of the Laboratory of Kidney and Electrolyte Metabolism may be divided into several broad categories. These will be discussed separately.

Renal Physiology

Micropuncture Studies

In recent years the reapplication of the micropuncture technique of Richards et al. has provided significant new information concerning the site and mode of transfer of water and electrolytes across the tubular epithelia in the amphibia as well as in certain small rodents. No comparable studies have been completed in the dog. Renal function in this species appears to resemble that in man more closely than in those species in which micropuncture data are available. Preliminary studies in this laboratory have established the feasibility of applying the micropuncture technique to the dog. An elegant system has been developed in which micropuncture data may be correlated with those obtained during clearance studies. It has been shown that reabsorption in the proximal tubule is accomplished by abstraction of isosmotic fluid. The ratio of the osmolality of tubular fluid to plasma remained 1.00 throughout the accessible portion of the proximal nephron during both hydropenia and water

diuresis. It is of interest that distal nephrons have not as yet been visualized in the accessible portion of the outer cortex. It is proposed to examine younger animals in which the proximal tubules are less elongated in an effort to determine whether the distal nephron will be accessible for puncture under these circumstances. Future studies are to involve measurements of the potential difference across the nephron, the in situ pH, etc.

Studies of acidification in the proximal nephron of the Necturus, initiated some time ago, continue. The purpose and difficulties of these experiments have been detailed in the previous report. In general, it is hoped to establish whether or not acidification is accomplished by direct reabsorption of bicarbonate or by secretion of hydrogen ions in exchange for sodium, as is generally accepted. The latter view, which involves conversion of luminal sodium bicarbonate to carbonic acid, requires that the rate of dehydration of carbonic acid be equal to the rate of bicarbonate reabsorption. In the absence of carbonic anhydrase the non-catalyzed dehydration of H_2CO_3 is rate limiting. In order for it to proceed at a rate equivalent to that of maximal bicarbonate reabsorption, the in situ pH must be approximately 1 pH unit less than that of plasma. In contrast if carbonic anhydrase is present in the luminal fluid or on the membrane the dehydration process will be virtually instantaneous and the in situ pH should approximate that of plasma. Carbonic anhydrase inhibitors should decrease rather than increase the pH under these circumstances. Though these considerations are amenable to experimental verification, the major deterrent has been the unavailability of pH sensitive microelectrodes. In recent months some of the difficulties have been eliminated and a pH sensitive microelectrode has been successfully prepared. In preliminary studies it appears that the in situ pH in Necturus does not differ from that of plasma. No studies utilizing carbonic anhydrase inhibitors have been completed. However, as an important check on the validity of the technique, the transtubular and transcellular potential differences have been determined. The values observed were similar to those reported by others.

Carbonic Anhydrase in Rat Renal Cortex

Methods for autoradiographic localization of carbonic anhydrase in renal tissue are being developed in collaboration with the Department of Anatomy of Georgetown University. It is hoped to determine the cellular localization of carbonic anhydrase as well as its presence or absence in the luminal membrane. The experimental approach is dependent on and takes advantage of the known high binding constant of acetazoleamide, a carbonic anhydrase inhibitor, and the enzyme carbonic anhydrase. Theoretically, it should be possible to localize autoradiographically the site of the enzyme-inhibitor complex if sufficient labeled acetazoleamide (H^3) is presented to the intact renal cortex.

Measurement of Medullary Blood Flow

In view of the important role of the countercurrent vascular system in the medullary and papillary portion of the kidney, in the concentrating and diluting process, studies have been directed at developing a technique for the estimation and evaluation of renal medullary blood flow in the intact dog. The purpose of these studies as well as the pertinent background information were discussed in detail in the last report. It is now generally accepted that the vascular countercurrent system is largely responsible for the maintenance of the osmotic gradient in the interstitial spaces of the kidney. In recent months it has been possible to estimate effective flow indirectly, by measuring the rate of accumulation of diffusible gas, in this instance, molecular hydrogen, by both cortex and medulla. A technique requiring implantation of a platinum electrode responsive to hydrogen tension has been devised. Hydrogen is administered intratracheally to the dog and effective flow to the medulla and cortex are estimated. In acute studies hydrogen is very effectively excluded from the medulla in antidiuresis, and its rate of entry is considerably increased when urine flow is elevated by administration of osmotic diuresis. These studies are now being extended to dogs in which electrodes for chronic studies have been implanted in both cortex and medulla.

Effect of Diuretic Agents in Vasopressin-Resistant Diabetes Insipidus

Studies concerning the influence of diuretic agents on urinary dilution in patients with vasopressin resistant diabetes insipidus have been completed. In the previous report it was noted that chlorothiazide, a diuretic agent, which interferes with sodium chloride reabsorption in the diluting segment, results in an expected rise in urine osmolality and a paradoxical fall in urine flow. The latter effect contrasts strikingly with that observed in normal individuals in whom an increase in urine flow is uniformly observed. It was suggested that the fall in urine flow may be conditioned by the resultant sodium depletion. The latter may provide the basis for a diminution in volume flow throughout the nephron either as a result of a decrease in filtration rate or a compensatory increase in proximal reabsorption. If sodium depletion per se is responsible for the decrease in urine flow, then antidiuresis should also be achieved with mercurial diuretics. This prediction proved correct in two patients examined.

Phosphate Secretion in the Dog

Investigations designed to attempt to demonstrate net phosphate secretion in the dog have been completed. Although such secretion has been reported by others, the evidence has not been convincing. Repeated attempts, utilizing all of the many experimental manipulations reported to result in phosphate secretion, have in our hands provided no evidence of this phenomenon in the dog. The present studies differ from earlier ones in an important respect. Steady state conditions insofar as possible were maintained throughout; that is filtration rate was relatively constant as was plasma phosphate concentration.

Electrolyte and Water Transport Across Biological Membranes

Renal Cortical Slices and Tubule Suspensions

For a number of years this laboratory has been engaged in a study of the transfer of K^{42} into and out of renal cortical slices. Although the results of these studies have generally been confirmatory of others it was recognized that the technique may provide little information concerning the transmembrane flux of K^{42} across the pertinent cell borders. The interposition of an interstitial space through which isotope must diffuse complicates interpretation of flux data. In general, studies such as these merely afford information concerning influx and efflux of isotope into and out of the whole tissue. In preliminary studies utilizing a constant flow technique in which isotope is passed continuously over a single slice immersed in the well of a scintillation counter, it was possible to establish conditions which provided reproducible data under a variety of circumstances. The technique was described in detail in an earlier report. Utilizing this method, it was observed that the cardiotonic steroid, strophanthidin, diminished the uptake of K^{42} into the slice, an effect analogous to that observed in other tissues including the red cell; however, in contrast to the red cell the washout of K^{42} was markedly accelerated. In view of the complications afforded by the presence of an interstitial space it was considered that the latter effect, the augmented K^{42} efflux, was an experimental artifact and that efflux across the cell membrane was either unchanged or diminished. In order to determine transmembrane fluxes with some degree of certainty it was necessary to eliminate the interstitial space. A technique for the preparation of a suspension of isolated proximal tubule segments was devised. The technique involves the dissolution of the interstitial space by perfusion of the intact kidney with collagenase (a proteolytic enzyme) and the subsequent collection of the short lengths of proximal tubule. The cell suspensions proved viable as demonstrated by normal respiration (oxygen consumption greater than in comparably studied slices), accumulation of PAH to a degree greater than in slices, and maintenance of normal tissue electrolyte composition. The effect of strophanthidin on K^{42} flux in this preparation supported the initial contention that augmented flux in the slice was an experiment artifact. As in the red cell and cortical slices, the drug interferes with the uptake of K^{42} but in contrast to the results in slices efflux of K^{42} is actually diminished. This last is similar to that observed in the red cell. The results cast considerable doubt on the validity of kinetic analyses of transport phenomena in intact cortical slices, and it is proposed to reexamine these in the tubule suspension system.

Sodium and Potassium Dependent ATPase in the Renal Cortex

A limited characterization of a sodium-potassium dependent ATPase in dog renal cortical tissue has been completed. The enzyme, which requires sodium and potassium for activation, has been observed in other tissues including crab nerve, red cells and guinea pig kidney. Since its activity has been shown to correlate with cation transport in red cells, it is presumed to be involved directly in the carrier mediated transport process. It is present in high, though variable concentration, in dog renal cortex. Homogenates require appropriate concentrations of sodium, potassium, magnesium and buffer for maximal activation. Of interest is the observation that the activity of the enzyme is depressed by as much as 75% following incubation with the cardiotonic steroid, strophanthidin. This may be cited as indirect evidence in support of the view that the enzyme is intimately involved in the cation transport process, since strophanthidin also interferes with active transport in this tissue. Aldosterone, the adrenal steroid which is thought to stimulate cation transport, had no effect on the activity of the enzyme, whereas the addition of either organic mercury or calcium effectively inhibited the activity.

Cation Transport in Red Cells

Alterations in Lipid Content. Studies of the mechanism of active cation transport in intact red cells and red cell ghosts comprise a significant segment of this laboratory's research activity. Attention has been focused on the influence of the lipid composition of the red cell membrane on cation transport. Three groups of red cells were studied—(a) rat red cells obtained from fatty acid deficient animals in which the fatty acid content of the cells was markedly altered; (b) human red cells from patients with acanthocytosis in whom the fatty acid content of the cells is altered in a fashion similar to (a) above; and (c) cells from vitamin E deficient rats. Despite significant changes in the lipid structure of the cell membrane the transport of sodium and potassium was unaffected.

Transport in Rat Red Cells. In the course of these studies a number of interesting observations were made relative to the kinetics of electrolyte transport in normal rat red cells. These proved of greater interest than the original purpose of the studies and may provide a tool for the examination of carrier mediated transport. It was noted that rat red cells contrast strikingly with those from other species studied. The rat possesses a sodium-potassium linked exchange pump, however the curve relating the activation of sodium outflux by external potassium does not resemble the classical Michaelis-Menten curve but rather is described by a sigmoid relationship; that is, negligible sodium transport occurs until the concentration of potassium is elevated above a critical level. Similar considerations apply to the curve relating potassium influx to external potassium concentration. It is also of interest that strophanthidin, a potent inhibitor of transport in human red cells, is relatively ineffective in this species, whereas scillaren is extraordinarily active. Furthermore, it was noted that exchange diffusion of sodium is inhibited by concentrations of scillaren which are without influence on active sodium-potassium exchange. An elevation of the external potassium concentration resulted in the reappearance of exchange diffusion. Although these studies are still in a preliminary stage, it may be reasonable to consider that active transport and exchange diffusion of sodium, though both carrier mediated, occur at different sites on the membrane.

Influence of Divalent Cations on the Permeability of the Red Cell to Na and K. In association with the above studies in intensive investigation of the effect of divalent cations and metabolic alterations on permeability to cations of human red cells had been undertaken. It has been known that the addition of calcium results in a marked increase in the passive permeability to potassium in red cells exposed to iodoacetate (IAA) and adenosine. This effect, which requires all three substances, served as a basis of the following studies. Normal and substrate depleted human red cells were examined with respect to the effects of (a) calcium, (b) IAA (and other inhibitors) and (c) adenosine (or other substrates) on the movement of potassium and sodium. It was noted in normal cells that the augmentation of potassium permeability required the presence of either calcium or strontium, that IAA could be replaced by iodoacetamide, NEM or BAL, with similar results, and

that inosine was the only compound which could substitute for adenosine. In contrast to the above effects in normal cells, energy depleted cells were responsive to calcium alone (i.e. without inhibitor or substrate). Furthermore, although a greater effect was obtained in partially depleted cells by addition of IAA and adenosine, adenosine exerted no effect when cells were depleted for 25 hours or longer. These cells respond maximally to calcium alone. Also preexposure of cells to adenosine or inosine prevented the subsequent action of calcium, IAA and adenosine on potassium permeability. Although final conclusions are not yet warranted it is sugegsted that potassium permeability is regulated both by calcium and a metabolic product which can be removed by a reaction involving adenosine (as limited by IAA) or by depletion of the cell. Removal of the metabolite predisposes the membrane to the action of calcium which is ineffective alone in non-depleted cells, but is effective in depleted cells even without the addition of IAA and adenosine. None of the above manipulations was capable of altering the passive permeability of the red cell to sodium. However, the inhibition of active sodium and potassium transport which occurs following addition of glycolytic inhibitors, such as IAA, is accentuated by the simultaneous addition of adenosine. Furthermore adenosine inhibits exchange diffusion in cells exposed to IAA. These latter observations support the view that an unknown product of metabolism is important in the maintenance of the integrity of the membrane with respect to carrier mediated transport as well as passive diffusion.

Water Movement Across the Toad Bladder

Vasopressin. The antidiuretic hormone, vasopressin, reduces urine flow and permits the excretion of a concentrated urine in the intact animal, by increasing the permeability of the distal nephron to water. The increase in permeability is thought to be accomplished by hormone-induced enlargement of aqueous channels (pores) in both the distal convolution and collecting duct. This view of the action of vasopressin is based on studies of water movement across the epithelial structure of frog and toad, skin and bladder. In all of these, addition of vasopressin to the serosal (blood surface) of the isolated membranes accelerates the osmotic flow of water across the structure. In association with this, net movement of sodium is also accelerated, as evidenced by an increase in the so-called short-circuit current. Little information is available concerning the metabolic effect of the hormone and it has been suggested that binding of the hormone by its SS bridge to SH groups on the membrane, and a subsequent interchange reaction, springs open pores in a mechanical fashion without the interposition of any known metabolic energy sources. The latter thesis is based on the observation that vasopressin binds both to renal tissue and toad bladder and that prevention of binding by either acidification of the bathing medium or the addition of sulfhydryl inhibitors prevents the permeability effect of the hormone. An alternative thesis developed in this laboratory involves the intermediacy of adenosine 3′5 phosphoric acid, a cyclic nucleotide (cyclic AMP). It is known that effects similar to those of ACTH in the adrenal and glucagon (or epinephrine) in the liver can be produced with vasopressin. ACTH and glucagon have in common the ability to stimulate the formation and accumulation of cyclic AMP in their respective receptor tissue. The nucleotide in both tissues accelerates the conversion of inactive to active phosphorylase, the enzyme which converts glycogen to glucose-1-phosphate, and is considered to be an integral factor in the development of the physiologically recognizable effects of the hormone. On the basis of these observations it was considered that vasopressin may simulate ACTH in the adrenal and glucagon in the liver by virtue of a stimulatory effect on cyclic AMP production. More pertinent to the present discussion was the assumption that cyclic AMP may also be an intermediate in the action of vasopressin in both the kidney and toad bladder. It was suggested that the hormone accelerates the conversion of ATP to cyclic AMP in these tissues and that the latter compound in some as yet undefined manner directly or indirectly increases the permeability of the membrane to water. It is of significance in this regard that theophylline, a methyl xanthine, prevents the degradation of cyclic AMP in other tissues and thereby conceivably could magnify the effect of endogenously formed cyclic AMP.

Evidence in favor of this view of the action of vasopressin was developed utilizing the toad blad-

der sac as an experimental model. Addition of vasopressin to the outer surface of the bladder sac markedly accelerates the osmotic flow of water across the membrane and stimulates active sodium transport. No other substances have been known to exert this unique effect. However, the addition of cyclic AMP to the serosal surface results in an immediate increase in water movement indistinguishable from that due to vasopressin. Short circuit current is also increased. Theophylline, which as noted earlier, interferes with the breakdown of cyclic AMP, exerted similar effects on water movement and sodium transport, as did vasopressin and cyclic AMP. N-ethylmaleimide, a sulfhydryl inhibitor, which prevents the action of vasopressin also interferes with the action of cyclic AMP and theophylline. Acidification of the bathing medium which interferes with the action of vasopressin in toad bladder also limits the effect of theophylline on water movement but does not inhibit the effect of cyclic AMP. Since acidification interferes with the conversion of ATP to cyclic AMP in other tissues a similar effect in the toad bladder could account for these results. In association with the above studies it has been possible to demonstrate an increase in phosphorylase activity in toad bladder following incubation with vasopressin. Though this is presumed to be mediated by an increase in the concentration of cyclic AMP, direct measurements of the cyclic nucleotide have not yet proved feasible. The latter project is being actively pursued in collaboration with the Department of Pharmacology of Western Reserve University.

Estimation of Vasopressin in Biological Fluids. Interest in the metabolism and action of vasopressin has provided a stimulus for initiating an investigation of the feasibility of a modified double isotope derivative method for the quantitative estimation of vasopressin in biological fluids. The method was originally developed for the measurement of aldosterone. At present vasopressin can be estimated only by means of a relatively nonspecific bioassay method. To date a technique for the isolation of pure arginine vasopressin from crude pituitary powder has been developed. Vasopressin has been acetylated successfully with nonlabelled acetic anhydride prior to adding the labelled compounds for measurement. The stoichiometry of the process is being examined at present.

Effect of Solute Concentration on Net Water Movement Across Toad Bladder. The laboratory has also been engaged in a detailed examination of the characteristics of osmotic flow of water across the toad bladder utilizing the toad sac technique. As indicated above, vasopressin uniformly augments the osmotic flow of water across this tissue. The imposition of a 220 milliosmolal gradient (serosal concentration greater than mucosal) results in a considerably greater net flow of water out of the sac than when the gradient is reversed and net flow is directed inward. This rectification phenomenon occurs over a significant range of osmolalities. Hypotonicity of the serosal bathing solution (osmolality below 220) exerts an independent effect since it decreases the permeability to H_2O in both directions without altering the rectification phenomenon. Hypertonicity of the serosa also appears to lower the permeability somewhat. In contrast hypertonicity of the mucosal bathing solution significantly lowers the permeability to water on that surface. These observations are being reexamined utilizing tritiated water in an effort to determine the changes in the unidirectional fluxes of water across both surfaces of the membrane as affected by variations in the imposed osmotic gradient. Similar studies are also being performed using labelled urea and thus far appear to suggest that the simple pore hypothesis involving bulk flow of water across the membrane, discussed in an earlier report, is inadequate to account for all of the results.

Water Movement Across an Artificial Liquid Membrane. The validity of the pore hypothesis has also been questioned in experiments utilizing an artificial membrane. The technique involves the use of a non-aqueous liquid membrane, mesityl oxide, separating two aqueous phases of differing osmolalitities. Preliminary results were discussed in the previous report. The studies were designed to test the existing hypothesis which states that a discrepancy between the ratio of the water activities on two sides of the membrane and the ratio of the unidirectional fluxes of water across the membrane are explicable on the basis of bulk flow of solvent through aqueous channel or pores. As noted in the previous report, the non-porous mesityl oxide membrane exhibited those characteristics previously ascribed to a porous biological structure. Thus, net flow of water along an osmotic gradient was greater than that predicted by

simple diffusion and the activities of water on two sides of the membrane. Further studies have confirmed and extended these original observations. Furthermore, solvent drag of urea, an augmentation in the unidirectional movement of the solute (initially equally distributed in both aqueous phases), in the direction of net water flow was observed in the mesityl oxide system. So-called solvent drag as defined above, has also been cited as unequivocal evidence for the presence of pores in biological membranes. It appears likely that the phenomena observed in the mesityl oxide system, which clearly is unrelated to pores or bulk flow of solute, is in part due to a differential partitioning of water in the mesityl oxide at the two interfaces separating it from the aqueous solution. Although the partitioning is greater in the boundary separating the phase of higher water activity from the mesityl oxide, the relationship between the activity of water and its partitioning is non-linear as are the unidirectional fluxes of tritiated water into the liquid membrane. This non-linearity may play a key role in understanding the basis for the discrepancies between the flux and activity ratios. Because of the non-linearity the unidirectional flux of water is greater from the more dilute solution into the mesityl oxide than from the more concentrated solution. On the basis of these studies it has been concluded that the concept of bulk flow and solvent drag as derived from the flux ratio-activity ratio discrepancy proposed by Ussing, are not necessarily evidence of the presence of pores. It is probable that the considerations developed above should also be applied to biological membranes.

Aldosterone

Aldosterone Stimulating Hormone

One of the more significant advances reported from this laboratory in the past year was the unequivocal demonstration that the kidney secretes a substance capable of stimulating the release of aldosterone from the adrenals. The original studies have now been confirmed and extended. Though unrecognized at the time, it is now apparent that so-called ASH (adrenal stimulating hormone) is renin, presumably formed in the juxtaglomerula apparatus in the renal cortex. Indirect evidence has also been obtained supporting the view that ASH is formed in all experimental situations associated with an increase in aldosterone secretion. Thus, nephretomy reduces aldosterone secretion in dogs in which the secretory rate was initially increased by acute blood loss, chronic sodium depletion or chronic thoracic vena caval constriction.

Renin-Angiotensin System

Recently studies have been completed which have further clarified the role of the renin-angiotensin system in experimental renal hypertension insofar as it relates to enhanced aldosterone secretion. The injection of renin into hypophysectomized-nephrectomized dogs increases the rates of secretion of aldosterone, corticosterone and Porter-Silber chromogens. Similar effects are also noted following injection of synthetic angiotensin II. It is also possible to administer a dose of angiotensin which, though incapable of elevating arterial pressure, stimulates the secretion of aldosterone without a physiologically significant change in either corticosterone or Porter-Silber chromogen release. Further support in favor of the thesis that renin represents ASH was the observation that the renin content of kidneys from dogs with malignant experimental renal hypertension is increased 10 fold above normal. These animals secrete abnormally increased amounts of aldosterone. In contrast the renin content of kidneys from dogs with the benign form of the disease is only double that of normals and these animals secrete aldosterone at relatively normal rates.

The Effect of Angiotensin on Vascular Resistance

In view of the evidence suggesting that angiotensin II may be a trophic hormone it is probable that a homeostatic mechanism is present which regulates the incidental action of angiotensin on the vascular resistance of the arterial tree. This is consistent with the observations that certain patients with enhanced aldosterone secretion in whom angiotensin II is presumably increased are not hypertensive, for example, cirrhotics, nephrotics, etc. It has been suggested that the vascular tree in these subjects is less responsive to the renin-angiotensin system, thereby accounting for the absence of hypertension. It has also been observed in this laboratory that the blood pressure

response to angiotensin II in animals with caval constriction is considerably less than that in normals. In an effort to obtain direct evidence in this regard the contractile response to angiotensin of arterial strips from normal dogs is being compared to that of animals with hyperaldosteronism and no hypertension.

Formation of ASH

The chemical nature of ASH has been studied in collaboration with the Laboratory of Chemical Pharmacology. Crude saline extracts of dog kidney were fractionated by heat, dialysis, and ammonium sulfate precipitation. Various fractions were tested biologically in dogs for evidence of ASH and pressor activity. It is notable that only those fractions separated in a manner similar to that required for the isolation of renin from renal tissue possessed steroidogenic and pressor activity.

Utilizing standard procedures for the separation of renin from kidney, it was also noted that tissue from animals with thoracic caval constriction contained considerably more renin than that from normal animals. Furthermore, the juxtaglomerular cells in the former animals were hypergranulated and hyperplastic, ancillary evidence in favor of increased renin production. Preliminary studies have also indicated that the stimulus for renin secretion may be related to a fall in arterial pressure and renal blood flow. Thus supra-adrenal aorta constriction of hypophysectomized dogs with reduced arterial pressure and renal blood flow resulted in a substantial increase in aldosterone secretion.

Humoral Sensitization of Renal Tubule Cells to Aldosterone

On the basis of studies reported last year it was suggested that caval constriction may sensitize the renal tubules to aldosterone. The bilaterally adrenalectomized and nephrectomized dog with one kidney transplanted to the neck, responded to caval constriction and DOCA administration by retaining sodium. When the caval ligature was released, sodium was no longer retained despite continuing desoxycorticosterone administration. These findings suggest that a humoral factor is responsible for sensitization of the tubule cells to salt-retaining hormone.

Disappearance of Aldosterone From Plasma

As reported last year chronic hepatic venous constriction markedly prolongs the half-time ($T\frac{1}{2}$) for disappearance of injected d-1 or d-aldosterone. In normal animals the $T\frac{1}{2}$ varied from 20 to 34 minutes, whereas in dogs with thoracic vena caval constriction the $T\frac{1}{2}$ for disappearance was 43 to 128 minutes. Hepatectomy in the former group resulted in a flattening of the plasma disappearance curve, indicative of the role of the liver in the metabolism of the steroid. Kinetic analyses of the data in association with the computer division of the N.I.H. supported the original contention that disappearance of aldosterone from plasma is largely attributable to degradation by the liver. Despite this, it was concluded that hypersecretion of aldosterone is of more importance in the development of hyperaldosteronemia than decreased turnover in the liver in dogs with hepatic venous congestion.

Effect of Cardiac Glycosides on Aldosterone Secretion

Studies of the acute effects of cardiac glycosides on aldosterone secretion in dogs with hyperaldosteronism secondary to chronic right heart failure have been completed. In these, improvement in hemodynamics following digitalization was uniformly associated with a decrease in aldosterone secretion. In animals in which digitalis did not result in hemodynamic improvement, aldosterone secretion either remained unchanged or increased in association with the deterioration in cardiovascular function.

Cardioglobulin and Related Studies

The Physiological Effects of Cardioglobulin on Mammalian Hearts

The nature of cardioglobulin, a protein system present in plasma of certain animals which exerts a cardiotonic effect on frog heart, has been discussed in detail in previous reports. This year much of the effort has been directed at examining its effects on isolated mammalian cardiac tissue. In association with these studies the mechanism of its destruction in plasma as evidenced by a decrease in the biologic activity of the preparation was also studied.

It has been established that right ventricular strips from guinea pig heart respond to cardioglobulin in a manner similar to that of frog heart. Restoration of contractility and the ultimate development of contracture were noted in both species. Less dramatic effects were observed when papillary muscle of cat or right ventricular strips from rat were used. In these contracture did not occur although contractility of the hypodynamic tissue was markedly improved. This is of interest since neither frog nor guinea pig plasma contains cardioglobulin. In contrast rat and cat plasma possess the system. The concentration of cardioglobulin used in the experimental studies in the latter animals was probably no greater than that normally present in their circulating blood thereby accounting for the less dramatic effects of the added substance.

Cardioglobulin Degradation

Studies on the degradation of cardioglobulin have centered on the observation that its activity on frog heart decreases rapidly under a variety of circumstances. Simple dilution in Ringer's solution at 37° C. is sufficient to eliminate cardioglobulin activity within 10 minutes. Of singular interest is the observation that destruction may be prevented entirely by addition of creatine phosphate, though not by ATP, ADP, UTP, creatine, inorganic phosphate, or 10% albumin. Furthermore, the inactive material may be reactivated by the addition of creatine phosphate at 37° for 10 minutes. It has also been found that inactivation of the cardioglobulin system is accomplished in undiluted plasma merely by the addition of a variety of tissue fractions, including crude tissue homogenates, pure myosin plus supernatant of tissue homogenates, or glycerinated muscle fibres. Under these circumstances inactivation is prevented not only by creatine phosphate but also by ATP or ADP. These provocative studies are being pursued actively since they may provide information concerning the chemical nature of the cardioglobulin system.

Ryanodine-Digitalis Antagonism

In the course of studies of isolated heart muscle it was observed that ryanodine, a water soluble plant alkaloid, was capable of depressing cardiac contractility in a manner similar to that effected by prolonged perfusion of the tissue with Ringer's solution. These studies were initially performed using rat right ventricular tissue. Unlike the hypodynamic state produced by perfusion with Ringer's, which is reversed by the addition of cardiac glycosides, the ryanodine effect was unaltered by the subsequent addition of digitalis-like compounds. Furthermore, the addition of ryanodine alone to a digitalized ventricle abolished the action of the glycoside, clearly demonstrating an antagonistic effect of these drugs. The diminution in contractility provided by ryanodine can be reversed by perfusing the muscle with a solution deficient in either potassium or sodium. It is presumed that this process reduces the cationic content of the tissue. The results have been interpreted in the framework of a hypothesis that an increase in cationic content of cardiac muscle is generally responsible for a diminution in contractility.

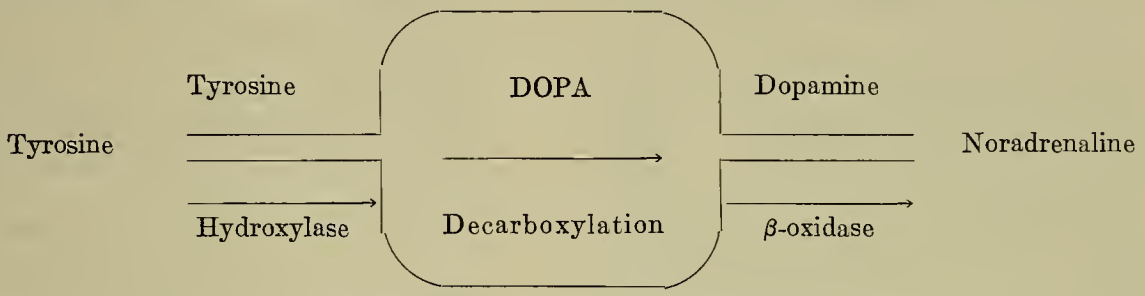
Of particular interest was the observation that ryanodine not only antagonizes the digitalis-effect on contractility but also is capable of abolishing ventricular arrhythmias characteristic digitalis toxicity in both the intact cat and dog. Appropriate concentrations of ryanodine were protective in animals to which otherwise lethal amounts of digitalis had been administered. In contrast to the antagonism with respect to ventricular muscle arrhythmias, ryanodine and digitalis in combination resulted in sinus node depression. The effect was abolished by atropine and it was concluded that the synergistic response to these derivatives on the sinus node was mediated by the vagus.

LABORATORY OF CLINICAL BIOCHEMISTRY

Amine Biogenesis and Metabolism

It is in this area of amine formation and metabolism that a rational approach to the development of pharmacologic agents is being attempted. For instance, from studies of the enzyme mechanism involved in the biosynthesis of norepinephrine it should be possible to devise inhibitors which by blocking one of the catalytic steps would produce a chemical sympathectomy. One of the enzymes involved in norepinephrine formation, aromatic L-amino acid decarboxylase, has now been well

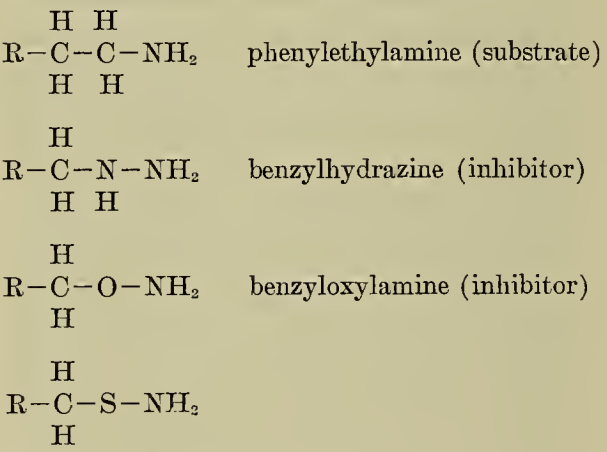
characterized and potent inhibitors have been prepared which are effective *in vivo*, even in man. However, it is now extremely doubtful whether the endogenous production of norepinephrine can be limited in intact animals even by the most active decarboxylase inhibitor. If one presents the sequence of reactions leading from L-tyrosine to norepinephrine in the form of a closed system flow diagram showing an estimated flow rate at each step, then it becomes apparent that the decarboxylase is far from being one of the rate-limiting steps in the reaction sequence.



Relative Activities of Catalytic Steps in Noradrenaline Biosynthesis

Obviously even if decarboxylation of dopa is inhibited over 90% the amount of dopamine, the end product of decarboxylation, would not become limiting. This explains why even the most potent of decarboxylase inhibitors, α-methyl dopa hydrazine (MK 485—Merck Sharp & Dohme), does not lower tissue levels of norepinephrine or alter the excretion of the hormone or its metabolites. Other decarboxylase inhibitors, Aldomet, α-methyl dopa and α-methyl meta tyrosine, have been shown to lower tissue norepinephrine levels but by a mechanism which influences the binding sites. It does not appear worthwhile to devote more time to the development of pharmacologic agents based on decarboxylase inhibition.

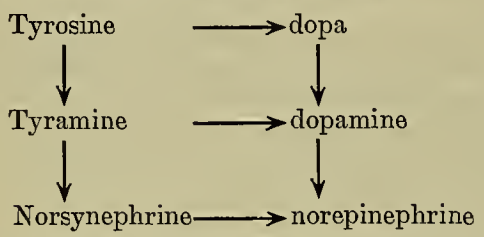
There are, however, two additional steps in the biosynthesis of norepinephrine, either of which can be made rate limiting. The ring hydroxylation is not well characterized but the side-chain hydroxylation is now known to be catalyzed by an enzyme, dopamine-β-oxidase, which appears to be distributed in sympathetically innervated tissues. Dopamine-β-oxidase has been purified and its requirements elucidated (Kaufman et al.). A simple and specific assay has been developed in our laboratories based on the oxidation of tyramine to octopamine. Following this it was shown that the enzyme is not highly selective in its substrate requirements. Phenylethylamines of all types can be hydroxylated to yield the corresponding β substituted alcohols. Even mescaline can be converted to mescalol. All of these substrates can inhibit dopamine oxidation in a competitive manner but since they have lower affinities would not be expected to be effective *in vivo*. It seemed that phenylethylamine isosteres with the following side chains would be more effective and perhaps irreversible inhibitors of the enzymes:



Several of these derivatives were therefore obtained and tested and found to inhibit the enzyme effectively at 10^{-5} M, the inhibition appearing to be of a non-competitive nature. It remains to be seen whether these are effective agents *in vivo*. One or two compounds related to those listed above were made available to the Laboratory of Chemical Pharmacology by a drug company for other reasons, and they have obtained evidence which suggests that they can block norepinephrine formation in animals *in vivo*. We have shown that

their compounds are potent inhibitors of purified dopamine-β-oxidase. Thus it would appear that another type of pharmacologic agent may become available for blocking sympathetic activity. Dr. Albert Sjoerdsma is already preparing methodology to carry such compounds into man should the animal biochemistry and toxicity data warrant it.

The non-specificity of dopamine-β-oxidase presents another interesting problem. It has been shown that tyramine is normally present in tissues and can be oxidized to norsynephrine. Recently we have administered tyramine and norsynephrine to animals and shown they are converted to norepinephrine and normetanephrine which are excreted in the urine. This is then an alternate route of norepinephrine biosynthesis.



All of these steps have been demonstrated to take place *in vivo* and all the metabolites have been found in tissues. In fact, the only one whose presence in tissues is still questioned is dopa. The significance of this alternate route of norepinephrine biosynthesis is being investigated.

Some of the same considerations which appear in norepinephrine formation also hold for serotonin biosynthesis. Although decarboxylase inhibitors can block conversion of 5-hydroxytryptophan to serotonin the rate limiting step *in vivo* is the hydroxylation of tryptophan to 5-hydoxytryptophan. For this reason endogenous formation of serotonin cannot be lowered by even potent decarboxylase inhibitors. As for tryptophan hydroxylase, investigators at the University of Wisconsin recently demonstrated that liver homogenates could hydroxylate tryptophan. We were able to corroborate this but found that the enzyme responsible for this is actually L-phenylalanine hydroxylase. Preparations of the phenylalanine enzyme, purified according to Mitoma and to Kaufman, were found to hydroxylate tryptophan. However, the affinity for tryptophan is about 0.01 that of phenylalanine. In fact, the affinity is so low that it requires 10^{-2} M solutions or greater of tryptophan to detect the activity. That one and the same liver enzyme hydroxylates phenylalanine and tryptophan is shown by:

1) The same two enzymes responsible for phenylalanine hydroxylation are needed for tryptophan.

2) The same pteridine cofactor is needed by both.

3) Pteridine antagonists inhibit both.

4) The ratio of phenylalanine to tryptophan activity remains constant during purification.

5) Competition can be shown between the two substrates, L-phenylalanine with the highest affinity being a very effective inhibitor of L-tryptophan hydroxylation.

These findings made it necessary to reinvestigate the localization of the hydroxylase in tissues. However, even with the most sensitive methods the hydroxylase could be demonstrated only in liver. If this enzyme were responsible for the serotonin found in brain, intestinal tract, lung, etc., it would mean that 5-hydroxytryptophan is transported to these organs via the blood from the liver. No evidence of circulating 5-hydroxytryptophan can be obtained. Furthermore, studies in many laboratories indicate that serotonin-containing tissues can form serotonin from tryptophan (malignant carcinoid, etc.). We have administered methotrexate, a potent pteridine antagonist of liver phenylalanine hydroxylase, and were able to achieve marked inhibition of the liver enzyme with respect to both phenylalanine and tryptophan hydrozylation. Under these conditions tyrosine tissue levels fell significantly. However, no changes in serotonin tissue levels were observed. These and other studies have convinced us that the liver hydroxylation of tryptophan is merely a non-specific activity which is observable with isolated purified enzyme preparations but which has little physiological significance. The tissue catalyst responsible for tryptophan hydroxylation in serotonin containing animal tissues must still be found.

As for amine metabolism the enzyme, monoamine oxidase (MAO) is still under investigation. It has been shown that MAO is unique among deaminases because it can act on N-dimethyl substrates and is inhibited by iproniazid and related

compounds. In spite of these unusual properties MAO has been shown to act in a manner similar to other deaminases, by dehydrogenation and addition of water to an imino intermediate. The enzyme has been purified to an extent where binding of hydrazine inhibitors may help elucidate the active site of the enzyme.

One final point of interest in the field of amines is the study of the vapor phase chromatography of amines. These procedures will make it possible to study normally occurring amines, such as phenylethylamine, for which chemical assay procedures are not available. Amine drugs such as amphetamine will also be made amenable to study.

Homocarnosine and Carnosine Metabolism

Since the finding of γ-aminobutyrylhistidine (homocarnosine) in brain the carnosines have been under active study. Although a "carnosine synthetase" has been reported this activity seems to be too weak (particularly in brain) to account for the large amounts of the carnosine in tissues. Another possible mechanism for homocarnosine formation would involve formation of γ-glutamyl-histindine which, on decarboxylation, would yield homocarnosine. Following this suggestion it has been possible to demonstrate a transpeptidase enzyme in brain which can form γ-glutamyl histidine from glutathione and histidine. It remains to be seen whether this peptide can undergo decarboxylation.

During these investigations the need arose for measuring other peptides. Procedures for vapor phase chromatography of some di and tripeptide derivatives have been developed.

Choline Biogenesis

This problem which was dormant for about a year has been reactivated. In order to facilitate enzyme studies on cephalin formation it was necessary to elaborate new analytical procedures. It has now been possible to develop procedures for phosphatidyl serine and phosphatidyl ethanolamine in tissue extracts which are specific and highly sensitive. Most important these procedures permit the assay to be made within a working day whereas former methods for these compounds, besides being less specific, required several days.

Collagen and Hydroxyproline

Studies with intact chick embryos have shown conclusively that collagen formation occurs in the microsome fraction. Following administration of proline-C^{14} the microsomal "collagen" was found to contain much more hydroxyproline-C^{14} than any other collagen fraction. Incorporation into the free hydroxyproline of the embryo was extremely low showing that it arose mainly as a decomposition product of the most slowly "turning over" form of collagen.

The studies were extended to cell-free systems and it has been shown that when chick embryo microsomes are incubated with proline-C^{14} in the presence of an ATP generating system, Mg^{++} and the soluble cell fraction, hydroxyproline-C^{14}, appear in the microsomes. This hydroxyproline is present in a protein which is non-dialyzable and is extractable with hot trichloroacetic acid as is collagen. Although the protein has not yet been more rigorously identified it would appear that it represents cell-free synthesis of collagen. It is of interest that even in this cell-free system proline is incorporated into collagen hydroxyproline far better than hydroxyproline itself. Preliminary studies indicate that it may be possible to dissociate the hydroxylation from the protein synthesis. It is hoped to increase the activity of the system and extend these studies to determine intermediates and the nature of the catalysts. Studies with O^{18} are nearing completion to determie whether the oxygen in hydroxyproline is derived from H_2O^{18} or from $O_2{}^{18}$. This will help in planning studies with the cell-free system.

Actinomycin I, which is elaborated by *S. antibioticus*, is the only other hydroxyprolyine peptide which can be obtained in quantity. Studies with this organism indicate that proline-C^{14} is converted to peptide hydroxyproline (actinomycin I) and peptide ketoproline (actinomycin V). It was found, however, that unlike the situation in collagen synthesis hydroxyproline can serve as a precursor for actinomycin I just as effectively as proline. Although this would indicate the presence of a hydroxyproline activating enzyme such

activity has not yet been demonstrated in cell-free preparations of the organisms. Attempts are being made to demonstrate conversion of proline to hydroxyproline by cell-free preparations of *S. antibioticus*.

Amino Acid Transport

Studies on the uptake of amino acids by brain have continued. It has been shown that the features which mark brain uptake as being under catalytic control are not found in peripheral tissues such as muscle. One of the consequences of this is that uptake of amino acids by brain is subject to competitive inhibition to an extent which is not characteristic of most other tissues. Thus some of the central manifestations of phenylketonuria and "branched-chain" amino aciduria may be due to the high concentrations of circulating phenylalanine or leucines which inhibit uptake of related amino acids that are essential to brain function.

Brain slices also differ from muscle slices in being able to concentrate α-amino acids. In addition to the requirement for sources of energy it has now been shown that certain metabolic inhibitors and digitoxin inhibit this uptake.

Biosynthesis of the B_{12} Coenzyme

Conversion of vitamin B_{12} to its coenzyme form involves the substitution of an adenosyl moiety in place of the cyanide and reduction of the cobalt.

Vitamin B_{12}

Coenzyme B_{12}

It has now been shown that cell-free extracts of Clostridium tetanomorphum catalyze this conversion in the presence of ATP, glutathione and a yeast extract. Studies with labeled cyanide-C^{14} indicate that the ATP starts a concerted reaction involving reduction of the cobalt, release of cyanide and attachment of the adenosyl moiety. The latter is derived from the ATP as is the ribose moiety. The enzyme system has now been purified many fold.

CLINICAL ENDOCRINOLOGY BRANCH

The work of the Clinical Endocrinology Branch has included chemical, physiologic and clinical studies related to the physiology of the adrenal cortex, biologic and clinical studies of calcium and phosphorus metabolism and of the physiology of the parathyroid glands, and several studies not closely related to these two areas.

Studies of methodology in relation to adrenal physiology include the development of gas chromatographic methods for the identification and measurement of aldosterone and other adrenal steroids, the biosynthetic production of aldosterone ring-labeled with C^{14} and its utilization for a double derivative isotopic method for analysis, studies in the degree of binding of aldosterone and other steroids to plasma proteins and to red cells and of various factors influencing this binding, and studies to improve methodology for measurement of aldosterone secretion rates and of the influence of various factors on these rates.

By converting C_{21} steroids to acetate derivatives it was possible to measure and identify them by gas chromatography. Studies in recovery and degradation of these steroids were begun with the use of isotopically labeled precursors. Biosynthetically prepared C^{14}-labeled aldosterone could be isolated in purified form, and proved to be moderately stable. The substance was adequate for use in isolation procedures where a ring-labeled tracer was desirable to allow complete measurement of hydrolysis and recovery; over the course of months the substance undergoes spontaneous degradation.

Red blood cells and red blood cell ghosts were found to bind aldosterone and hydrocortisone, and the binding was readily reversible by washing with saline. Hydrocortisone was less bound to red cells than aldosterone as a result of a stronger affinity of plasma protein for hydrocortisone. In man, plasma protein is more effective in binding hydrocortisone than in the dog. There is weak binding to albumin and very strong binding to corticosteroid-binding globulin. Aldosterone showed only the weak type of albumin binding. It was found that corticosterone, compound S, cortisone and prednisolone resembled hydrocortisone in their method of binding, whereas progesterone, desoxycorticosterone and 17 ketosteroids resembled aldosterone in the absence of

specific binding. A method of titration of plasma with a given steroid was devised to measure and compare degree of binding as between steroids and as between clinical states. It was shown that whereas treatment with estrogen markedly increases the amount of corticosteroid-binding protein (or the number of available sites), it is decreased in patients with hypoproteinemia.

Secretion rates of aldosterone determined by measuring the decrease of specific activity of urinary steroid after intravenous administration of tracers confirmed in every respect conclusions derived from studies of urinary aldosterone excretion. This method was used to study the effect of expansion of intravascular volume and the effects of ACTH in extended studies. Preliminary results suggest that secretion rates determined over six hours may give information comparable to that acquired over twenty-four hours.

Factors controlling adrenal steroid secretion were extensively studied. The role of the kidney was investigated, and the metabolic actions of renin and angiotensin were studied. In other studies the effect of changes in magnesium intake, of sustained treatment with ACTH, and of fasting were investigated and diurnal fluctuations of adrenal secretion were measured. Patients with primary aldosteronism were studied with a special reference to the effects of factors known to influence aldosterone secretion physiologically. A new syndrome, characterized by aldosteronism, hyperplasia of the juxtaglomerular apparatus and normal blood pressure was extensively studied in two patients, and a number of patients with the secondary aldosteronism of renal tubular acidosis were studied on metabolic regimen.

Aldosterone, corticosterone and cortisol secretion, which are all lowered by hypophysectomy, fell to values significantly lower than those found with hypophysectomy alone when the kidneys were removed from dogs. When renal ischemia was produced by constriction of the renal arteries or of the lower aorta, secretion of aldosterone, corticosterone and cortisol was increased. With moderate constriction it was possible to induce increases of corticosterone and cortisol secretion without increases in aldosterone secretion. In animals in which aldosterone secretion had been increased by caval constriction, removal of the kidneys from the circulation lowered aldosterone, corticosterone and cortisol secretion, and the restoration of the kidneys to the circulation restored the secretion to control values. These studies suggested that renal ischemia might have its effect through production of renin, and the effect of renin was accordingly measured. It was found that renin would induce increases in the secretion of aldosterone corticoserone and cortisol in the hypophysectionized, nephrectomized dog. Quantitatively the effects on the secretion of corticosterone and cortisol were relatively greater than those on the secretion of aldosterone. Synthetic asparagine 1 valine 5 angiotensin II was shown to reproduce the effects of renin, even at doses too low to induce changes in blood pressure: secretion of aldosterone, corticosterone and cortisol was increased, but the relative increase of secretion of corticosterone and cortisol was much greater than that of aldosterone. When angiotensin was given in small doses to normal human subjects over periods of 16 hours or more, they showed an increase in urinary aldosterone and moderate retention of sodium. At comparable doses, angiotensin did not produce sodium retention in patients with Addison's disease. In large doses (sufficient to induce hypertension) angiotensin induced sodium retention in normal subjects and in patients with Addison's disease. Direct measures of the effect of angiotensin on the isolated frog skin suggested that has no direct influence on sodium transport. It is likely that the sodium retention observed with angiotensin in patients with Addison's disease is secondary to the hemodynamic changes in the renal circulation.

Changes in magnesium intake and total fasting had little effect on aldosterone excretion despite marked sodium loss with the latter procedure. Secretion of aldosterone, like the excretion of aldosterone, was increased with ACTH but spontaneously fell despite continuance of the ACTH. In some patients the secondary fall was associated with expansion of the intravascular volume, but in others the fall occurred without change in intravascular volume. It is concluded that the secondary fall of aldosterone secretion does not require an increase in intravascular volume or in arterial pulse pressure. Excretion of aldosterone and of Porter-Silber steroids, as well as that of water and electrolytes were shown to undergo marked fluctuations during the day. For aldos-

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terone, Porter-Silber steroids, sodium and potassium, the maximum excretion occurred towards the middle of the day with minimal figures at night. The phase relationships were such that aldosterone might control the diurnal excretion of potassium, but was 12 hours out of phase to offer an explanation for diurnal changes in sodium excretion.

Patients with primary aldosteronism were studied for the effects of restoration of body potassium, and of expansion of intravascular volume on aldosterone secretion and excretion. It was found that expansion of intravascular volume had little effect on aldosterone excretion or secretion. In contrast this procedure effectively decreased excretion and secretion of aldosterone in patients with secondary aldosteronism. Two patients with primary aldosteronism and normal blood pressure were found to have marked hypertrophy and hyperplasia of the juxtaglomerular apparatus. It was found that these patients manifested increased circulating levels of angiotensin and resistance to the hemodynamic action of antiotensin, a finding which suggests that this syndrome may result from a vascular insensitivity to angiotensin. Patients with renal tubular acidosis were studied and found to have secondary aldosteronism, which was thought to be responsible for the potassium loss. Metabolic studies suggest that the primary defect in this condition is related to the inability of the renal tubule to produce a gradient in hydrogen ion concentration between interstitial fluid and tubular lumen. There results a secondary inability to reabsorb Na with consequent aldosteronism and increased secretion of potassium.

Studies on calcium and phosphorus metabolism and on the physiology of the parathyroids included studies of the metabolic fate of vitamin D, studies of the effects on reabsorption of phosphate of sustained phosphate loading and measurements of the role of the parathyroids in the release of bone calcium and the reabsorption of calcium by the renal tubules.

Clinical studies included measurements of the defect in calcium metabolism in sarcoidosis, evaluation and development of tests for hyperparathyroidism, and studies of the metabolic defects in renal osteitis and in idiopathic osteoporosis.

Labeled vitamin D was obtained and studies were instituted to determine its biologic fate in bile fistula rats. Noraml and parathyroidectomized dogs were studied for phosphate reabsorption and maximal tubular phosphate reabsorption during the administration of large loads of phosphate orally over periods of several weeks. The reabosrption of phosphate decreased consistently in the normal dogs. A similar change was noted in the parathyroidectomized dogs but this was associated with marked clinical deterioration and with decreases in the glomerular filtration rate. Parathyroidectomy was shown to decrease the availability of bone calcium to restore serum calcium ion concentrations which had been acutely depressed with chelating agents. Whereas calcium feeding or vitamin D could restore serum calcium values to normal after parathyroidectomy, they did not restore the normal availability of bone calcium. It is concluded that the parathyroids exert a measure of control over the physicochemical release of calcium ion from bone. Preliminary results suggest that parathyroid hormone may increase renal tubular reabsorption of calcium.

Studies in patients with sarcoidosis confirm the finding of very low fecal calcium with high urinary and occasionally high serum calcium, changes which suggest hyperabsorption of dietary calcium. This effect could be grossly exaggerated by administration of vitamin D in small doses and could be prevented by administration of prednisolone in large doses. Prednisolone, indeed, would reverse the effect even when given together with vitamin D. It was shown that the defect does not result from increased levels of vitamin D in the circulation, a finding which suggests that it represents a true state of hypersensitivity to the vitamin.

Tests of phosphate clearance, of calcium absorption and of the effect of calcium infusion were evaluated in normal subjects and in subjects with hyperparathyroidism. Of the available tests, only the increase of calcium excretion on a low phosphate intake and the failure of urinary phosphate to fall with calcium infusion or to "rebound" after calcium infusion proved reliable for the diagnosis of primary hyperparathyroidism. Patients with the osteitis of renal failure were shown to have a moderate or marked block in gastrointestinal absorption of calcium. It was found that vitamin D in large doses might cure the osteitis, and studies are in progress to deter-

mine whether it restores also the defect in calcium absorption. Patients with idiopathic osteoporosis were studied with especial reference to calcium and phosphorus balance under the influence of steroids and parathyroid hormone. It was found that strontium did not induce a positive calcium balance in three subjects.

Investigations in other areas included studies of the pathogenesis of atherosclerosis, with special reference to permeability of vessel walls, studies on the interaction of catechol amines and corticosteroids on blood pressure, studies in the defect of free water excretion in Addison's disease, experimental induction of a physiologic state similar to that seen with inappropriate secretion of antidiuretic hormone, measurements of the metabolic fate of albumin in patients with hypoproteinemia and studies of the effects of hypercalcemia and hypercalciuria and of amphotericin on renal function.

Studies in atherosclerosis involved measurements of the rate of passage of plasma protein into the wall of the dog aorta. It was found that the rate of passage of labeled albumin, like that of cholesterol, was directly related to the arterial blood pressure. The essential findings could be reproduced *in vitro*, and it was thus possible to show that pulsation is not necessary to induce the acceleration of transfer brought on by hypertension. It was shown furthermore that it was not dependent upon tension in the arterial wall, as there were no significant differences between the findings in small and those in large aortas. The finding suggested that passive filtration of large particles plays a part in the development of atherosclerosis. The effect of catechol amines in raising the blood pressure was measured quantitatively in subjects with Addison's disease. Each patient was studied with no treatment, with saline alone, with saline and desoxycorticosterone and with hydrocortisone. The findings do not suggest a dependence of the magnitude of response to catechols upon the presence of adrenal cortical steroids. The defect in water metabolism was studied in patients with Addison's disease under a similar protocol. In addition, some patients were subjected to expansion of intravascular volume with salt-poor human albumin. Significant increases in free-water clearance could be produced with saline or with albumin, with little further effect added by addition of steroids. It thus appears that contraction of intravascular volume is of relatively greater importance than steroid deficiency in producing the defect of water excretion in Addison's disease.

Normal subjects on fixed sodium intake were subjected to stepwise reduction in plasma tonicity by progressive increment in water intake during the constant administration of pitressin tannate in oil. The syndrome of inappropriate secretion of antidiuretic hormone could be reproduced in all its essentials, and the findings did not support the notion that normal subjects differ from patients with this syndrome in having an ability to "escape" from the effects of pitressin. The metabolic fate of human serum albumin given intravenously was followed in patients with the hypoproteinemia of gastrointestinal protein loss and in patients with analbuminemia. The former group was shown to degrade administered albumin abnormally rapidly whereas the latter group degraded it significantly less rapidly than normal subjects. In the former group there was less nitrogen excreted than the amount predictable on the assumption of total degradation of administered albumin with deamination of the amino acids therein, and it was concluded that albumin degraded in the gastrointestinal tract is reformed into new tissue protein. In the latter group nitrogen excretion actually decreased with albumin suggesting that the albumin provided a stimulus to the oxidation of fat. Hypercalciuria and hypercalcemia were found to induce reversible decreases in urinary concentrating ability, and occasionally to impair sodium reabsorption and hydrogen ion secretion as well. Amphotericin was found to induce reversible decreases in glomerular filtration rate and in renal plasma flow.

EXPERIMENTAL THERAPEUTICS BRANCH

A biochemical approach to cardiovascular pharmacology and therapeutics has again been emphasized in investigations of this laboratory. In addition, increasing use has been made of conventional physiologic techniques in studies on the actions of drugs in experimental animals and man. Various findings will be considered under the fol-

lowing headings: 1) biosynthesis and metabolism of aromatic amines, 2) action and metabolism of drugs, 3) metabolism of hydroxyproline and collagen and 4) miscellaneous.

Biosynthesis and Metabolism of Aromatic Amines

Using our specific assay procedure for urinary histamine, several aspects of the metabolism of this amine in man were studied. Normal excretion values were found to be 21–65 μg/day for males and 15–90 μg/day for females. Oral administartion of L-histidine produced an increase in urinary histamine and high levels were found in a patient with gastric carcinoid tumor and in another with uticaria pigmentosa. Oral administration of nomycin or succinyl sulfathiazole produced no change in urinary histamine indicating its origin from tissues rather than intestinal flora. It was shown that the specific histidine decarboxylase of mouse mast cell tumors could be inhibited by the hydrazino analogue of α-methyl-dopa (MK485); preliminary studies are underway to determine whether this drug will lower urinary histamine in man.

Tyramine has been found to be present in some mammalian tissues and in human urine. From *in vitro* studies it is known that this amine is an excellent substrate for monoamine oxidase and that it may undergo β-hydroxylation to yield norsynephrine. The metabolism of tyramine in the intact animal is conjectural. Accordingly the fate of tyramine administered intravenously to 5 human subjects was studied. About 90 per cent of the intravenous dose was recovered in the urine as p-hydroxyphenylacetic acid and only a few per cent as unchanged amine. A minor increase in p-hydroxymandelic acid was observed and surprisingly a significant increase in dopamine also. The latter finding requires further evaluation since a role for tyramine in the synthesis of norepinephrine is suggested.

A highly sensitive technique for the assay of monoamine oxidase (MAO) was developed, using tryptamine as substrate. An extensive survey of MAO activity in human tissues was performed. In general the results paralleled those found in animals. In contrast to the situation in most species, however, the human heart was found to be rich in MAO activity. Successful application of the method has been made to specimens of human jejunal mucosa obtained by peroral biopsy. Hypertensive patients were found to have normal levels of MAO; marked decreases in jejunal MAO were demonstrable following introvenous or oral administration of MAO-inhibiting drugs. Diminished jejunal MAO was found in patients with thyrotoxicosis, in correlation with previously demonstrated elevations in urinary amines. These findings do not explain the enhanced sensitivity to catecholamines in thyrotoxicosis.

Further attempts have been made to decrease serotonin synthesis in carcinoid patients by use of inhibitors of aromatic-L-amino acid decarboxylase. The use of α-methyl-dopa for this purpose was found to be limited in most patients by hypotensive effects. A marked chemical effect has now been achieved with its hydrazino analogue (MK485) in two patients. Further studies will depend on availability of the compound.

Action and Metabolism of Drugs

The effects of several sympathomimetic amines on plasma levels of free fatty acids (FFA) and blood glucose were studied in normal subjects. The following alterations in the basic phenylethylamine structure were found to be associated with maximal potency in mobilizing FFA's: hydroxyl substitution in para position and on the β-carbon and the presence of a primary or secondary amine. Effects on FFA and blood glucose were dissociable. No difference in FFA response in normotensives and hypertensives to infusion of norepinephrine could be shown, in spite of obvious differences in vascular reactivity.

Following the observation that intravenous injections of several different sympathomimetic agents produced typical flushes in carcinoid patients and that this effect could be blocked with phentolamine (Regitine), the latter compound was evaluated for its effects on spontaneous flushes. Marked symptomatic improvement has been noted in four patients with Regitine in oral doses up to 100 mg every 4 to 6 hours.

Effective inhibition of monoamine oxidase in human hypertensives is associated with an orthostatic lowering of blood pressure. Hemodynamic studies in nine patients receiving the MAO in-

hibitor, MO–911 (Pargyline, Abbott), point to drug-induced diminution of compensatory vasoconstriction in the erect position and during exercise, with resultant decrease in calculated peripheral resistance. As a possible corollary in animals we have demonstrated blockade of transmission through sympathetic ganglia in intact cats following administration of several different MAO inhibitors. However, by assaying directly the MAO activity in ganglia it could be shown that onset, duration and degree of enzyme inhibition do not parallel the ganglionic blocking effects of these drugs. It seems unlikely that the ganglionic effects in animals explain the blood pressure effects in man.

Therapeutic evaluation of Pargyline has now been extended to thirty hypotensive patients who have received the drug for periods up to 15 months. The agent has the advantage of combining useful mood-enhancing effects with potency and freedom from sympatholytic effects. In a few patients marked weight gain secondary to increased appetite, and interference with sexual functions in the male have made the drug unacceptable. In twelve patients in whom enzyme inhibition has been followed for up to six months by frequent measurements of urinary tryptamine, no appreciable loss of chemical effectiveness has been noted. The agent continues to be a promising antihypertensive drug in situations where lowering of blood pressure is not required urgently.

Another area of therapeutic interest with MAO inhibitors, the alleviation of angina pectoris, is receiving increasing attention. Detailed studies in several patients indicate the increases of pulse rate and blood pressure occurring during treadmill exercise may be markedly obtunded with impunity by careful administration of an MAO inhibitor. Diminished cardiac work resulting therefrom is associated with increased exercise tolerance. Principles derived from these studies may explain some of the discordant results reported in the literature.

Following the demonstration that the hypotensive and decarboxylase inhibiting activity of α-methyl-dopa resided in the levo-rotatory form (Aldomet), we have adopted this form for further study in patients. Our initial impressions of usefulness and potency have been confirmed. Advantages include effectiveness against all degrees of hypertension, frequent lowering of recumbent as well as standing blood pressure, smoothness of effect and low incidence of tolerance. A possible limitation is the occurrence of febrile reactions in four of approximately one hundred patients studied. In each case the reaction developed within two weeks of initiating therapy. In two cases reversible abnormalities in liver function were observed after the onset of fever.

From studies with other decarboxylase inhibitors in man, it now appears that the lowering of blood pressure with Aldomet is unrelated to enzyme inhibition. The hypotensive effect may be due to direct depletion of central or peripheral stores of catecholamines, as suggested by studies in LCB. In experiments on the uptake of serotonin and norepinephrine by human platelets *in vitro* marked inhibition of uptake could be produced with reserpine but little if any effect was observed with α-methyl-dopa or its major metabolite, α-methyl-dopamine. Thus the amine depletion resulting from administration of α-methyl-dopa may be on a different basis from that produced by reserpine. No clues to account for variations in blood pressure responses in different patients have been found in studies on metabolism of the drug. Addition of MK485 to treatment with Aldomet failed to alter the blood pressure response in several patients though formation of urinary α-methyl-dopamine was completely inhibited.

Metabolism of Hydroxyproline and Collagen

Utilizing a specific method developed in this laboratory for assay of hydroxyproline (HPr), it has been established that peptide-bound HPr in urine is a useful index of collagen degradation. However, both dietary and renal factors must be considered to obtain valid information. That HPr-peptides may be absorbed from the gastrointestinal tract was shown by marked rises in plasma and urinary levels following ingestion of large amounts of gelatin by normal volunteers. This is the first demonstration of absorption of a peptide in man. Furthermore, considerable day to day variation of urinary HPr has been observed in normal controls and patients unless the dietary intake of HPr was greatly restricted. Study of several patients in severe renal failure has revealed

marked elevation of plasma peptide-HPr under this circumstance.

Measurements of HPr excretion of a large group of normal volunteers have been made with these factors in mind. Each subject was required to ingest a special diet extremely low in HPr. Preliminary findings show a progressive decrease in the amount of HPr excreted in successive decades of life, suggesting that urinary HPr may be an indication of the "metabolic age" of an individual's collagen.

At least three different metabolic pools of HPr have been demonstrated in rats by measurements of the specific activity of HPr following administration of radioactive proline. Similar studies appear feasible in man using highly active H^3-labelled proline. Knowledge of turnover rates of HPr in man would be useful in setting up experiments designed to study the effects of drugs on collagen metabolism. We have recently set up an extensive screening program using weanling mice to test the effects of various drugs. HPr intermediates and analogues on the synthesis of total body protein and collagen.

Miscellaneous

A technique was developed for surgical denervation of rat spleen. The catecholamine content of this organ was shown to be completely dependent in an intact innervation.

A fibrinolysis assay model was devised using urokinase as an activator. Other constituents were also of human origin. Testing of a large number of sera from normal volunteers and patients with various diseases revealed eight instances of elevated fibrinolysis inhibitor activity. Of these eight, two were from patients with essential hyperlipemia and three from patients with thyrotoxicosis.

CARDIOLOGY BRANCH

1961 was the first full year of operation of the Cardiology Branch. The research efforts of this Branch were divided into two sections, Clinical Physiology and Clinical Biophysics, but the clinical efforts of both sections were combined. The research activities of the section of Clinical Physiology can be divided into four chief areas.

Dynamics of Ventricular Contraction

The basic principles of muscular contraction have heretofore been related to the human heart only in indirect fashion, primarily because the precise measurement of ventricular volumes, ventricular dimensions, the force of ventricular contraction, ventricular contractility, and aortic blood velocity have not been possible in man. Efforts have been directed first to the development of methods to measure these important parameters and then to the utilization of these measurements in the study of the dynamics of ventricular contraction.

During 1961 the applicability of Starling's law of the heart to unanesthetized human subjects was studied by means of serial biplane-angiocardiography utilizing injections of contrast material into the left ventricle. The end-diastolic and end-systolic volumes of the left ventricle were determined from the angiocardiagrams and stroke volumes calculated by subtracting the end-systolic from the end-diastolic volumes. In patients in whom beat-to-beat variations in left ventricular end-diastolic volume occurred, these were accompanied by corresponding changes in stroke volume and arterial pulse pressure. However, in patients in whom the left ventricular end-diastolic volume remained almost constant, the stroke volume showed little or no change. From these observations it was concluded that the ventricular stroke volume is a function of the end-diastolic volume and that therefore Starling's law of the heart is applicable to man.

The mechanical properties of human ventricles in situ were studied at the time of operation by sewing a specially designed strain gauge to the ventricle of 12 patients. This device permits the measurement of the resting (diastolic) and active (systolic) tension exerted by that segment of myocardium to which it is attached. Measurements of tension can be carried out with the muscle at varying lengths. The resting length-tension curves of the human myocardium were hyperbolic and convex to the length axis. As the myocardial fibers were stretched from resting length to approximately 150% of resting length an increase in the developed tension and in the rate of development of tension were observed. With greater stretching there was a decrease in these variables.

A method for the precise, continuous measurement of ventricular dimensions in closed-chest, intact human subjects has long been sought. Small silver clips were sutured to the surface of either the right or left ventricle of 17 patients undergoing cardiac surgery. During the postoperative period cineradiography was carried out and the distances between the clips were then measured on each individual frame of the film which had been obtained at a rate of 30 frames per second. During the Valsalva maneuver there was a marked decrease in right ventricular size, which averaged 12% of control values. With release of the Valsalva and a deep inspiration, there was evidence of a "rebound". Cardioactive amines, such as isoproterenol and norepinephrine, given to patients during the course of this study usually resulted in a decrease in ventricular end-diastolic distances. Exercise also produced decreases in end-diastolic and end-systolic dimensions. This cineradiographic technique thus permits, for the first time, accurate and reproducible measurements of ventricular dimensions in man. It is anticipated that this approach will prove to be a useful one for this detailed study of ventricular dynamics in unanesthetized intact patients.

Since observations in experimental animals have indicated that the rate of ventricular pressure rise is a function of myocardial contractility, it was thought that determination of the rate of change of ventricular pressure (dp/dt) would permit study of myocardial contractility in intact man. In order to eliminate artifacts, ventricular pressures were recorded by means of a catheter with a high fidelity micronmanometer mounted at its tip. The first derivative of ventricular pressure was continuously computed by means of an electronic differentiating analogue computer. Normal values for the maximum dp/dt in both ventricles were determined and the effects of cardioactive amines, exercise, atropine-induced tachycardia, emotional stimuli, myocardial failure, and of myocardial depressant drugs have been studied. This technique affords a convenient and accurate assessment of myocardial contractility in intact human subjects.

Studies on the determination and significance of the instantaneous pulsatile blood velocity in patients by the double-lumen catheter technique devised by Fry have been continued. The use of operational amplifiers (Donner Computer Units) has replaced passive electric analog circuits and tape recording techniques for recording prime data have been adopted. The use of tape recording units has made possible the recomputation of the instantaneous velocity and permitted more accurate assessment of the contributing factors for the terms involved in the hydrodynamic equations. Studies have now been done in 35 patients with meaningful data and useful correlations being available from the majority.

Although it is generally agreed that Starling's law applies to the heart-lung preparations and open-chest anesthetized dogs, there is considerable disagreement about the relevance of the Frank-Starling mechanism to the intact organism. An electromagnetic flow meter was placed around the ascending aorta of dogs and changes in left ventricular dimensions and left ventricular diastolic and intrapleural pressures were measured simultaneously. The chest was closed and the dogs permitted to recover from the anesthesia. Left ventricular stroke work, mean and peak values of aortic blood velocity and acceleration, and left ventricular power, all increased as left ventricular size and filling pressures were augmented. During infusion of norepinephrine at any given filling pressure and ventricular size all of these indices of ventricular performance increased. These experiments support the view that Starling's law and the ventricular function curve concept are applicable to the heart of the closed-chest, unanesthetized dog with an intact autonomic nervous system.

Adrenergic Nervous System and Myocardial Function

The roles of the two enzymes, catechol-o-methyl transferase and monoamine oxidase, in the inactivation of norepinephrine have not been fully elucidated. In preliminary studies on the isolated heart of the dog utilizing H^3-norepinephrine, it was shown that the major metabolic product is normetanephrine, which demonstrates the importance of catechol-o-methyl transferase as an inactivating mechanism in cardiac tissue.

Recently, on the basis of pharmacologic observations it has been suggested that the action of some

sympathomimetic amines is mediated by release of endogenous norepinephrine from adrenergic nerves. Since there has been little direct evidence to support this hypothesis, a study was undertaken to determine whether such vasoactive amines release norepinephrine from the heart. Tyramine, phenylethylamine, tryptamine, p-hydroxyamphetamine, amphetamine, ephedrine, mephentermine, and guanethidine, on intravenous administration, all produced release of norepinephrine into the coronary venous blood. Phenylephrine, methoxamine, and serotonin, however, had no such effect. After infusion of tyramine for one hour the norepinephrine content of the heart was consistently reduced. These observations lend direct proof for the hypothesis that a number of vasoactive amines affect the cardiovascular system by means of release of endogenous norepinephrine.

It has been established previously by investigators in the Laboratory of Cardiovascular Physiology, NHI, that stimulation of the stellate ganglion results in an efflux of norepinephrine into coronary sinus blood. In studies carried out in this laboratory, it was demonstrated that the myocardial norepinephrine content is not altered at a time when the physiologic effects of supramaximal cariod-accelerator nerve stimulation are markedly reduced, although at this time the postganglionic action potentials are unaltered. Hence, despite a normal tissue catecholamine store and delivery of the stimulus to the adrenergic neuro-effector junction, the response of the effector organ is diminished. These data provided indirect evidence for a high rate of local amine synthesis and/or myocardial extraction of circulating plasma catecholamines.

The acute effects of guanethidine and bretylium on heart rate, myocardial contractile force, arterial pressure and cardiac output have been studied in normal dogs and dogs with denervated hearts in which the myocardial catecholamines have been depleted. The strikingly positive inotropic, chronotropic, and pressor effects as well as the increase in cardiac output produced by guanethidine in normal dogs were markedly reduced in the dogs with denervated hearts. Denervation of the heart reduced the positive chronotropic effects as well as part of the increase in cardiac output produced by bretylium in the normal dogs. These data indicate that a large part of the acute hemodynamic effects of guanethidine and bretylium are produced as a result of the sudden release of myocardial catecholamines.

Although it has been generally accepted that the hemodynamic effects of tyramine are produced by sudden catecholamine release, the source of the catecholamine which produces the effects on the heart has not been determined. Experiments carried out on normal and reserpinized dogs and dogs with denervated hearts indicate that the release of *myocardial* catecholamine stores is primarily responsible for the cardiac effects of tyramine. Furthermore, these studies with tyramine, guanethidine and bretylium demonstrate the physiological potential of stored myocardial amines.

Experiments were carried out in order to determine how the myocardial norepinephrine content modifies the effect of a digitalis glycoside on the heart. In experiments in which the myocardial responses to ouabain in normal and reserpinized dogs were compared, no differences either in the inotropic or the arrhythmogenic properties of the glycoside were observed in these two groups of animals. However, it was noted that the functional refractory period of the A–V node was markedly greater in the reserpinized than in the normal animals and that although ouabain was capable of prolonging the refractory period in normal dogs it failed to do so in reserpinized animals. The infusion of norepinephrine into reserpinized animals shortened the refractory period of the A–V node, suggesting that the heart of the reserpine pretreated animal can bind circulating norepinephrine, and that this bound store is potentially active physiologically.

In spite of the widespread clinical use of antiadrenergic drugs, such as reserpine and guanethidine, little is known of the hemodynamic mechanisms underlying their hypotensive effects. The possibility was considered that one of the important mechanisms of their action was through their effect on blocking venous venoconstriction. Utilizing the major vessel occlusion technique for measuring reflex venoconstriction, it was found that both acute and chronic administration of guanethidine and of reserpine blocked this reflex. Norepinephrine infusion failed to restore the reflex venoconstrictor response. These observations

suggest that the action of these drugs on the venous bed may play a prominent role in their clinical effects.

In order to assess the contribution of the autonomic nervous system to the hemodynamic response to exercise, normal subjects were studied at rest and during steady-state leg exercise in a control period without drugs, and after pharmacologic interference with the autonomic nervous system. The latter was accomplished by the administration of guanethidine and atropine. Following the administration of these drugs, muscular exercise resulted in significantly smaller increases in heart rate, stroke volume, cardiac output and left ventricular work than during the control period. However, the arterio-mixed venous oxygen differences were substantially greater following autonomic blockade than in the control state. These data indicate that combined pharmacologic inhibition of the sympathetic and parasympathetic nervous systems interferes with the normal cardiovascular response to exercise.

Attempts have also been made to evaluate the contribution of adrenergic reflexes and myocardial catecholamine stores to cardiac homeostasis in man by measuring the effects of guanethidine administration upon patients in borderline congestive heart failure. In 4 such patients guanethidine produced sodium retention and weight gain and three of these showed concomitant increases in venous pressure, hepatic enlargement, dyspnea, orthopnea and peripheral edema. These effects disappeared after discontinuation of guanethidine treatment, and were produced by doses of guanethidine which did not cause significant hypotension. These results indicate that guanethidine administration can aggravate congestive heart failure in patients with overt decompensation and that interference with the adrenergic component of the autonomic nervous system is deleterious to patients with reduced cardiac reserve.

Previous studies in this laboratory have shown that guanethidine reduces the tremor, restlessness and tachycardia associated with tri-iodothyronine induced hyperthyroidism in normal control subjects. These results were interpreted as evidence that the adrenergic reflexes are important in many of the manifestations of hyperthyroidism. These observations have been extended to include measurement of cardiac outputs in clinical hyperthyroidism and in normal subjects with tri-iodothyronine induced hyperthyroidism. In 8 subjects with high cardiac outputs associated with endogenous or exogenous thyroid excess, guanethidine lowered the outputs markedly in only two. This suggests that the high cardiac output in hyperthyroidism is not dependent upon sympathetic reflexes.

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Cardiovascular Diagnostic Techniques

Transseptal left heart catheterization, a technique previously developed in the Heart Institute, was modified considerably during 1961. The procedure is now performed by percutaneous puncture of the femoral vein. Left atrial puncture is carried out with a needle having a 21 gauge tip, thus decreasing the hazard of accidental puncture of a structure other than the atrial septum. A radiopaque catheter is then passed over the needle into the left atrium and across the mitral valve into the left ventricle. The lumen of the catheter is large enough to permit rapid injection of a large quantity of radiopaque dye and the accurate measurement of pressures in the left atrium and left ventricle. The numerous technical advantages of this modified technique have led to the almost universal acceptance of the transseptal method as the technique of choice. Investigators from numerous laboratories learned the technique on visits to Bethesda. More than 500 such procedures have been performed here. Transseptal left heart catheterization in infants and children with heart disease, in children and adults without cardiovascular disease and transseptal left heart angiocardiography received particular attention this year.

In spite of the widespread applications of left heart catheterization, the pressures in the left side of the heart in subjects without cardiovascular disease and in basal physiologic states have not been known. Transseptal left heart catheterizations were carried out on 18 individuals without organic heart disease. The mean left atrial pressures ranged between 2 and 12 mm Hg, with an average value of 7.9 mm Hg. The mean left atrial pressure exceeded the mean right atrial pressure by an average of 3.9 mm Hg. The left atrial V peak averaged 12.8 mm Hg and the left ventricular end-diastolic pressure ranged from 5 to 12 mm Hg

with an average value of 8.7 mm Hg. It is anticipated that these values will serve as normal standards for these parameters in future studies of left heart dynamics.

It was shown previously that the inhalation of an inert foreign gas and the determination of its concentration in blood sampled simultaneously from the right side of the heart and the systemic arterial bed form the basis of a sensitive test for the characterization of left-to-right circulatory shunts. Radioactive krypton (Kr^{85}) has been found to provide substantial advantages over other gases and it has therefore been employed routinely in this laboratory for the past several years. In 1961 the results of inhaled Kr^{85} tests, carried out in 323 patients in whom the diagnosis was firmly established, were analyzed. In the 161 patients subsequently proved to have left-to-right shunts the results of the test ranged from 13% to 113%. In the 162 patients without cardiac shunts the results ranged from 0.9% to 12.2%. The Kr^{85} test may thus be employed with confidence for determining the presence or absence of a left-to-right shunt. In addition, when the test is successively performed in the pulmonary artery, right ventricle, and right atrium, the site of entry into the right side of the heart may be correctly localized.

A simple technique for the recording of indicator-dilution curves from the pulmonary vascular bed and its applications to the detection of left-tor-right circulatory shunts was developed. Diodrast labeled with I^{131}, a gamma-emitting isotope, was injected intravenously and its activity in the pulmonary vascular bed determined with a scintillation detector placed over the upper lung field. In the patients in whom the presence of a left-to-right shunt was subsequently proved, the descending limb was prolonged. Simple analysis of the curves in a total of 33 patients allowed determination of the presence or absence of a shunt. Placing the detector over the lung fields, rather than the precordium, markedly simplified the analysis of the curves and resulted in increased sensitivity. The clinical value of this simple technique in the study of patients following cardiac operations and in the screening of patients with heart murmurs of uncertain etiology was demonstrated. This technique is now used routinely for clinical studies at the NHI and at a number of other institutions.

A technique has been developed for determining the fraction of left ventricular end-diastolic volume which is ejected during each cardiac cycle. Radioiodinated Diodrast was rapidly injected into the left ventricle at the time of transseptal left heart catheterization and the fraction of isotope which was discharged from this chamber per beat was determined with a well-shielded scintillation probe placed on the chest wall over the left ventricle. Left ventricular end-diastolic volume was estimated from the stroke volume determined by the dye-dilution method, and the fraction of isotope discharged per beat. Difficulties resulting from inadequate mixing of isotope in the left ventricle were minimized by this technique since the probe detected indicator in the entire left ventricular cavity. The accuracy of this technique was first demonstrated in a circulatory model and then in open-chest dogs in which ventricular dimensions were continuously monitored by means of mercury-in-rubber gauges. In 21 patients without detectable abnormalities of left ventricular functions the fraction of left ventricular volume discharged averaged $37 \pm 8\%$ per beat and the end-diastolic volumes averaged 89 ± 26 ml/M^2 B.S.A. In 21 patients with heart failure and/or valvular regurgitation, in whom left ventricular function was compromised, the fraction of left ventricular volume discharged into the aorta averaged $16 \pm 5\%$ per beat and the end-diastolic volumes averaged 209 ± 75 ml/M^2 B.S.A. This technique has been found to be sensitive to changes in left ventricular function and practical to apply routinely in the course of left heart catheterization.

The most acurate technique available for the measurement of blood oxygen content is the manometric technique of Van Slyke. Unfortunately this method is a tedious one, and can be carried out only by a trained technician. In collaboration with the Laboratory of Technical Development, NHI, the rapid analysis of blood oxygen content by a gas chromatographic technique is being explored. A rugged and very sensitive ionization detector designed to detect minute (less than 0.1 microliter) quantities of O_2 and other gases has been developed. This detector has been shown to have excellent accuracy and reproducibility over a relatively wide range. The extraction of oxygen from very small samples of blood has proved to be the major problem which is incompletely re-

solved thus far. The perfection of this technique would be of considerable importance to cardiac catheterization laboratories.

The accurate measurement of systolic arterial pressure in infants and young children may be a formidable task. A modification of the Whitney mercury strain gauge with an Elsner impedance matching circuit and a recording galvanometer has been developed into a simple and sensitive device with which to record systolic arterial blood pressure in patients of all age groups. This technique has been shown to be accurate to within 5 mm Hg of the directly recorded systolic arterial pressure. The simultaneous recording of systolic blood pressures from the upper and lower extremities by the use of two plethysmographs has proven useful in the pre- and postoperative evaluation of patients with coarctation of the aorta. This simple but accurate device can be constructed for approximately $40.

The presence of an atrial (fourth) heart sound has been observed frequently in patients with severe aortic stenosis. This observation led to a careful phonocardiographic-hemodynamic correlation of this sound. In a study of 50 patients with aortic stenosis it was found that the absence of this sound signifies that the aortic valve obstruction is relatively mild, with a gradient less than 70 mm Hg. This sound, which may be detected clinically or recorded phonocardiographically, thus appears to be very helpful in the assessment of patients with aortic stenosis.

The time interval between the two components of the second heart sound and the changes in this interval during respiration constitutes one of the most important physical signs in the bedside diagnosis of congenital heart disease. The mechanisms responsible for the behavior of this sound are still under debate. The characteristics of the second heart sound were analyzed in phonocardiograms recorded from 350 patients in all of whom the diagnosis was proved either at operation or by detailed catheterization studies. Normal values for the time intervals between aortic and pulmonic valve closure, for the duration of right and left ventricular systole and for the effects of respiration and of the Valsalva maneuver on these intervals in patients without heart disease and with a variety of congenital lesions were established. These studies showed that careful auscultatory and/or phonocardiographic analyses of the effect of respiration on the two components of the second heart sound may help considerably in the diagnosis of all of the major forms of congenital heart disease.

Clinical Cardiology

During 1961 a number of clinical studies on patients with congenital heart disease were conducted in conjunction with the Clinic of Surgery, NHI.

The clinical and hemodynamic features of a previously unrecognized variant of partial A-V canal were described. Two patients with evidence of massive mitral regurgitation due to cleft mitral valves were discovered to have small associated atrial septal defects of the ostium primum variety. The interaction of these two lesions resulted in a decompression of the distended left atrium by the defect, and the anomalous hemodynamic effects of the repair of these defects were described.

The clinical, hemodynamic and angiocardiographic findings in two other unusual congenital cardiovascular anomalies were described in detail. Seven patients with isolated congenital mitral regurgitation and six patients with aortopulmonary septal defects were studied. Comprehensive studies on these patients permitted definitive description of these two lesions, about which relatively little clinical or hemodynamic information was heretofore available. The decline in the increased pulmonary vascular resistance following surgical closure of the aortopulmonary septal defects in four patients were particularly interesting.

Five patients with tetralogy of Fallot who developed pulmonary hypertension following creation of an aortopulmonary anastomosis were described in collaboration with members of the Department of Medicine, Johns Hopkins Medical School. Thus, for the first time the development of an elevated pulmonary vascular resistance in man could be related directly to increased pulmonary blood flow. The characteristic radiologic features in the five patients should permit ready recognition of this complication of sugical treatment for tetralogy of Fallot.

A total of 27 patients with idiopathic hypertrophic subaortic stenosis have now been studied. A number of new variants have been recognized.

These include: 1) patients in whom the left intraventricular pressure gradient is present on some occasions and absent at other times; 2) familial forms of the disease in which some members of the family have obstruction to left ventricular outflow, others obstruction to right ventricular outflow and others with obvious left ventricular enlargement but without hemodynamic evidence of obstruction. The possibility was suggested that the same basic disease process may, in different patients, be responsible for a variety of clinical and hemodynamic pictures.

It is now clear that electrocardiographic evidence of a disturbance in right ventricular conduction with QRS prolongation develops in the majority of patients in whom an isolated ventricular septal defect is repaired or in whom the teralogy of Fallot is completely corrected. The time intervals between the onset of ventricular depolarization and of right ventricular contraction were determined both before and after operation in 17 patients who developed electrocardiographic evidence of right ventricular conduction disturbance. It was concluded that the mechanical delay in the onset of right ventricular contraction which develops in the majority of patients following closure of ventricular septal defects and complete correction of tetralogy of Fallot results from interruption or of other trauma to the right bundle branch.

Circulatory Physiology

Myocardial oxygen consumption is being studied in the dog utilizing a preparation in which the external work of the myocardium is maintained near zero while coronary blood flow and oxygen delivery are varied. The heart is hemodynamically isolated from the systemic circuit and the root of the aorta is perfused by a separate pump. The entire coronary venous return to the right side of the heart is diverted to a separate reservoir and coronary flow can be accurately determined. Myocardial oxygen consumption is determined from the product of coronary blood flow and the coronary arteriovenous oxygen difference. In 12 experiments it has been demonstrated that as coronary blood flow is increased from low levels there is a progressive increase in myocardial oxygen consumption despite maintenance of constant external left ventricular work. These observations demonstrate that myocardial oxygen consumption may vary with varying coronary blood flow (and varying oxygen delivery) while the external work of the heart is kept constantly near zero and suggest that previously described determinants of myocardial oxygen consumption may be incomplete because this phenomenon had not been appreciated.

The effects of hypoxia on the capacity of the total systemic vascular bed, venous return, systemic vascular resistance, and myocardial contractile force were studied in dogs. The effects of hypoxia on each of these aspects of the cardiovascular system was evaluated separately. In addition the role of the chemoreceptors in the mediation of these responses was investigated by hemodynamically isolating or denervating these structures. Generalized systemic hypoxia resulted in striking venoconstriction. Since this effect could be prevented by chemoreceptor denervation it was concluded that it was mediated by the chemoreceptor reflex arc. An increase in systemic vascular resistance occurred when hypoxemia was localized to the carotid arterial bed. However, a decline in systemic vascular resistance was observe when the entire systemic vascular bed was made hypoxic and the chemoreceptors denervated or perfused with oxygenated blood. These results indicate that the direct effect of hypoxia is to produce arteriolar dilatation but that this response is opposed by the arteriolar constriction which is mediated by the chemoreceptor reflex arc. Myocardial contractile force increased when intact chemoreceptors were perfused with hypoxic blood, regardless of whether the heart received well or poorly oxygenated blood. However, myocardial contractile force decreased whenever poorly oxygenated blood perfused the heart and the chemoreceptors were either denervated or perfused with well-oxygenated blood. It therefore appears that the augmentation of myocardial contractile force which occurs during generalized hypoxia is dependent upon an intact chemoreceptor reflex arc which opposes the direct myocardial depressant effects of hypoxia.

Hypothermia has been produced in dogs on complete cardiopulmonary bypass at a constant systemic perfusion rate. Cooling to 15–25° C. has been effected. During the cooling phase a de-

crease in peripheral resistance (28%), a decrease in venous return, and an augmentation of systemic blood volume (24 ml/kg body weight) have been noted. In addition to direct measurement of the systemic blood volume changes, an indirect dye dilution technique was utilized. A decrease in calculated circulating blood volume during hypothermia was demonstrated with the latter technique. These observations indicate that during hypothermia, significant arteriolar dilatation occurred and that substantial volumes of blood were trapped in the peripheral vascular bed. The latter resulted in the striking decreased in venous return and an increase in systemic blood volume.

The acute hemodynamic effects of ouabain were studied in patients with valvular aortic stenosis and in patients with idiopathic hypertrophic subaortic stenosis. Left atrial and left ventricular pressures and cardiac output were measured. In the patients with valvular aortic stenosis, ouabain either improved left ventricular function or had no discernible effect on it, but in no patient was left ventricular function depressed. In the patients with hypertrophic subaortic stenosis, the left ventricular end-diastolic pressure and mean left atrial pressure rose significantly following ouabain adminstration; cardiac output either fell or remained unchanged and the systolic pressure gradient between the left ventricle and the brachial artery rose. These actions of ouabain in hypertrophic subaortic stenosis are considered to result from a sustained increase in left ventricular contractile force which increased the obstruction produced by the muscular outflow tract.

Clinical Physiology

A substantial fraction of the professional and technical efforts of the *Section of Clinical Physiology* of the Cardiology Branch is devoted to clinical activities which are unrelated to its research program. These non-research activities include: 1) Recording, mounting and interpretation of all of the electrocardiograms for the Clinical Center. Approximately 6,000 tracings were handled in 1961. In addition a course in electrocardiagraphic interpretation was given and personal instruction in ECG interpretation was provided to Clinical Associates from other laboratories of NHI and from other Institutes. 2) Clinical cardiology consultations for the Clinical Center. 3) Cardiology consultations to the Clinic of Surgery, NHI. 4) An average of 3 postoperative cardiac catheterizations weekly, carried out for the Clinic of Surgery. 5) Consultations in pulmonary physiology, and performance of pulmonary function tests for the Clinical Center.

Clinical Biophysics

A major activity of the *Section of Clinical Biophysics* has been the development of realistic mathematical models of the circulatory and pulmonary systems. To the extent that this can be accomplished new hypotheses can be tested experimentally and computer techniques can be brought to bear on solutions of many physiologic problems in health and disease. Progress in this area depends on an active program in instrumentation development as well as comprehensive studies of the physical properties, anatomy, stresses (including pressures), tissue reactions to these stresses, and general system behavior (including feedback phenomena) of the circulatory and pulmonary systems. Therefore, the activities of this section may be summarized under three general headings: 1) instrumentation, 2) circulatory studies, 3) pulmonary studies.

Instrumentation

The measurement of stresses in general and pressure in particular have continued to constitute a major effort of this section. A set of standards for the accurate measurement of vascular pressure and the axial blood vessel pressure gradient have been established. Results show that currently available pressure transducers can be made to meet minimum standards for both of these measurements. Moreover the estimation of the pressure gradient is essentially independent of pressure tap separation up to values of 5 cm.

The performance of the Kolin electromagnetic flowmeter and the computed pressure gradient technique were compared to a monitored pulsating flow. Both the Kolin electromagnetic flowmeter and the pressure gradient technique were shown to have excellent recording fidelity for pulsatile flow up to at least 10 cycles per second. The pressure gradient technique was originally developed in this laboratory for the estimation of instantaneous flow

in man since the necessary pressures are attainable by conventional catheter techniques. Preliminary results from studies in conjunction with investigators working at the Mt. Alto Hospital indicate that the method gives reasonable values for blood flow and distribution of blood flow in man.

Considerable effort was made to apply analog computer techniques to various systems of equations describing particular biological systems. It it concluded that although analog computer techniques are fruitful for relatively simple systems, their lack of flexibility seriously limits the application of analog techniques to the more common complicated systems represented by the lung and circulation. Therefore, greater effort must be made to facilitate the use of digital computer techniques.

Circulatory Studies

Statements describing the circulatory system can begin with a description of the hydraulic load on the ventricles. An essential part of this description is the hydraulic input impedance of the vascular bed. Although studies in this area continue, results to date indicate that the systemic circulation is a distributed system, behaving in many ways like an electrical transmission line. The spectrum of input impedance to the aorta shows an amplitude that waxes and wanes with frequency and an associated phase angle that oscillates between minus 90 degrees and plus 90 degrees.

Similar studies in the pulmonary artery reveal that this system also behaves like a distributed system, in spite of its relatively short length. The explanation lies in the observation that the pulse wave velocity in the pulmonary artery is much lower than that in the aorta. Interpretation of hydraulic impedances in the pulmonary artery are complicated by the presences of nonlinear terms in the equations of fluid motion. The significance of these terms, which are the result of the highly nonuniform geometry of the pulmonary fluid boundaries, has been evaluated.

Concomitant with the foregoing studies of the cardiac load, attempts have been made to define the manner in which myocardial contraction will deal with this load. The force-velocity equation of A. V. Hill was transformed into a pressure-flow equation applicable to the heart. On the basis of preliminary experiments, it appears that there exists a unique instantaneous pressure and flow for a given instantaneous fiber length.

Pulmonary Studies

Theoretical considerations have been developed which indicate that abnormal stress distribution within the structure of the lung may be an important factor in the production of the disruptive lesions of emphysema. Using an improved intraesophageal pressure measuring system, the intrathoracic pressure has been measured simultaneously at three different sites along the esophagus. The differences between oral pressure and the intraesophageal pressures have been studied under static conditions in several normal subjects. It has been possible to describe this relationship as a function of balloon volume, balloon position, and lung volume. The relationship seems to be independent of the rate of change of pressure or of the amplitude of pressure changes. This initial part of the project, which is nearing completion, is preliminary to studying the pressure differences among the three balloons during conditions of air flow, particularly during cough. If it can be shown that abnormal stress distribution occurs in subjects known to have a high incidence of pulmonary emphysema, indirect evidence in support of the foregoing theory will be obtained.

Theoretical considerations based on anatomical and biophysical studies of excised bronchi indicate that the relationship between the maximum expiratory flow at any given degree of lung inflation should be controlled by the density and viscosity of the gas breathed as well as the dimensions and physical properties of the intrathoracic airways. If it can be shown experimentally that the effect of either density or viscosity on this relationship causes changes in the flow-volume relationship which would be predicted by the equations describing the theory, evidence in support of the theory would be gained. The findings thus far are in general agreement with the theory; however, computational methods must be developed to quantify the degree of agreement.

SURGERY BRANCH

The investigative projects of the Surgery Branch have, as in past years, largely centered

around the development of new methods for the surgical treatment of patients with congenital or acquired heart disease. In addition, the majority of projects carried out in the Experimental Surgery Laboratory have been designed to elucidate the physiologic changes associated with reproduction, in experimental animals, of various forms of heart disease and of the operations designed to correct them. The results of the experimental work have been applied in the continuing clinical program in which opportunity has been taken to obtain physiologic measurements in the course of both open and closed cardiac operations.

Aneurysms of the left ventricle, when seen in patients, almost invariably have resulted from coronary atherosclerosis and a subsequent myocardial infarction. Under these circumstances, the hemodynamic effects of the aneurysm are difficult to distinguish from those of the underlying myocardial disease. To determine the hemodynamic effects of left ventricular aneurysms on the normal circulation they were created in dogs attaching an excised urinary bladder to the left ventricle and creating a communication between this sac and the ventricular cavity. Effective left ventricular function and cardiac output were determined in these animals when the aneurysm was open and closed. It was found that significant acute depressions in effective left ventricular function and in forward cardiac output occurred when the aneurysm was opened.

In many open operations for the correction of both the tetralogy of Fallot and pulmonic stenosis it is necessary to induce pulmonary regurgitation and sometimes to enlarge the outflow tract of the right ventricle with a plastic prosthesis. The physiologic effects of various surgical procedures on the pulmonic valve and right ventricle were evaluated. Effective right ventricular function was only slightly depressed when the right ventricle was incised. When pulmonic regurgitation was produced by any means, however, additional impairment of function occurred and total excision of the valve usually resulted in such severe depression of function that the curve could not be inscribed. Following these operations a more detailed study of the physiologic effects of pulmonic regurgitation was undertaken in which the volume of regurgitant blood flow was measured, either by an electromagnetic flowmeter or by the application of indicator dilution methods. The excision of one pulmonic valve leaflet was found to cause a regurgitant flow equal to about half of net forward cardiac output and in experiments in which two or three leaflets were removed regurgitant flows in excess of forward output were measured. When allowance was made for the volume of regurgitant flow in the stroke volume of the ventricle it was found that true or corrected ventricular function was impaired by these valve lesions, but that effective ventricular function was severely impaired. The results of these studies will have immediate clinical application in the operative treatment of patients with obstruction to right ventricular outflow.

In the treatment of a variety of congenital lesions it is necessary to increase pulmonary blood flow and this is usually accomplished by the creation of a systemic to pulmonary artery shunt. In the conventional Blalock operation, in which the left subclavian artery is anastomosed to the pulmonary artery, it has been observed that as the patient grows the shunt presumably stays of the same magnitude and with increasing age the benefit derived by the patient from the procedure diminishes. A number of Russian surgeons have modified the Blalock operation and construct the anastomosis by means of a large vascular graft inserted between the side of the subclavian artery and the side of the pulmonary artery. Thus, the size of the shunt is not limited by the size of the anastomoses but by the size of the subclavian artery, and, on clinical grounds, the Russians believe that as an infant or young child grows the size of the shunt and consequently the increase in pulmonary blood flow will remain relatively constant. In order to test this hypothesis, the Russian operation is being carried out in newborn puppies and in newborn lambs. The relationship of shunt flow to total cardiac output is being measured in the animals with growth. In this anastomotic operation, as well as those carried out for the reconstruction of obliterative arterial disease, vasopressor agents are frequently used in the immediate postoperative period either to counteract hypotension or to increase flow through the anastomosis or prosthetic artery. A detailed study is in progress in which blood flow in the aorta and various peripheral arteries is measured by means of an electromagnetic flowmeter and the effects on

regional blood flow of various vasopressor drugs is being evaluated. The preliminary results of these studies at this time, contrary to clinical impression, indicate that the majority of pressor agents, although raising blood pressure, cause a fall in systemic arterial flow.

Generalized or local cardiac hypothermia is being employed throughout the country with increasing frequency in conjunction with open cardiovascular operations. A number of projects in the past year have dealt with physiologic studies of hypothermia. In dogs previously subjected to total cardiac denervation, it was found that there was a far more striking fall in the heart rate with cooling than in normal animals and when denervated dogs were subjected to acute hemorrhage no significant change in the heart rate or contractile force of the ventricle occurred. In an extension of this work normal dogs were subjected to acute hemorrhagic shock as contractile force was measured. It was found that down to an arterial pressure level of 90 mm. Hg contractile force was unimpaired, but below this pressure level a rapid decrease in heart rate and a decline in contractile force occurred. These, and the previous observations, indicated that both neurogenic and intrinsic cardiac effects are operative in the early stages of acute hemorrhagic shock. The observations were then extended in normal dogs in which the systemic and coronary arterial circulations could be perfused separately by two extracorporeal systems. In these animals it was possible to "shock" all of the body except the heart in which normal flow and pressure were maintained. In the first stage of acute systemic hypotension the heart rate and contractile force increased slightly after which there was a steady gradual decline in both measurements. Thus, all of the above observations indicate that, during early acute hypovolemic shock, para-sympathetic stimulation occurs and primary cardiac effects are not evident at this time. With prolongation of the shock state, however, a second mechanism, probably a humoral one becomes operative and is responsible for the decline in cardiac efficiency.

Surgical opinion remains varied as to the best means for maintaining the functional integrity of the heart following long periods of aortic occlusion, such as are necessary in partial or total replacement of the aortic valve. A comparative study was carried out to determine the technic which would minimize impairment of ventricular function after aortic occlusion. As found previously, simple cardiopulmonary bypass did not depress ventricular function and aortic occlusion and anoxic arrest could be tolerated by the normal dog for only 20 minutes. Intermitten perfusion of the coronaries with warm oxygenated blood improved subsequent function but marked depression usually resulted nevertheless. When the heart was first rendered hypothermic, anoxic arrest was better tolerated. However, only when local hypothermia of the heart was supplemented with intermittent coronary perfusion was satisfactory subsequent function indicated in the function curves. In spite of the results of these experiments, it is still felt that insufficient information is available concerning the effects of hypothermia on the heart and local cardiac hypothermia is not employed in patients.

When the heart is cooled, by any means, it has long been recognized that the rate slows and an increase in ventricular contractile force occurs. In order to determine whether this response was due to the direct cardiac effects of cooling or was the result of neurogenic stimulation, experiments were carried out in which the temperature of the heart and the remainder of the body could be independently varied during extracorporeal circulation. Initial experiments indicate that when the body is cooled and the heart remains normothermic that little change in its rate or strength of contraction occurs, indicating that the effect is in all likelihood a local rather than a central one.

Extreme degrees of hypothermia are sometimes applied when it is necessary to interrupt the circulation completely during the repair of particularly complex intracardiac anomalies. When the temperature is lowered to profoundly hypothermic levels (12–15°C.) it has been postulated by others that the hemoglobin dissociation curve is shifted so far to the left that oxygen may not be available to the tissue from oxygenated hemoglobin. On this basis numerous surgeons have advocated that acidosis be induced during cooling to shift the curve toward a more normal position. Acidosis is known to have deleterious effects of many types on the circulation and an experimental investigation of the desirability of acidosis during hypothermia was undertaken. Dogs were cooled to 12°C. and

in some the blood pH was lowered during cooling by the administration of HCL and in others it was raised by the administration of bicarbonate. The total body oxygen consumption was unaltered by these changes in blood pH indicating that the shift in the hemoglobin dissociation curve is of little importance at these temperatures. The aortas of these dogs were occluded for 30 minutes after they had been rendered hypothermic and either alkalotic or acidotic. Function curves were then inscribed after the animals had been rewarmed. It was found that the dogs rendered acidotic showed severe depression of subsequent ventricular function, as did dogs with a normal pH, but that dogs previously rendered alkalotic had distinctly better ventricular function. It was concluded that in clinical practice acidosis during hypothermia serves no useful purpose and, since it has a deleterious effect on the heart, it is probably undesirable.

In the large number of open operations that have been carried out at the National Heart Institute, it has been our clinical observation that following cardiopulmonary bypass patients often require supplemental digitalis in the first 24 hours. To determine the effect of cardiopulmonary bypass on the digoxin content of the heart a supply of tritium labeled digoxin was obtained and given to normal dogs. Biopsies of the heart and other tissues were obtained and the animals were then subjected to 30 minutes to cardiopulmonary bypass after which biopsies and blood samples were again obtained and analyzed for radioactivity. It was found that 30 minutes of cardiopulmonary bypass caused the heart to lose approximately ¼ of its radioactivity and that the radioactivity of the blood in the dog and heart lung machine rose strikingly. By means of paper chromatography it was shown that at least ⅓ of the radioactivity which left the heart was unchanged digoxin and the remainder presumably its metabolic products. By using tagged digoxin with a higher specific activity preliminary observations have been made in seven patients subjected to open operation and similar decreases in radioactivity of the heart have been observed. Continuing studies on the mechanism by which digoxin is lost from the heart are being carried out in both patients and animals.

In the past year much progress has been made throughout the country in the problem of prosthetic replacement of the aortic and mitral valve. Following the initial encouraging results with the foam plastic mitral valve developed here, its use has been abandoned until further refinements can be made. Modifications have been made in the configuration of the artificial leaflets and the method of attaching the artificial chordae tendineae to them. The modified valve is presently being tested in experimental animals to determine its hemodynamic effects and in an attempt to obtain chronic survival. The same plastic molding technics which were developed in the design and fabrication of the prosthetic mitral valve are now being applied in the development of prosthetic total aortic valves and individual aortic valve leaflets. The total prosthesis has been constructed in a size suitable for implantation into the descending thoracic aorta of dogs and is now being evaluated in this anatomic position in animals in which aortic regurgitation has been produced. Information concerning thrombosis and ultimate mobility of the prosthesis will be obtained before attempts at insertion in the subcoronary position are made.

Prosthetic mitral or aortic valves must be constructed of some plastic or metal substance and work continues on the evaluation of various prosthetic materials in the circulation, with particular regard to clotting. Dacron cloth has been coated with polyurethane foam with both open and closed cell surfaces. Pieces of the material have been implanted into the wall of the heart and also suspended within the cavities of the heart by means of artificial chordae tendineae of various types. Chronic preparations of this type over a period of a year or more will allow a comparative evaluation of the surface which is most desirable and the material most suitable for the suspending chordae tendineae. Preliminary observations indicate that substances with closed cell surfaces are more desirable but they do not, of course, allow for tissue ingrowth. Attachment of the chordae tendineae to the wall of the ventricle by means of aluminum buttons has been found to be satisfactory even when extreme tension has been placed upon them. Chordae of teflon or polyurethane coated silk apparently function better than those of wire or other substances.

An outgrowth of the work on prosthetic valves has been the discovery that a plastic adhesive (Eastman 910 monomer) is a useful adjunct in both experimental and clinical cardiovascular

surgery. This agent is a rapidly polymerizing material which adheres with ease to dry living tissues and causes little undesirable foreign body reaction. It was evaluated as a method of controlling hemorrhage from the heart and great vessels. Incisions were made in the heart or in the aorta and, utilizing the adhesive, a patch of prosthetic material or skeletal muscle was cemented over the site of injury. Satisfactory hemostatis was obtained by this method in virtually all experimental animals, but before clinical application is extensive it will be necessary to obtain further long term information about the ultimate histopathologic fate of the material. The monomer solidifies almost instantly on contact with blood and when it was accidentally injected into the vascular tree it was found to promote widespread vascular clotting. This suggested that occlusive lesions of the arterial or venous system could be experimentally produced by selective injections of the monomer. This has proved true. With left atrial injection complete occlusion of the aorta could be produced as well as occlusions of the coronary, renal, celiac or femoral arteries. Similarly, by injection of the monomer into the pulmonary artery or the portal vein, occlusive lesions resulting in pulmonary or portal hypertension were easily produced. It would seem that this technic may prove to be a valuable one in the experimental production of various occlusive arterial lesions.

Immediately after the correction of an intracardiac lesion acute right or left sided heart failure may supervene and be responsible for early postoperative death. Under these circumstances it would be desirable to be able to support the circulation by some mechanical means until the heart was able to recover from the acute trauma of the operation and again support full cardiovascular function. Various investigators have demonstrated that the work and oxygen requirements of the failing heart may be decreased by partial extracorporeal circulation but partial bypass has almost invariably resulted in severe metabolic acidosis. This finding has been confirmed in laboratory experiments on partial cardiopulmonary bypass for periods of an hour or more. In addition, abdominal distension and engorgement of the abdominal viscera were noted. In the system evaluated no oxygenator was employed and venous blood was returned through a pump to the femoral artery of the animal. It seems likely that the "pooling" of blood within the abdomen may be related to the fact that unoxygenated blood is delivered to the lower half of the body from the perfusion system and this has been confirmed by arterial oxygen determinations in the descending aorta. Continuing work on this important adjunct to cardiovascular surgery and the role that THAM and other buffer agents may play in the amelioration of the acidosis is underway.

The orthopedic resident assigned to the Surgery Branch has continued collaborative work with the National Institute of Arthritis and Metabolic Diseases. The hyaline sclerosis of synovial blood vessels described in the previous report has been subjected to further study. This heretofore undescribed pathologic lesion was found in the vast majority of all normal joints studied. The significance of the lesion remains unknown and continuing investigations of the etiology and possible significance of the finding are underway. The osseous changes resulting from arterial oxygen desaturation are well known clinically but little information is available concerning the method by which the pathologic bone changes develop. Arterial oxygen unsaturation has been produced in dogs by various surgical procedures, such as pulmonary arterial-left atrial fistula and pulmonary arterio-venous fistula. Arterial oxygen saturations as low as 50 percent have been obtained in chronically surviving animals and they are being subjected to detailed radiographic study. Inequality in the length of long bones is a common and distressing orthopedic problem, particularly in children. Many operations to stimulate bone growth selectively have been evaluated but none has stimulated sustained growth sufficiently to totally equalize limb length. For this reason the best clinical results have been obtained from operations in which bone growth was retarded in the normal limb. A new mechanical method for stimulating bone growth is being evaluated. A pair of compressed coil springs, anchored to a metal bar, are attached to the diaphysis in such a manner that there is constant tension between the diaphysis and the growing epiphysis. The effectiveness of this method of stimulating bone growth is being evaluated in growing puppies. Chemical adhesives

have also been found of interest in the orthopedic field and two experimental polymers were evaluated in the repair of osteotomies in dogs. Pathologic studies revealed that the compounds were unsatisfactory for this purpose since they were exothermic in tissue, causing burns, and did not bond sufficiently well to bone to permit stabilization of a fracture unless they were supplemented with metal support. Neither was found as effective as polyurethane foam for this application.

GERONTOLOGY BRANCH

The research program of the Gerontology Branch is directed toward (1) describing the biochemical, physiological and psychological changes that take place with increasing age in man, and (2) investigating the basic biological changes that contribute to aging in order to understand age-dependent alterations in the performance of humans. During the past year, primary emphasis has been placed on investigations of basic biochemical processes which are essential in maintaining life in the cell.

In this connection the enzymatic reactions associated with the utilization of oxidative energy for the synthesis of high energy compounds like ATP for subsequent use in muscle contraction and synthesis of tissue constituents have been investigated. The work carried out during the previous year indicated that the key reaction in the energy trapping system in mitochondrial electron transport may involve a dithiol grouping. The current work on this problem has established with reasonable certainty that the system does indeed contain a functional dithiol group. The uncoupling of ATP synthesis from oxidations by arsenite under well-defined conditions has been observed now in mitochondrial fragments prepared from rat liver and beef heart mitochondria. The physical changes in mitochondria (swelling) in the presence of the uncoupling agent have been shown to occur subsequent to the uncoupling.

The oxidative decarboxylation of α-ketoglutarate to succinyl CoA is another reaction in which the oxidative energy is used for ATP synthesis. This system has two functional dithiol compounds—the dihydrolipoate associated with the primary oxidation of the aldehyde to the level of the active acyl and the unidentified dithiol compound present in the highly purified dihydrolipoyldehydrogenase flavoprotein. The participation of the latter dithiol, previously inferred from inhibition of the enzymatic activity by arsenite, has been established by direct titration with p-chloromercuribenzoate.

The results clearly indicate that dithiols have unsuspected key roles in oxidative and energy trapping reactions, and have opened up a possibly rewarding approach to the study of the mechanism of oxidative phosphorylation.

Since developmental processes set the stage for senescent changes investigations on cellular changes during the process of maturation have been carried out. The developmental studies are designed to test the hypothesis that:

(a) A continuation of developmental processes beyond functional maturity may lead to less viable rather than more viable cells.

(b) The loss of developmental plasticity characteristic of cells during normal development continues during later maturation and senescence.

Evidence obtained during the past year has confirmed that the formation of muscle syncytia in tissue culture as reported earlier from this laboratory occurs by cellular fusion rather than by nuclear division unaccompanied by cytoplasmic division. This fact was shown both by time-lapse photomicrographic studies and by the measurement of DNA in syncytial nuclei and mononucleate cells using a specially built and designed microspectrophotometer. All syncytial nuclei were found to be diploid in their DNA content, whereas mononucleate cells were found to contain diploid, tetraploid and intermediate quantities of DNA.

The kinetics of the differentiation process in muscle syncytia have also been followed by measuring the concentration of creatine-kinase in such preparations at various stages of development as well as *in vivo*. The concentration rises in a manner characteristic of an autocatalytic reaction over a considerable portion of the period of differentiation. More recently it has become possible to inhibit or accelerate the differentiation process by control of specific environmental factors.

The effect of age on the capacity of human muscle explants to give rise to new outgrowths in tissue culture is being examined. Successful culture ap-

pears feasible using present methods only in a small fraction of cases, but the important variables differentiating successive experiments have not yet been worked out.

Studies on the senescent phase of cell life have concentrated heavily on the chemical nature of the so-called lipofuscin age pigment of human cardiac tissue and the possibly related inclusion bodies occurring in the short lived experimental animal, *Campanularia flexuosa*. Improved methods of isolating age pigment in highly concentrated and purified form from human myocardium have been developed. The preparations obtained have been subjected to systematic examination by infra-red analysis, fluorometry, microspectrophotometry, amino acid and elementary analysis as well as silicic acid and paper chromatography of the constituent lipids and gas chromatography of the fatty acids derived from methanolysis of chromatographic fractions. Two major fluorescent bands are typically obtained from chromatography of the lipid soluble components. These bands are eluted from silicic acid columns along with the cardiolipin and cephalin fractions. The residue remaining after extraction with lipid solvents yields free amino acids and an insoluble residue upon hydrolysis in either base or acid. This residue contains further lipid soluble pigments and an insoluble component. Enzymatically, such preparations are essentially inactive, containing only minor amounts of either the lytic enzymes associated with lysosomes or of the respiratory enzymes of mitochondria. This absence of lysosomal enzymes is not consistent with results obtained using less drastic methods of preparing age pigment (an early part of this present procedure is sonication). Since histochemical studies by Gedigk and Bontke and by Essner and Novikoff had indicated a close association between lipofuscin and lysosomal enzymes, the low activities found in the most purified pigment preparations suggest either that the pigment of aged human myocardium is no longer associated with lysosomal enzymes or that treatment during isolation has liberated previously associated enzymes.

The occurrence of increasing numbers of acid phosphatase positive granules in aging *Campanularia* suggests that the normal senescence and death of individuals of this species occurs through the action of lysosomal enzymes. The histochemical studies on cardiac lipofuscin referred to above furnish a link between the "primitive" senescence of *Campanularia* and that occurring in human fixed postmitotic cells.

Estimates of cathepsin activity have been made on various fractions of cell particulates separated by differential centrifugation and density gradients. Catheptic activity in percent of total recoverable activity is distributed as follows: nuclei, 10%; mitochondria, 36%; lysosomes, 35%; supernatant, 19%.

Stabilized cultures of *Euglena* cells have been used as a model to study age effects. The results so far show that *Euglena* cells can survive for about 12 days without any external source of carbon or energy and without loss of viability. During this time the cell consumes most of its glycogenstarch reserves, half of its protein and half of its RNA, its DNA appears to become haploid, and its oxygen consuming capacity becomes exceedingly small. The content of carotenoids, however, does not decrease demonstrating that not all components of the cell are available for utilization. The time course of resynthesis of these components has also been determined when the cells are resupplied with acetate after 5, 8, and 12 days of starvation. Oxygen consumption capacity rises very sharply before any net synthesis of protein occurs. This points to the existence of a control mechanism in this cell and we intend to examine the cell for the nature of this mechanism (feed back or enzyme repression or both?). The system also is of interest since it provides a nondividing culture in which the resynthesis of 50% of the RNA and protein of the cell occurs, and hence the system can be used for studying some aspects of ribosome formation.

Work has also progressed on the study of the synchronized division of *Astasia*. The system has been improved so that division occurs in a rather reproducible fashion every day, and with sufficient rapidity so that the culture actually exists in the haploid state for a short time during the peak of mitotic activity. It has been found that 8-azaguanine, an analogue of guanine that was originally used for blocking nucleic acid synthesis, has an effect on the synchronized system, provided it is present in the cold period, but has little effect on logarithmically growing cultures. Thus we

have demonstrated the existence of a key reaction in the cold period which is essential to the timing of mitosis. Present evidence strongly suggests that the 8-azaguanine is *not* interfering with nucleic acid synthesis, and we intend to pursue this study and attempt to characterize the process that is being blocked by 8-azaguanine.

In the field of protein studies, we have finished a project on actomyosin aimed at elucidating some of the relations between the role of the sulphydryl groups in ATPase activity and their role in contractility. When small amounts of certain −SH reagents react with actomyosin, the ATPase activity is greatly enhanced. We have shown that when this happens, contractility is not interfered with and, furthermore, the ability of a relaxing factor system to cause relaxation is also not interfered with. Thus the first −SH group to react is not essential for contraction or relaxation, although it exerts a considerable influence on ATPase activity. Further treatment with −SH reagents reverses the activation of ATPase activity and it is found that contractility is lost before there is any *net* inhibition of the ATPase. This suggests that the integrity of mechano-chemical coupling requires the integrity of a particular −SH group on the myosin and does not depend on the binding of ATP or the overall rate of hydrolysis of ATP.

A cold-precipitable protein obtained from a patient whose diagnosis was essential cryoglobulinemia has been studied. Samples of blood were obtained from the patient's wife, daughter, sons and grandchildren. Two of the sons had a cryoglobulin in their blood serum. The proteins were partially characterized by ultracentrifugation, paper electrophoresis and amino acid analysis. The evidence suggests the possibility that cryoglobulinemia is an inherited molecular disease.

The most important project of the Section on Molecular Biology at the present time is the one designed to study the nature of the reaction of metal ions and other substances in the nucleic acids and their derivatives. The ultimate goal is to provide a chemical method for the determination of sequences of nucleotides in nucleic acids, a goal of great importance because this sequence is believed to constitute the coding of the hereditary information of the cell. The studies are of interest *per se*, however, since they provide clues to the way in which the structure of complicated macromolecules is regulated by the interactions with small substances such as metal ions.

Two questions are of primary importance in an investigation of the binding of metal ions to nucleic acids. (1) Do the metals bind to the outside of the molecule, neutralizing the charge on the phosphates, or to the inside coordinating to the hydrogen-bonded bases? (2) If the metals bind to the inside, do they bind equally to the four nucleotides, or do they differentiate among them?

The first question has been answered for a large number of metal ions; some bind to the outside, and some to the inside. Binding to the outside is important because stabilization of the molecule is accomplished; the metals that bind in this way can be ruled out in sequence determination studies. Binding to the inside means competition with hydrogen bonding and consequent weakening of the macrostructure.

The second question is more difficult to answer because each metal ion seems to react in a different manner. The question is important to sequence determination studies, since a reaction is useful in such a determination only if it can pick out one of the four code-producing nucleotide bases, and leave the other three alone.

The summation of the many studies that have been carried out is that metals do differentiate among the nucleotides, but in general the differentiation is quantitative, rather than qualitative. Nevertheless, by manipulating conditions, such as pH, a metal can be made to prefer one of the four nucleotides very substantially over the others. It has been possible to obtain a great deal of specificity in a number of cases.

The second project, that of the function of metal ions in enzymatic reactions, has led to the further elucidation of two reaction mechanisms. In both cases, the same question has been answered in the same way. The question: Does the metal function to bring enzyme or coenzyme in contact with substrate, or does it function as the catalytic site for the reaction? The answer: the latter. Studies on models for the vitamin B_6 catalyzed reactions have shown that metals actually tend to prevent contact between coenzyme and substrate. Studies on the reaction of metal with the aconitase substrate indicate that the metal is the prime agent

responsible for the essence of the reaction; namely, the removal of a hydroxyl group for citric acid.

During the past year efforts have been made to identify age changes in terms of various biochemical and physiological parameters and to determine the extent to which these changes may be explained on the basis of loss of cells. These experiments have been performed on adult and senescent rats.

It was shown that, except for a greater loss of muscle mass, the senescent loss in organ weights and changes in enzymatic activities observed in the tissues of 34 as compared to 14 month old wild rats were similar to, but of no greater magnitude than, those detected when 12 and 24 month old domesticated rats were examined.

In other experiments, designed to estimate the maximal transport of PAH by kidney slices, no age differences were found in the initial rate of accumulation of PAH (μg PAH/gm wet weight—μg PAH/ml. medium after 15 minutes incubation). However, the maximum accumulations (μg PAH/gm wet weight—μg PAH/ml medium after 60 minutes) observed in 14 and 30 month old rats were 18% and 39% lower respectively than that found in 3 month old animals. Since these decrements exceeded the agewise reduction in the number of cells, as estimated by the concentration of DNA, these data suggest that aging is accompanied by an alteration in the function of proximal tubule cells, assuming that there is no selective loss of tubule cells from the total population of renal cells.

Other studies were carried out to estimate age changes which occur in collagen by measuring the rate of change in the chemical contraction and relaxation of rat tail tendon fibers immersed in 2.5 M sodium perchlorate. Although differences of 30–40% were found between young growing and mature rats, differences of only 5–10% were observed from maturity to 2 years, after which no changes were evident.

Other data failed to demonstrate any differences in the response to atherogenic diets as estimated by changes in the concentrations of liver succinoxidase, plasma or liver cholesterol or in the extent of vascular sudanophilia when 12 and 24 month old male rats were compared. However, the vascular sudanophilia was less in young growing animals than in the old.

As an attempt to test the hypothesis that reduced dietary intake results in increased life span by prolonging the growth phase, experiments were carried out to compare some biochemical and physiological characteristics in normal animals and those subjected to caloric restriction of the diet.

An increased concentration of succinoxidase in liver and of alkaline phosphatase in kidney was observed in weanling male rates offered 50% of the amount of diet consumed by *ad lib.* fed animals for periods of 5 weeks to 10 months. In female animals, however, the increase in the latter enzyme was not evident. Adult female rates, so restricted for 8 weeks, showed the same pattern of change observed in young growing females subjected to dietary restriction. These data fail to support the concept that dietary restriction prolongs the growing phase in animals, since the reduced intake resulted in higher concentrations of selected enzymes which are low in chronologically younger animals. The similarity in response to dietary restriction in adult rats and young growing animals suggests that life span may be increased by a reduced dietary intake in adult animals. Thus, retardation of normal growth processes may not be necessary for increased longevity.

However, measurements of the chemical contraction and relaxation of fibers isolated from the tail tendons of both male and female restricted rats were similar to chronologically younger animals. Estimates of the total activity of *ad lib.* fed and restricted rats made in the suspension type cage demonstrated a lower activity in restricted rats. In the wheel cage, where an increased activity due to hunger drive was found by a comparison of *ad lib.* fed animals and acutely restricted rats of the same age, rats chronically restricted during growth had a lower activity. At present, it is not possible to evaluate the latter findings in terms of growth retardation due to incomplete data regarding the effect of body weight on activity measurements in the suspension cage and the quantitative aspect of the hunger drive in terms of wheel running.

Taken as a whole, these data do not completely support the concept that reduced dietary intake results in a retardation of the temporal sequence of changes accompanying normal growth and thereby increases life span.

A method for determining lung compliance in the intact human has been developed and standard-

ized. Data on the effects of aging on this parameter are being collected.

A method for the quantitative estimation of erythrocyte agglutinability has been developed and a study of the agglutinability of B type cells from old and young donors is in progress. This study is aimed at determining whether aging is associated with a change in the synthetic processes involved in the formation of red cells. Measurements of the effects of epinephrine and norepinephrine on pulmonary blood volume in anesthetized dogs (closed-chest) have been completed. Intravenous infusion of these drugs in doses ranging from 0.5 to 2.0 μg/kg/min increased pulmonary blood volume significantly. A simultaneous and proportional increase in the effective pulmonary artery and left atrial pressures suggested that the increase in pulmonary blood volume was predominately passive. Since these drugs have been shown to have an active, direct, constrictor effect on some pulmonary blood vessels, the observed response in the intact dog appears to be an example of passive distention outweighing the effects of active constriction.

Infusion of isoproterenol (2.0 μg/kg/min) and of histamine (5 μg/kg/min) also increased pulmonary blood volume, with either no change or a decrease in transmural vascular pressure. This suggests that these drugs cause an intrinsic dilation of some part of the pulmonary vascular bed, larger in volume than the arterioles alone.

Investigation of the effects of negative pressure breathing on pulmonary blood volume in man has given inconclusive results. Further studies in which both extra-thoracic and air-way pressures are controlled will be carried out.

Investigation of the effects of negative pressure breathing on pulmonary blood volume in man has given inconclusive results. Further studies in which both extra-thoraric and air-way pressures are controlled will be carried out.

The rate of disappearance of the hemoglobin-haptoglobin complex from the plasma was linear with time in 15 subjects. The rate of disappearance from the blood was independent of the level of the complex in the plasma.

Urine osmolality of samples collected at 6:00 p.m. in subjects permitted their usual water intake diminished from 950 milliosmols/L in subjects under 40 years of age to 850 milliosmols/L in subjects over 70. When no fluid intake was permitted after 6:00 p.m., the young subjects showed an increase in urine osmolality by 12:00 m., whereas there was a slight decrease in osmolality in the old subjects. This differential response of old and young subjects may be due to the fact that the kidneys of the old subjects are operating near their maximum concentration capacity under most circumstances or that the tubules of the aged kidney are less sensitive to ADH or that the old subjects liberate less ADH following the mild fluid deprivation than do the young. Further experiments are planned.

The pattern of solute excretion during the night also shows age differences. The youngest subjects excreted 60% of their total 14 hours solute excretion in the first 6 hours (6:00 p.m.–12:00 m.), whereas the old subjects excreted only 45% of the total during this time.

Two independent estimates of body fat have been compared in a group of 100 subjects. An equation using our estimates of total body density and of age reduction in creatinine excretion and bone density estimates a decrease in fat in older subjects which averages 0.12% fat per year. An anthropometric index which summarizes the major circumferences and diameters of the body estimates a decrease in fat in older persons which averages 0.2% fat per year. Thus, the lower weight found in these older persons represents a lesser amount of both fat and lean tissue.

Preliminary results of a sensory-motor test of tapping between similar targets for speed and accuracy indicate that persons over the age of 60 take additional time to perform the tasks involved and that the slope of the curve relating movement time to movement distance and target width is different for old and young. The relation between movement time and overall information transfer

$$\left(\text{movement time} = \frac{\text{effective movement distance}}{\text{target width}}\right)$$

gives a curve for persons under 60 years of age which must be corrected by adding time for errors, in order to extrapolate to zero time for zero information transfer. In persons 60 years of age and over, additional time for all tapping tasks results in a residual time of 0.05 to 0.10 seconds at zero information transfer when the curves of the movement time, information transfer relationship is extrapolated to zero information transfer. These

residuals can only be increased by correction for errors. Thus the "increased times for all tasks" does not lend itself to the interpretation that increased difficulty of a task alone places the older person at a relatively greater disadvantage but rather that some factor which works at all levels of difficulty is responsible for part of the additional time taken by the old.

Lung volume and maximum breathing capacity measurements were compared between an indigent group in an old peoples home and a self-supporting community residing group. Even when individuals with clinical ratings of moderate or marked ventilatory functional impairment were excluded from the comparisons, differences were found between the two groups. The community residing group (50–69 years old) had significantly ($P=<.001$) better maintained vital capacity and maximum breathing capacity than the indigent group (50–69 years old) when subjects without or with only slight ventilatory functional impairment were compared. Residual volume was correspondingly lower for the community residing group than for the indigent group when subjects in the sixth and eighth age decades were compared.

It is therefore clear that inferences drawn about age changes in certain physiological characteristics may be biased when based on observations made on institutionalized subjects.

Investigators in the Psychology Section are providing information on the psychological performance and personality characteristics on a group of subjects (aged 20–101) selected from upper educational and socioeconomic levels. In addition to standard tests of intellectual performance, standard questionnaires to evaluate personality characteristics are being administered. Laboratory tests of learning ability under a variety of experimental conditions have been developed and applied to subjects over the age range of 20 to 101 years. Estimates of reaction time, α-frequency of the electroencephalogram (EEG), spinal reflex amplitude, heart rate variability and motor time have been recorded under standardized conditions.

In the psychophysiological program, a relationship has been found between age and brain-wave frequency of the electroencephalogram. The observed correlation of 0.59 ($N=55$) shows that the period (the reciprocal of frequency) of an individual's brain potentials is strongly associated with his age, and that chronological age accounts for 35% of the variance in brain-wave frequency.

A low positive correlation of 0.23 ($N=55$) was obtained between age and mean reaction time, while a correlation of 0.71 between mean reaction time and mean brain-wave period of the electroencephalogram was observed. The latter correlation is a significant and interesting finding. By means of a partial correlation analysis, the relation between age and reaction time was determined under conditions where brain-wave frequency is held constant for all subjects. This partial correlation, which proved to be *negative*, shows that brain-wave frequency can account for the increase observed in reaction time with age. Thus, an age-associated, central nervous system factor has been identified which may be *the* factor behind age-associated slowing in motor responses.

Thus far, in the two verbal-learning experiments in which subjects (selected from upper educational and socioeconomic levels) participated the performance of the younger group was superior to the older (60 and above) and the slower the pace the better the performance. In addition, age differences were greatest at the fastest pace and smallest at the slowest pace. Results of the two verbal-learning experiments with subjects of lower educational and socioeconomic levels were somewhat equivocal. Further investigation is in progress.

The extent to which judgments of stimuli are affected by preceding stimuli (context effects) have been implied as a possible source of age differences in recent perceptual studies. Using time estimation of visually perceived intervals to provide a measure of context effects, substantial context effects were produced but no age differences were found for this perceptual task.

Preliminary cross-sectional analyses of the data collected on subjects from the upper educational and socioeconomic levels showed: (1) Four of the ten factor scores of a personality questionnaire (Guilford-Zimmerman Temperament Survey) were related to age. General activity, ascendance, and sociability scores decreased with age, and the restraint measure increased with age. (2) Of the four verbal fluency measures (Southern California Tests of Creative-Thinking Abilities), only Word

Fluency (how rapidly words containing a specified letter are produced) was related to age ($r = -0.21$). (3) A measure of reproducing geometric designs from memory (Benton Revised Visual Retention Test) was most highly correlated with age ($r = -0.44$). (4) On a vocabulary measure, subjects above age 60 were somewhat superior to the group below 60. Almost every subject was above the national average based on extensive adult norms.

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

INTRODUCTION

The direct research activities benefited this year by availability of additional space in Buildings 5 and 8 and renovation of a number of laboratory areas. Next year will see additional space readjustment which will permit more adequate utilization of resources. The staff of the Laboratory of Biology of Viruses moved into new quarters on the third floor of Building 5, releasing additional space in Building 7 for expanded work in acute respiratory viral diseases. Additional space is also available for the Laboratory of Parasitic Diseases and Laboratory of Parasite Chemotherapy in Building 5. The Laboratory of Tropical Virology occupied enlarged laboratories renovated for both highly infectious agents and for ordinary virologic procedures.

After twenty-five years of distinguished research in the Public Health Service, Dr. Harry Eagle retired to accept an academic position. The Laboratory of Cell Biology of which he was Chief was incorporated into the Laboratory of Biology of Viruses under Dr. Karl Habel as a Section. It remains essentially intact and continues productive studies in cell biology.

The Laboratory of Germfree Animal Research established a new Experimental Pathology Section under Dr. Edwin Lerner. This Section will support ongoing studies in germfree life and also provide histopathologic consultation to NIAID investigators.

The Laboratory of Tropical Virology in which the Middle America Research Unit is administratively located secured expanded space in Bethesda, as well as improved facilities in the Laboratory in the Panama Canal Zone. Dr. Alexis Shelokov, Chief of the Laboratory, returned to Bethesda in July and relinquished his duties as Director, MARU to Dr. Henry K. Beye formerly with the Laboratory of Clinical Investigation. An Epidemiology Section was established in MARU in addition to other previously established sections.

On his return from a year of study in Europe, Dr. Carl L. Larson was assigned to the Office of the Director, NIAID, to conduct an evaluation of the total NIAID research program emphasizing particularly the extramural training program. Dr. Cornelius B. Philip succeeded Dr. Larson as Director of the Rocky Mountain Laboratory and Dr. Herbert Stoenner became Assistant Director. Dr. William Hadlow rejoined the staff of RML as Head of the Pathology Section. Plans were initiated for the construction of an animal house and insectary with new funds provided by the Congress.

Dr. Don Eyles of the Laboratory of Parasite Chemotherapy established a field station in Kuala Lumpur, Malaya on the grounds of the Medical Research Institute to investigate more intensively the problem of simian malaria. He is assisted by two NIAID staff members and local technical help.

Although the position of Chief, Laboratory of Immunology has not been filled, research work of that laboratory is proceeding successfully under the able guidance of Dr. John Tobie, Acting Chief. One of the successful ventures of the Laboratory of Immunology which influences work throughout the NIH is the series of weekly seminars in immunologic subjects by staff members or outside scientists. A highlight was a special seminar in honor of the late Dr. Jules Freund, the first Chief of the Laboratory of Immunology, presented in December by Dr. Merrill Chase.

As in the past, the Institute participated in the NIH Research Associate Program. Recent medical graduates who can fulfill their military duty by a commission in the Public Health Service are selected and assigned to research laboratories. Seminars and lectures provide a scope of interest but the principal value is in actual research ex-

perience under the guidance of a preceptor. This is the fifth year of the program. It is already clear that these young medical scientists form an important reservoir of talent for the staff of this Institute and other research centers in universities and medical schools.

As a part of the United States effort to establish better-scientific communication with the USSR, this Institute sponsored the U.S. Infectious Disease and Microbiology Exchange Mission. A group of well known virologists, headed by Dr. Robert J. Huebner, Chief, Laboratory of Infectious Diseases, NIAID visited research institutions and laboratories in Russia for four weeks in the spring. Other members of the delegation were: Dr. Robert M. Chanock, Laboratory of Infectious Diseases, NIAID; Dr. Fred M. Davenport, University of Michigan; Dr. W. McD. Hammon, University of Pittsburgh; Dr. Edwin H. Lennette, California State Department of Public Health; and Dr. Alexis Shelokov, Laboratory of Tropical Virology, NIAID. As a further step in the international exchange of scientific information, this Institute was host to Dr. Nicolai Petrovich Yelinov, Deputy Director of the Leningrad Chemical-Pharmaceutical Institute, from September until December, who worked in the laboratory of Dr. Chester Emmons, LID, investigating mycotic diseases.

The Institute has continued to assign carefully selected staff scientists to other research laboratories either to pursue their own line of investigation or to learn new concepts and techniques. Dr. Eugene C. Weinbach, Laboratory of Parasitic Diseases worked at the Wenner-Grens Institute, University of Stockholm, Sweden. Dr. Philip McMaster, Laboratory of Immunology is at Pasteur Institute, Paris. Dr. Maryjane K. Cook, Laboratory of Infectious Diseases continues her studies at Max-Planck Institut für Virusforchung, Tubingen, Germany. Dr. Kelsey C. Milner, Rocky Mountain Laboratory, began work at The Karolinska Institut, Stockholm, Sweden in October. Dr. Sanford Stone started a year's work at the Hôpital Broussais, Paris. Dr. J. Frederick Bell was awarded a Guggenheim Fellowship which provided him an opportunity to visit research laboratories in Middle Europe interested in tularemia. In December, Dr. William J. Jellison was assigned to the Pan American Sanitary Bureau to conduct investigations at the Zoonoses Center, Azul, Argentina.

Once again the Institute was host to many scientific guest workers who remained from several days to several months. Five foreign scientists joined the Institute in the Visiting Scientist program to engage actively in curernt investigations. Six scientists supported by Fellowships worked with members of the Institute staff.

Each year there is increasing demand and need for training oportunities at the Institute. Approximately 25 Visiting Scientists, Fellows, Research Associates, and Clinical Associates at the postdoctoral level engaged in research activities in a training capacity. It is clear that the various Institutes at NIH have the opportunity to play a major role in the postdoctoral degree education of biomedical scientists in this country. It is a role which this Institute can pursue effectively, because of its resoures and interest in a wide variety of research problems in infectious and allergic diseases.

A long felt need in the study of viruses as a cause of disease is a readily available supply of uniform reference antisera and antigens. Virologists find that with the increasing number of isolates (now numbering in the hundreds) their investigations are curtailed by lack of identifying reagents. To fill this need, the Director, NIH requested this Institute to establish a Viral Reagent program under guidance from selected scientists and representatives of interested Institute Councils. This effort extends the support which the National Cancer Institute already has given for reagents of the enteroviruses and this Institute for the adenoviruses. Panels of experts were established for enteroviruses and adenoviruses and will be established for the arthropod-borne viruses. The panels will write specifications for reagents and determine procedures for monitoring the quality of the material produced under contract.

As new laboratory methods for uncovering viruses are developed, and their role as a cause of acute respiratory disease determined, it has become clear that vaccines for immunization will be needed as soon as possible. This Institute has initiated a Vaccine Development Program which is intended to fill the gap between the fundamental research conducted in laboratories and the production of tested prophylactic vaccines suitable for

use by practicing physicians. The rapid advancement of research accomplishments in respiratory diseases demands equal effort to translate these into practical usefulness. The Institute will be advised by a Board of outside scientists and staff members on specific developmental problems and the particular competence of various laboratories to fulfill the project requirements.

The following pages describe the accomplishments of scientists working in Institute laboratories. They comprise a wide spectrum of research endeavor. All projects have been significant and contributed in various measure to scientific knowledge and to research techniques. Of special interest has been the development of procedures for utilizing inmate volunteers in the studies of minor respiratory illnesses and malaria. The excellent clinical facilities at the Clinical Center and the carefully controlled laboratory procedures insured a high degree of precise clinical measurement and safety. In a short time with relatively little cost the project has given valuable information which otherwise would have been impossible or inordinately time consuming and expensive.

Advancements which are particularly timely and influential are: the demonstration that the Eaton agent, an important cause of respiratory illnesses, is a pleuropneumonia-like-organism; the description of the first cases of Venezuelan equine encephalitis in Central America; the axenic culture of *Entamoeba histolytica;* demonstration by immunofluorescence of antibody production in human malaria; and the evidence for a new "foreign" cell antigen produced by polyoma virus as it transforms normal cells to tumor cells which is rejected by the immunologically competent adult animal without tumor formation, whereas immunologically tolerant suckling animals cannot reject the new antigen and tumors develop. Other equally important findings are included in the following report of activities.

LABORATORY OF CLINICAL INVESTIGATION

The program of the Laboratory has been enlarged by the development of projects in viral respiratory disease, immunology, mechanism of fever, and malaria. In contrast to previous years when optimum bed occupancy was achieved only with difficulty, the daily census has been maintained near 100 percent by reason of the volunteer program, and there is now a waiting list for other cases which permits a more careful selection of those to be admitted.

Viral Infection of Volunteers

The largest project of the laboratory has been the study of viral respiratory disease in prisoner volunteers. During the year, the organization and function of this activity has become more efficient. A total of 189 volunteers have been inoculated with one of 14 different respiratory viruses or the Eaton agent. The principal purpose of these studies has been to determine the capacity of the agents to cause illness and to study the virological and immunologic response to inoculation. The viruses tested were, with exception of influenza, suspected of causing significant amounts of respiratory viral infection, but their role had not been defined. Thus, a determination of their capacity to cause illness represented an important step in assessing their role in the total picture of respiratory disease in the general population.

A classical type of moderately severe viral influenza has been produced in susceptible volunteers while a milder disease or no illness occurred in those with specific antibody. Illness and viral excretion began by the second day after inoculation and virus was still present in some cases 5 or 6 days later, and after recovery from illness. These studies have demonstrated a type of infection and illness which lends itself to many kinds of clinical study.

Despite many close biologic and biochemical similarities it was found that Coxsackie A–21 (the Coe agent) caused an acute, febrile flu-like illness while Coxsackie A–24 (the Pett virus) caused no illness. Virus was repeatedly isolated from the throat of volunteers given A–21, while A–24 was found only in rectal swabs. Several higher-type adenoviruses have been shown to produce disease when inoculated by cotton swab into the conjunctiva beneath the lower lid but not when given intranasally or by spray into the throat. In all situations, however, viral infection occurred with both throat and anal swabs positive. The higher type agents appear to be less virulent than lower

type adenoviruses, as judged by comparison with earlier studies.

Reo-viruses obtained from human sources failed to produce illness although viral infection occurred in susceptibles. Recently, a common cold syndrome with rhinorrhea, malaise, and in some cases, fever, has been produced by three new and presently unclassified enteroviruses. The incubation period was only a few hours.

Salmonella Carriers

Studies of 26 chronic salmonella carriers revealed that gallstones present in each case contained salmonella (most were *S. typhosa*). When these stones were incubated in high concentrations of antibiotics, salmonella persisted and grew in subsequent cultures not containing antibiotics. Consistent with these findings was the observation that antibiotic treatment alone was not effective in curing the carrier state. On a basis not yet fully explained, was the further observation that cholecystectomy alone was often not effective in curing carriers, suggesting that infection in sites other than gallstones perpetuate the carrier state. A few instances of positive cultures from liver tissue were found. Finally, it was found that lasting cure occurred if antibiotic treatment was combined with cholecystectomy. It was estimated that over 300 cases and 3 deaths from typhoid fever occurred in contacts of this group of patients.

Malaria in Volunteers

Following demonstration of human infection with the simian vivax-type malaria, *Plasmodium cynomolgi bastianellii*, several prisoner volunteers infected with this agent, and others inoculated with human vivax strains were studied at the Clinical Center. In addition to the usual parasitologic determinations, radioactive chromium survival tests, of erythrocytes excretion of adrenal steroid metabolites, liver function, a number of other parameters were measured. Among other findings was the observation that a significant reduction in serum haptoglobin concentration took place in association with hemolysis of the red blood cells.

The most significant observation, however, was the demonstration of fluorescent stainable malaria antibody appearing approximately two weeks after onset of illness and reaching a peak about one month later. Titers as high as 1/5120 were frequently reached. Following recovery the titers slowly decreased. In connection with these studies, it was found that the concentration of circulating gamma globulin in the malaria patients increased during infection, and the highest values of gamma globulin coincided closely in time with the occurrence of the highest titer of malaria antibody.

Mechanisms of Fever

A new program this year has been the investigation of host response to fever, and the causes of fever and related phenomena in certain febrile diseases of obscure origin. The studies are presently centered around the disease known as familial Mediterranean periodic fever or familial polyserositis. It is an inherited disease of certain persons of Jewish, Armenian and sometimes of Turkish descent. It is characterized by many years of progressive, disabling attacks of noninfectious fever and peritonitis unrelieved by any presently known treatment. Studies here have been concerned with the effect of the recurrent fever on mechanisms of heat loss when the patients are stressed in a low temperature chamber. It appears now that patients with polyserositis have lost some responsiveness to cold stimulation as compared with normals.

Fungus Disease

The program in systemic fungus infection has continued in a vigorous way. A number of clinical reports have been made of treatment of various kinds of fungus disease with amphotericin and the new agent of Hoffman-La Roche, X5079C. Both drugs are extremely useful in treatment of fungus disease as described in the following table:

	Amphotericin	X5079C
Blastomycosis	Good	Good.
Histoplasmosis	Good	Good.
Sporotrichosis	Good	Good.
Cryptococcosis	Good	Doubtful.
Coccidioidomycosis	Slight	Doubtful.
Candidiasis	Moderate	Doubtful.

Since amphotericin causes significant renal toxicity and local irritation, especially when introduced into the spinal canal these features of treatment have been studied. It was found that a persistent renal tubular lesion characterized by prolonged depression of para-amino hippurate clearance is caused by amphotericin. Furthermore, recent studies of biopsies of kidney from patients under treatment reveal necrosis of glomeruli. This is an unusual renal lesion and is under detailed investigation in dogs. So far no effective means has been found to control the irritative effects of the drug on the central nervous system after intrathecal instillation. The nerve damage encountered has been so severe that local use of the agent in meningitis (cryptococcal) will henceforth be administered only to those cases in which it is considered an absolute essential.

The compound X5079C was found to cause a prompt rise in bromsulfalein retention despite only slight evidence of interference in other kinds of liver function. This received careful examination in dogs and the effect is now revealed to be a competition of the antibiotic for the excretory route for bromsulfalein. The exact mechanism has not been determined. Prolonged treatment with the agent causes some liver damage but it appears to be sufficiently slight to permit therapeutic use of the drug.

Penicillin Derivatives

Two new penicillin derivatives have been tested in staphylococcal infection. The agents, Prostaphlin, manufactured by Bristol Laboratories, and SKF No. 12141, manufactured by Smith, Kline & French, are similar in that they are substantially resistant to destruction by penicillinase, they are significantly more active on a weight basis against sensitive staphylococci than dimethoxyphenylpenicillin, the first of the penicillinase resistant compounds (see last year's report), and they are active after oral administration. Limited clinical studies have revealed that these agents are effective in the treatment of staphylococcal infection, including those resistant to older forms of penicillin. Biochemical studies on these compounds have also been made and will be described elsewhere in the report.

Penicillin-Resistant Staphylococcal Infections

Work in the past three years on the problem of penicillin-resistant staphylococcal infections has developed into two distinct problems. The first phase of the investigation dealt with the nature of the resistance of staphylococci to penicillin. It was established that naturally-occurring pathogenic strains of *Staphylococcus aureus* which are resistant to penicillin, are resistant by virtue of their containing an enzyme, penicillinase, which rapidly destroys penicillin G. A series of papers have been published on the biochemical properties of this enzyme as it occurs in *S. aureus* and on its role in penicillin resistance.

The second phase of the investigation has been concerned with the many new penicillins that have become available in the past two years as the result of a technologic development in the field of penicillin chemistry; namely, the ready availability of 6-aminopenicillanic acid as the immediate precursor of a wide variety of penicillins previously unknown. When it was determined that resistance of staphylococci was based on their ability to destroy penicillin G at a rapid rate, it became evident that a possible solution to the problem would be to find a modification of penicillin which would be resistant to the destructive action of penicillinase and yet retain antibiotic activity. When 2,6-dimethoxyphenylpenicillin was given to the Laboratory for clinical trial, it was very soon found that this new penicillin met the essential biochemical criteria and offered therapeutic promise. Our laboratory, by virtue of being the first to obtain staphylococcal penicillinase in large amounts in concentrated and purified form, was able to establish the absolute rate of destruction of the new penicillin by staphylococcal penicillinase and show it to be more resistant than penicillin G by a factor of about 200. The consistently good therapeutic results obtained with this drug on extensive clinical usage in the past year have tended to verify the conclusions drawn from the laboratory findings.

Similar techniques have enabled us to evaluate all the new penicillins which have been offered to us for clinical trial including an isoxazolyl penicillin which is not only resistant to penicillinase but is also sufficiently stable to acid to permit its

oral use. Such a penicillin would have an advantage over 2, 6-dimethoxyphenylpenicillin which is not effective after oral administration and must be given intramuscularly or intravenously.

Clinical Immunology

Association with the Clinical Immunology Section of the Laboratory of Immunology has provided the Laboratory of Clinical Investigation with an increasing number of laboratory techniques to support clinical studies. The immunoelectrophoresis technique, agar gel diffusion methods, and fluorescent antibody techniques have been used to study the serum of patients with the nephrotic syndrome, lupsu erythematosus, and other disease entities under study by members of the laboratory. A group of 60 patients with the nephrotic syndrome studied over the past eight years are being followed to evaluate the effect of steroid therapy. Similar studies are being made with a group of patients with lupus erythematosus and in addition, these patients are being evaluated for the degree of skin test hypersensitivity to autologous leukocytes.

Mouse Plasma Cell Tumors

Two years ago it was found that mice injected intraperitoneally with Freund's adjuvant and certain antigens developed chronic ascites containing a high titer of antibody to the administered antigen. Later it was shown that a certain number of animals, which were strain-related, developed rapidly fatal plasma cell tumors. Further, it was observed that the tumor-bearing mice were poor producers of antibody. These tumors have been found to produce predominantly one or another types of globulins (alpha, beta, gamma) and currently studies are under way to measure the relative responsiveness to antigenic stimulation of mice with these tumors.

LABORATORY OF IMMUNOLOGY

Passive Transfer of Allergic Encephalomyelitis

The passive transfer of allergic encephalomyelitis was accomplished when lymph node cells from sensitized Strain 13 inbred guinea pigs were transferred to normal Strain 13 recipients. Furthermore, this exprimental autoimmune disease was transferred from sensitized adult Strain 13 guinea pigs to newborn Strain 13 animals. Strain 2 inbred guinea pigs are relatively resistant to allergic encephalomyelitis and the disease is not transferred to the F1 generation animals of a Strain 2–13 cross. The F1 animals evidently inherit the Strain 2 resistance. Passive transfer with viable lympoid cells represents an important tool in the study of immune phenomena. In histocompatible animals, the variable of transplantation immunity is eliminated, the transferred cells thrive, and the cells go on to immune maturity in the recipient.

Antibody Production and Gamma Globulin in Human Malaria

For the first time, the course of antibody production has been followed in human malaria. It has been possible not only to detect but to titrate antibody by the fluorescent antibody technique and to correlate this with the appearance of the parasites in the peripheral circulation. In 4 sporozoite-induced *Plasmodian vivax* infections in human volunteers antibody was detectable several days after the parasites appeared. In a typical case, antibody rapidly rose to a serum dilution level of 1:2,560, was maintained at this level for about 40 days after which is gradually fell and remained at a low level for the period of observation (121 days). In 5 patients infected with the B strain of *P. cynomolgi* essentially the same results were obtained and certain of the patients still had detectable antibody 1 year after infection. The patients showed gamma globulin levels above the normal limits. The maximum gamma globulin rise tended to occur at the same time as the maximum antibody titer with a median difference of 3 days. The ability to follow antibody production and increases in gamma globulin levels in controlled malaria infections provides some very basic information necessary in the program of worldwide eradication of malaria.

Proteolytic Enzyme of Human Spleen Purified

A method has been established for the isolation and purification of a proteolytic enzyme from human spleen which cuts human albumin into large

precipitating fragments. By column chromatography the enzyme has been shown to be a single substance. Its electrophoretic mobility, pH activity curve and catheptic activity has been determined. Four large fragments of human albumin resulting from the degradation of the native protein by the human enzyme have been detected by immunoelectrophoresis. This study combines several fundamental problems bearing on the nature of antigenicity and the possible roles of enzymes in the degradation of antigens as a primary step in antibody formation.

Lupus Serum Reacts With Mammalian Chromosomes

Fluorescent antibody studies have demonstrated that sera from certain patients having lupus erythematosus when applied to mammalian chromosome preparations, possessed anti-nuclear factors. In the case of human chromosomal preparations all 46 chromosomes reacted and became fluorescent. When sera from three patients with Sjögrens syndrome were utilized, the chromosomes did not become fluorescent even though the sera had anti-nuclear factors. It may be possible to find sera or fractions of sera which are more specific in their chromosomal reactions. The anti-nuclear factors found in the sera of patients with the so-called autoimmune diseases are of importance for a better understanding of the mechanisms involved.

Genetic Control of Gamma Globulin Allotypes

In studies on the genetic control of serum allotypes in rabbits, it was previously shown that RGG–I and RGG–II allotypic determinants are under the genetic control of an allelic pair of autosomal genes. Further studies have revealed three other gamma globulin allotypic determinants which are under the genetic control of a three allelic system at a second gene locus. Furthermore, two allotypic determinants were either both present or absent from all sera tested indicating genetic control by a single allele or two closely linked alleles. In addition a gamma globulin at a third locus has been found. The distribution of allotypes varied considerably among several rabbit colonies. The selective breeding of rabbits is in progress to establish genetically defined lines of rabbits based on the known gamma globulin allotypes.

Allergic Thyroiditis

Delayed hypersensitivity to thyroid extract has been successfully transferred within Strain 13 inbred guinea pigs. Intact lymph node cells from animals actively immunized to thyroid extract were injected into normal recipients. Successful transfer was accomplished only if the lymph node cells were taken early after immunization of the donors. Cells taken 5 days after sensitization successfully transferred skin test hypersensitivity. Lesions of the thyroid were produced by the transfer of lymph node cells in about 30 percent of the recipients.

LABORATORY OF BACTERIAL DISEASES

The research program in the Laboratory of Bacterial Diseases has continued to develop in the same general areas noted in last year's report. The major emphasis has been on the projects on Intracellular Parasitism and Staphylococcus.

In Vitro Cell Antibody

In the course of the project on Intracellular Parasitism a study was undertaken of the production of specific antibody by mammalian cells *in vitro*. This study has been reasonably rewarding and offers considerable promise. Accordingly, at present, the major effort on this project is devoted to this line of investigation.

Specifically, cells are obtained from the peritoneal cavity of laboratory animals, usually the guinea pig. A method has been developed which yields a high proportion of mononuclear cells. Further separation of the cell types results in explants which are composed almost entirely of this type of cell. Cells removed from immune animals and properly handled and cultured elaborate specific antibody *in vitro* for days or even several weeks. The explants result in growth of sheets of cells which are derived from the mononuclear cells. On glass they become stellate cells but retain some of the characteristics of macrophages. Such tissue cultures can be maintained for long periods and be

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carried through at least a number of subcultures. The antigen usually has been egg albumen; the test procedure, hemagglutination of specifically sensitized red blood cells. While production of antibody *in vitro* has been sporadic, significant titers have been obtained, and in a number of experiments antibody has been detected for periods of several weeks. In comparison with initial cell homogenates and with supernates from cells maintained under non-optimum conditions, the amount of antibody produced exceeds that which can be accounted for by release of preformed antibody and certainly represents new protein synthesis after removal of the cells from the animal. There is still a question as to the type of cell which is producing antibody and also whether these particular cells have undergone multiplication even though general growth of the tissue culture has occurred. This system allows further study of the factors involved in continued antibody production.

Staphylococcus Virulence

In the project on Staphylococcus there is continued emphasis on the factors responsible for virulence of the organism and on basic studies of methods of identification of pathogenic strains of the organism. Gel-diffusion immune precipitation techniques have been applied to standard reference strains of Staphylococcus. Employing methods which tend to eliminate minor and nonspecific cross reactions the antigenic mosaic of such strains has been characterized. This technique seems promising for the development of a system of identification. If reference spectra consisting of precipitation patterns formed with specific reference antigens and antisera can be established for staphylococci, this would form a solid basis for the identification of strains.

Brucellosis Diagnosis

We continue to collaborate with other Brucella research centers throughout the world in the development and standardization of laboratory tests for species identification, and for diagnosis of Brucella infection. Brucellosis is still a disease of world wide importance in relation to the welfare of mankind. Control of the disease in domestic animals rests in part upon regional epidemiological studies which require precise identification and classification of strains. A unified effort is currently under way in an attempt to clarify Brucella taxonomy. This undertaking is under the aegis of the Subcommittee on Taxonomy of the Genus Brucella of the International Committee on Bacteriological nomenclature, of which the Chief, Laboratory of Bacterial Diseases, is a member.

LABORATORY OF BIOLOGY OF VIRUSES

Two major changes in the Laboratory of Biology of Viruses during 1961 have resulted in an expansion of its activities. Between July and September, all units of our original laboratory were moved from their scattered locations in Building 7 to be together on one floor in Building 5. The second change was the transfer as a section to the Laboratory of Biology of Viruses of the remainder of the Laboratory of Cell Biology after the retirement of Dr. Harry Eagle.

Virus-Host Relationship

Investigations of relationships between the virus and its host cell have continued to be the greatest effort of the laboratory. Four different units are working in this important general area, attempting to define the biochemical events, their sequence, site and inter-relationships. These studies represent both basic and practically oriented research. The basic and more direct goal is to determine the mechanism of protein and nucleic acid syntheses in the normal cell, the virus being used only as a self-replicating identifiable biochemical macromolecule. The other more remote goal is the development of knowledge concerning virus invasion and multiplication which might give a lead as to a logical point of attack in chemotherapy of virus diseases. Studies on poliovirus in HeLa cells have shown that viral protein and viral RNA syntheses are closely coordinated in time and increase only a short time before whole infections virus can be demonstrated. By the use of inhibitors there is evidence that the synthesis of viral RNA may be at least partially dependent upon protein synthesis. Viral protein antigens appear in both the nucleus and cytoplasm, but evidence is lacking as to whether this is the result of synthesis in both areas of migration after synthesis. Studies on

vaccinia virus—a DNA virus—suggest that the synthesis of viral DNA and viral protein are dissociated. Work with Coxsackie virus—a RNA virus—shows precursor viral RNA present in the nucleus early after infection, declining by 3 hours and again increasing at 6 hours. Viral RNA in the cytoplasm decreases until 2 hours, then rises continuously to eventual high levels.

Virus Structure

Three units have carried out studies on chemical and physical structure of virus as such. The protein coat of poliovirus has been isolated and found to contain no free N-terminal amino acid residues. The subunit of this protein appears to have a higher molecular weight than the predicted 20,000. A base analogue—5 fluorouracil—has been successfully incorporated into the RNA of poliovirus to the extent of replacing 30% of the normal uracil, but with retention of biological properties of the virus. This "abnormal" virus continues to have the properties of the parent normal virus in regard to infectivity and host range susceptibility. Coxsackie virus has been shown by electron micrographs of purified virus to have an outer envelope and a dense inner core.

Virus Inhibitors

An important practical contribution to virus research technology has been made by the group studying virus inhibitors. The previously demonstrated inhibitor of plaque formation by certain genotypes of certain viruses has been identified as a sulfated polygalactone. Two different practical methods for its removal from agar have been developed as well as materials found which can neutralize the inhibitor. This has made it possible to obtain plaques with strains of ECHO viruses, myxoviruses, arbor viruses, adenoviruses and rabies virus, all of which produced no plaques by standard methods.

"Foreign" Cell Antigen

A study of the mechanism of oncogenesis by polyoma virus has developed evidence that when the virus transforms normal cells to tumor cells it changes the genome of the cell in such a way that a new "foreign" cell antigen appears. This "foreign" antigen is rejected by the immunologically competent adult animal and tumor development is suppressed, whereas the suckling animal being immunologically tolerant cannot reject the new antigen and tumors develop. This demonstrates two important phenomena: 1) the interaction of a virus with a mammalian cell can induce a genetically stable change in the antigenic components of the cell, and 2) immunological competence may be the limiting factor in the progressive development of a tumor after transformation has occurred.

Melanin Granules and Mitochondria

Important contributions have been made in the general area of normal cell metabolism unrelated to virus infection. In the application of the finding made last year that tetracycline antibiotics localized specifically on mitochondria and can be demonstrated by fluorescent microscopy, it has been found that melanin granules in melanoma tumors act like mitochondria thus adding another bit of evidence for the mitochondrial nature of these granules.

The development of a mutant line of HeLa cells resistant to the toxic effects of a glucose analogue has led to the demonstration that this drug resistance is due to the presence of an inhibitor of hexose phosphorylation. This inhibitor may be related to akaline phosphatase since the latter is 8 to 10-fold higher in the resistant cells. Studies using a DNA inhibitor in HeLa cells have produced evidence that synthesis of protein and RNA in the nucleus is DNA-dependent, whereas their synthesis in the cytoplasm is not. In a continuation of studies on inborn metabolic defects, initiated by another investigator, current studies of cell cultures from gout patients show these cells to produce and release large quantities of purines compared to normal cells.

LABORATORY OF PARASITE CHEMOTHERAPY

The United States is an active participant in world-wide malaria eradication. Our principal contributions to the effort arise from basic and

clinical studies on the disease. In addition, we are committed to the same kind of investigations involving parasitic infections in general, with special emphasis on schistosomiasis and intestinal parasites. Because of the zoonotic character of simian malaria, as demonstrated here in 1960, and a large interest by the senior staff in overall problems of malaria, the main research effort during the year has been in that field.

Malaria—Simian

Man-mosquito-man transmission was accomplished consistently with two different strains (B & M) of *Plasmodium cynomolgi*, even with low gametocytemias. Man-mosquito-monkey transmission was accomplished with each strain also.

Infections produced by the inoculation of blood parasitized with the B or the M strain of *P. cynomolgi* exhibited no significant differences in clinical manifestations. However, when these infections were induced in volunteers by mosquito bites, several differences were noted; in the M strain, the duration and height of the fevers were greater, the tertian patterns were more numerous and typical, and splenomegaly more frequent.

Malaria Studies in Malaya

In Malaya, approximately 33 percent of pig-tailed macaques (*Macaca nemestrina*) and about 40 percent of the long-tailed macaques (*Macaca irus*) are infected with a malaria. A new species of malaria was isolated from this latter species and was designated *Plasmodium fieldi* in honor of Dr. John Field. *M. irus* also harbored *P. knowlesi* and *P. inui*. *Hepatocystis semnopetheci* was discovered for the first time in *M. nemestrina*.

Malaria parasites were also demonstrated in *Macaca irus* and *M. nemestrina leonina* from Thailand and from the latter monkey from East Pakistan. *Plasmodium knowlesi* and *P. inui*, along with a species of *Hepatocystis*, were found in *M. irus lacta* from an isolated island off the east coast of Malaya.

A significant proportion of all three Malayan species of leaf monkeys (*Presbytis cristatus*, *P. obscurus*, and *P. malalophos*) were shown to be infected with malaria. Exact species determination of the parasites have not been made.

Anopheles hackeri was shown to be a natural vector of *P. knowlesi* and at least two, possibly three, tertian-type parasites so far unidentified. *A. maculatus*, *A. sundiacus*, *A. leucosphyrus*, *A. umbrosus* and *A. letifer* were found to bite simians but so far there is no evidence that any transmit malaria. Experimentally, it was shown that *A. maculatus*, *A. sundiacus*, *A. philippinensia*, *A. kochi* and *A. hackeri* are susceptible to infection with *P. cynomolgi*.

Malaria—Human

Plasmodium falciparum from Colombia, South America was found to be resistant to several members of the important 4-aminoquinoline group of drugs, i.e., chloroquine, amodiaquine, and hydroxychloroquine. Because of the wide-spread use of these drugs, such resistance, if prevalent, could be of major significance. Resistance to mepacrine was present also. Resistance has now been shown, by certain malaria parasites, to each of the important groups of synthetic antimalarial drugs developed in the last 30 years.

A drug (BW–377C54), with reported activity against human malaria similar to that of chloroquine, failed to cure *P. falciparum* malaria. Also, the Colombia strain of *P. falciparum* appears to be resistant to this compound.

A study of the life-pattern of Venezuelan vivax malaria indicates that it has an intermediate-type latent period following treatment of the initial attack. This differs from the short-term (Chesson) or long-term (St. Elizabeth) latent period of other strains.

The chloroquine-resistant strain of *P. falciparum* contains a characteristic component which can be detected in hydrolyzed RNA. This suggests a chemical method for identifying drug resistant strains of malaria.

Biochemical Studies

Analysis of ribonucleic acid derived from *Plasmodium gallinaceum*, *P. berghei*, *P. cynomolgi*, *P. inui* and *P. gonderi* revealed the presence of nucleotides of the usual purine and pyrimidine bases: adenine, guanine, cytosine and uracil with adenine most abundant and cystosine the least abundant. In addition, nucleotides of hypoxanthine are found indicating the activity of adenosine deaminase.

Significant binding of chloroquine to a component or components in plasma, but not to pure serum albumin, was demonstrated. The degree of binding of this drug to blood cellular elements in vitro could be altered by a number of physical means. Antimalarial drugs, energy sources, and metabolic inhibitors were studied to determine their effect on the incorporation of amino acids into malaria protein. None of the antimalarial drugs had an effect on this process or on glycolysis.

Nucleic acid extracted from a pyrimethamine-resistant strain of *P. gallinaceum* hastened the development of resistance in a normal strain exposed to both drug and the extract. A decrease in chloroquine sensitivity of *P. berghei* has been observed following exposure of infected donor mice to 1,000 roentgens of X-radiation.

Nutrition and Malaria

Nutritional deficiencies that favor the development of peak parasitemia in mice infected with *Plasmodium berghei* include folic acid, pantothenic acid, and niacin.

Deficiencies suppressing parasitemia include para-aminobenzoic acid and riboflavin. Aminopterin, a folic acid antagonist, also suppressed peak parasitemia. Thiamine deficiency appeared to have no effect. Deficiencies were studied both in the absence and presence of suppressive levels of chloroquine. In only one case did deficiencies have a reverse effect; pyrodoxine deficiencies appeared to favor the parasite in the presence of the drug but suppressed the parasites in the absence of the drug.

Intestinal Parasites

Bephenium chloride, given for three or five days, was highly effective against hookworm infections and also removed 68 and 88 percent, respectively, of *Trichuris trichiura worms*. A rural population heavily infected with *Ascaris* lost 90 percent of their worms when given single doses of piperazine. Hookworm and *T. trichiura* infections were shown to persist for at least seven years in institutionalized mental patients.

Tapeworms (*Hymenolepis diminuta*) exposed to mepacrine (Atabrine) *in vivo* were resistant to the action of the drug *in vitro*. This is interesting to follow up because it suggests a type of resistance not based on selection but upon physiologic adaptation of individual organisms.

Evidence suggests that three anthelmintics, bephenium, mepacrine, and dichlorophen, are effective by inhibiting the enzymes located on the outer surface of tapeworms.

Insect Tissue Cultures

Lepidopteran tissues have undergone growth or extended maintenance in culture. In cultures of caterpillar hemocytes, St. Louis encephalitis (SLE) virus has been maintained up to 10 days.

Prepupae and pupae fat body cells readily proliferate in hanging drop culture for 10–14 days; SLE virus persists in such cultures and may increase between the 6th and 8th day.

Virus-Mosquito Larvae Associations

Larvae of *Aedes aegypti* and *Culex pipiens* mosquitoes acquired SLE viruses from an experimentally contaminated aquatic environment. The virus persisted through the subsequent pupae stage and into the adult insect. There have been nine confirmed transmissions of the virus by bites of the *Aedes* and nineteen by the *Culex* mosquitoes.

Virus-Parasite Combinations

Plasmodium gallinaceum reduced the quantity of circulating encephalomyocarditis virus in chicks. Additional experiments have continued to verify the role of *P. berghei* in the transport of SLE virus to the central nervous system in mice when the virus is given intraperitoneally.

LABORATORY OF PARASITIC DISEASES

Axenic Culture of Entamoeba Histolytica

Work on the cultivation of *Entamoeba histolytica* in the absence of any other living organisms has progressed steadily. Most important is the demonstration that the technique of developing such axenic cultures is applicable to more than one strain. A strain of the amoeba very recently isolated from a human patient has been established in axenic culture. Secondly, the culture medium

has been further simplified, with the elimination of chick embryo extract. Also, it has been possible to eliminate agar from the liquid overlay, a technical factor of importance in that it will eventually aid in evaluating culture growth.

Freezing Protozoa

Freezing of protozoa in liquid nitrogen has been developed to the point of practicability. *Entamoeba histolytica* has been kept viable for at least 10 months and *Trichomonas vaginalis* for six months; there have been no losses in viability by the use of the technique. Other tests underway clearly demonstrate that *Entamoeba invadens*, *Trichomonas hominis*, *T. gallinae*, and *T. foetus* can be maintained viable in liquid nitrogen. These experiences indicate that indefinite storage of protozoal strains is feasible.

Filariid Larvae in Vitro

The development of techniques for freeing microfilariae completely from blood cells and the demonstration of development of microfilariae to the sausage stage are significant advances. This is the first time that sausage stages have been found with any regularity in *in vitro* preparations. Additional work can now be expected to result in further development of filariid larvae *in vitro*. Survivial of adult *Dirofilaria uniformis* in chemically defined tissue culture nutrients (NCTC 109+10% serum) has been obtained for as long as 35 days. Microfilariae were discharged from the females during the first 2 to 3 weeks, only when serum was present in the medium. Under these conditions for maintenance of the filariids, glucose was transformed quantitatively to lactate.

Nematode Cycle in Germfree Animals

In the study of the development of *Nematospiroides dubius* in germfree animals, the nematode has now been carried through its entire life cycle three times in the absence of bacteria. Larvae from the feces of germfree mice have been cultivated axenically to the infective stage and then used to reinfect new germfree mice. It is noteworthy that infections thus produced in germfree animals are not as extensive as in conventional animals; worm burden, worm size, and egg-production appear reduced. This may represent an important lead in determining the factors responsible for alterations in host-parasite balance. The possibility of a steroid action, which is in some way dependent upon the microbial activity in the intestinal tract, is suggested by the differences between conventional and germfree animals in respect to the worm recovery in male versus female mice.

Nutrition and Schistosomiasis

The study on the relation of nutrition to schistosomiasis in Puerto Rico is almost complete. Liver function tests returned to normal in all schistosome-infected patients who were placed on the high protein, high caloric diet. Stibophen (Fuadin) levels were higher and maintained longer in the same patients.

Studies on the mechanisms which produce higher blood levels of stibophen in human beings on high protein high calorie diet have been accomplished in mice on complete semi-synthetic diets in which enhanced activity of stibophen is observed. The higher levels of drug are attributable to the acid-base relations of salts in the diet. With acidic salts Fuadin activity is enhanced. Similarly the efficacy of antimony potassium tartrate, antimony (III) sodium meso-2 3-dimercaptosuccinate (Astiban) and pararosaniline pamoate for killing mature *Schistosoma mansoni* in mice has been found to be increased up to 16× in mice maintained on the semi-synthetic diet compared to mice on the commercial pellet diet.

Experimental Schistosomiasis

Continued work on the pathology of schistosomiasis has revealed differences in response among mice, hamsters, and multimammate rats (*Mastomys*) as to the vascular lesions seen in the liver. Mice and *Mastomys* develop more lesions than hamsters. The explanation for this may be that the lesions are at least partly due to allergic reactions and the hamster is notably less reactive in this respect. The development of hepatosplenic schistosomiasis occurs in mice even with very small worm burdens; a longer period of time is required for the syndrome to appear. The cor

pulmonale picture produced in schistosome-infected mice by partial portal vein ligation provides another experimental model for studying clinical schistosomiasis.

Biochemical Studies

Investigations on *Trypanosoma cruzi* have revealed the fixation of carbon dioxide by this organism, with the incorporation of the CO_2 into succinic acid. Degradation of succinic and acetic acids from incubates containing C^{14} labeled glucose has established that most of the carbons of these acids are derived from glucose. Preliminary fractionation of the phospholipids of *T. cruzi* has yielded serine and ethanolamine as well as an unidentified ninhydrin-positive fraction. CO_2 fixation is known to occur in a number of free-living and parasitic protozoa, and some parasitic helminths. It appears that CO_2 functions as a metabolite as well as appearing as an excretory product.

The studies on the loss of sugars from *Cysticercus fasciolaris* and *Taenia taeniaeformis* in sugar-free solutions and the absorption of glucose and galactose when these are available are basic observations in work that is being continued on the kinetics of absorption of nutrients through the cestode surface. It can be expected that further work in this direction may lead to demonstration of active and selective transport. In connection with the cestode studies it is of interest to note that the data show the metabolism of larval and adult worms to be proportional to fractional powers of the body weight intermediate between those characteristic for animals with increase in metabolism proportial to surface area. This was an unexpected finding because in tapeworms surface and body weight increase in the same ratio, and hence an increase in metabolic rate paralleling weight increase had been anticipated. Also, the quantitative, rather than qualitative, nature of differences in metabolism of adult and larval worms is noteworthy, since in other forms of parasites, e.g. trypanosomes, there are qualitive differences between various forms.

Additional work on the calcareous corpuscles of *T. taeniaeformis* in comparison with artificial mixtures of appropriate salts indicates that the dolomite structure must be preformed in some way in the corpuscles, although they are definitely amorphous until heated. This is of considerable biological and geological interest.

Biochemical studies on intact rat liver mitochondria have shown that a reversal of DPN-flavin-linked oxidative phosphorylation is involved in the reduction of acetoacetate to succinate, with an energy transfer equivalent to one high-energy bond per molecule of acetoacetate reduced. This energy can be supplied by one or both of the two terminal respiratory chain phosphorylations without the intermediary of extramitochondrial ATP. Oxaloacetic acid inhibits oxidation of succinate by DPN-depleted mitochondria. The inhibition is not of the classical competitive type but can be overcome by added ATP. Mitochondria inhibited by oxaloacetate gradually regain ability to oxidize succinate after the oxaloacetate is removed. These findings support concepts previously advanced that there is a compartmentation of substratelevel phosphorylation within mitochondria. This work, on basic biochemical pathways, is of general importance and obviously may be useful in explaining mechanisms for succinate metabolism in parasites.

Virus Potentiation by Trichinella

The potentiation of encephalomyocarditis virus by *Trichinella spiralis* in mice is closely related to the size of the worm inoculum and the time in the worm infection that the virus challenge is given. A similar phenomenon, of enhanced multiplication of Coxsackie A–9 virus in murine muscle, is again related to *T. spiralis* infection.

The studies on *Taenia taeniaeformis* infection in mice, which seem to equate natural resistance with an accelerated response of acquired resistance, are of general interest. The demonstration of a possible immune tolerance in animals receiving *T. taeniaeformis* antigen shortly after birth, is of considerable significance; this phenomenon has not been demonstrated previously in relation to parasites.

Toxoplasmosis

Toxoplasma gondii has been found in the ovaries and oviducts of healthy chickens. The organism is present in the cyst form that can survive digestion. The possibility that it can be found in shelled

eggs is important in regard to the epidemiology of toxoplasmosis. Also, it has a technical importance relative to the use of chick embryos for cultivation of viruses and rickettsiae.

In sheep, resistance to congenital transmission of toxoplasmosis is manifest only against moderate challenges. No protection is manifested against high challenge doses. This is of interest in human medicine. Exposure to large numbers of *Toxoplasma* can occur in women of particular population groups that customarily enjoy raw meat, and under such circumstances it is possible that habitual abortion may occur. This has been reported from Germany but not from the United States.

Electron microscopy of *Toxoplasma* has revealed a schizogonic reproductive process, sometimes with the production of two filial parasites within a parent cell and sometimes apparently with the production of small rosettes. A third process, in which two organisms are involved, may represent longitudinal fission or a sexual fusion. These new observations lend support to the concept that *Toxoplasma* belongs among the Sporozoa of the Protozoa, probably in a new sub-class along with organisms such as *Besnoitia* and *Sarcocystis*.

Under the electron microscope, the formation and character of the cyst wall of *Toxoplasma* has been studied. The wall arises from interaction of the parasite and the host cell. The nature and development of the cyst are important in understanding chronic infection. Although some activity of cysts is evident in chronic infection, long-term treatment with pyrimethamine begun as early as three weeks after infection can produce some diminution in residual brain infection but cannot eliminate it.

Progress has been made in the fractionation of *Toxoplasma* antigens. Separation of hemagglutinating antigen on a hydroxyl apatite column yields an initial fraction that induces hemagglutinating (HA) antibodies but not dye test antibodies when injected into rabbits. This fraction has spectrophotometric activity indicating a high proportion of nucleoprotein, presumably from the cytoplasm of the parasite. Attempts to get a "clean" cell wall preparation to induce dye test antibody formation in the absence of HA antibody have thus far been only partially successful, in that such fractions have stimulated dye test antibodies more rapidly. If the complete separation can be attained, one can conclude that dye test antibodies are produced against the cell wall and that hemagglutinating and complement fixing antibodies are stimulated by the intracellular components of the parasite.

LABORATORY OF TROPICAL VIROLOGY

The third year of the existence of the Laboratory has seen continuation of field-laboratory investigation on viral disease at the Middle America Research Unit (MARU) in the Panama Canal Zone and supportive laboratory studies in Bethesda.

Venezuelan Equine Encephalitis (VEE)

The first isolation of VEE virus in Panama was accomplished in April from specimens obtained from a dying Panamanian boy. Three senior laboratory staff members became infected as a result of the exposure (with virus isolation and serological confirmation in each case). The entire MARU contingent was then immunized with live attenuated VEE virus vaccine. Mild reactions were often encountered, and the attenuated virus was isolated from some of the vaccinees. Several aspects of practical application of this still experimental preparation were thus explored.

Epidemiologic and ecologic studies in collaboration with Gorgas Memorial Laboratory (MGL) of the rural community where the case had occurred, failed to demonstrate the VEE virus in wild, domestic or sentinel animals, or in the mosquitoes collected. Serological evidence of VEE infection (CF, HI, NT) was established for a significant percentage of residents of the area.

A remarkable urban epidemic of VEE occurred in another part of Panama near the site of the Gorgas Memorial Laboratory field station. Approximately 350 cases were registered during a two months' period. Important new information on the epidemiology and the clinical spectrum of epidemic infection with this virus is being developed by GML and MARU.

Vesicular Stomatitis Virus (VSV)

Last year we reported repeated isolation of Indiana type VSV from phlebotomus sandflies collected in the course of a collaborative project

with Gorgas Memorial Laboratory on the ecology of arthropod-borne viruses. Approximately 700 human sera were collected from the population of a town near the field station. One-fourth of the 490 sera tested had neutralizing antibodies to Indiana type VSV. One-fourth of the neutralizing sera also possessed CF antibodies.

Interest in this virus prompted investigation of an outbreak of vesicular stomatitis in cattle. The causative agent was found to be the New Jersey, rather than Indiana, type of VS virus. Antibody patterns of humans in close or remote association with the infected cattle suggested that direct contact, rather than arthropod vectors, may be involved in the transmission of this infection to man. It would seem that the ecology and epidemiology of the two types of VSV are quite dissimilar.

Virus From Rain Forest Arthropods

During the second year of the study on the ecology of arthropod-borne viruses in the tropical rain forest of Panama, conducted by GML in collaboration with MARU, the number of virus isolates was doubled: present two-year total 38–28 from mosquitoes and 10 from phlebotomus sandflies. The isolation rate from sandflies continues to exceed by far the overall isolation rate from mosquitoes.

The first isolate of Indiana VSV from a *non*-sandfly source was recorded (mosquito *Cultex nigripalpus*). Many of our mosquito isolates have been identified with collaboration of the Trinidad and Belem laboratories of the Rockefeller Foundation: six strains closely related to *Una* virus of Group A (work with four other similar agents has not been completed); one strain of Ilheus virus of Group B; several members of the *Bunyamwera* group-one *Guaroa* and four *Wyeomyia* viruses; *Guama* group, represented by six isolates, all from the same mosquito (*Culex vomerifer*). A few other agents remain unidentified.

Attempts To Recover Viruses From Parasitic Mites

Nearly 500 pools of acarines (including 25 species of chiggers, 35 of parasitoid mites and a few ticks from over 350 wild vertebrates) were inoculated into suckling mice and hamster kidney cell cultures. Not a single viral agent was isolated, attesting to the uncommon presence of at least the recognizable viruses among the common tropical mites. Many ectoparasite pools were shipped to the Rocky Mountain Laboratory for attempted recovery of rickettsia. One positive isolation of the *Coxiella burnetii*-like agent was made from a species of intradermal chigger, parasitic on the tropical spiny rat.

Mycoses

Repeated isolation of *Histoplasma capsulatum* from bat liver and spleen suspensions was a most important finding, providing further evidence for the suspected role of bats in the ecology of histoplasmosis. The first human case of actinomycosis (cervical-facial type) caused by *Actinomyces bovis* was recorded demonstrating the existence of this disease on the Isthmus of Panama.

ROCKY MOUNTAIN LABORATORY

Investigations have continued with the same general objectives as previously; those of one large group are oriented toward the zoonoses and arthropod-borne diseases, and those of another major group are chiefly concerned with problems related to allergy, immunology, and resistance to disease. Both divisions involve basic laboratory research, some of which could lead ultimately to development of applied and control measures. While investigations of various vector-borne illnesses have never actually diminished, occasional changes in emphasis have occurred in the last few years with new leads, such as provided in encephalitis ecology by the mosquito-garter-snake relationship, discovery of new foci of Powassan and California viruses, and variations in spotted-fever-like isolates. On the other hand, attacks on phases of such nonvector-borne disease problems as tuberculosis, *Salmonella*, pertussis, poliomyelitis, influenza, and certain mycoses have been intensified in varying degrees.

Tularemia

While it was reported under *Pasteurella tularensis* last year that "the protection afforded by

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live organisms was not effective for long periods of time," mice later vaccinated with a living Russian attenuated strain had a high level of immunity for more than 30 weeks.

Further comparison of isolates from different sources and geographical localities has strengthened the evidence that a "fully virulent" organism in North America causes severe disease in sheep, hares, rabbits, horses, and man. A less virulent form is common to North America and also to Europe and Asia.

Disease of Wildlife

Corynebacterium pyogenes was cultured from 3 dead mountain sheep during an epizootic and from 1 deer and 1 elk. These findings suggest that this may be a pathogen of consequence to certain wildlife, as well as to domestic animals.

Three more isolations of rabies from bats this year bring the local total to 24. Contrary to most concepts, rabies can be chronic in mice. A consequent discovery was that recovered mice with limb movement impaired by rabies became heavily infested with ectoparasites. Mechanical interference with combing and grooming, caused by amputating limbs of normal mice, resulted in similar excessive parasite populations. Another unidentified, nonfatal virus was incidentally isolated from bats and passed in tissue cultures and mice.

In the absence of a usable skin test for detecting the protozoon, *Toxoplasma microti* in *Microtus*, an effective method that does not cause permanent injury to the animal was devised for observing brain lesions through a slit in the scalps of mice. Tolerance for this organism may result from *in utero*, or very early postnatal, infection in native mice.

A fungus, *Emmonsia crescens*, found in certain indigenous small animals of several continents, may also infect man, as indicated by positive passive cutaneous anaphylaxis tests applied to human serums.

Arthropod-Borne Viruses

Western equine encephalitis (WEE) virus was isolated from *Culex tarsalis* at Vale, Oregon during the midsummers of 1960 and 1961, but not in the months from October to May or from bird tissues collected during this period. The overwintering virus mechanism still remains illusive. Recovery of WEE virus in the spring of 1961 from captive garter snakes that had been bitten before hibernation by infected mosquitoes is suggestive of one method of overwintering in spite of failure, as yet, to find naturally infected snakes. This lead has been a popular one and has caused examination of reptile-virus relationships in many countries.

Three isolates of California virus have been made in the Bitterroot Valley from ticks off a snowshoe hare, a golden-mantled ground squirrel, and a chipmunk. Antibodies occurred in a low percentage of these local animals. This naturally leads to an inquiry into the role of ticks as vectors.

An additional isolation of Powassan virus from ticks in western South Dakota has resulted in intensified ecological studies in this area with consequent finding of antibodies in serums from local chipmunks and wild mice. A human serum from western Minnesota and one from central North Dakota (of 1,058 miscellaneous samples tested) showed high neutralizing indexes against this virus. Regional field work will be focused on ecologic and public health aspects of this virus, in view of the known significance of the related Russian spring-summer encephalitis.

Q Fever

Field investigations this year were oriented toward a more objective evaluation of the potential public health problem associated with the vast reservoir of infection among dairy cattle. As determined through continuous surveillance, even minor illness among persons exposed to infected dairy cattle could not be attributed to Q fever. However, 87% of persons exposed to infected herds have acquired antibodies detectable by the mouse-neutralization test and the radioisotope precipitation test.

Investigations on developing a safe method of vaccinating humans with conventional Q fever vaccine were continued at Montana State Prison. Most of the skin-test negative volunteers (97%) who last year received from 1 to 3 doses of 10 complement-fixing (CF) units of antigen subcutaneously reacted positively on a skin test given

10 months after vaccination. However, the agglutinin response among groups of vaccinees varied from 45 to 91% and was directly proportional to the number of doses of vaccine received. Studies on a comparable group of volunteers who received from 1 to 3 doses of 1 CF unit of antigen intradermally are not complete, but results to date are similar to those obtained when vaccine was given subcutaneously. Although the resistance induced in human volunteers could not be challenged, vaccination appears to have been effective, as judged by available immunologic and serologic data.

Significant progress has been made in investigations directed toward fractionating *C. burnetii* into its component antigens. The separation of nontoxic immunogenic fractions from those responsible for inducing or eliciting the hypersensitive state remain a major objective. All antigens of any consequence were determined to be located in the cell wall. Chemical extraction of whole phase I rickettsias with dimethyl acetamide, dimethyl sulfoxide, or a combination of trichloroacetic acid and phenol have yielded protective antigens whose toxicity and ability to induce an allergic response were decreased about 100-fold. Similar extracts of phase II organisms were nonimmunogenic and, in preliminary comparisons of whole phase I and II rickettsias, phase I organisms were more potent vaccines.

Rocky Mountain Spotted Fever

Because of the results of recent studies on rickettsial variants and observations made possible by fluorescent microscopy, interest in the biology of spotted fever rickettsias has been rekindled. Particular attention has been given to the elucidation of factors which affect the natural infection rate among ticks and to the characterization of spotted fever antigen in naturally infected ticks. Of ticks collected in nature, up to 25% contain specific antigen detectable by fluorescent microscopy but only 1 to 3% contain pathogenic rickettsias.

The ecology of *R. rickettsii* appeared to be the same in 2 local areas which had similar natural features. Heaviest infestations of immature *Dermacentor andersoni* occurred on golden-mantled ground squirrels and chipmunks, and virulent organisms were isolated from these rodents for the first time west of the Mississippi.

Heretofore, 4 variant types of *R. rickettsii* in *D. andersoni* have been recognized. The spectrum of pathogenicity of these types varies from an ability to cause overt disease and death to a limited ability to cause only immunizing, inapparent infections. From ticks collected in eastern Montana, many isolates of a fifth type have been recovered. These strains are nonpathogenic for guinea pigs and mice and appear to be antigenically different from other types of *R. rickettsii*. The relationship of the fifth type to the ecology of spotted fever rickettsia remains to be clarified.

By the use of the fluorescent antibody technique, the gross discrepancy between the incidence of pathogenic rickettsias among *D. andersoni* and the prevalence of specific antigen in this tick has been tentatively explained by the demonstration of a rickettsia like organism that is noninfectious for chick embryos as well as for guinea pigs. This agent behaves like *R. rickettsii* because it is transovarially transmitted by *D. andersoni*, has a similar distribution in tick tissues, and is indistinguishable from *R. rickettsii* by fluorescent microscopy. Studies will be continued to determine whether this organism may influence the prevalence of pathogenic rickettsias among ticks in nature.

Mechanisms of Allergic Phenomena

A particular skin protein which had been altered by combination with a simple chemical plays an important role in contact allergy. When a conjugate of a purified heterologous protein and hapten was used, antibody but not contact hypersensitivity to the hapten developed. However, when a conjugate of a simple chemical and a soluble fraction from guinea pig skin was used as a sensitizing agent, contact hypersensitivity to the protein developed. This clearly shows that contact hypersensitivity, such as poison ivy rash, is dependent upon combination of chemicals with substances in the skin.

Since the delayed hypersensitive state can not be quantitated by conventional tests in the skin of sensitized animals, an attempt was made to use the cornea as a site for measuring response to the antigen because this tissue is not vascularized. In

animals sensitized with a conjugate of protein and hapten, the reactions in the cornea occurred only to the protein moiety, although circulating antibody to the hapten and associated allergic reactions could be demonstrated in other tissues. In the corneal response, which is primarily cellular polymorphonuclear leucocytes from surrounding tissue and blood vessels were chemotactically attracted to the antigen.

Although delayed shock is thought to be a systemic manifestation of delayed hypersensitivity, the reaction apparently is dependent also upon circulating antibody. In guinea pigs sensitized with hapten-protein conjugates, only injection of the protein causes the systemic delayed reaction when only delayed hypersensitivity to the conjugate is present. However, after antibodies have appeared, intraperitoneal injection of the conjugate causes a brief initial hyperthermia followed by a distinct hypothermia and lymphopenia typical of delayed shock. At this time delayed shock can not be precipitated by the injection of protein alone but it can be produced by injection of hapten combined with a heterologous protein.

Although many immunologic reactions seen in comparative studies of allergic phenomena in newborn and adult guinea pigs were similar, the delayed response evoked by intradermal injection of antigen could not be elicited before 14 days after birth. This unresponsiveness is attributed to the inability to excite a normal inflammatory response, but the basis for this inertia has not been fully clarified.

To date, these and related studies on tolerance and the effect of hormones and vitamins on delayed allergy support the thesis that delayed hypersensitivity and antibody formation are but phases of the same process. Fundamental knowledge of the mechanisms involved should provide some insight into the study of allergic phenomena associated with infectious and autoimmune diseases.

Endotoxins

Although the exact chemical composition of endotoxin has not been defined, the biological activity of endotoxin complexes, contrary to previous conceptions, cannot be associated with the lipid fractions, and the active principle is probably a polysaccharide.

In further studies, in which endotoxin complexes were depolymerized by treatment with dilute acid, biological activity, including pyrogenicity, tumor damage, lethality, and immunogenicity, was associated with the size of the molecule. When depolymerization had progressed to the extent that the haptenic units were about $\frac{1}{100}$th the size of the original endotoxins, all biological activity was eliminated. These studies indicate that one of the major requirements for endotoxin to elicit host reactions is a macromolecular complex of critical size.

Other methods of depolymerizing and recombining the haptenic units will be used to clarify the relationship between molecular size and biologic activity.

Morphological Elements of Microorganisms

Through collaboration with designers and engineers of a firm specializing in the manufacture of laboratory instruments, a self-contained refrigerated cell-fractionator patterned after the prototype made at RML was tested and developed for the market. The machine is designed so that biological materials can be subjected to any selected pressure up to 60,000 psi and then by sudden release of material through a cooled orifice, organisms or tissue cells can be disintegrated without denaturation of any of the cellular components. During the past year, 12 guest workers and visitors came to RML specifically for the purpose of using this instrument or learning more about it. Since cellular material, varying from the size of small bacteria to large mammalian cells, can be completely disintegrated without denaturing any of the chemical constituents, this instrument will have varied and extensive application in driverse problems in bio-medical research.

Radioisotope Precipitation

The development and application of a radioisotope precipitation technique (RIP test) to serology of virus diseases and the study of virus synthesis received major consideration. This technique is based on the principle that radioactive

particulate antigen (25 mμ to 300 mμ in diameter) sensitized by specific antibody is agglutinated after species-homologous antiglobulin is added to the reaction. No removal of excess globulin is necessary as it is in conventional Coombs type tests where visible precipitate, rather than removal of radioactivity from suspension, is the indicator. The test has been standardized so that it possesses a high degree of sensitivity and specificity. In the determination of specific antibody, the test is 30-fold more sensitive than the tissue-culture neutralization test for poliovirus and 100-fold more sensitive than agglutination or complement-fixation tests for Q fever. By the use of an inhibition-type modification, it is possible to detect as little as 0.1 to 0.001 ug of antigen. The inhibition-type modification has been found particularly useful in studies of viral metabolism wherein it is necessary to detect specific viruses, viral components, and enzymes which may be stimulated by RNA and DNA of plant and animal viruses.

LABORATORY OF GERMFREE ANIMAL RESEARCH

Interest in the use of germfree animals in an increasing variety of research projects continues to grow. More and more, members of our staff are asked to lecture on germfree animal research, techniques, and applications, at scientific sessions and before university staff. At present, in addition to collaborative work with the staff of other laboratories in our own Institute, we are engaged in projects with scientists of the Cancer Institute, CDC, and three universities. It is anticipated that this type of cooperation will continue, especially when the cooperating groups have materials, techniques, and tests which will be helpful to us, but which would not be worthwhile for us to attempt to attempt to set up.

The possibilities offered by germfree, virus-free, or virus-defined animals for serologic, tissue cul- or virus-defined animals for serologic, tissue culture and perhaps pharmacologic standards will, it is felt, receive increasingly more attention, and has been the subject of discussion among scientists working with germfree animals.

Susceptibility by Sex

In the collaborative studies with the Laboratory of Parasitic Diseases the early impression that the sex effect known to occur in certain helminth infections in conventional animals does not occur in germfree animals has been corroborated several times. Whatever the mechanism involved, the lack of a flora reverses the effect normally shown in conventional females. The latter are poor hosts for *Nematospiroides dubius*, for example. We are currently studying mono-infections with individual species of intestinal bacteria in an effort to see if we can pinpoint this interesting relationship. Studies by others working with mono-infected animals have shown that individual species of bacteria can bring about a specific physiologic effect. Among these relationships has been a demonstration of the effect of bacteria on steroid compounds *in vivo*.

Dietary Effect on Helminth Infections

An effect of diet on the course, and duration of certain helminth infections has been observed. This effect appears to be holding up in germfree animals as well as in those reared under conventional circumstances. These infections do not do as well in an animal on a semi-synthetic diet of casein, carbohydrate, vitamins, etc., as they do on the stock Purina-type laboratory animal feed. However, the germfree state appears to compound the difference.

"Natural" Antibodies

In studies on the occurrence of so-called "natural" antibodies in uninoculated animals, both germfree and conventional, several interesting findings have been made. We have found that mice from a colony which has been reared for at least 7 years free from viable *E. coli*, *S. typhosa*, etc., show about the same reactivity to Gram-negative organisms and their products as do conventional animals. No differences were obtained in levels of bactericidal antibody, resistance to endotoxin and phagocytic response. This is of particular interest inasmuch as it had been postulated by

others (in theoretical discussions of modes of action of endotoxin) that germfree animals might be considerably more resistant of endotoxin because of their lack of exposure to Gram-negative organisms. It would appear that even though there are no viable, metabolizing, Gram-negative organisms in the animal's intestinal tract, repeated ingestion of small doses of the heat-stabile endotoxin material inevitably present in the diet is sufficient to stimulate the animals to form "natural" antibodies.

A similar situation seems to prevail with respect to antibodies or antibody-like activities against *Staphylococcus* antigen. In a variety of tests involving serum-gel-diffusion, agglutination, and fluorescent antibody techniques, uninoculated animals not harboring any bacteria that could be detected still demonstrated staphylococcal antibodies. It was noted that the germfree animals did not develop these "natural" antibodies at as early an age as did the conventional animals. Animals 2–3 months of age were negative, whereas those 7–8 months of age showed considerable activity. Again, one must conclude that prolonged ingestion of small amounts of heat-stabile staphylococcal antigenic material (it has recently been demonstrated that such substances exist) can stimulate the formation of antibodies. These findings point to the source of some of the "natural" antibodies that occur in uninoculated animals. Eventually, we may have to resort to animals raised on soluble diets of small-molecule materials prepared under conditions which (hopefully) would preclude the presence of these, apparently, ubiquitous heat-stabile antigenic materials.

Mouse Colony Free of Virus

In our continuing search for evidence of the presence of viruses in our mouse colony, the results continue to be essentially negative for most of the viruses tested. In samplings of well over 125 animals there has been the suspicion of the presence of only one virus in a few instances—Reo 3. Attempts to isolate the agent from bedding, etc., from colony units have not been successful. Among other viruses tested for which negative results were obtained are included mouse adenovirus, poloyma, GD VII, hepatitis and K virus. These studies will continue until we are reasonably sure of the viral state of our colony. However, thus far, it would appear that our germfree mice are free of some of the more common mouse viruses plaguing viral studies in conventional animals.

Thyroiditis in Guinea Pig

In collaborative studies with the Laboratory of Immunology, thyroiditis has been produced in some guinea pigs as early as 5 days after immunization. This constitutes the earliest recorded observations of this autoimmune disease in experimental animals. The lesions are found in all animals 16 days after immunization, and at 7 weeks the disease is severe and extensive in all animals. This has persisted as long as 6 months, at the end of which time some decrease in severity has been found. In studies on mechanisms involved in this autoimmune disease, antibody levels, as well as tests for delayed hypersensitivity to thyroid extract, have been followed. Findings thus far suggest that the presence of the disease is correlated with delayed hypersensitivity, but not necessarily with circulating antibodies. Inasmuch as autoallergy is felt, at present, to be the underlying process in several human diseases, information derived from these studies as experimental models can be of potential importance.

Trauma in Amoebic Infections

In continued studies aimed at an understanding of the role played by bacteria in aiding *Endamoeba histolytica* to establish infection and produce lesions in the intestine, efforts were directed at trying to determine whether trauma or damaged mucosae were essential. As we reported previously, it was possible to get some chronic-type lesions in the intestinal wall of germfree guinea pigs with amoebae that were prepared by techniques which apparently resulted in more vigorous organisms than had been used in the past. However, most of the lesions seemed to occur at the site of the inoculation. Recently we have tried inoculations via the terminal ileum whereby the amoebae were delivered into the cecum at some distance away from the puncture wound. Under these conditions the number of germfree animals developing lesions in the cecum was significantly reduced. Thus, at least in the case of the type of lesion obtained in the absence of a bacteria flora,

trauma or some frank tissue break appear to be necessary for the amoebae to establish infection.

Second Generation Guinea Pigs

Some further studies on the biology of germfree guinea pigs were conducted. The lack of bacteria seems to have more profound and diverse deleterious effects on guinea pigs than on other laboratory animal species that have been reared. We were able to obtain two litters of second generation germfree animals. Only rarely and recently has this been possible. The growth rate and general physical condition of these young was better than has been experienced with Caesarean-derived animals. The fact that they received maternal milk perhaps may explain their better performance.

Germfree Chickens

In further studies to ascertain activities of the intestinal flora, it was found that 6-week-old germfree chickens did not contain valeric acid in their intestinal tracts. This substance is a common constituent of gut contents in conventional chickens and, therefore, these findings indicate that it arises from microbial fermentation. Of further interest was the fact that chromatographic analyses of gastro-intestinal material from germfree chickens failed to reveal any fermentation acids in the crop. However, lactic acid was demonstrated to be present throughout the lower GI tract in amounts which are normally found in conventional birds. This rather unexpected finding in the absence of a demonstrable flora must be explored further.

LABORATORY OF INFECTIOUS DISEASES

The work of the virus and epidemiology sections continued to be aimed at better definition and understanding of numerous human and animal virus infections, particularly of their roles as etiologic agents in respiratory disease, cancer and birth defects. In other words, the chief concern is to find the true natural history of viruses, and define their importance in various disease states. In order to do this it has been necessary to develop appropriate tests and survey methods and in 1961 we were able to achieve comprehensive surveys of viral infections not only in man but also special studies of domestic and commensal animals. We have continued field and clincal studies in collaboration with research personnel of the Bureau of Medicine and Surgery, U.S. Navy; D.C. Children's Hospital Research Foundation; the D.C. Welfare Department; the National Institute of Neurological Diseases and Blindness; the National Cancer Institute; and the Laboratory of Clinical Investigation of the National Institute of Allergy and Infectious Diseases.

Respiratory Virus Disease

Previous annual reports and publications have documented the key role of LID scientists in the discovery of adenoviruses, parainfluenza viruses and respiratory syncytial viruses, and in defining their importance in causing respiratory illnesses, especially in children. In 1961 the role of these agents in causing 35 to 45 percent of the acute respiratory illness of infants and children was confirmed. In addition, the contribution of the enteroviruses (Coxsackie and ECHO viruses) to the acute respiratory syndrome was partly elucidated. Studies of outbreaks of Coe virus (Coxsackie A–21) in military recruits, Pett (Coxsackie A–24), Coxsackie B 3 and 5 viruses suggest that these enteroviruses (and new unclassified ones similar to the British Salisbury viruses) will soon be found to contribute significantly to the overall respiratory disease problem.

Eaton Agent is PPLO

The most significant finding since the beginning of our studies of Eaton's "virus" (Primary Atypical Pneumonia) was the demonstration in 1961 that it is not a virus but a PPLO (pleuropneumonia-like organism) which can be grown in synthetic media.

This discovery is exciting for three reasons. First it opens the door for the development of much simpler techniques for diagnosing primary atypical pneumonia. Secondly, it provides a ready source of material for preparing a preventive vaccine, and thirdly, it suggests that additional serologically different PPLOs can be expected to turn up as causes of acute respiratory illnesses.

The fact that pneumonia due to the Eaton agent responds readily to tetracycline therapy and the fact thta PPLOs are known to be generally very susceptible to broad spectrum antibiotics furnished previously undreamed of possibilities in the therapy of respiratory illnesses.

Enteroviruses in Acute Respiratory Disease

The enteroviruses (Poliovirus, Coxsackie and ECHO viruses) are well known as causes of certain specific illnesses such as poliomyelitis, herpangina, pleurodynia, aseptic meningitis and myocarditis. Less well appreciated are their importance in causing acute upper respiratory illnesses. It is evident from our long term studies of Junior Village children (where we observed nearly 2,000 persons infected with many different enteroviruses) that acute respiratory illnesses often with fever represent the most common clinical manifestations of most enteroviruses. Recently using more sensitive tissue culture isolation procedures, we have encountered numerous additional viruses having the properties of Coxsackie A viruses. The most common group of similar isolates are related to Coxsackie A–24 Pett virus which is related to Coxsackie A–24 virus.

Information on the role of enteroviruses as etiologic agent of adult respiratory illness, particularly the common cold syndrome has been derived chiefly from outbreaks of ECHO 28 (2060 virus) reported at different times chiefly from Chicago by Magabgab several years ago and in 1961 by Hamre and the outbreaks of Coxsackie A–21 in Marines observed last year. It appears that viruses can be recovered from about 20 to 25 percent of adults with mild respiratory illnesses, thus providing new dimensions for study of the "common cold."

Antibiotics in Acute Respiratory Disease

We completed studies on the value of tetracyclines in the treatment of primary atypical pneumonia due to Eaton's (PPLO) agent in adults and on the value of oral penicillin in preventing acute febrile respiratory illnesses in children. The first study of pneumonia which was carried out in Marine recruits at Parris Island (in collaboration with the Bureau of Medicine and Surgery, U.S.N.) showed that declomycin, a synthetic of tetracycline, was very effective in the treatment of primary atypical pneumonia, reducing significantly the duration of fever, pneumonitis and hospitalization.

The daily use of oral penicillin for nearly a year in a proportion of children at Junior Village failed to significantly alter the overall illness rate despite the fact that hemolytic streptococcal infections were virtually eliminated from the throats of those in the test group. Only during brief outbreaks of several typable hemolytic streptococci was it possible to show any measurable beneficial effects from the drug. No effect was observed on viral infections.

Respiratory Viruses in Human Volunteers

Many studies of numerous respiratory agents (viruses and PPLO) in volunteers are scheduled to be carried out in cooperation with the Laboratory of Clinical Investigation and the Federal Bureau of Prisons. To date studies of the pathogenesis of several parainfluenzas, RS, and Coe (Coxsackie A–21) viruses, and Eaton's PAP agent grown in tissue cultures have been completed. All were found to cause acute respiratory illnesses. Eaton's agent produced not only primary atypical pneumonia but also middle ear infections; in the latter case providing one of the first specific clues to the cause of nonbacterial serious myringitis.

One of the more interesting findings from the volunteer studies was the fact that many of the virus infections produced illnesses despite the presence of pre-existing antibodies; but the antibodies also were responsible for the general mildness of the illnesses observed.

Studies in volunteers of Coxsackie A–21 virus (a Coe-like strain isolated from a military recruit) revealed that growth of this enterovirus was almost entirely restricted to upper respiratory tract. This is interesting since other Coxsackie A–21 strains are commonly found in the stools of children. Carefully designed volunteer studies provide a wealth of high order information on host-parasite relationships virtually unattainable in any other way. Hence they will be continued and expanded.

Vaccine Development

One of the chief purposes of the volunteer study program is to test live viruses for attenuation and for inclusion as candidates in experimental vaccines. Of course, killed vaccines can also be tested in volunteers—the efficacy of the vaccine can be measured by challenge with a pathogenic variant of the virus as previously done by LID scientists in developing the first effective adenovirus vaccine.

Sero-Epidemiology of Human Viral Diseases

During 1961 antigens against more than 100 common viruses (6 myxoviruses, 28 adenoviruses, RS, 3 reoviruses, 59 enteroviruses, and 10 mouse viruses) were evaluated in the micro-CF and micro-HI test systems. This program represents collaboration between the cerebral palsy study group of NINDB and all of the research units in the Virus and Epidemiology Sections of LID.

Extensive serologic-epidemiologic surveys are now in progress on the prevalence of antibody responses against more than 100 viruses in women giving birth to abnormal babies, and in children and adults with acute respiratory illnesses, neurological diseases, and cancer. The prevalence of specific adenovirus and myxovirus antibodies in sick children at Junior Village and Children's Hospital is now under study, thus adding a new dimension to our knowledge of the natural history and pathogenesis of these agents.

Similarly serological surveys of these populations have revealed new and wholly unexpected information on the enteroviruses. Previous data from Junior Village which suggested that enteroviruses were important causes of acute respiratory illness, and that this may be the most frequent illness caused by enteroviruses, was confirmed by serologic studies of patients from D.C. Children's Hospital. These tests showed in 1961 that enterovirus infections in children with acute respiratory disease were eight times as frequent as in age-matched control children without respiratory illness.

Cancer Viruses

Studies of animal cancer viruses have proceeded satisfactorily both in the field and in the laboratory. Perhaps our most important function is to focus attention on the importance and feasibility of studying the occurrence and behavior of cancer viruses in nature, to help develop laboratory tools which make such studies possible, and to continue to help define the extraneous viruses in cancer virus study systems which are producing almost insuperable obstacles to the development of reliable information about cancer viruses.

Natural History of Polyoma Virus

The natural cycle of polyoma virus was found to be well established in *Mus musculus* living in farm industry ecologies in Maryland and in Indiana. There is extensive evidence that the age-old association of the house mouse first mentioned in relation to grain storage in the Mediterranean area (recorded in ancient Egyptian and Greek records and the Old Testament) now persists in modern grain storage facilities, on farms and in central depots all over the world. Although *Mus musculus* is now indigenous to all parts of the world, it originated in southern Asia and the Middle East, from whence it was carried along lines of trade to other parts of the world reaching northern Europe and England by 900 A.D. and becoming well established in the New World only within the last two centuries. Studies in southern England and nationwide surveys in the United States show that infestation of grain storages are extensive, almost universal, and frequently very intensive. For instance, a recent 29 state U.S. survey revealed an average count of 80 mouse pellets per peck (about 15 lbs) of shelled corn.

LID studies in the field to date show that polyoma virus infection could be demonstrated in *Mus musculus* on 4 of 15 Maryland dairy farms, in 6 of 6 Maryland cereal grain mills and 3 of 4 Indiana grain mills. Polyoma virus was isolated from cereal grains used chiefly for livestock feed (but also for laboratory mouse and human consumption) wherever such grains showed objective evidence of mouse contamination and where the mice were found to be positive for polyoma. This was not surprising, since infected mice are known to excrete large amounts of virus for long periods in the urine and infestation with polyoma-positive mice was very heavy in most of the ecology studies. Thus, as reported last year, the polyoma cancer

virus is widely distributed in all the ecologies in which commensal house mice live—in laboratories, in production colonies of laboratory mice, in densely populated urban tenements, an in rural environments.

Extraneous Viruses in Cancer-Virus Study Systems

This problem has if anything become worse. Additional viruses were found in production colonies of laboratory mice, including PVM, new strains of hepatitis and Riley's plasma agent. For instance, Riley's agent, together with reovirus 3 and hepatitis virus turned up successively in our passage lines of Moloney's leukemia.

On the credit side we have been able to develop satisfactorily *in vitro* survey tests for mouse hepatitis, PVM, Theiler's virus, and LCM, thus adding these to our test battery which already included tests for polyoma, K virus, mouse adenovirus and mouse salivary gland virus. Thus by working with commercial producers of laboratory mice, we hope to achieve rather soon supplies of mice that are free of infections with these agents.

Immunological studies of mouse reoviruses showed them to be indistinguishable from those isolated previously from man, cattle and monkeys. An interesting sidelight was the demonstration that Stanley's encephalohepatitis virus is identical with reovirus type 3 and that the various other hepato-encephalitis agents isolated from mice and described by Cheever and others are serologically related to MHV and other strains of hepatitis virus.

SPF and Germfree Mice

The obvious answer to the background noise problem is the use of susceptible SPF (specific pathogen free) mice and germfree mice. The main difficulty with the latter is that they simply are not available in large numbers. The SPF mice as suggested above, are of value only when they can be kept in isolated virus-proof quarters, which currently are not available in our virus building.

We have tested two methods of isolation—both with considerable success. The first method made use of isolated house trailers which are chemically sterilized between experiments. These have been very useful for studies of polyoma virus in such field specimens as mouse tissues, excreta and mouse contaminated grain.

Recently, successful experiments have been performed in the Lobund plastic isolators. Although these require much attention and high caliber animal attendants, they may well be the answer to the production of pedigreed, clean and certified cancer virus pools—a prime requirement in cancer virology just as it is for respiratory virology.

Fungus Disease

Mycology studies have followed a multidisciplinary and multifacted research program. In the field the work is focused on isolation and identification of new fungi and on ecologic studies on various fungi known to be pathogenic for man. The studies of new fungi are done in collaboration with the hospitals and diagnostic laboratories; the ecologic studies in collaboration with the Rabies Control Unit, Trinidad, West Indies. New antimycotic drugs and antibiotics are tested for efficiency and safety, in collaboration with the Medical Physiological Bacteriology Section of LID and with the Laboratory of Clinical Investigation. The clinical and field studies are supported and supplemented by basic work in laboratory on the physiology, toxicity and immunology of pathogenic fungi. The search continues for serological methods capable of recognizing mycotic infections in humans and in various animal hosts, and for improved techniques designed for growing and identifying fungi. *Histoplasma capsulatum* was isolated in Washington, D.C. for the first time from a congested urban area, from soil adjacent to houses in Trinidad, W.I., which harbor bats, and from soil under roosting sites of starlings.

Penicillinase

A highly interesting observation concerning staphylococcal penicillinase has been made. It seems probable that this enzyme is a non-specific cyclic peptidase, splitting various cyclic peptides to straight chain compounds—this observation offers at least a partial answer to the hitherto disturbing question of the role and function of penicillinase in bacteria in their natural habitat.

Under normal ecological conditions, it is highly unlikely that bacteria ever comes into contact with penicillin, hence the value of the possession of penicillinase to the total economy of the cell has been dubious. By offering the possibility that penicillinase is primarily a specialized peptidase, its role is conceivably enlarged to a more important function in cellular metabolism.

Antibacterial Marine Waters

Significant observations have been made on the problem dealing with the antibacterial activity of marine waters. It is now quite clear that the activity is due to a large organic molecule with molecular weight greater than 10,000 rather than due to the inorganic ion content of the sea water. This observation, obtained as a result of exhaustive dialysis experiments, settles in large part a controversy of 75 years amongst biological oceanographers. Partial isolation and purification of the active factor has been obtained. Further, the activity seems to be quite specific for gram-positive bacteria, thus offering an attractive hypothesis in explanation of the fact that the vast majority of bacterial genera in the oceans are gram negative. The activity is always present in ocean water, varying only quantitatively and this finding answers in part the hitherto disturbing observation that activity of raw sea water varies temporally and seasonally. From the point of view of isolation and purification of an antistaphylococcal factor, particularly active against penicillinase-producing staphylococci, this is, of course, decidedly advantageous.

Staphylococcus Iron Metabolism

Highly significant observations have been discovered this year dealing with metabolic differences occurring between iron-deficient and iron-sufficient strains of *Staphylococcus aureus*. The degree of iron depletion or enrichment was controlled by the ratio of free Fe- to iron-bound siderophilin in the medium of growth of the organisms. Using radioactive tracers, it is now quite clear that iron-deficient cells lack the ability to metabolize the six carbon of glucose when compared with iron-sufficient cells and further that the deficient cells also have decreased ability to incorporate carbon into protoplasm. These observations are a highly important beginning in a project designed to understand the biology of staphylococci. Since production of the various staphylotoxins presumably mirrors the overall metabolic processes of the cells and since, as indicated above, iron plays such a highly important role in metabolism, the hope remains that by juggling iron availability *in vivo* (and this can be done by proper use of siderophilin), one will be able to render invading staphylococci less toxic.

In the development of the above thesis, it has been essential to devise methods for purification of physiologically active siderophilin and for assaying such activity. This has finally been done by various ingenious biochemical techniques and the problems can now be attacked forthrightly.

Cell Division in Streptococci

Study of the M antigen of Group A, beta-hemolytic streptococci had yielded a surprising but highly important dividend of basic significance. A controversy which has been going on for decades over whether synthesis of the cell wall in dividing bacteria occurs by random intercalation or at specifically polarized growing loci has been all but settled, at least for the Group A streptococci. Using, alternately, periods of growth in culture media with and without fluorescein-labelled homologous anti-M protein, the issue has been definitely decided in favor of the latter hypothesis, i.e., polarization. The observation is of significance to all studying cytokinesis and morphogenesis in bacteria and fungi as well as to those interested in cell-wall metabolism in general. Interest, of course, in cell walls has been high in recent years since many important inhibitors, including penicillin, presumably act by virtue of inhibiting cell-wall synthesis.

Further improvement of the "long-chain" test for streptococcal type specific antibody has been made and its simplicity and accuracy appear likely to make it a most valuable tool for the answering of many clinical and epidemiological studies. Currently, it is being used in the study of pre- and post-isolation sera from a type 12 streptococcal outbreak among institutionalized nursery-school-age children.

The mechanism of the long-chain reaction has been under investigation and the possibility exists that the phenomenon is a form of agglutination requiring bivalent antibody and may not be enzymatic as suggested by other workers.

Antibacterial Substances in Mollusks

Of vast potential import is the isolation, characterization and partial purification of antibacterial and antiviral substances from mollusks, particularly oysters. Two fractions, decidedly different in behavior on columns, but both presumably glycoproteins, have been found to be active against bacteria, including staphylococci and viruses (polyoma and influenza). The significance of this is obvious for an effective chemotherapy of small virus infections is of great moment.

Hydrogenomonas

Marked advances have been made in an understanding of the electron transport of *Hydrogenomonas* and as a consequence, relying on the thesis of comparative biochemistry, the main energy-yielding systems of mammalian tissues. The system in the autotrophic *Hydrogenomonas* is ideal for these studies since it is, in distinction to systems in all other forms studied, at least partially soluble. This fact obviates the extreme difficulty of working with particulate and hence highly complex material. A further indication of the relative simplicity of the complex in this organism has been the finding that it contains only one cytochrome. It now seems obvious that flavoproteins are active in this system in the transport of elutions and judicious thesis can be presented for the formation of reduced pyridine nucleotides via the hydrogenase system. However, a new intermediate, different from others heretofore described, apparently is involved in the formation of reduced pyridine nucleotide. This, of course, has vast theoretical ramifications and would indicate important differences between bacterial and human energy-yielding systems and might well indeed be a basis for selective attacks on bacteria by antibacterials.

Eosinophilic Meningitis

Field investigations in French Polynesia uncovered an extensive outbreak of eosinophilic meningitis. The epidemiologic evidence suggests the disease is associated with the consumption of raw fish infected with a helminth. While these cases were being investigated, two patients died of a similar condition in Hawaii. From the brain of one of these, young adult nematodes identified as *Angiostrongylus cantonensis*, the lung worm of the rat were found in the brain substance and in the meninges. Intermediate hosts of this parasite are terrestrial slugs and snails. In spite of the clinical similarity of cases in French Polynesia and Hawaii, there are epidemiologic differences which remain to be explained.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

BASIC RESEARCH

Introduction

The review by Laboratories and Clinical Branches of the scientific accomplishments of the National Institute of Arthritis and Metabolic Diseases for the calendar year 1961 is herewith presented. To the casual reader, this summary may appear to be a discoordinated series of individual reports of the activities of many individual scientists. A more intimate contact with the participating scientists reveals currents and cross-currents running through the several administrative segments of NIAMD and, indeed, through the several Institutes of N.I.H. Big areas of common interest are apparent. The origins of such interests, the nature of the infective foci and the mechanism of the spread is a rewarding epidemiologic study.

Genetics

Perhaps the most prominent such epidemic is the disseminated interest evident this year in phenomena related to genetics. As one reads the attached summary it is clear that almost every segment of NIAMD has been contaminated with an interest in one or another aspect of the genetic problem.

In the patient, a considerable list of genetically transmitted defects continues to be studied. These include phenylketonuria, alkaptonuria, cystinosis, gout, galactosemia, cystic fibrosis and the glycogenoses. In the intact animal, genetic studies of the transmission of joint disease and of obesity are described. The chromosome, which is the ultimate visible unit of genetic transmission, is the subject of special scrutiny. Abnormalities in the number and morphology of chromosomes are being correlated with abnormal phenotypes. Genetically transmitted defects in the amino acid sequences of individual proteins which are under study include variations in hemoglobins and in lactoglobulins.

The information transfer which is fundamental to the genetic process rests upon the structure and biosynthesis of the nucleic acids. Accelerating activities in the area of the chemical structure and physico-chemical properties of RNA will be found described herein. The properties of these compounds in solution as well as in the solid state are being examined by the most sophisticated methods. The enzymes which generate these polymers and degrade them are being purified and studied.

Of unusual interest are the mechanisms whereby information contained in nucleic acid structure is transferred into the amino acid sequence of protein. A remarkable step forward in the understanding of this so-called "code" has been taken in the year just completed. Working with cell-free protein synthesizing systems, NIAMD scientists have deciphered the first words of this cryptogram. Thus "polyuridylic acid" in the language of RNA means "-phenylalanyl-" in the language of polypeptides. We like to consider this accomplishment, like that of Champollion in unscrambling the cartouches of the Rosetta Stone, as initiating a new era.

Enzyme-Catalyzed Reactions

Another area of scientific activities which transcends administrative divisions is the study of regulatory mechanisms. It is becoming increasingly apparent that one of the advantages which results from the near-universal enzyme catalysis of biochemical events is the opportunity thus provided for regulation. In various portions of the succeeding report will be found references to such mechanisms. Thus, analysis reveals that the effect of hormones of the anterior pituitary gland upon the end-organs, the effects of posterior pituitary extracts upon mammary gland, are mediated through effects upon enzymes. The specific effects

of certain steroids upon aldehyde deyhdrogenase have been carefully studied and documented. Feedback and repression mechanisms have been studied in the regulation of histidine synthesis and evidence has been presented that the enzymes involved in urea synthesis are adaptive. The enzymes concerned with insulin destruction in the liver are likewise regulated by feedback mechanisms.

Perhaps the most completely analyzed example of regulation of a specific enzyme is that of hepatic glutamic dehydrogenase. It has been clearly shown that this enzyme exists in at least two aggregation states. The larger aggregates are active against one amino acid, the smaller units are active against another amino acid. The process of aggregation-disaggregation is reversible and is exquisitely sensitive toward various reagents such as estrogens, pyridine nucleotides, certain amino acids, and various other compounds of biological interest.

Intrinsic to the nature of enzyme catalysis is the capacity for the great variability which is an essential condition for survival. Examples are now coming to light in increasing numbers wherein we can define, with ever greater precision, how this variability is accomplished.

General

Representatives of our Institute have spent prolonged periods, in the past year, in laboratories in England, Scotland, Switzerland, France and Japan. Visiting scientists from 14 countries are presently in residence at Bethesda.

Educational activities are growing. The Research Associate program continues to provide training in laboratory science to selected physicians and this operation is clearly attracting the most capable people of appropriate age group and background. In the opinion of those participating, this program has become an important part of the way of life in NIAMD.

LABORATORY OF EXPERIMENTAL PATHOLOGY

Cell Structures in Aging

Studies of certain cytoplasmic bodies demonstrable by histochemical techniques in aged animals have been extended. As a result the widespread distribution of cytosiderin and of oxidation-aldehyde-fuchsin reactive particles has been determined in retired breeder animals of four rodent species. The epithelial inclusions containing Prussian blue reactive iron, according to the most likely interpretation, derive from breakdown products of the electron transport system. This cytosiderin and the morphologically similar bodies in the same or different histologic sites which can be visualized by an oxidation-aldehyde-fuchsin stain, may be examples of de Duve's lysosomes, since in the instances thus far investigated they also contain hydrolytic enzymes. Cells containing cytosiderin inclusions examined in the electron microscope by Dr. Bruce Wetzel have shown variably vacuolated dense bodies with dark granules possibly similar to ferritin morphologically.

Cytogenetic Studies

During the past year studies have been continued on chromosomes of neoplastic cells for a critical evaluation of the de BOVERI concept of carcinogenesis. Additional studies have been concerned with a comparison of the relationship between chromosome constitution on the one hand and human hereditary diseases and developmental defects on the other. A project investigating the biosynthesis of polio virus by euploid fibroblast cells of non-neoplastic origin has been completed as has also a collaborative study of certain abnormal human sera with mammalian chromosomes employing the Coon's fluorescent antibody technique.

Degenerative Joint Disease

Studies of the pathogenesis of degenerative joint disease and of the descriptive pathology of human rheumatism have been extended. Osteoarthritis was found to occur less frequently in female than in male STR/1N mice. Orchiectomy did not reduce the amount of osteoarthritis; thus, the arthritis enhancing effect of maleness was presumably due not so much to a testicular contribution as to an absence of an ovarian one.

Studies of the occurrence and nature of sclerosis of blood vessels in normal human joints are nearing completion in collaboration with Dr. Stanley

Elmore of the N.H.I. These lesions have not been described previously although they are very common. The significance of the findings, for the most part, lies in their recognition as non-rheumatic changes. In addition, however, they are associated with small areas of infarction in the pulvinar acetabuli of older individuals. It is suggested that this may be a cause of a presently unrecognized syndrome of hip pain in elderly individuals who apparently have no arthritis.

An interesting by-product of studies of joint disease in mice has been the recognition of genetically governed obstructive uropathy in male STR/1N mice. The lesions were prevented by castration and never occurred in females. They are a major cause of mortality in this group of animals and probably unrecognized in certain others as well.

Hematology

It has been shown that erythropoietine promotes the differentiation of stem cells into erythroid elements. The hypothesis was advanced based on studies on irradiated animals that the turnover rate of the stem cell compartment is governed by a negative feedback; differentiation of stem cells, then, serves as a stimulus for stem cell production. Intense erythroid stimulation whether due to severe anemia, hypoxia, or erythropoietine leads to the production of macrocytes, which have a shortened life span. The cause of the macrocytosis is as yet unproven but is most likely explained by a shortening of the emergence time together with skipping of divisions.

The growth potentiality, function and life span of the small peripheral blood lymphocyte have been the subject of controversy for many years. Using H^3 thymidine it was demonstrated that in culture the small lymphocyte of the peripheral blood cell undergoes transformation after which it begins to synthesize DNA and divide (MacKinney, Stohlman and Brecher). Using H^3 thymidine, it was conclusively demonstrated that the small lymphocyte has a life span of more than 100 days while the large lymphocytes have a life span of 60 days. (Brecher)

Hemoglobin

The genetic abnormality in Hb I, which produces a hemolytic anemia, is in the α chain, where there is a substitution of aspartyl for lysyl in the 16th amino acid residue. A unique optical property of sickle cell hemolysates was demonstrated in these hemolysates. There is a reversible increase in the positive Cotton effect of 3.5 fold suggesting that there is a dynamic conformational change of the hemoglobin molecule. (Dr. Murayama)

The constant for the mercapto-mercapto interaction in the α chain of fetal hemoglobin is similar to that of the α chain of adult hemoglobin but the constants of the γ chain differ from those of the β chain of adult hemoglobin (fetal hemoglobin $\alpha_2\gamma_2$, adult hemoglobin $\alpha_2\beta_2$). The energy barrier due to steric hindrance in fetal hemoglobin is 2 kilocalories/mole. This value is somewhat less than that of adult hemoglobin, indicating a "loosening" of the molecule, which results in a greater affinity for O_2 as well as for $Ag+$ ions. (Dr. Murayama)

Although the formation of Heinz bodies and the inhibition of heme enzymes by phenylhydrazine have been observed for many years, the mechanism of action of this compound has not been clear. It now apears that the active molecule is not phenylhydrazine itself, but is an unstable oxidation product, monophenyl, diimide. (Dr. Itano)

Histochemistry

The first conclusive evidence that carotid body-like tissue in the human contains norepinephrine and the first report of hypertension-producing carotid body-like tumor has been reported from this laboratory. Proof of the presence of norepinephrine in this chemoreceptor tumor was demonstrated by histochemical methods and verified by chromatographic and biochemical analysis. Norepinephrine was localized to unique and characteristic argentaffin cells bounding the non-argentaffin cell nests of normal carotid bodies and carotid body tumors. Evidence from this study suggests that the argentaffin cells in chemoreceptor tissue originate from the neural crest and participate in the sympatho-adrenal system, perhaps as local modulators of baroreceptor response or as norepinephrine stores responsive to local or generalized tissue anoxia. These findings also suggest that the hypertension of hypoxia may be at least partially of local reflex origin and imply a direct regulative effect on vasomotor control by chemoreceptor tissue. (Dr. Glenner)

A study of enzyme systems involved in the synthesis and breakdown of tissue proteins has revealed by histochemical methods the presence in the islets of Langerhans of the guinea pig of an enzyme hydrolyzing α-glutamyl peptides. An enzyme of this type has not previously been described either biochemically or histochemically. Also described for the first time histochemically is the precise tissue localization of a γ-glutamyl transpeptidase enzymic activity believed to be of significance in the synthesis of specific proteins, and a peptidase activity in peripheral and central nervous system myelin, active in the maintenance of protein structure in nervous tissue. By these histochemical methods defects of protein metabolism in specific cells can be detected and their relationship to pathologic processes determined. (Drs. Glenner, Hopsu, McMillan and Spatz)

Efforts have been directed toward development of methods for identification of specific mucosubstances. The histochemical classification of mammalian mucins thus developed includes types not previously distinguishable histologically as well as certain mucopolysaccharides not recognized biochemically. Histochemical methods have been fairly well established for differentiating sulfated from non-sulfated acid mucins. A technique for identifying most of the latter as sialomucins has been devised in collaboration with Dr. L. Warren. In combination with other procedures this technique distinguishes sialomucins which are digestible by *Vibrio cholerae* sialidase from non-digestible sialomucins. A third type of acid mucopolysaccharide has been recognized which lacks sialic acid and sulfate esters and contains an unknown acid group. Histochemical methods have also been worked out for demonstrating periodate unreactive sulfomucins (e.g. heparin and chrondroitin sulfates in mast cells and cartilage and sulfomucins in some glands and goblet cells) as well as procedures for localizing biochemically unrecognized, periodate reactive (hexose containing) sulfomucins in various glands and goblet cells. Application of these methods to characterization of mucins in human tumors has shown that benign and malignant lesions of the breast and tumors in patients with pseudomyxoma peritonei contain sialomucins, that mixed tumors of the major salivary glands secrete periodate unreactive sulfomucins and mucoepidermoid tumors of the oral minor salivary glands secrete a periodate reactive sulfomucin. (Dr. Spicer)

A histochemical method has been worked out which employs an acid dye in differentiating basic proteins according to their relative basicity. It is thought that in this method the dye specifically combines with ε amines or guanidino groups of proteins and that the staining of these two groups can be differentiated. In its application the procedure has visualized histone type protein with unique histochemical properties in chromatin and nucleoli. It also reveals the presence of a similar protein (?mucohistone) in sites containing acid mucopolysaccharides such as goblet cells and mucous glands. (Dr. Spicer)

Human Pathology

The opportunities offered to this laboratory through consultative and diagnostic studies of surgical and autopsy specimens from the Division of Indian Health and other facilities of the Public Health Service have continued to stimulate interest in aspects of geographic and environmental pathology. Following visits to a number of the Indian Hospitals, the quality of the material submitted for study has improved. There has also been a substantial increase particularly in the number of autopsy specimens submitted. Problems related to sarcoidosis, to diabetes, to dietary hemosiderosis, to cholecystitis, and to atherosclerosis and its complications are of particular interest. Consultative services to two Korean Charity Hospitals has resulted in the collection of 37 cases of fatal pneumocystis carinii infection in children.

In addition to the research projects summarized separately, certain scientists in other laboratories received advice from members of our staff, particularly in pathologic anatomy. Our histopathological preparation unit also took part in this cooperative effort by cutting and staining tissue sections from about 2,000 animals this year for seventeen investigators not in laboratories of NIAMD.

Hypersensitivity

Rat marrow cultures are used to study *in vitro* the mechanism of the interaction between rat marrow cells and mouse lymphocytes sensitized to rat marrow. The interaction is in the realm of delayed hypersensitivity and may aid in elucidating the mechanism involved in graft rejection. (Drs. Demopoulos and Gesner)

Another study dealing with the generalized Schwartzman reaction induced in rabbits by two injections of bacterial endotoxin is being conducted. Prominent features of this reaction include altered vasomotor reactivity, leukocyte damage, and intravascular coagulation. Since the basophilic leukocyte is believed to contain histamine, 5-hydroxytryptamine and heparin, substances which may effect vascular tone and blood coagulation, the possible role played by circulating basophilic leukocytes in the Schwartzman reaction is being examined. (Dr. Horn)

Immunochemistry

In collaboration with Dr. Marian Webster a study has been completed of the antigenicity of human kallikreins from urine and pancreas after inoculation into rabbits. Precipitin reactions were readily demonstrated with homologous antisera by the Ouchterlony technique and in test tubes. Rabbit antiserum to human kallikrein inhibits the vasodilator activity of human kallikrein injected into dogs, but fails to precipitate or inhibit either dog or hog pancreatic kallikrein.

A study of the localization of glyceraldehyde-3-phosphate dehydrogenase with fluorescent antibody to the enzyme has been completed. The enzyme has been demonstrated in high concentration in mitochondria surrounding the I band of the wing and leg muscle of the cockroach. Further studies with tissue cultures of human and chicken myoblasts have demonstrated the presence of this enzyme in specific mitochondria close to the nucleus while other mitochondria are completely negative. Studies on sections of mouse and rat kidney have shown the specificity of the enzyme for certain cells and specific areas of the tubules.

A study of the antigenicity of prolactin in rabbits included precipitins in gels and in test tubes. These studies have been coordinated with Drs. Condliffe and Bates with fractionations of prolactin.

Marrow Cultures

A new technique for culturing human and rat marrow cells is employed for the purpose of studying *in vitro* transmissibility of leukemias. The cells are grown at the bottom of a closed miniature well with a restricted oxygen supply. This slows the mitotic rate enough to permit the cells to differentiate. Survival of cultures is thus greatly prolonged offering a tool for the study of leukemogenesis *in vitro*.

Melanomas

It was previously found that phenyllactic acid inhibited the growth of pigmented melanomas but not that of non-pigmented melanomas. Studies are continuing to determine further the effect of phenyllactic acid and of other tyrosinase inhibitors on the growth of pigmented and non-pigmented melanomas in mice.

Nutritional Deficiencies

Pathologic studies on conventional and germ-free animals given various deficient diets are continued. A recently completed study demonstrated that supplementation with selenium compounds markedly delays the onset and decreases the incidence of hepatic necrosis in conventional rats fed a diet based on 4% casein as the protein source. Studies are currently in progress to evaluate the ability of selenium compounds to prevent this lesion in germ-free animals.

Serum Enzyme Changes After Stress

Previous studies showed that large doses of catechol amines or a 4-hour exposure of dogs to hypoxia (32,000 feet altitude) caused, in addition to hyperglycemia, a transient sharp rise in serum glutamic oxalacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), serum lactic dehydrogenase (SLD), serum alkaline phosphatase (SAkP), and serum aldolase (SAld). Studies with adrenergic and ganglionic blocking agents suggested that the hyperglycemia produced

by hypoxia was due to the released of catechol amines from the sympathetic adrenal system and that the rise in serum enzymes after catechol amines or hypoxia was due to an increase in cellular permeability. To shed further light on the mechanisms causing rise in serum enzymes and the relation between stress and the release of catechol amines, serum enzymes were studied in animals subjected to other types of stress.

In one study, dogs were exposed to a simulated altitude of 32,000 feet for 4 hours 5 days weekly for 7 weeks. They showed a continuing gradual increase in SGOT and SLD values during the 7 weeks, while SGPT stabilized by the 2nd week at slightly above initial control values. SAkP and SAld values stabilized after the third week at 2–3 times the initial values. After cessation of exposures, values dropped sharply during the first 2 weeks and approached normal in 6 weeks. A subsequent single 4-hour exposure to 32,000 feet evoked the usual transient sharp rise in serum enzyme values, but not the typical hyperglycemic response, observed before acclimatization. These studies indicate that the alterations in cellular permeability are reversible. Residual pathologic lesions, attributable to the repeated exposures, may account in part for the slow restoration of normal enzyme values.

After exercising rats for 16 hours a transient 2-fold increase in BUN and SLD, a 4-fold increase in SGOT and SGPT, and a 6-fold increase in SAld was found. SAkP declined 30%. Nearly all the rats showed moderate to severe fatty changes in the liver, kidney and thigh muscles and slight fatty changes in the heart. About one-fourth showed a few foci of inflammation and necrosis in the muscle.

The effect of cold was studied in dry and wet rats exposed 16 hours to 1.7 and −5° C and 5 hours on two successive days to 1.7° C respectively. There was a transient significant rise in SGOT, SAld and BUN, most pronounced and persistent in the wet rats, which showed a more profound hypothermia. Concurrently, there was a transient decrease in mean hematocrit, a depletion of liver and muscle glycogen and adrenal lipid, and a marked leukocytosis with a relative decrease in lymphocytes. Fatty changes were noted in the liver and, less frequently, in the kidney and heart.

It is postulated that hypoxia, prolonged exercise and exposure to cold increase serum enzyme levels by increasing cellular permeability, permitting cellular enzymes to escape and accumulate in the blood. These findings are of practical importance since they indicate that serum enzyme elevations need not be due to myocardial infarction or other serious organic disease, and that exposure to various physical stresses must be considered in the differential diagnosis of such diseases.

Pneumocystis Carinii Infection

A second focus of frequent pneumocystis carinii infection in small children in Korea was established during this year. From this new location, massive infection with pneumocystis carinii was found in 18 out of 25 fatal cases sent to the laboratory for diagnosis.

Our attempts to propagate the organism in cortisonized germ-free rats and mice after intrapulmonary injection of material obtained from heavily infected conventional rats have failed. Concentrates of organisms are now being prepared by washing and careful digestion of lung tissue from donor rats and by exposing such cyst suspensions to feeder cultures.

The only important information which our experiments in germ-free rats and mice appears to have produced is that such rats and mice are indeed free of pneumocysts in spite of prolonged injections of cortisone and antibiotics which would have produced severe pulmonary involvement by this organism in conventional rats within a corresponding period of time.

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Carbohydrate Metabolism

Polymers

The polymer, alginic acid, contains a repeating unit consisting of D-mannuronic and L-guluronic acid. Continuing studies on the pattern of its enzymic degradation have led to the elucidation of a new pathway for the bacterial utilization of uronic acids. The initial monomeric reaction product, 5-keto-4-deoxy-D-mannuronic acid, was

found to react with a DPNH-linked dehydrogenase to form 2-keto-3-deoxy-D-gluconic acid. This, in turn, was phosphorylated in the presence of ATP and subsequently cleaved to yield pyruvic acid and triose phosphate. The enzymes, alginase and ketodeoxygluconic acid dehydrogenase, have been purified and their properties examined.

Work carried out in Switzerland in the laboratory of V. Prelog relates to structure determination of Avilamycin, an antibiotic elaborated by *Streptomyces viridans*. It consists of an aglycone, the partial structure of which has been determined, and 8–10 monosaccharide units. Two of the sugar components were characterized as 6-deoxyhexoses. The third sugar varies from one batch of Avilamycin to another. Some preparations contain L-lyxose, a sugar not heretofore reported to occur naturally. Others contain an O-methylpentose.

Small Molecules and Coenzymes

Further studies on the biosynthesis of L-fucose by *Aerobacter aerogenes* have substantiated the finding that GDP-4-keto-6-deoxy-D-mannose is an intermediate in the formation of this sugar. The GDP-heptose reported last year has now been isolated and completely characterized as GDP-D-glycero-D-mannoheptose. In addition, the enzymic synthesis of this compound has been demonstrated to be due to a pyrophosphorylase present in yeast which catalyzes the reaction of GTP and the heptose-1-phosphate to form GDP-heptose and inorganic pyrophosphate. A tentative identification of thymidine diphosphate N-acetyl-D-fucosamine from *C. violaceum* has led to an attempt to demonstrate enzymic synthesis of this nucleotide. Also, an enzyme system from *S. paratyphi A* has been found to synthesize a new sugar nucleotide, cytidine diphosphate glucose. This may be a precursor of the dideoxyhexose, paratose, which is one of several unusual sugars present in the surface antigenic lipopolysaccharides of certain microorganisms.

An investigation of the nucleotide bound sugars in hen oviduct was undertaken in an attempt to demonstrate the existence of an iduronic containing nucleotide. During the course of this work, a new class of nucleotide-linked disaccharide was discovered and identified as UDP–N–acetyl–glucosamine–6–phosphate–1–galactose. This material was isolated in pure form and subjected to a sequential enzymic degradation accompanied by recovery of the reaction product at each step. From the same source, a family of AMP–sugars has been found to exist as a labile complex. This material is being currently studied.

An alternate pathway of L–ascorbic acid catabolism in molds has been demonstrated whereby a new 3–keto hexonic acid (3–keto–L–idonic acid) is formed and rapidly decarboxylated to yield L–xylulose. This mechanism differs markedly from that found in mammals which has been shown previously to result in the formation of two pentonic acids, L–xylonic and L–lyxonic.

Biosynthesis

Thiamine

The formation of thiamine phosphate is catalyzed by an enzyme from baker's yeast discovered in this laboratory, thiamine phosphate pyrophosphorylase. This enzyme catalyzes the condensation of the two ring moieties according to the following equation:

$$\text{"pyrimidine"}-\text{PP}+\text{"thiazole"}-\text{P} \xrightleftharpoons{Mg^{++}} \text{thiamine}-\text{P}+\text{PP}$$

The reaction is readily reversible, having an apparent equilibrium constant of 6 at the pH optimum, 9. 2. Procedures have been devised for the enzymic determination of each substrate. The crystalline cyclohexylamine salt of the pyrimidyl pyrophosphate has been prepared and the acid lability of the allylic pyrophosphate ester linkage has been studied.

Lipid Synthesis

The determination of the absolute rates of lipid synthesis in liver by means of proton incorporation has been undertaken. It has been shown that when palmitic acid is synthesized from acetate 12.2 protons should be incorporated per molecule synthesized. On the other hand, the synthesis of palmitate from acetyl–CoA derived from glucose requires the participation of 15.9 protons per molecule. Fatty acid synthesis from acetyl–CoA derived from fatty acid catabolism utilizes 16 protons per molecule synthesized. Thus in the absence of exogenous acetate 16 protons per molecule

of fatty acid synthesized should represent the number used in determining fatty acid synthesis in complex systems. Consistent with the fact that glucose and/or fatty acids contribute equally with acetate in the formation of acetyl–CoA, 14 protons per molecule fatty acid synthesized are taken up when liver slices are incubated with 5×10^{-3} M acetate. It has been calculated that 0.4 μmole of fatty acid is synthesized per gram liver per hour.

Regulatory Mechanisms and Hormones

Insulin

Studies relating to the enzymic degradation of insulin by mamalian liver have been continued using perfused liver and broken cell preparations. Neither ACTH nor glucagon are effective competitors of insulin-I^{131} degradation by perfused rat liver. This is to be contrasted to the substantial competition which these protein hormones exhibit in liver homogenates. Thus, while the same enzyme system present within the liver cell may carry out the ultimate degradation of a number of protein hormones, access to this system in intact cells may be limited by binding to specific loci on the cell membrane. This conclusion is consistent with previous evidence for the binding of insulin-I^{131} on or in the cell membrane as an obligatory first step in the catabolism of this hormone by liver.

A highly purified liver enzyme preparation, homogeneous according to electrophoretic and sedimentation studies, was found to catalyze the reductive cleavage of the disulfide bonds of insulin in the presence of glutathione as the hydrogen donor. Studies on the physiological role of this enzyme could implicate it in the over-all "insulinase" system for the hepatic breakdown of insulin. Of significance also would be its possible relation to the mechanism of action of insulin, prior reduction of insulin being postulated by some to precede physiological action.

Humoral Agents in Uremia

Studies on the chemical nature of the humoral agents which accumulate during uremia and the chemical basis for the changes in cerebral function in uremia have been extended. The assay for an agent in human uremic serum which alters the metabolism of nervous tissue is based on the fact that normal rat brain cells when suspended in uremic serum generate greater yields of $C^{14}O_2$ from glucose C^{14} than do the same cells suspended in normal serum. The agent is unaffected by trypsin, trichloracetic acid or heat, is partly dialyzable and is removed by cation and anion exchange resins.

Pyrophosphatase

Many critical, reversible, biosynthetic reactions also result in formation of inorganic pyrophosphate and for this reason it becomes of interest to study the properties and possible control mechanisms for enzymes that split inorganic pyrophosphate. There are at least two such enzymes in mammalian liver. One is present in the soluble fraction of the tissue. It requires Mg^{++}, has a pH optimum of about 7.8 and resembles yeast pyrophosphates. The other occurs in microsomes, has a low pH optimum and does not require Mg^{++}. This particulate enzyme has transphosphorylation as well as hydrolytic activity. Glucose, mannose, fructose, and galactose have been found to act as phosphate acceptors, while ribose, creatine, and maltose and inactive.

Galactosemia

Eight subjects who had typical symptoms of congenital galactosemia in infancy have been studied. The bed blood cells of each are essentially devoid of P-gal uridyl transferase enzyme and are incapable of oxidizing galactose-1-C^{14} to $C^{14}O_2$. However, two of these subjects, one prepubertal and one post-pubertal, are, at present, near normal in their capacity to oxidize galactose *in vivo*. Thus the usual erythrocyte diagnostic test for this disease does not necessarily reflect the state of the whole organism. Furthermore, it appears that in some galactosemic patients a tissue (or tissues), other than red cells, may acquire a pathway for galactose metabolism in the course of maturation. The biochemistry and genetics of this remain to be elucidated.

Steroid Hormones

More and more evidence is accumulating on the remarkable effects of steroid hormones on enzyme structure and enzymic catalysis. With respect to structure, the method of intensification of fluorescence of bound pyridine nucleotides was employed

to determine the state of aggregation of the glutamic dehydrogenase molecule under various conditions. Evidence was found for the fact that hormones do unfold the molecules, exposing additional pyridine nucleotide bind sites, and from this evidence, the approximate molecular weight of the DPNH binding units was determined. The method of light scattering was also used and confirmed the fact that there is a rapid equilibrium between the sub-units and the aggregate, and that the position of the equilibrium is influenced by the steroid hormones.

The bacterial induced enzyme β-galactosidase was used to investigate the effects of steroids on the synthesis of this enzyme, and it was found that there was a stimulatory effect by the hormones on this synthesis. This effect was most readily observed when normal enzyme synthesis was inhibited by puromycin.

A possible regulatory function for steroids was discovered in their curious effects on *aldehyde dehydrogenase*. A partially purified aldehyde dehydrogenase for rabbit liver has been found to be inhibited by one group of steroids and either stimulated or inhibited by another group, depending on the concentration of aldehyde. Since further efforts to purify the enzyme were unsuccessful, the less direct approach was utilized of examining the effect of reagents which are known to alter the configuration of proteins. A low concentration of urea, for example, was found to produce an effect similar to diethylstilbestrol (stimulation) with high aldehyde concentration—inhibition with low aldehyde concentration, and at a higher concentration of urea, one similar to progesterone (inhibition). It thus appears that steroids may act on the enzyme system by altering the secondary or tertiary structure of the enzyme molecules, although lack of effect of steroids on the sedimentation velocity suggest that this influence is not accomplished through dissociation of the enzyme into subunits.

Another enzyme system subject to control by steroids involves the oxidation of *retinene*. Retinene is oxidized irreversibly to vitamin A acid by a pyridine nucleotide-linked aldehyde dehydrogenase present in liver, kidney, and small intestine. Diethylstilbestrol, dehydroisoandrosterone, estrone, and cortisone stimulate the reaction rate at optimal substrate levels; diethylstilbestrol also inhibits when substrate is below 3×10^{-6} M. Progesterone, desoxycorticosterone, testosterone, and androsterone inhibit at all substrate levels. The Km for retinene is 1.4×10^{-6} M. The energy of activation for retinene is 14.6 kilocalories per mole, and is descreased to 11.9 by stilbestrol and increased to 20 by progesterone. The stimulation produced by stilbestrol is reversed by dialyzing away the hormone.

Nucleic Acids

Striking progress was made in the purification of polynucleotide phosphorylase from *Micrococcus lysodeikticus* and now, for the first time, the enzyme is available in high purity, and with a rigid primer requirement. There is a nearly absolute requirement for oligonucleotides of the type pApApA both for making polymer and for catalyzing the exchange reaction between inorganic phosphate and nucleoside diphosphate. This work will make possible a really definitive study of polynucleotide phosphorylase and also has practical value in the synthesis of polyribonucleotide chains all of which are built on small primer units as a nucleus. Such materials has already been used in studies on the genetic code (see below).

Alkaline phosphomonoesterase from *E. coli* and a number of other non-specific phosphatases were purified, and each of them contained a low but definite ribonuclease activity. By many criteria this nuclease activity was shown to be part of the same protein enzyme. This led to the generalization that non-specific phosphatases have an intrinsic component of ribonuclease activity, that is differentiated from true nucleases by being inhibited by low concentrations of inorganic phosphate. Such a significant generalization is satisfying, but has unfortunate consequences for the problem of nucleotide sequence in RNA. All schemes for nucleotide sequence depend on sequential removal of terminal phosphate from RNA, and non-specific phosphatases have been widely used for this purpose, due to the mistaken impression that they were safe reagents.

Polyguanylic acid of large size than formerly available has the interesting property of being just about completely resistant to every enzyme tried on it. Presumably this reflects a high degree of interaction of guanylic acid units along the

polynucleotide chain, thus protecting the polymer from enzymatic attack. Incorporation of the base analogues, azaguanine, into RNA of *Bacillus cereus* has been studied and doubt is cast on earlier reports concerning the way in which this base is arranged in the nucleic acid molecule. Polyazaguanylic acid has been made with polynucleotide phosphorylase and its properties studied.

We realize today how important is the secondary structure of nucleic acids. Specific hydrogen bonding interrelationships provide the mechanism for hereditary transmission, and this accounts for the interest in physical studies, such as the following: The determination of tautomeric forms (i.e., the positions of the hydrogen atoms responsible for hydrogen bonding) in polynucleotide helices has permitted this structural feature to be used in order to rule out a recently proposed, crystallographically feasible alternative to the Watson-Crick model for DNA. Where products of the interaction between different nucleic acids differ only in the positions of the exchangeable hydrogens on the bases, knowledge of these positions of attachment can be used as a criterion of macromolecular structure.

Cell-Free Protein Synthesis

A cell-free system for protein synthesis was obtained from *E. coli* and stabilized so that the enzyme extracts could be stored frozen without undue loss of activity. This system has been used to provide a cell-free assay for messenger RNA and to show that polyuridylic acid can serve as a specific carrier of information for the incorporation of phenylalanine and polycytidylic acid for proline. Various copolymers have since been found active for the incorporation of other amino acids into polypeptide structure. It is clear that the genetic code is yielding to an experimental approach. It was also found that ribosomes from one species can make protein from another species if the proper conformational RNA is added, and that "phenylalanine soluble-RNA" is the intermediate through which phenylalanine is incorporated into protein.

Lysogeny

Biochemical studies of the metabolic events occurring during bacteriophage infection have been fruitful in many laboratories. Because of its great genetic interest, infection of *E. coli* K_{12} cells by lethal mutants of the phage λ was investigated. The DNA polymerase present after infection shows minor differences when compared to the DNA polymerase of uninfected cells. However, the deoxyribonuclease activity of *E. coli* K_{12} is increased about 10-fold by bacteriophage infection. This increase in nuclease activity is under examination as a possible control mechanism in the induction of lysogenic organisms. Additionally, it has been found that the glucose repression of λ production in K_{12} is due to the faulty adsorption of the bacteriophage by cells which are grown in glucose. Presumably a receptor site for λ has been affected.

Histidine Biosynthesis

Continuing investigation of the biochemistry of histidine formation and the control mechanisms regulating this pathway (feedback inhibition) has resulted in the isolation of the enzyme catalyzing the first step in histidine biosynthesis. ATP and 5-phosphoribosylpyrophosphate are condensed by this enzyme to form N-1-(5-phosphoribosyl)-ATP. The enzyme has been purified approximately 1,700 times over wild-type levels and exhibits a K_i for L-histidine of 5×10^{-5} M at pH 7.5. The inhibition is strongly pH dependent with a pK at 9.0 suggesting that inhibition of the enzyme by histidine is dependent upon the presence of a charged α-amino nitrogen.

Model Compounds

The enzyme, thiooxidase, has been isolated from the fungus, *Piricularia oryzae*, and purified to the stage where it sediments as a homogeneous protein in the ultracentrifuge. The preparations catalyze two reactions: 1. The oxidation of compounds bearing −SH on ethylenic or aromatic carbons to their corresponding disulfides. 2. The oxidation of catechols. Based on constant ratios of the two activities throughout fractionation, elution pat-

terns from resins and sedimentation patterns upon ultracentrifugation, it is concluded that thiooxidase and catalase are functions of one enzyme.

LABORATORY OF CHEMISTRY

Carbohydrates

The chemistry of 2-deoxy-D-ribose has been explored in a variety of directions thanks to the ready availability of this sugar through a simple preparation developed earlier by H. W. Diehl. A key intermediate used earlier by R. K. Ness for the synthesis of deoxynucleosides has now been obtained in crystalline form by R. K. Ness. The substance, 2-deoxy-3, 5-di-*O*-*p*-nitrobenzoyl-D-ribosyl chloride is a representative of a comparatively little known class of sugar derivatives and, because of its utility in nucleoside synthesis, has been examined in some detail. Reactions involving replacement of the chlorine atom are, as expected, non-stereospecific. Means have been found, however, to influence the proportions of stereoisomeric products in a useful manner and this finding was of importance in the synthesis of the two anomeric 2-deoxy-D-ribofuranose phosphates. The chloride was found to decompose readily to furfuryl *p*-nitrobenzoate, a reaction reminiscent of the formation of kinetin from deoxyribonucleic acid.

The 3-deoxyaldohexoses having the *arabino* and *ribo* configurations are of current interest in certain biochemical studies but the recorded synthetic methods for making these compounds are cumbersome and difficult. A method was devised by H. B. Wood, Jr., for making both of them in a simple two-step synthesis from 2-deoxy-D-ribose.

A very labile sugar phosphate of intermediary metabolism is 2-deoxy-D-ribose 1-phosphate, one anomeric form of which has been made by enzymatic methods and is the intermediate in the biosynthesis and breakdown of deoxyribonucleosides. This exceedingly sensitive substance has now been synthesized by D. L. MacDonald; by appropriate modification in the synthetic pathway, the other anomer (not, as yet, reported in biochemical systems) was also made.

An unusually simple method for the chemical preparation of aldose 1-phosphates has been devised by D. L. MacDonald, the first step being simply fusion of a fully acetylated aldose with anhydrous phosphoric acid. α-D-Glucopyranose and α-D-galactopyranose 1-phosphates were synthesized in the course of this research.

In continuation of studies on the chemistry of ribofuranose derivatives R. K. Ness has investigated the behavior of a number of 1-*O*-acyl-2-*O*-*p*-nitro-benzenesulfonyl-3, 5-di-*O*-benzoyl-β-D-riboses with various acyloxy ions. In each case three steps in a concerted reaction took place: (1) the acyloxy ion made an α-attack at carbon one, (2) the β-acyloxy group at carbon one migrated to carbon two and (3) the *p*-nitrobenzenesulfonyloxy group was ejected from carbon two with inversion of the configuration of that carbon.

In the course of the above work 3,5-di-*O*-benzoyl-2-*O*—*p*-nitrobenzene-sulfonyl-β-D-ribosyl bromide was prepared. In subsequent research this substance was found to undergo an elimination when treated with sodium iodide in acetone solution. The product, 3,5-di-*O*-benzoyl-1,2-dideoxy-D-*erythro*-pento-furanos-1-ene, is the first known sugar glycal having a furanose ring. Its structure was confirmed both by conventional chemical means and through its nuclear magnetic resonance spectrum. The exceptional reactivity of the substance suggests its possible utility in the synthesis of derivatives of biochemical importance.

The Committee on Biological Chemistry of the Division of Chemistry and Chemical Technology of the NAS–NRC has been engaged for the last few years in efforts to formulate specifications for organic substances of biochemical importance. As members of the Sub-Committee on Carbohydrates, H. G. Fletcher, Jr. and H. B. Wood, Jr. have carried out an exhaustive study of a wide variety of carbohydrates and evolved criteria and standards of purity which have now been published by the NAS–NRC in the hope that they will prove useful both to the producers and users of biochemicals.

Condensation of nitromethane with the periodate oxidation product from methyl α-D-glucopyranoside yields 3-nitronates principally with the D-*manno* and D-*gluco* configurations. Spontaneous epimerization of the 3-nitronates in aqueous solution yields principally glycosides with the D-*talo* and D-*galacto* configurations. From the former, crystalline 3-amino-3-deoxy-α-D-talose

hydrochloride has been obtained to complete the series of the eight possible 3-*amino*-3-deoxy-D-hexoses. (H. H. Baer)

Further studies of the sugars in the avocado and *Sedum* species have proved the first known naturally occurring nonulose to be D-*erythro*-l-gluconulose. Several octuloses were synthesized, and D-*glycero*-D-*gulo*-octulose became the first crystalline representative of this series. (N. K. Richtmyer and H. H. Sephton)

Medicinal Chemistry

The immunochemical program of the section (T. D. Perrine) has been continued along three main lines: synthetic antigens, Vi antigen and TB cell-walls. Copolymerization of styrene and *p*-acetoxystyrene has been successful. The resultant copolymer was coupled with a diazotized napthylamine disulfonic acid to yield a colored polymer which showed some antigenic activity when tested in rabbits. Eleven other test antigens based on polyvinyl alcohol have been prepared and an interesting versatile, intermediate, *p*-hydroxyphenylpyrrolidone has been synthesized in a 3-step sequence for possible selective N-vinylation. Gas chromatographic assay for acyl groups in the Vi antigen studies has been worked out. The American Instrument Company, in collaboration with Mr. Perrine, has produced a prototype of the cell-wall press developed by Mr. Perrine.

The pharmacology unit (N. B. Eddy, consultant and advisor) continues to act as a clearing agency for all narcotic-analgesic type substances prepared in the U.S., and abroad in their complete evaluation for analgesic effect, toxicity and addiction liability. In fulfilling this function some 80 new compounds have been assayed by this unit (Mrs. Louise Atwell assisted by Mrs. Josephine Goodwin) for both oral and parenteral effect in the mouse, while for at least 20, acute toxicities have been determined. On the basis of the results many of these compounds have been recommended for addiction evaluation in the monkey (Dept. of Pharmacology, University of Michigan). More promising substances have been further studied at the Addiction Research Center, Lexington, Kentucky. Before granting a license for marketing, the advice of these groups is sought by the Food and Drug Administration. In addition, the Pharmacology unit has this year completed their portion of a two-year cooperative standardization study of the hot-plate method for assaying quantitatively narcotic analgesics. All data obtained in these studies and in our own program, including acute toxicity experiments, have been subjected to careful probit analysis. (Mrs. Wendy Ness)

Following completion of a 3–5 year study, data have been assembled which corroborate earlier preliminary findings. Doses of 20 mg./kg. (10 times the analgesic dose) of morphine repeated at intervals of 2.5–3 months over a period of 15 months induces a degree of tolerance to analgesic effect in the rat almost as great as that induced by 70-day addiction periods in which animals were given as much as 100 mg./kg. twice daily. One year after withdrawal some tolerance to analgesic effect still persists. On the other hand, recovery as judged by another parameter, swimming capacity, is complete at the end of six months. (J. Cochin)

A reproducible, sensitive, rapid method of detection and identification of narcotic analgesics and metabolites in the urine of patients has been developed (J. Cochin in collaboration with J. Daly, Metabolites). The method is based on thin layer chromatography and may prove applicable to barbiturates, phenothiazines, etc.

Reduction of diethyl 2, 5-diketohexahydroterephthalate (I) (J. Murphy) with lithium aluminum hydride has led to a variety of products from which 1, 4-cyclohexadiene-1, 4-dimethanol could be isolated in 26% yield. Catalytic reduction of I in acetic acid produced α-1, 4-dicarbethoxy-2, 5-dihydroxycyclohexane (all *cis* configuration based on infrared data and mode of formation) in 62% yield. These compounds are of interest because of their resemblance to intermediates in carbohydrate metabolism and they offer possibilities of preparing analogs with carbocyclic rather than heterocyclic structures.

Alkaline hydrolysis followed by enzymic dephosphorylation of the teichoic acid present in the cell-awls from Lactobacillus arabinosus, gave 4-0-(α-D-glucopyranosyl)-D-ribitol (II) as a major product. The synthesis of this α-anomer has been achieved (L. J. Sargent) in eight steps from D-ribonolactone. Systematic protection by, and removal of, various functional groups was necessary. Furthermore, inasmuch as a mixture of the anomeric glucosyl ribitols was obtained it was ex-

pedient to incubate this mixture with β-glucosidase and remove monosaccharides. The resultant crude material was purified by preparative paper chromatography to give crystalline II which proved to be identical with the cell-wall degradation product.

The acid cyclization of 3,4-dialkyl 2-(*p*-methoxybenzyl)-1-methyl-1-,2,5,6-tetrahydropyridines leads invariably to *diastereoisomeric* (at carbon 9) 5,9-dialkyl-2'-hydroxy-2-methyl-6,7-benzomorphans in yields of 70–80% for the α-isomer and 2-8% for the β-isomer the yield and proportion depending on the temperature and the cyclizing agent. Methiodide rate-formation studies (based on non-aqueous titration of unreacted base) (S. E. Fullerton, E. L. May) complemented by NMR data in the dimethyl series (E. D. Becker, Lab. of Physical Biology) prove conclusively the valadity of our initial assumption that in the predominant (α) isomers, the 5,9-dialkyl radicals are in *cis*-relationship to each other, the 9-alkyl being oriented away from nitrogen (axial for the hydroaromatic ring) in this azabicyclo system. In chloroform at 25° the α-isomers are converted to their methiodides 10–20 times faster than the β-compounds where the orientation of the 9-alkyl substituent is close enough to the nitrogen to provide steric hindrance. This correlates the stereochemistry of the α-isomers with that of morphine and the morphinans at the three common asymmetric centers, and the analgesically more potent β-isomers with the rarer but more powerful isomorphinans. New alkyl-2'-hydroxy-6,7-benzomorphans (several of which were degraded to alkyl-2-dimethylamino-7-methoxy-1,2,3,4-tetrahydronaphthalenes (J. H. Ager, S. E. Fullerton, S. Saito)) found to have diuretic properties synthesized from alkylpyridines *via* the Stevens modification (E. M. Fry) or the Grewe synthesis and evaluated as analgesics or as tranquilizers are: 5-ethyl-2-methyl-;5-ethyl-2-phenethyl- (S. Saito); 5-propyl-2-methyl-; α- and β-5,9-dipropyl-2-methyl (J. H. Ager); α- and β-5-ethyl-2,9-dimethyl; α- and β-9-ethyl-2,5-dimethyl (S. E. Fullerton). Several of the benzomorphans were also degraded to previously unknown naphthalene derivatives, to serve as reference compounds in structure elucidations. Also synthesized in eight steps (H. Kugita, S. Saito) from a simple benzene derivative was 5-carbethoxy-2-methyl-6,7-benzomorphan, in essence a hydrid of the benzomorphan and meperidine classes of analgesics. Cyclization experiments (S. E. Fullerton, J. H. Ager, E. L. May) designed to improve the yields of the more interesting β-5,9-diakyl compounds (some of which are 25 times more potent than morphine and will not support morphine addiction in the monkey) have been moderately successful. An ingenious alternative 7-step synthesis for β-2'-hydroxy-2,5,9-trimethyl-6,7-benzomorphan, not applicable to higher homologs, has been developed (S. Saito), starting material 7-methoxy-β-tetralone. Basic research in the pyridine series (E. M. Fry) is being conducted with a view of preparing intermediates which might undergo ready cyclization to produce practicable amounts of the above-mentioned β-isomers. The decomposition of the hydrochloride salt of 2-benzyl-6-cyano-1,3,4-trimethyl-1,2,5,6-tetrahydropyridine gives as a major product, a basic compound (λ 303 mu) which, based on analogy with simpler systems, could be the desired 2-benzyl-1,3,4-trimethyl-1,2,3,6-tetrahydropyridine with the 3-methyl and 2-benzyl groups in *trans*-juxtaposition.

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Metabolites

A review, as well as preview, of the rapidly expanding program on "Non-Enzymatic Methods for the Preferential and Selective Cleavages and Modifications of Proteins" has been completed for Volume 16 of Advances in Protein Chemistry. A useful dichotomy of the methods involved has been introduced and defined by the terms "preferential" and "selective," the former involving competitive and hydrolytic conditions, the latter noncompetitive, non-hydrolytic and oxidative cleavage. Selective cleavage so far has been realized with peptide bonds following tryptophan, tyrosine, methionine, and to some extent histidine, as well as γ-aminobutyryl, γ-glutamyl and δ-amino-adipyl peptides. (B. Witkop)

The selective cleavage of γ-aminobutyrylglycine with nitrous acid, which has been elaborated by J. E. Francis, has now found a practical industrial application in the conversion of the novel and important cephalosporin C to 7-aminocephalosporanic acid.

The nonenzymatic selective cleavage of bovine pancreatic ribonuclease in the hands of E. Gross

has led to a major revision of the primary sequence of this enzyme. In accordance with these findings the group at the Rockefeller Institute, as well as Anfinsen's group in the Heart Institute, formulate the crucial, and probably labile, sequence 11–18 as follows: Gn (11)—His (12)—Met (13)—Asp (14)—Ser (15)—Ser (16)—Thr (17)—Ser (18). A major methodological improvement has been the fractionation of cyanogen bromide-treated ribonuclease on a column of Sephadex G–50. This procedure permits the clean separation of the "chemical tail peptide" (ribonuclease positions 1–13) and of homoserine (lactone) from the cleavage of Met (29)—Met (30) from the core of ribonuclease.

The new fractionation on Sephadex has made it possible to prepare the so-called *S-peptide* obtainable from ribonuclease by the action of subtilisin in a dependable manner in high yield. The preparation of this S-peptide has been a major problem in the laboratories of Richards at Yale and Anfinsen in the Heart Institute. The availability of S-peptide has made it possible for E. Gross to study the nonenzymatic cleavage with cyanogen bromide at the peptide bond following Met (13). The cleavage products, a tridecapeptide and a heptapeptide, have been purified, characterized and analyzed. The new preparation method for S-peptide foregoes the use of trichloroacetic acid. It will now be possible to study the suspected lability of the S-peptide under acid conditions in detail and to explore the possibility of rearrangements or even sequence tautomerism.

C. M. Foltz has studied the influence of alkyl groups on the cleavage of sulfonium salts derived from ethyl N-acetylmethionylglycinate. The best percentage yields in peptide cleavage were obtained with iodoacetamide as alkylating agent. The course of the alkylation at 35–40° was followed by argentometric titration. A comparison of yields in the cleavage of iodates, acetates and nitrates of methioninesulfonium peptides showed that the cleavage yield was independent of the nature of the anion.

By means of N-bromosuccinimide oxidation, S-carboxymethyl ribonuclease has been cleaved selectively next to the 6 tyrosine C-peptide bonds to release 6 new NH_2-terminal amino acids. The results obtained by L. A. Cohen and J. G. Wilson are in full accord with the sequence proposed by investigators at NHI and at the Rockefeller Institute.

The program on enzyme fragmentation has been started by C. M. Foltz with pepsin, which has been cleaved by nonenzymatic methods under conditions which did not lead to denaturation but to fractions retaining 60–80% of the original activity corrected by control runs. The major problem, which has been surmounted, is the preparation of pure samples of pepsin without concomitant autolysis. The aim is the preparation of an enzyme fragment which is still fully active without necessarily being fully specific, and which is of a size small enough for study and synthesis of the active center. (A. Arens)

The antibiotic gramicidin A, homogeneous by the criteria of 1,000-transfer countercurrent distribution, has been resolved by S. Ishii into several components of the use of analytical and preparative thin layer chromatography. The presence of isoleucine in gramicidin A has been observed for the first time. The successful structural elucidation of gramicidin A is awaiting the preparation of sufficient amounts of these new congeners.

The intramolecular participation of amide groups in peptides of unsaturated amino acids has been studied in some detail by N. Izumiya and A. V. Robertson. Amides and peptides of N-acylated DL-allylglycine or DL-methallylglycine reacted in aqueous buffer systems or water with N-bromosuccinimide with liberation of ammonia or the peptide component. The liberation of ammonia, glycine, or glycinamide was a linear function of the added reagent and after the addition of 1–2 moles of N-bromosuccinimide 60–80% of the amine component was liberated, as measured by ninhydrin assay. On a preparative scale the primary amides gave products different from the lactones obtained from the allylglycine peptides. Their insolubility in water, solubility in nonpolar solvents, analytical data, and content of one covalent and one active positive bromine led to their formulation as N,C-dibromoiminolactones. Unsaturated amino acids which contain the double bond in a 6- or 5-membered ring participated only in the release of ammonia, but not of, *e.g.*, glycine, from the respective precursors.

In collaboration with N. Izumiya, a stereospecific approach to the synthesis of *allo*-hydroxy-L- and -DL-proline and *threo*-hydroxy-L- and -DL-ornithine has been worked out based on the reaction of N-carbobenzyloxyallyl-L- and -DL-glycine with N-bromosuccinimide.

Professor Izumiya is continuing this approach at the Laboratory of Biochemistry at Kyushu University in order to make accessible the racemic and optically active forms of hydroxylysine and *allo*-hydroxylysine. The new synthetic route will be utilized for the preparation of L-hydroxyarginine and L-*allo*-hydroxyarginine and relative rates of enzymatic deguanidination by arginase, and furthermore for the synthesis of homoarginine, arginine and lysine peptides containing hydroxy groups for the study of their substrate specificity with trypsin and papain.

Detailed nuclear magnetic resonance studies by A. V. Robertson in collaboration with E. Becker have confirmed the structure of the oxidation product of N-carbobenzyloxydehydroproline amide to be the unusual dicarbinolamide, formally the condensation product of benzylcarbamate with maleic dialdehyde. In a cooperative study Professor G. Fodor of the Hungarian Academy of Science in Budapest is testing this new dicarbinolamide as a partner in the Mannich reaction which should lead into the difficulty accessible tropinone series.

J. E. Francis and A. V. Robertson succeeded in preparing the two isomeric betaines derived from dehydroproline differing only in the position of the double bond. The unstable 3,4-dehydro-DL-stachydrine rearranged easily to the isomeric optically inactive betaine which was found to be identical with the water elimination product from the two diastereoisomeric 3-hydroxy-stachydrines from *Courbonia virgata*.

3-Hydroxyproline, a novel hydroxyamino acid occurring as a regular constituent in collagen to the extent of 1% of 4-hydroxyproline, has been synthesized by A. V. Robertson from 3,4-dehydroproline by the hydroboration reaction. A. Robertson, now professor at the University of Sydney, is carrying on this research by trying to prepare the *cis*- and *trans*-diastereoisomeric DL- and L-3-hydroxyprolines.

Poly-3,4-dehydro-L-proline has been prepared by two different routes. Like poly-L-proline the new polypeptide has two metastable interconvertible forms which have been characterized by their rotational values, supplemented by rotatory dispersion and infrared studies. This investigation has been a joint one with Dr. Arieh Berger from the Weizmann Institute of Science.

Selectively 4-tritiated natural hydroxy-L-proline has been synthesized by A. V. Robertson *via* 4-keto-L-proline by reduction with $NaBT_4$. A novel procedure for the separation of diastereoisomeric hydroxyprolines, developed by S. N. Birnbaum, NCI, has been utilized to advantage, employing controlled buffer systems and ion exchange columns. E. Katz, jointly working in the Laboratory of Clinical Biochemistry, NHI, and LC, has made a detailed study of the incorporation of selectively labeled hydroxyproline and proline into the peptide part of the various actinomycins elaborated by *Streptomyces antibioticus*.

A comprehensive synthetic program on 3- or 4-fluoro-, 3,4-dihydroxy-, and 3,4-epoxyprolines and the respective halohydrins has been started by A. V. Robertson. These compounds are being supplied to the laboratories of S. Udenfriend and A. Sjoerdsma, where screening tests are being developed for compounds specifically inhibitory to the incorporation of (hydroxy)proline into collagen.

A novel tricyclic metabolite of serotonin has been discovered in dehydrobufotenine, a major indole component from the South American toad (*Bufo marinus*). A new isolation procedure utilizing ion exchange columns has made possible the easy preparation of dehydrobufotenine from excised toad parotid glands. In the elucidation of its structure some new chemical reactions have been helpful, but a complete and detailed analysis of the nuclear magnetic resonance spectrum of dehydrobufotenine, compared with bufotenine, furnished rigorous proof for the proposed structure, whose biosynthesis is under investigation. The synthesis is in progress in the laboratory of Professor a Burgstahler, Univ. of Kansas, who started this project as an NIH visitor during the summer of 1961.

In addition to the biosynthesis of dehydrobufotenine in toad parotid glands, F. Märki, with H. Weissbach, has started an investigation of the biosynthesis of adrenoglomerulotropin in beef pineal gland. A highly sensitive assay for this interesting tetrahydroharman derivative has been worked out. Evidence has been secured for the occurrence of other tetrahydroharman derivatives occurring in the glands.

In collaboration with J. Axelrod, F. Märki has completed a detailed study of the O- and

N-methyltransferases occurring in the parotid glands of *Bufo marinus*. In the course of this investigation he discovered epinine for the first time in the animal kingdom.

John Daly, in collaboration with the Laboratory of Clinical Biochemistry, has made a careful study of the mechanism of action, assay method and substrate specificity of the enzyme that converts dopamine to norepinephrine. These studies have made use of substrates labeled with C^{14}, tritium and O^{18}. In collaboration with Dr. David Samuels of the Weizmann Institute, who is holder of a special NIH grant, methods for the micro assay of O^{18} are being worked out.

John Daly, in collaboration with Professor L. Horner of the Univ. of Mainz, has shown that the addition of methanol to quinones of dopamine obeys the 1,2-principle followed by allylic rearrangement. Using nonenzymatic and enzymatic methods all three isomers of the monomethyl ethers derived from 2,4,5-trihydroxyphenethylamine have been synthesized. 2,4-Dihydroxy-5-methoxyphenethylamine has been discovered as a new significant metabolite of dopamine in rats.

The metabolic fate of mescaline *in vivo* and *vitro* has been further studied by John Daly, who discovered mescalol as a new hydroxy derivative resulting from the action of norepinephrine synthetase on mescaline, which is 3–5% as good a substrate as dopamine.

Y. Kanaoka completed the difficult synthesis of 6–hydroxynnorepinephrine, which was found to be a substrate for O–methyltransferase, yielding 2,4-dihydroxy-5-methoxyphenethanolamine, which is not formed from 2,4-dihydroxy-5-methoxyphenethylamine by the action of norepinephrine synthetase. The possible confluence of the metabolism of mescaline with the metabolism of dopamine is under investigation.

In collaboration with J. Cochin, John Daly has applied thin layer chromatography to the separation of alkaloids, barbiturates, phenothiazine and related compounds and their identification as such or as metabolites in urine. The method is unusually sensitive and promises to be of forensic value.

B. Witkop, while Visiting Professor at the Univ. of Kyoto, collaborated with S. Senoh, Institute of Food Chemistry, Osaka, Japan, on the comparative role of metal cations in enzymatic and nonenzymatic O–methylation reactions. The nonenzymatic and enzymatic mono-O–methylations of certain catechol derivatives produce mixtures of isomeric mono-O–methyl ethers in a ratio dependent on pH and on the type of added bivalent metal cation. In enzymatic O–methylations with O–methyltransferase the total yield of O–methylation products as a function of metal ions *decreases* in the following order: $Mg^{++} > Zn^{++} > Mn^{++} > Co^{++} > Ni^{++} > Fe^{++} > Cu^{++}$. The effectiveness of bivalent cations in promoting nonenzymatic *m*–O–methylations with dimethyl sulfate *increases* in the following order: $Mg^{++} < Zn^{++} < Mn^{++} < Co^{++} < Ni^{++} < Fe^{++} < Cu^{++}$. These observations are discussed in terms of the 2:1 catechol-metal complex and the enzyme-metal bridge complex operative in the enzymatic and nonenzymatic O–methylation reactions.

In collaboration with S. Udenfriend and J. Pisano, L. A. Cohen has synthesized several dipeptides of γ-aminobutyric acid which have been identified with natural peptides occurring in brain tissue. The significance of such peptides in neurochemistry is currently under investigation.

In collaboration with G. Glenner, L. A. Cohen and W. M. Jones have prepared a variety of histochemical substrates designed to locate new proteolytic enzymes in specialized tissues. To date, the study has demonstrated a trypsin-like enzyme which is not inhibited in the same manner as trypsin. Of particular interest is the fact that the enzyme shows considerably higher activity in some carcinoid tissues than in normal.

In an attempt to elucidate the role of Coenzyme Q in oxidative phosphorylation, W. Dürckheimer and L. A. Cohen have undertaken the preparation of a new class of labile compounds, cyclohexadienone phosphates. Oxidation of α-tocopherol with N-bromosuccinimide and acetate ion has yielded a cyclohexadienone acetate, an analog of the desired dienone phosphate.

A detailed study of the reaction of ninhydrin with various classes of amines and amino acids has suggested that the generally accepted mechanism for the reaction may require modification. On the basis of new information, L. A. Cohen and W. M. Jones hope to apply the ninhydrin method to the quantitative assay of various phenolic amines present in physiological systems.

In collaboration with E. Mosettig and J. Steele, L. A. Cohen has applied nuclear magnetic reso-

nance spectroscopy to the elucidation of structure and sterochemistry in rearrangement products of dehydroergosterol. The findings have provided a considerably simplified method for the stereochemical analysis of ring B aromatic steroids.

Steroids

Steviol-Isosteviol

The inversion with butyl lithium at carbon atoms 8,13 of the "nor keto acid" (from steviol) to an "iso nor keto acid" and the Cotton effects of these two acids confirm our previous conclusion that in steviol-isosteviol the 9–H is β-oriented. The identity of stevane A with (-) α-dehydrokaurene (of Briggs *et al.*) established the absolute configuration of centers C_9, C_8 and C_{13} in the latter. The identity of stevane B with the degradation hydrocarbon from garryfoline (Djerassi *et al.*) confirms the absolute configuration of these centers in the garrya alkaloids, and therefore also in the alkaloids of the atisine group.

A number of physical measurements of appropriate and comparable degradation products from garryfoline, atisine and steviol-isosteviol confirm the tentative assumption (by Edwards *et al.*) of the "wrong" A/B juncture in these three groups. Thus the absolute configuration of all asymmetric centers (5–7) in the above groups and the kaurenes is established. This in turn brings to conclusion the combined efforts (starting about 1953) of Wiesner (Fredericton, N.B.), Edwards (Ottawa), Pelletier (New York), Djerassi (Stanford), Briggs (Auckland) and our laboratory. (E. Mosettig, U. Beglinger and J. A. Waters)

The C_{17}, C_{20}-Isomers of Cholestane

Δ^5-Cholestane-3, 16, 27-triol and the corresponding 5, 6-dihydro compound, give on tosylation and detosylation, among others, Δ^5-cholesten-16β-ol and the corresponding dihydo derivative respectively. On dehydration of the latter $\Delta^{5,16}$-cholestadiene and Δ^{16}-cholestane were obtained in pure form (*ca.* 35%). The corresponding Δ^{15}-isomers were formed but could not be isolated in crystalline form. The locations of the double bonds were established by N.M.R. spectroscopy. The reduction of the Δ^{16}-enes, in conjunction with previous work (20-iso-Δ^{16}-cholestane), allowed assigning the definite structures 20-iso cholestane and 17- and 20-isocholestane respectively to the reduction products. These compounds are of practical and theoretical interest in view of stereospecific biosynthetic pathways of natural sterols and tetracyclic triterpenes isomeric at the C_{17}-, C_{20}-centers. (G. Nair and E. Mosettig)

Microbiological Hydroxylations of Steroid Alkaloids and Steroid Sapogenins

By employing *Helicostylum piriforme* and a medium consisting of peptone, cornsteep liquor, dextrose and tap water it has been possible to hydroxylate solasodine and tomatidine. The reaction products obtained are respectively: 11α-hydroxy- and 7β-hydroxysolasodine, 9α-hydroxy-, 7α-hydroxy- and 7α,11α-dihydroxy tomatidine. By the same means diosgenin was converted to 7β,11α-dihydroxy- and 11α-hydroxy-7-oxo-diosgenin. These hydroxylated derivatives may serve as starting materials for hormonal steroid analogs not accessible by conventional chemical procedures. Theoretically significant is the decisive effect of the configurations of the C_{25} asymmetric center upon the course of microbiological hydroxylation. (Y. Sato and S. Hayakawa)

Biogenesis in the Slime Mold: The slime mold *Dictyostelium discoideum* incorporates C^{14}-acetate or C^{14}-mevalonate into Δ^{22}-stigmasten-3β-ol. Studies on this incorporation during the various stages of the life cycle revealed that it occurred selectively in the vegetative stage. These findings are significant since this stage precedes the aggregation of the single cells into a pseudoplasmodium, and Δ^{22}-stigmasten-3β-ol has been shown to be involved in this phenomenon. An unknown radioactive compound was found to be attributable to *E. coli* used in the growth medium. Chromatographic evidence suggests that this material may be a sterol. (D. F. Johnson and E. Heftmann)

Biogenesis of Plant Steroids: Mevalonic acid was incorporated into stigmasterol isolated from tomato fruits, while sodium acetate was not. The reverse pattern was reported earlier in *Dioscorea* tuber homogenates. Stem feeding of young D. *spiculiflora* plants with radioactive mevalonic acid resulted in a high incorporation into steroidal sapogenins and β-sitosterol, the latter having a very high specific activity. Degradation work is in progress to establish the pattern of labeling, and the biosynthetic relationships between the sapo-

genins and β-sitosterol. (R. D. Bennett and E. Heftmann)

Steriod Sulfur Analogs: Two new approaches to the synthesis of 11-thiol steroid analogs failed: (1) The hydroboration of $\Delta^{9,11}$-steroids and subsequent treatment with hydrogen polysulfide did not introduce the SH group. (2) Microbiological hydroxylation of appropriate 19-norsteroids with a variety of fungi (which introduce the 11-hydroxyl group in normal steroids) gave only minimal amounts of 11-hydroxy compounds while the main products proved to be 10-hydroxylated derivatives. This closes an important avenue to $\Delta^{9,(11)}$-19-norsteroids. It is assumed that the absence of the methyl group at C_{10} enhances the reactivity of the 9,11-double bond or the corresponding epoxy ring, and thus allows the introduction of an S-group at position 11. (Y. Ueda and E. Mosettig)

Steroid Anlyzer: The gradient system of the automatic steroid anaylzer developed in our laboratory, permits programing any desired solvent ratio for gradient elution chromatography of steroids in a mixture. Preliminary studiles with several different gradients indicate that this feature will facilitate the separation and quantitative estimation of closely related steroids in a mixture. Experiments are continuing on the effects of these gradient changes on chromatographic separations. (D. F. Johnson, E. Heftmann)

Spectroscopic and Spectropolarimetric Studies

(a) The decision between $\Delta^{2,3}$ and $\Delta^{1,2}$ in arborine could not be made on the basis of chemical results. U. V. and I. R. spectra were equally inconclusive. Finally, N.M.R. spectra established the structure of arborine as 1-methyl-2-benzyl-1,4-dihydroquinazol-4-one. (H. K. Miller, L. A. Cohen and R. N. Chakravarti). (b) 784 infrared spectra have been made in support of investigations in this and other laboratories. (c) An infrared analytical procedure for the evaluation of cis-trans olefinic fatty acid compensation in materials submitteed is under design. (d) The precision of the Rudolph spectropolarimeter was greatly increased by stabilization of the high pressure xenon arc lamp, replacement of the polarizing and analyzing prisms, replacement of the high voltage power supply and the installation of a magnetic commutator system. (H. K. Miller and Mrs. A. W. Wright)

Microanalytical Services

Approximately 8,000 analytical determinations were carried out for 140 of the NIH research staff and for several scientists in other Government agencies. These included 7,800 routine microanalyses and approximately 225 non-routine analyses requiring varying degrees of laboratory and literature investigation.

LABORATORY OF MOLECULAR BIOLOGY

The preparations for occupancy, in Building 2, of the newly organized Laboratory of Molecular Biology are approaching completion. Four sections will be assembled. These will be:

Section on Metabolic Enzymes. This section is the Laboratory of Biochemistry and Metabolism (q.v.). This section is headed by Dr. Gordon Tomkins and includes Drs. Miles, Nirenberg and Maxwell, together with research fellows and supporting staff.

Section on Chemical Genetics. This section is headed by Dr. Harvey Itano, presently in the Laboratory of Experimental Pathology (q.v.) Dr. W. J. Dreyer, presently of NHI, will join this Section.

Section on Physical Chemistry. This group is headed by Dr. Gary Felsenfeld, who has been brought to NIAMD from the University of Pittsburgh. The studies carried out in the Section on Physical Chemistry will primarily be concerned with the relationship between structure and function in nucleic acids and proteins. Major projects which are now being established deal with the role of the negatively charged backbone of nucleic acids in the stabilization of ordered structures, the kinetics of DNA denaturation, the structure of the active site in copper proteins (Felsenfeld, Sandeen, Smith) the kinetics of polynucleotide interaction and the transport behavior of polyelectrolytes (Ross). Two studies presently in progress (Felsenfeld, in collaboration with P. Von Hippel, Dartmouth Medical School) are concerned with the dependence of the apparent denaturation temperature upon the wave length used to detect the loss of ordered structure of nucleic acids and with the specificity of deoxyribonuclease for various structural forms of deoxyribonucleic acid.

Section on Molecular Structure. This Section is headed by Dr. David R. Davies, formerly of NIMH. The members of the Section on Molecular Structure (Davies, Gellert, Sigler and Skinner) have been concerned during the past year with structural studies on nucleic acids and proteins using x-ray diffraction methods. In the area of nucleic acid structure a variety of investigations has been undertaken concerning structural aspects of both natural and synthetic nucleic acids. A systematic study of the synthetic polydeoxyribonucleotides has been commenced with a demonstration of the structural similarity of the synthetic polymers containing adenine plus thymine, and adenine plus 5-bromouracil with natural DNA. The mode of binding of the mutagenic acridine-class of dyes to DNA has been investigated with results which rule out some present hypotheses concerning the mechanism of the mutagenic action of these dyes. The organization of DNA, in the T_4 bacteriophage has been studied by flow birefringence methods, and it has been shown that only part of the DNA is perferentially oriented parallel to the long axis of the phage.

In the area of protein structure attention has been focussed on the proteolytic enzymes. Preliminary crystallographic investigations have been carried out on several of these and a systematic search for isomorphous heavy atom substitutions in γ-chymotrypsin is now in progress.

LABORATORY OF NUTRITION AND ENDOCRINOLOGY

Folic Acid

The intact rat, deficient in vitamin B_{12}, is unable to metabolize Carbon-2 (ring) of histidine and consequently excretes formiminoglutamate. Immediately following the injection of methionine, the rat acquires the ability to metabolize Carbon-2. Examination of the folate distribution patterns of the livers of such rats has shown (1) that N^5-methyltetrahydrofolate accumulates in the liver of the B_{12} deficient rat and (2) after methionine administration, the methylfolate concentration is greatly reduced and the liver now contains large stores of N^{10}-formyltetrahydrofolate. It thus appears that methionine provides an acceptor for the methyl present in the folate, freeing the latter for the metabolism of Carbon-2 of histidine. It is concluded that in vitamin B_{12} deficiency, folic acid is "trapped" in the tissues as methyl folates and functional folic acid is released only after a suitable acceptor for the methyl group is provided.

Prefolic A has been identified as methyltetrahydrofolic acid with the methyl group most probably in the 5-position. On enzymatic oxidation prefolic A was converted to 5, 10-methylenetetrahydrofolic acid which disassociates to yield formaldehyde and tetrahydrofolic acid. Menadione (vitamin K_3) functioned as the hydrogen acceptor. Prefolic A was enzymatically synthesized using reduced di- or triphosphopyridine nucleotide as the hydrogen donor in reducing 5, 10-methylenetetrahydrofolic acid. Prefolic A was synthesized chemically by reduction of the 5, 10-methylenetetrahydrofolic acid with sodium borohydride.

The gas gland of the Portuguese Man of War (*Physalis physalia*) excretes CO into its float. Serine appears to be the primary source of the CO. The gland responsible for its secretion contains a high concentration of folic acid (Wittenberg, 1960). In collaboration with Wittenberg (Albert Einstein College of Medicine, N.Y.) the gas gland has been examined for its folate pattern. The results indicate the folates consist essentially of N^{10}–formyl derivatives of tetrahydrofolic acid, diglutamyl-tetrahydrofolic acid, and as yet, an uncharacterized polyglutamyl derivative of folic acid. No methyl derivatives are detectable. These observations are consistent with the view that the CO formed by the gas gland originates from the B carbon of serine by way of an N^{10}-formyltetrahydrofolate derivative.

Purification of the enzyme from chicken liver catalyzing the reduction of N^{10}-formylfolic acid to the tetrahydro-level has led to the conclusion that this enzyme is probably identical to folic reductase. However, the observation that this reduction is stimulated by the presence of folic acid itself has suggested a second reaction occurring in the absence of TPNH: that N^{10}-formyldihydrofolate, produced as an intermediate product via TPNH, is reduced to the tetrahydro-level by tetrahydrofolic acid. Preliminary observations indicate the reaction occurs at a rate much higher than the reduction of N^{10}-formylfolic acid by TPNH.

Vitamin B_{12}

The vitamin B_{12} deficient chick excretes formiminoglutamic acid (FGA) when given a supplemental dose of histidine only if the diet contains a borderline level of methionine. That methionine is not all-important in determining the excretion of FGA is shown by the observation that chicks deficient in methionine under similar conditions do not excrete the compound unless they are simultaneously deficient in vitamin B_{12}. These observations may explain the contradictory reports in the literature concerning the excretion of FGA by pernicious anemia patients. The variations in the methionine content of the diets of these patients are probably associated with differences in FGA excretions. Chicks deficient in folic acid excrete large amounts of FGA and the rate is little affected by the methionine content of the diet. Patients deficient in folic acid also excrete large amounts of FGA.

Chicks deficient in vitamin B_{12} and receiving a minimal intake of methionine show only a slight reduction in FGA excretion when fed homocystine (or homocysteine thiolactone-HCl) and a source of labile methyl groups (choline, betaine). Methionine produces a marked reduction in FGA excretion during the first day of supplementation. These and other studies suggest that vitamin B_{12} facilitates the transfer of methyl groups from choline or betaine to homocystine.

Dietary Liver Necrosis

Studies of the respiratory decline in liver homogenates of rats with dietary liver necrosis indicate that a variety of compounds, tocopherol metabolites, menadione and short-chain coenzyme Q compounds, are protective. Although some antioxidants were very potent, lipid peroxidation was not involved in the respiratory decline since peroxidation can be prevented by levels of the compounds which do not alter the rate of peroxide formation as shown by the thiobarbituric acid reaction. Further support for this comes from the finding that some compounds prevent peroxidation but have no effect on the respiratory decline reaction.

Previous work showed a release of deleterious trace elements from deficient microsomes as a possible cause of the respiratory decline. Combination of microsomes and mitochondria produced respiratory decline which was prevented by EDTA, GSH and dimercaptopropanol.

Factor 3-active selenium compounds were ineffective in preventing respiratory decline when added *in vitro* to the homogenate or to liver slices. However, when supplied to the animal either in the diet or by injection, they were effective in preventing respiratory decline in liver slices. In the homogenized liver, no such effect was seen. A comparison of the potency of various selenium compounds is under way, together with a dose and time exposure study. The difference between liver slices and the homogenate system indicates that the effect of Factor 3 selenium is either located at the cell membrane, or that it is mediated by an active co-factor which is diluted out during homogenization. Both possibilities are under investigation.

Factor 3-Selenium Compounds

When various synthetic selenium carboxylic acids were assayed for Factor 3 activity, it was observed that the activity increased as selenium was shifted from the alpha to the beta to the gamma or delta position. Phenyl derivatives were inactive while benzyl substitution increased activity. Bromine substitution in the para position of the benzene rings increased potency whereas methyl substitution decreased it.

The injection of radioselenium into rats fed Torula yeast diets resulted in the urinary excretion of about 30% of the radioactivity within the first two weeks. Chromatography and radioautography showed the presence of five different organic selenium compounds in the urine. One of these, representing 3% of the radioactivity, appears to be a high molecular weight compound; another minor component is a compound that is unstable to storage. A major component after chromatographic concentration is stable to evaporation and H_2O_2 treatment but is destroyed when autoclaved in a 0.1 N NaOH solution. It has been isolated in radioautographically pure form. The major radioselenium compound excreted by the rat contains 42.5% of the radioactivity. Attempts to concentrate this compound have resulted in large losses of radioactivity. The properties of the compound resemble those of α-Factor 3

whereas the other major constituent resembles β-Factor 3. These compounds are being used as "markers" in attempts to isolate large amounts of the metabolites.

The observation that sulfur amino acids have a strong potentiating effect on α-tocopherol led to a study of the action of other amino acids. Tryptophan at a dietary level of 0.2% was the only amino acid which showed a significant protective effect. Methionine and tryptophan are synergistic with a combination of these two amino acids permitting the rats to survive 2 to 3 times as long as normal on the Torula yeast diet without developing liver necrosis. Studies are under way to determine the mode of action of the amino acids.

Vitamin E Antioxidants and Selenium

Vitamin E can be replaced in some species by certain antioxidants while in other species, these compounds appear to have only slight vitamin E-like activity. An explanation for this variability comes from the observation made in this laboratory that there is a direct correlation between the absorption of the antioxidants by the cells of the body and their efficacy in preventing vitamin E deficiency symptoms. For the chick, low levels of 1,2-dihydro-6-ethoxy-2, 2,4-trimethylquinoline (ethoxyquin) and N,N′-diphenyl-p-phenylenediamine (DPPD) added to a vitamin E and selenium deficient diet completely prevented all deficiency symptoms. Antioxidants such as nordihydroguaiaretic acid (NDGA) and di-tertiary amylhydroquinone (DAH) were inactive even when fed at high levels in the diet (0.5%) white butylated hydroxytoluene (BHT) and di-tertiarybutyl-4-methylphenol (DBPC) showed intermediate activity. The inactive compounds were shown to be unavailable to the tissues and conversely, the effective compounds exerted a significant antioxidant action in the tissues as measured by *in vitro* lipid peroxidation tests.

The antioxidant-like action of tissues which results from feeding trace amounts of selenium is associated with the tissue proteins and is not due to any changes in the lipid components. Dietary selenium was found effective in significantly reducing the *in vitro* hemolysis of erythrocytes from vitamin E-deficient rats (another manifestation of antioxidant deficiency).

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Other Fat Soluble Vitamin Studies

Vitamin A acid in the rat has been shown by others to replace vitamin A alcohol for growth functions but not for visual and reproductive processes. In the chick it has been found that vitamin A acid is slightly less active than the alcohol in promoting growth and preventing neurological disorders. High doses of the acid (50 to 100 μg daily), however, did not prevent infection of the conjunctiva in otherwise normal birds. Toxic doses (2 mg daily) produced excessive secretion of the tear glands, fissures in the corners of the mouth, poor growth and an unthrifty appearance.

There have been three possibilities considered for the origin of the benzoquinone moiety of ubiquinone (coenzymeQ) in animals: (a) dietary, (b) intestinal flora, (c) endogenous. To resolve this problem, a chemically defined diet devoid of ubiquinone was fed to young germfree rats. They were found to accumulate ubiquinone in their livers at a rate comparable to that of conventional rats. These results show that the tissues can synthesize the entire ubiquinone molecule *de novo* and no exogenous source of the benzoquinone ring is required.

A screening program of patients with malabsorption syndrome with respect to abnormalities in the metabolism of vitamins A and E and carotene and ubiquinone, has turned up two patients with a complete absence of serum vitamin E. Tests to date on one patient indicate that certain symptoms are responding to therapy with α-tocopherol.

Plasmalogens

Plasmalogens are phospholipids which contain a vinyl ether group. A specific procedure has been perfected for analyzing quantitatively as little as 0.02 μmole of plasmalogen. This amount of plasmalogen is present in the small size of samples required for enzymatic studies involving the metabolism of plasmalogens.

Protein

Studies have been carried out on the response of various liver components of the adult rat to prolonged protein deficiency followed by repletion. Variables studied in this investigation may be

separated into two general classes: (1) gross and structural changes in the liver and liver cells and (2) functional changes in the liver cells as represented by the individual enzyme components of the succinic oxidase system which was used as a model of an important oxidative system.

While the components of the electron transport chain are considered by many investigators to be spacially arranged in the mitochondrion in one particle, each of the individual components responds entirely differently to a protein deficiency. Recently others have shown that the half-life of the liver mitochondrion of the rat is about 10 days. The rat must synthesize mitochondria with varying levels of electron transport enzymes, rather than simply losing the enzymes from the mitochondria. Perhaps the actual mechanism is a combination of both synthesis of incomplete mitochondria and loss of enzymes from the mitochondria during development of the protein deficiency.

Refeeding the protein depleted rats with an adequate diet results in a rapid rate of liver cell synthesis. Within five days, the total number of cells have been restored but the cells are as low in nitrogen as they were prior to the repletion program. The formation of new protein-poor cells takes precedence over the repletion of the protein in the cells. This contrasts with the situation in the rats pair-fed an adequate ration for the 72 days of the depletion part of the experiment. These animals also had a reduced number of liver cells but when fed on an *ad libitum* basis, they showed no increased production of liver cells until much later than was observed with the protein deficient rats.

Maximum changes in many of the variables did not occur until 30 days after the start of the depletion regimen. The slow rate at which maximal changes occurred in both the protein-depleted rats and their pair-fed controls emphasizes the importance of maintaining animals and subjects on a dietary program for sufficiently long periods if it is desired to study the situation as it exists when equilibrium has been established.

Experimental Obesity

Genetic studies (with Dr. L. Sokoloff) with various strains of mice indicate that the gene governing osteoarthritis was separate from those which controlled body weight and plasma lipids. Plasma total lipids, phospholipids and cholesterol levels showed little evidence for recessiveness or dominance of the genetic factors.

A hybrid strain of rats (S5B/N) does not become obese when fed the high fat diet which made both parent strains obese. Lipid (FFA) mobilization by the hybrid rats was much less than that of the parent strains during fasting or following epinephrine injection. Blood glucose levels respond to these conditions in all three strains of rats.

Marked increases occur in the concentration of total lipids, neutral fats, cholesterol and phospholipids in the plasma of scorbutic guinea pigs. A decrease in the ester to total cholesterol ratio was found in scorbutic but not in fasting guinea pigs.

Germfree Animal Research

Germfree rats and mice require a dietary source of folic acid whereas conventional animals receive adequate amounts of this vitamin from their bacterial flora, except when fed diets very low in protein or when subjected to some stress such as administration of sulfa drugs. By inoculation of germfree rats with single strains of bacteria (*E. coli*, *Aerobacter*, *Alkaligenes* and *Proteus*) sufficient folic acid is supplied to the animal to prevent or cure folic acid deficiency. When coprophagy is prevented in these monoinfected rats, there is still sufficient folic acid formed and made available to the rat to cure a folic acid deficiency indicating that coprophagy is not necessary to maintain a flora or to obtain vitamins synthesized by this flora. Studies are in progress to determine what contribution, if any, are made by the oral flora with respect to vitamin synthesis.

Experiments are in progress to study the development of dietary liver necrosis in the rat with both Tourula yeast diets and low protein diets, and to determine what effects, if any, bacteria may have upon the relative activity of various forms of selenium with respect to prevention of necrosis.

Experiments have been initiated with Dr. Pearson of the University of California, Los Angeles, to study experimental arthritis in rats with respect to any role played by bacteria.

The effect of biotin on the synthesis of folic acid by the germfree rat and the relationship of vita-

min B_{12} and folic acid in the germfree rat is being studied.

Guinea Pig Nutrition

The guinea pig shows a normal rate of growth when either casein or a purified soybean protein are fed at a dietary level of 60%. At a level of 70%, animals fed the soy protein ration showed excellent growth whereas those fed the casein ration gained weight slowly and a number of them died. Tests are under way to determine whether the high methionine content of the casein produces an amino acid imbalance.

The methionine requirement of the growing guinea pig was shown to be 5.75 g/kg of diet. This is much lower than that required by most other animals. The guinea pig also differs from other animals in its reduced ability to utilize the D-isomer of methionine for growth.

Glucose Tolerance Factor (GTF)

Previous work showed that small amounts of insulin were required to elicit a "chromium response," the increase in glucose uptake by muscle in response to added trivalent chromium ions. It has recently been shown that glucose uptake and galactose entry rates into rat diaphragm and adipose tissue cells were not increased by low levels of insulin—such increases occurred only when small amounts of chromium were added to the insulin containing media. Although small amounts of chromium were detected in commercial insulin samples, replacement of the zinc with chromium produced a reduction in the activity of the insulin. Further evidence that chromium and insulin do not interact directly came from the finding that glucose uptake by epididymal fat pads was increased only when the two substances were added separately.

Although chromium increases the amount of insulin bound by adipose tissue cells, it would have to do so four times more if this were its only action. It is suggested that chromium enhances the effect of the insulin, possibly as a glucose transport factor.

A paradoxical phenomenon has been discovered in rats fed certain purified diets. The animals respond to an intravenous glucose tolerance by maintaining blood sugars of 200 to 300 mg% for hours. This probably results from the release of glucose by the liver.

Studies are under way on the assay and isolation of a water-soluble, heat-stable factor in liver which can function in the eviscerated, liverless, GFT deficient rat.

High Calcium Intakes and Hemoglobin Determinations

In a study of the efficacy of dietary phosphate supplment as a means of reducing the incidence of dental caries in the human, large amounts of calcium were also added to the diets of the children under study. Since various reports of animal work indicated that high intakes of calcium interfered with the absorption of a number of trace elements, especially iron and copper, it became important to check such parameters as hemoglobin levels in the children. The cyanmethemoglobin procedure was chosen for this purpose since it was reportedly the best for field studies. The first results suggested that many children in both the supplemented and control groups had low hemoglobin levels. After extensive laboratory checks it became apparent that the cyanide-ferricyanide reagent became decolorized when frozen and remained so even after thawing. During the field studies, the reagent was usually kept in a station wagon where it could have frozen due to the low external temperatures. The reaction produced by freezing involved the reduction of the ferri- to ferrocyanide and the oxidation of the cyanide to cyanate.

Studies carried out under conditions which eliminated the above difficulty showed that even though the children received as much as 2.5 g of calcium per day for almost two years, their hemoglobin levels were the same as those of the children in the control schools. This suggests that high intakes of calcium added to a diet typical of that found among the poorer segments of the American population has no effect on the absorption of those minerals involved in hemoglobin formation.

Riboflavin

Previous workers have reported a correlation between the urinary excretion of riboflavin and

nitrogen during periods of negative nitrogen balance. Both animals and man show pronounced increases in urinary riboflavin excretion starting with the first 24 hour urine samples collected during a starvation period. Preliminary work in this laboratory suggested that when human subjects were starved for periods of 18 hours, there was no increase in riboflavin excretion. For this reason, studies were initiated to determine when the increase in excretion occurs and its relation to nitrogen excretion. Fasting schedules ranging in duration from 29 to 45 hours have been followed by normal adults. Preliminary evidence reveals a 2- to 4-fold increase in riboflavin excretion about 18 hours following the beginning of a fast, though with some individuals this increase is not seen until 30 hours. These data will be correlated with nitrogen excretion.

Adipose Tissue Metabolism

A technique has been developed for perfusing isolated adipose tissue from the parametrium of the rat. When this tissue was removed from fed rats no free fatty acids (FFA) were released during a two-hour perfusion. Adipose tissue removed from fasted rats released FFA at a rate of 4.5 microequivalents per gm during the first hour and 2.0 during the second hour. Addition of small amounts of insulin to the perfusate (2mU per ml) immediately arrested the release of FFA, even in the absence of glucose. Addition of glucose (240 mg%) had no effect on the release of FFA. Epinephrine stimulated the release of FFA into the blood stream but to see this effect it was necessary to use a vasodilating drug such as papaverine (the latter had no effect on FFA release). Caffeine enhanced and prolonged the effects of epinephrine. Caffeine also stimulated lipolysis. Adipose tissue taken from rats pancretectomized for one day released FFA twice as fast as adipose tissue from unoperated controls.

Development of the technique for perfusing adipose tissue provides another method for studying the metabolism of body fat. This procedure not ony facilitates study of the isolated tissue but makes possible study of the role of the vascular bed in the response of the tissue to hormones, especially to those that have pronounced effects on blood vessels, such as epinephrine and norepinephrine.

Studies with incubated fat pads have shown that metabolism of glucose by adipose tissue is decreased in rats fed a diet which causes obesity. This diet, which has a high fat content, also decreases the sensitivity of the fat pad to insulin as measured by uptake and conversion of radioglucose to CO_2 and lipids. Efforts are being made to determine the reason for the difference in behavior of adipose tissue between rats fed the high fat and those fed a high carbohydrate diet.

Glucocorticoids injected into hypophysectomized-pancreatectomized rats produced severe ketosis, hyperlipemia and fatty livers. Incubation studies with adipose tissue have confirmed the suggestion made earlier, as a result of the above findings, that glucocorticoids may have a direct action on adipose tissue. Addition of very small amounts of glucocorticoids such as dexamethasone or triamcinolone to the incubation media (0.02 γ per ml) increased the rate of FFA release and decreased the uptake of glucose.

Protein Hormones

To date no one has succeeded in isolating a pure homogeneous thyroid stimulating hormone (TSH) preparation. The previous concentrates secured in this laboratory have contained several active components differing in their mobilities in starch-gel electrophoresis. Recent work confirms this and supports the earlier findings that TSH can exist in several active forms. A TSH preparation assaying 34 U per mg has been secured by purification on DEAE–C columns.

The hormonal activity of TSH preparations is unaffected by prolonged papain digestion. Physical measurements indicate that such treatment breaks very few peptide bonds. The activity is relatively stable in a solution at pH 2.6 if the temperature is 2° C but at 25° C, 90% of the activity is lost within five hours. Considerable activity remains after standing at pH 11 for three hours at room temperature but spectrophotometric data suggest that some alteration of the protein occurred.

Preparations of human TSH with an activity of 2 to 3 U per mg have been obtained by gel filtration on Sephadex and chromatography on CMC carboxymethyl-cellulose. This work indicated that human TSH may be more acidic than is the bovine preparation. The plasma of normal young

men contains 1 to 12 milliunits of TSH per 100 ml. Plasma from patients with simple goiters or thyroiditis had normal TSH levels while hypothyroid patients had levels of 40 to 100 milliunits per 100 ml. Hyperthyroid patients had normal or elevated levels (up to 40 milliunits per 100 ml plasma).

Rats with transplantable pituitary tumors (MtT) have in their blood a 100 times higher concentration of growth hormone and prolactin than normals and 10 times more ACTH. Thyrotropin and gonadotropin levels are apparently not elevated. Animals with these tumors show a 10 to 20% increase in body weight, a 50% increase in gut weight, a 3-fold enlargement of liver and kidney, a doubling of heart weight, a 5-fold increase in preputial gland weight and mammary glands fully developed and filled with milk. On the other hand, the thymus and body fat stores are absent and the ovaries and uteri are half normal size. The preceding changes are similar to those seen in hypophysectomized pigeons injected with growth hormone, prolactin and ACTH. Similar changes are not seen in the rat under comparable conditions probably due to the fact that sufficiently high blood levels are not attainable in this species by injection.

There was 50 to 100 times as much growth hormone and ACTH in the tumors as in the blood while there was only twice as much prolactin in the tumors. This finding is compatible with the theory and data of others that the hypothalmus inhibits release of prolactin and contact with the hypothalmus is necessary to permit storage of prolactin in pituitary tissue.

The adrenals of rats with the transplanted MtT tumor increase in size about the time the tumor begins to appear. At this time, the adrenals no longer respond to stress or ACTH injections with elevated blood levels of corticosterone and depletion of adrenal ascorbic acid. The high blood level of endogenous ACTH has the adrenal functioning already at maximum capacity.

Incubation of the adrenals of rat fetuses in a Krebs-Ringer solution produced corticosterone in proportion to the amount of ACTH added. The corticosterone production per mg of adrenal was much higher for the fetal adrenal than for the adrenals from 250 g rats suggesting a five times greater sensitivity in the former to ACTH.

LABORATORY OF PHARMACOLOGY AND TOXICOLOGY

Spermidine and Spermine

These polyamines have been investigated in this laboratory for several years in collaboration with Dr. S. M. Rosenthal. They are widely distributed in a variety of biological materials, and previous reports have been concerned with their distribution, biosynthesis, and metabolism.

Recently, our work has been concerned with the stabilizing effects of these amines on a variety of biological materials. These polyamines will stabilize protoplasts, spheroplasts, bacteriophage, and deoxyribonucleic acids. These stabilizing effects have been shown against a variety of agents, including hypotonic lysis, heating and mechanical stress (shearing). Very low concentrations of the polyamines are effective, and suggest that these stabilizing effects are indicative of a physiologic function for these amines. The most likely mechanism for these effects is the formation of a complex between the amine and both phospholipids and nucleic acids.

We have also continued our studies on the biosynthesis and metabolism of the polyamines. As part of these studies, a new enzymatic reaction has been described in extracts of *Serratia marcescens* that converts spermidine stoichiometrically to Δ'-pyrroline, 1, 3-diaminopropane, and H_2O_2.

Sialic Acids

The term, sialic acid, refers to a group of compounds that are derivatives of neuraminic acid, a 9-carbon carbohydrate. They are usually found linked to various carbohydrate polymers, and, as indicated in part by the work summarized here, appear to be of importance in a variety of biologic areas.

. One example of the sialic acids is *N*-acetylneuraminic acid, and a major contribution during the past year has been the elucidation of the enzymatic biosynthesis of *N*-acetylneuraminic acid. Three separate enzymes have been purified from rat liver and bovine submaxiallary gland, which carry out the following reactions:

1. *N* - Acetyl - D - mannosamine + A T P → *N*-acetyl - D - mannosamine- 6-phosphate + ADP

2. *N*-Acetyl - D -mannosamine-6-phosphate + phosphoenolpyruvate → *N* - acetylneuraminic acid-9-phosphate + Pi
3. *N*-Acetylneuraminic acid-9-phosphate → *N*-acetylneuraminic + Pi

Another aspect of these studies has been concerned with sialidase, an enzyme that splits the sialic acid moiety from the polymer to which it is attached. The enzyme has been purified from plasma proteins. Sialidase activity has also been found in purified diphtheria toxin, and is inhibited by diphtheria antitoxin. Sialidase has also been detected in rat thyroid (with Dr. S. Wollman).

The sialic acid studies have also included (1) histochemical studies of sialomucins in human mammary carcinoma with Dr. S. S. Spicer, (2) a phylogenetic study of sialic acid distribution, (3) discvovery of new forms of sialic acid in star fish and sea urchin eggs.

Sulfur Amino Acids

Previous work from this laboratory has presented evidence that three enzymatic steps are required for the reduction of methionine sulfoxide by TPNH. It has now been shown that at least one of these enzymes (and possibly two) are flavoproteins.

During this study it was also found that yeast glutathione reductase is also a flavoprotein containing −SH groups; this aspect of this well-known enzyme had not been previously observed.

Enzyme Levels

Considerable work has been reported from various laboratories on the factors controlling the level of various enzymes in bacterial systems. Very little work, on the other hand, has been done on these control mechanisms in mammalian systems.

We have now been able to demonstrate clearly that *each* of the enzymes involved in urea synthesis (carbamylphosphate synthetase, ornithine transcarbamylase, arginosuccinate synthetase and cleavage enzyme, and arginase) increases when the protein intake and urea excretion increase, and decreases when the protein intake decreases. Various studies have been carried out, including enzyme isolation, to show that these changes reflect actual changes in enzyme levels, and are not the result of activators or inhibitors.

Isotopic studies have shown that the enzyme level is regulated by active control of *both* enzyme and synthesis *and* of enzyme degradation.

Burns

Both laboratory and clinical studies on burns have concentrated mainly on the role of infection, since the work of the past few years has indicated how important infection is in the mortality following burns. In the Peru project *Pseudomonas* has been the organism most often involved.

In the laboratory a potent antiserum against Pseudomonas has been developed, and has been shown to be effective *in vivo* against 23 different isolates of *Pseudomonas aeruginosa.* In other studies preliminary experiments have shown (1) an increase in survival of burned mice upon treatment with convalescent serum and (2) the presence of an antigen in burned mouse serum different from those found in normal serum.

Associated with these laboratory studies, similar data are being collected from clinical burns in the Peru project. Since *Pseudomonas* infections were seen frequently in these cases, the cases were treated prophylactically with gamma globulin, plasma, or albumin, and the effect of these treatments noted on early and late mortality. No effect above that due to saline solution alone was noted in adults, but in burned children a definite protective effect was observed with gamma globulin and plasma that was not observed with albumin. Pseudomonas antiserum is also being tested in the therapy of human burns, but insufficient data are available yet on the efficacy of this antiserum. At present, the preferred treatment for burns in young children is saline solution plus plasma and gamma globulin.

Histidine, Histamine, and Related Imidazoles

The most interesting development in this area has been the demonstration by exchange reactions, that an amino-enzyme is an intermediate in the reaction: *L*-Histidine → urocanic acid + NH_3. In addition, further purification studies have been carried out on this enzyme, as well as on urocanase and diamine oxidase.

Further work has also been carried out on the identification of the imidazoline and imidazolidine compounds that have been obtained by the catalytic reduction of imidazole compounds in acetic anhydride. These are new derivatives as this reduction represents a new chemical reaction. The organic chemical studies on the synthesis and characterization of the ribosides of imidazoleacetic acid and other imidazole compounds has also been continued.

Leprosy

The chemotherapeutic studies on mouse leprosy that have been conducted for many years are continuing, but have now been supplemented with chemotherapeutic studies on human leprosy. For the latter studies, in collaboration with Dr. C. Shepard, C.D.C., the technique growth of organisms in the mouse footpads has been used.

In the mouse studies a new phenazine derivative has been the most active chemotherapeutic drug yet found. Two ethylmercaptan derivatives were also studied; these drugs were almost as effective as isoniazid, but showed the additional advantage in slower development of resistance.

The human leprosy studies show that diaminodiphenylsulfone, isoniazid, para-aminosalicylic acid, and cycloserine were effective; studies of other agents are in progress.

Improvements have also been made in the conditions for growth of *Mycobacterium leprae murium*, the agent of mouse leprosy.

Mevalonic Acid

Studies in this area have been directed towards discovering new pathways for mevalonic acid utilization. As part of these studies *Lactobacillus acidophilus* has been grown on C^{14}-mevalonic acid, and a large incorporation of label has been demonstrated in an unidentified neutral lipid, containing a free hydroxyl group, that does not appear to be one of the common steroids.

Enzyme Activity

Hydroxylysine has been previously reported by Drs. Viswanatha and Irrevere as a constituent of trypsin and chymotrypsin. An activating enzyme (detected by pyrophosphate exchange) for hydroxylysine has now been found in pancreatic and yeast extracts.

Enzymatic Conversion of Vitamin B_{12} to Its Coenzyme Form

Vitamin B_{12} has been enzymatically converted to its active coenzyme form by an enzyme from *Clostridium tetanomorphum* in a reaction mixture containing glutathione, ATP, and yeast extract (in collaboration with Dr. H. Weissbach of the National Heart Institute).

LABORATORY OF PHYSICAL BIOLOGY

Basic Cellular Induction Mechanisms

Dr. Park's discovery that sexual differentiation in *Hydra* may occur cyclically under uniform mass culture conditions and that sexuality is not specifically induced by CO_2, bicarbonate or versene buffer, or by aeration was shown to be true in a second strain of *Hydro*. Stirring the culture 8 hours per day has been shown to prevent differentiation for at least 4 weeks.

Protein Synthesis in Insect Metamorphosis

Citrate oxidation is shown to be indicative of Krebs cycle activity in adult development. It is about three times as great after emergence as just prior to emergence. During adult development there is a nine-fold change in citrate oxidation compared with only a three-fold change in citrate turnover. A study of blowfly muscle amino acid composition shows tropomyosin from larval and adult flies to be similar but to have different physical properties.

Factors Influencing Insect Respiration

The alkaloid ryanodine has been shown to: stimulate respiration in roaches and grasshoppers but not in adult flies; paralyze fly leg muscles but not wing muscles; and to cause flaccid paralysis in the legs of flies but tonic paralysis in fly larvae. Cole exposure which easily damages the mecha-

nism responsible for pupation of bee-moths is shown to be ineffective in army-work larvae.

Biological Triggers

An extensive review of the biophysics of insect respiration and the first paper of a series on triggering of firefly flash have been completed.

Physical Factors Influencing Breathing

Pressure-flow lag shown in earlier work to exist in active breathing patterns was found to be influenced by subjective "effort" in carrying out the breath cycle. A mechanism is not yet known for this phenomenon.

Physiological States That Affect Tolerance of Hypoxia

It is seen that prolonged exercise and subjection to hypoxia each bring about changes in serum enzyme levels which may be related to pathologic changes. Such relationships may aid in the diagnosis of diseases through exercise tolerance studies where biopsy may be impossible or undesirable.

Circulatory Reaction to Plasma Expanders

Edema does not form in anesthetized rats after minimal dextran doses in the presence of hyperglycemia although the blood pressure is normal. Significant edema occurs in conscious rats with vaso-depression but no hyperglycemia. Primary factor in transudation of fluid thus is blood glucose level rather than hemodynamic condition.

Evaluation of Molecular Organization at Interfaces

In monomolecular films of lipids containing phosphate groups, but not those containing $-OH$, $-NH_2$ or ester groups, procaine acts as a crosslinking agent with the ionic portions of the film lipids; veratrine on the other hand reacts with the lipid.

Muscle Fiber Energetics

Refined methods of measuring the ATPase activity of glycerol-treated muscle fibers have revealed that the activity is almost linear with time. The diffusion of ATP into fiber-bundles was found to be about 0.5×10^{-6} cm^2/sec., 100 times greater than the formerly accepted value.

Muscle Proteins

Evidence has been presented showing that the tropomyosin-like component of myosin is the analogue of the paramyosin of invertebrate muscle.

Enzyme Reconstitution

Studies on pyruvate and alpha-ketoglutarate dehydrogenase complexes from *Escherichia coli* show that subunits separated in various ways can be recombined to restore the original "multi-enzyme" units with the natural activity.

Small Ion Binding in Tertiary Structure of Proteins

From ion binding studies it has been shown that the peptide chain of ribonuclease is folded in a manner to create two clusters containing two imidazol groups and one ammonium (or guanidinium) group in each cluster, each cluster thus acting to hold the chain in a specific fold.

Genetic Differences in Bovine Beta-lactoglobulins A and B

By amino acid analysis and "peptide mapping" it was shown that bovine β-lactoglobulin A differed from β-lactoglobulin B in two residues. Since no lactoglobulin differing in a single amino acid has been discovered, it is suggested that this double substitution may have arisen from a single mutational event. The possibility of two successive mutations with loss through selection of the intermediate form can not be ruled out.

Blood Proteins

A detailed study of both bovine and human thrombin by ultracentrifugal assays have shown a very low sedimentation rate for the DFP-inactivated thrombin confirming the low molecular weight previously reported from this laboratory. The entire sequence of peptide B of Co-fibrin has been elucidated. It contains 21 amino acids in a single polypeptide chain, in which the free alpha-amino group is blocked by N-acetyl substitution. It was found possible to oxidize the carbohydrate component of fibrinogen by periodate without destroying any of the amino acid residues and such

treatment renders fibrinogen incapable of polymerization, indicating that the carbohydrate moiety is essential in keeping the fibrinogen clottable.

Gas Chromatography of Amino Acids

Chromatographic procedures for the analysis of the amino acids have been under continued study. Ammonia has been introduced as a carrier gas to make practical the use of the ester in gas phase analysis.

Unusual Amino Acids Isolated From Biological Materials

The quest for novel amino acids in natural products has been continued. 3-hydroxyproline in sponge and in the antibiotic Telomycin was demonstrated.

Macromolecular Protein Synthesis in Yeast

The use of fluorouracil, an analog of the normal metabolite uracil, demonstrates that the so-called "inner pool" of amino acids which are precursors of the proteins built by the yeast, differentially accumulates four of the acids without effect on the protein synthesis other than rate or total quantity produced. This fivefold accumulation is of interest in delineating the possible template mechanism permitting such behavior.

Cellular Reaction to Sera From Carious Disease States

Hela cells adapted to pooled human sera are found to undergo recognizable form changes in the presence of test sera from a variety of patients whose diagnosed disease state falls into the catagory of renal diseases and autoimmune conditions. Investigation of the possible factor or factors interfering with the normal growth of Hela cells is under way.

Transmission of Light by the Crystalline Lens

Both transmitted and scattered light studies of the rabbit lens have been made showing that backscattering increases with age and that diffuse opacity developed in tissue culture of such lens is due to scattering losses from the transmitted beam. The physics of the scattering process pertinent to the crystalline lens has been reviewed indicating that a paracrystalline state of the soluble lens proteins can be inferred to explain transparency and that the larger proteins of the cell walls minimize scattering by their regular spacing.

Source for Neutron Irradiations

Irradiation in a mixed flux of known composition has been made available. Its use for activation analysis either with fast neutrons or thermal neutrons is feasible. Dosimetry for use in irradiation studies have been worked out.

Graded Electron Beam Ionization Effects on Protein Denaturization

Basic globular proteins subjected to dry, *in vacuo*, electron bombardment acquire the ability to induce excess turbidity in polynucleotide solutions. This turbidity is characterized as a nonmonotonic, protein characterizing function of the ionizing radiation dose. A comparison of the functions obtained for chymotrypsinogen, ribonuclease, and lysozyme shows that the isoelectric pH of, the molecular weight of, and the number of sulfur bridges possessed by a protein are important determinants of the differences observed.

Electrochemistry of Membranes and Interfaces

Thermosmosis, the transport of liquids across a membrane which separates two liquid phases of identical composition but unequal temperature has been re-examined. The process requires the presence of electrolytes and electrically charged membranes. It is thus shown to be a special case of electro-osmosis. It has been shown also to be strikingly similar to the same parameters which control anomalous osmosis. A study of silicon carbide crystal electrodes which should be highly inert and independent of the composition of the solutions to be measured has been made to test the feasibility of their use as reference electrodes. The loss of photovoltaic sensitivity and its restoration by anodization seem to control the required properties of these crystals as reference electrodes. Studies of collodion matrix ion-exchange membranes were extended to test the "contractility" effect. A model was considered which satisfactorily accounted for the variation of electro-osmotic permeability with concentration; it passes through a maximum as the polyelectrolyte uncoils or swells in the membrane pore. Studies of the

nonstructural homogeneous (oil) membranes have progressed despite great experimental difficulties to show both cation and anion selective oil membranes. The low conductivity of the oil membranes indicate that the ions which permeate across the membrane must be present in the oil in a non-ionized form.

Macromolecular Organization of Substances of Biological Interest

The analysis of the crystal structure of protein crystals using electron micrographs together with models has been applied to the diamond shaped parasporal bodies formed in *Baccillus Thuringiensis* showing them to be essentially composed of cubic close-packed spheres about 80 Å in diameter which form a face centered cubic structure having a tetramolecular unit cell of about 110 Å on an edge. Analysis of the periodicities appearing on organic crystal images in the electron microscope show them to be associated with a single resolvable molecular plane, but the condition attending the microscopy may show dimensions which may be any of several multiples or fractions of the true value. An analysis of these effects has been made for the dye indanthrene olive T.

NMR Characterization of Molecular Structure

Nuclear magnetic resonance has now established itself as a powerful spectroscopic technique. During the past year this was used to extract information of fundamental importance to the elucidation of the molecular structure of a series of porphyrins and lignans and permitted the exclusion of certain alternative interpretations. Similarly the technique was an invaluable aid to the structural elucidation of a number of naturally occurring alkaloids, steroids, and amino acids.

Optical Activity of Molecular Structures

The origin of the major contribution to the optical activity of the optical isomers of organic compounds containing a conjugated diene system has been shown to be due to the diene itself if the four carbon atoms of the diene are skewed out of a single plane. On the basis of a number of such substances studied it has been shown that several types of compounds not before amenable to investigation by the method of rotatory dispersion can now be studied. The possibility of elucidating absolute configurations of dienic compounds by a simple measurement is of particular significance.

Ergosterol-Vitamin D Mechanism and Other Photochemical Studies

Data representing the only comprehensive investigation of the action of monochromatic radiation on ergosterol and its photoisomers are being analyzed. Experimentation with the recording of total information on magnetic tape has been initiated and will be extended to other sources of data such as gas chromatography and ultraviolet absorption. Considerable interest attaches to the limitations on computor approach to these determinations. The effect of structural changes in substituted diazophenones on photochemical constants has been investigated, and consideration of the molar extinction coefficient yield an equation which permits the development of a theoretical and mechanistic basis for this relationship.

Structure as Related to Chemosynthesis by Radiant Energy

The use of image converters for direct observation in the infrared spectral region and polarizing optics to detect polarized fluorescence and dichroism have yielded direct evidence of a high degree of orientation of the pigment molecules in the chloroplast of *Euglena*. The significance of the laminar structure in this and other biological structures is seen to be critical to the specific biological functions dealing with energy transfer.

Bioelectric Phenomena of Photoreceptors

Microelectrode recordings from isolated slices of squid retina during direct observation via image converters in infrared light have permitted the examination of dark and light adapted units to demonstrate that the mechanism of excitation is a depolarization of the whole photoreceptor by a flow of membrane current through only that part of the cell which is illuminated. Effects of light adaption and the relationship of sodium and

potassium ions to the membrane currents have also been studied.

Temperature Dependence of Form and Virulence of Hemoflagellates

Studies on a number of parasitic hemoflagellates has shown that where there is a different optimum temperature for the development of the form the host tissues attacked will be those normally carrying the optimal temperature without regard to the type of host cell. Temperature is also seen to affect the organization of the outer form of the organism and it is inferred that similar effects on internal organization account for the activity and viability of the cell.

OFFICE OF MATHEMATICAL RESEARCH

Work has continued along the broad lines indicated in the report of last year: Development of mathematical and computational methodology for mathematical models based on differential equations; mathematical formulation and analysis of neurophysiological problems; general mathematical problems arising from the rate behavior of biological systems; mathematical studies of visual perception. Progress in these areas is summarized below in terms of highlights and examples.

Solutions have been obtained for a set of problems designed to answer the question: Is the contribution made to the integrative properties of a neuron by synapses distributed over the dendritic surface significant and, if so, what are the effects of various dendritic distributions of synaptic excitation and inhibition? The solution of a multi-region boundary-value problems, based upon a transformation reducing the appropriate differential equations to a canonical form and expression of synaptic activity as step conductance changes in the nerve membrane, yields the transient potential distribution for any assumed distribution and relative intensities of excitation and inhibition. Quantitative calculations suggest that dendritic synaptic activity dominates the slowly changing background level of neuronal excitatory state, while central synaptic activity produces rapid soma changes especially appropriate for "triggering" of impulse discharge by the neuron. Independent experimental support for this suggested functional distinction has recently been obtained by the neuro-anatomy group at Oslo. The method of computing extraneuronal action potentials, reported last year, has been extended to include the time course as well as spatial dependence and programmed for the IBM–650 digital computer. Calculations, based on assumed passive dendritic membrane, give predictions in excellent agreement with the observations of Drs. K. Frank and P. Nelson (NINDB). Previously the shape of the transient action potential curves and apparent conduction velocities have been claimed to support the assertion that nerve impulses are propagated into the dendritic tree. The remarkable agreement of experiment with predictions based on a theory explicitly denying this assertion calls for a reconsideration of this question and its not inconsiderable physiological implications. (Dr. Wilfrid Rall)

The computer program for routine analysis of data and the formulation and assessment of models has been extended to handle larger (more variables) and more complex systems. The methodology has been improved to secure more rapid convergence for iterative solutions. A general formalism has been advanced for analysis of data in terms of linear compartmental systems which embodies much of the flexibility and versatility of analogue computer methods but which retains the precision, ability to assess uniqueness, goodness of fit, etc., of the digital computer program. A postulate regarding perturbations in linear systems has been advanced and explored quantitatively in hypothetical as well as actual systems. When a perturbation can be described by maximal changes in a minimal number of parameters, comparisons of the original and perturbed system permit a unique prescription of the model when information on the original system alone is insufficient to do this. The above program and methodology have been applied to a number of problems in collaboration with other investigators. (Dr. Mones Berman and Mrs. Marjory Weiss)

A formal model for brightness perception has been formulated and shown to give good agreement with experimental results under a wide variety of conditions. The essential features of the model are an averager, which measures average illumination, I_o; a differencer which forms the

difference between I_o and the illumination at any point; and a variable gain amplifier with gain linear in I_o. Under certain circumstances, increased illumination may increase, decrease or leave unchanged the perceived brightness of an object. This much discussed phenomena is accounted for qualitatively, in a simple way, by the brightness equations derived for the model which shows good quantitative agreement with experimental results from two independent sets of studies. The basic model can be refined by including a local averager, which yields well known edge effects, and a coupling capacitor, which yields the stabilized retinal effect. (Mrs. R. B. Marimont, NIMH, Associate member OMR)

It has been shown that if $x(t)$ is a solution of a linear second-order differential equation with negative characteristic roots and exhibits a maximum or minimum at $t=T$, then $x(t)$ has the property that $T\,|x(T)|/|A| \leq 1/e$ where A is the area under the entire $x(t)$-curve. The inequality is strict unless $x(O)=O$ and the characteristic equation has a double root. The theorem provides a means of distinguishing between critical and overdamping in any damped second-order system as well as a test of the hypothesis that a given chemical or metabolic product is separated from the original precursor by two or more intermediates. It has also been shown that if a matrix, A, of order $n+1$ is sign-symmetric and of the form

$$\begin{bmatrix} a & r \\ c & D \end{bmatrix}$$

where D is diagonal of order n, then the roots of A are real and separated by the diagonal elements of D. Practical and theoretical consequences of this theorem in terms of blood-gas exchange, tracer kinetics and chemical kinetics have been derived including conditions for a repeated root and conditions under which a n^{th} order system will behave as a system of order n-k, $1 \leq k \leq n-1$. A theorem with closely related consequences is this: If $u=(1, 1, \ldots, 1)$ is an eigenvector of the matrix A then the sums along any row of the cofactors of $adj(A-xI)$ have a common value for all x. This theorem establishes necessary and sufficient conditions under which the sum of the species in a linear chemical or metabolic system will behave as a single species and under which the amount of an injected material remaining in the body at any time is independent of the site of injection. (Dr. John Z. Hearon)

CLINICAL INVESTIGATIONS

This is the first year that the number of beds (70) allocated to NIAMD and clinical units has remained constant. The patient census (occupancy) for 1961 has averaged 83%.

A total of 480 in-patients was admitted during the 12-month period from December 1, 1960 to November 30, 1961. The total patient-days was 19,856, an increase of 722 over the preceding year. In the Admissions and Followup Department 1,881 patients were examined and studied, an increase of 296 over the past year. The average inpatient stay at the Clinical Center was 41 days.

Investigations related to the diseases studied at NIAMD have resulted in 93 publications in scientific journals, monographs, annual reviews and medical textbooks. Dr. James Field and Dr. Ira Pastan were awarded the Van Meter Prize by the American Goiter Association in recognition of their elucidation of the mechanism of action of the thyroid-stimulating hormone secreted by the anterior pituitary. Dr. Paul di Sant'Agnese and his associates, Dr. Gibson and Dr. Landauer, were awarded a prize by the American Medical Association for their exhibit on Cystic Fibrosis of the Pancreas. Dr. Joseph J. Bunim gave the annual A.O.A. lecture at Yale University College of Medicine. At the International Congress on Rheumatic Diseases in Rome he was elected as Honorary Member of the Italian Rheumatology Society.

Our staff scientists have derived substantial benefit from the association of distinguished Visiting Scientists and Guest Workers who have worked at our Institute during the past year: Dr. John Lawrence of Manchester University, Director of Field Unit of Empire Rheumatism Council; Dr. Mario Andreoli of University of Rome, Italy; Dr. Mehroo Bharucha of Basel University, Switzerland; Dr. Tetsuo Shiba of Osaka University, Japan; Dr. Othmar Gabriel and Dr. Lewis Gibson, Fellows of the National Cystic Fibrosis Research Foundation; Dr. Harry Keen, PHS Fellow from England; Dr. Mario Werner, Basel City Hospital, Fellow of Swiss Academy of Medi-

cal Science; Dr. Nicholas Halmi of Iowa State University, Fellow of National Science Foundation; and Dr. David Matthews of Royal Free Hospital and School of Medicine, London, Fellow of British Postgraduate Medical Federation.

ARTHRITIS AND RHEUMATISM BRANCH

Demography of Rheumatic Diseases

The earliest studies on prevalence of rheumatic diseases were done only within the past 25 years. Most of these were sociologically oriented and are of little value to the biomedical investigator interested in gaining some insight into the causes and predisposing factors of rheumatoid arthritis, osteoarthrosis, systemic lupus erythematosus, gout and other rheumatic diseases. The principal deficiency in these prevalence studies is that they are based on subjective complaints of joint symptoms. Only since 1956 have reports appeared that were based on acceptable methods for determining prevalence in population surveys. In the recent surveys, prevalence of specified types of arthritis have been determined by objective and internationally standardized criteria which include clinical findings, radiographic abnormalities, and serological tests for rheumatoid factors. Seven such surveys have been reported to date in Northern Europe (England, Wales, Scotland, Holland, and Finland), and only one in the United States (Pittsburgh). These reports represent a promising start, but as is to be expected from any pioneering effort in clinical investigation, the results so far have served to sharpen the focus on certain specific questions which require that new studies be undertaken that are so designed and directed as to yield, hopefully, the desired information.

The studies in which we are currently engaged have been designed to answer several questions. Based on standardized criteria and comprehensive examinations of a random sample of this nation's population, what is the prevalence of rheumatoid arthritis and osteoarthrosis in the U.S.? How does prevalence of these diseases vary with age, sex, geographic location, environment (urban and rural)? What is the influence of age and environment on the occurrence of rheumatoid factors in the serum of rheumatoid and non-rheumatoid respondents? In population groups of similar racial, ethnic, cultural and economic background (the American Indians) what effects do a widely varying climate (e.g. 54°, 48°, 33° and 15° latitude north) and altitude have on occurrence of rheumatoid arthritis, osteorarthrosis, on erosive articular changes (by x-ray) in cervical spine, sacroiliac joints, hands and feet, and on rheumatoid factors? Studies done in our laboratories and by others suggest that there are at least two rheumatoid factors: one that complexes with human aggregated gamma globulin (demonstrated by the bentonite flocculation test), and another with antigen-antibody complexes (demonstrated by the sheep cell agglutination test). There is evidence to suggest that the latter is genetically determined and the former is influenced by environmental factors. This hypothesis can now be tested by a *family* study on the Indian population now under survey by our unit. If the occurrence of abnormal circulating antibodies to tissue components—which some workers regard as evidence for development of auto-immunity—is a result of genetically deranged immunological mechanism, appropriate study of the relatives of patients with a rheumatic disease such as Sjögren's syndrome (which is associated with this phenomenon) should contribute significant information. This study has been undertaken by our unit and is near completion (vide infra).

What has been said thus far will serve as introduction to summaries of three studies now underway in the Arthritis and Rheumatism Branch: (1) National Health Examination Survey for prevalence of arthritis, (2) survey of American Indians at widely different latitudes for prevalence of arthritis, and (3) family studies on Sjögren's syndrome.

National Examination Arthritis Survey

The National Health Survey Division (Mr. Theodore Woolsey and Dr. Alice Waterhouse and staff) is currently conducting a Health Examination Survey in which 42 representative areas selected in a stratified random sampling pattern throughout the U.S. will be visited by two mobile examining centers, each staffed by teams of 10

persons and operating simultaneously in two specially constructed, large truck trailers. A probability sample of 6,000 adults aged 18 to 79 years selected from the population of 93 million civilian adults (non-institutional) will be examined. The survey was begun July 1960 and is scheduled for completion by July 1963. NIAMD is collaborating in the arthritis component of this survey. The mobile team takes a complete history of musculo-skeletal complaints covering especially symptoms of arthritis and rheumatism, and performs a standardized examination of the joints. Radiographs of both hands and feet are made and a blood sample taken. The x-rays and serum samples are shipped to NIAMD in Bethesda. Bentonite flocculation tests are done in our laboratory and the films are read independently by three observers (Drs. Bunim, Burch and Black). To date (November 1961) 3,421 serum samples collected from 26 of the 42 areas have been tested. Of these, 118 (3.4%) gave a positive bentonite flocculation test. When the survey is completed more detailed analysis will be made and the relation of various environmental factors, as well as age and sex, to the presence of rheumatoid factor will be determined. In the aforementioned surveys in northern Europe, the prevalence of positive tests for rheumatoid factor (sheep cell agglutination test) varied from 2 to 5%, being higher in the urban areas than in the rural ($P = <.01$).

Thus far (in three areas completed) radiographic evidence of osteoarthrosis (grade 2 or higher) has varied from 28.1% in Valdosta, Georgia to 48.6% in Akron, Ohio. The frequency of changes consistent with rheumatoid arthritis varied from zero in Valdosta to 1.5% in Philadelphia. (Drs. Bunim, Burch & Black)

Indian Survey

This survey was undertaken for several reasons. The principal one, to test the hypothesis that prevalence of rheumatoid arthritis and possibly of osteoarthrosis was related to climate. This relationship first became apparent from results of recent population surveys in England and Wales (Lawrence 1960 and Miall 1958). Prevalence rates of rheumatoid arthritis in four towns going from north to south are listed below:

Town	Latitude (degrees)	Prevalence Rheumatoid Arthritis (age 15 and over), percent
Wensleydale, Yorkshire	54–55	5. 7
Leigh, Lancashire	54	3. 9
Glamorgan, Wales	51–52	2. 0
Rhondda, Wales	51–52	1. 3

A survey made of U.S. Indian school children by Paul (John R.) and Dixon in 1937 for prevalence of rheumatic heart disease showed the same phenomenon.

Area	Latitude (degrees)	Prevalence Rheumatic Heart Disease (percent)
Montana and Wyoming	44–46	4. 5
Arizona (North)	36–37	1. 9
Arizona (South)	32–33	. 5

Of more pertinent interest are results of examinations of Indians done by the Division or Indian Health in 1955, when the operation of hospitals and health facilities was transferred from the Department of Interior to DHEW (USPHS). The health examination records re-analyzed by Dr. Burch reveal a significant difference in prevalence of rheumatoid arthritis and osteoarthrosis between northern and southern reservations.

Area	Latitude	Prevalence of Arthritis (age 15 and over), percent
Montana and South Dakota	46–48	27. 6
New Mexico and Arizona	32–36	11. 2

To test the influence of climate on arthritis it was thought best to select a relatively homogeneous population that lived at widely different latitudes for several successive generations. The Blackfeet tribe (currently being surveyed) living in Browning, Montana at the northern border of U.S. at 48th parallel and the Pima tribe (to be surveyed in September, 1962), living near Phoenix, Arizona at 33rd parallel seemed especially suitable for comparison. These tribes have much in com-

mon: stable population, economic status, living habits, occupation, culture and racial background.

For the Blackfeet survey, excellent cooperation and valuable guidance was obtained from the Indian Health staff, both at Billings Area Office and the Blackfeet Reservation, as well as from the Area Engineering staff. The Blackfeet Tribal Council was receptive and helpful. We were fortunate in recruiting Dr. John Lawrence of Manchester, England, Director of the Field Unit of the Empire Rheumatism Council as an NIAMD Visiting Scientist for ¾ of the duration of the actual survey in Montana. Dr. Lawrence had pioneered the northern European surveys referred to above and his participation in the Montana survey, as well as in all seven European surveys, should help standardize observations and criteria in both continents. The NIAMD mobile unit consisted of the following staff members: Drs. Burch and O'Brien, Miss Watson (statistical clerk and laboratory aid) and Mr. Aldridge (radiographer). Six natives were employed as receptionist, chauffers, interpreters and coordinator. Two 21-foot trailers were used: one as reception office and examining room and the other as x-ray and laboratory unit. These trailers were obtained as surplus from another government agency and then repaired, renovated and equipped by NIAMD.

The sample to be examined consisted of about 1100 subjects randomly selected from 2,000 adult Indians above age 30. The survey was begun on October 2, 1961 and is scheduled to terminate about December 10, 1961. A satisfactory completion rate of 85% has been achieved thus far (November 30, 1961). Results and analysis of their significance will be included in the 1962 annual report. (Drs. Burch, Bunim, O'Brien and Lawrence)

Relatives of Patients with Sjögren's Syndrome

In a study on the clinical features and serological reactions in a group of forty patients with Sjögren's syndrome an unexpectedly high frequency of several abnormal serologic factors was found. Rheumatoid factor tested by the F II bentonite flocculation reaction was present in every case even though half the cases did not have rheumatoid arthritis or connective tissue disease. Antinuclear factor tested by the immuno-fluorescent technique was found in 77%. Complement fixing antibodies reactive with normal human tissue constituents, e.g. liver, were present in 49%. Thyroglobulin antibodies tested by tanned red cell technique were found in 27%. The serum gamma globulin concentration measured by paper electrophoresis was elevated in 75%.

Since studies on rheumatoid arthritis and on systemic lupus erythematosus have indicated that many of the above factors may be hereditary, a family study was undertaken to determine what role genetic factors may play in the occurrence of clinical and serological changes observed in Sjögren's syndrome.

Propositi—Fourteen patients who had been admitted to the Clinical service of NIAMD served as propositi. After discharge from the hospital, nine of these propositi have been visited thus far at their homes and the closest neighbors of the same race, sex and approximately the same age were selected as "control" propositi.

Relatives—The patients furnished a list of 281 and the control propositi of 121 blood relatives. To date 84% of the parents, siblings and children, 43% of other relatives of patients and 55% of the control relatives have been examined.

	Sjögren Relatives		"Control" Relatives and Propositi
	Group I (first degree rel.)	Group II (all other rel.)	Group III
Total	99	192	130
Examined	83	83	72
Completed (percent)	83. 8	[1] 43. 2	[1] 55. 4

[1] Efforts will be made to bring completion rates up to 80% during 1962.

Only two individuals have refused to participate in the study. In this report the parents, siblings and children of the patients will be designated as group I, other relatives of patients as group II and relatives of the control propositi plus the control propositi themselves as group III. The age and sex distribution was as follows:

	Under 45 Years			45 Years and Over		
	Group I	Group II	Group III	Group I	Group II	Group III
Male	18	32	16	18	1	15
Female	29	41	24	18	9	17
Total	47	73	40	36	10	32

The majority of the relatives were visited in their homes by Dr. Burch who used a small house trailer equipped as a clinic. A few relatives were examined at the Clinical Center of NIH.

A clinical history with special emphasis on rheumatic complaints, ocular and oral symptoms suggestive of the sicca complex and of thyroid disease was obtained. The physical examination was limited to the joints, eyes, mouth, skin and thyroid. A Schirmer test utilizing a strip of Whatman number 41 filter paper (5×35 mm.) was done to measure the rate of lacrimal secretion. Wetting of less than 15 mm. in five minutes was considered as positive. Roentgenograms of the hands and feet were made on all subjects, except pregnant females, and approximately 30 ml. of blood was drawn in the majority of cases for various serological and biochemical tests.

The following tests were performed on the sera when the amount permitted: a) Rheumatoid factor by the FII bentonite flocculation test. A dilution of 1:32 was considered as the minimum positive titer; b) Thyroglobulin antibodies as tested by agglutination of tanned human group O Rh+ red blood cells coated with highly "purified" thyroglobulin; c) Thyrotoxic thyroid tissue antibodies by complement-fixation reaction; d) Complement-fixing antibodies reactive with thymus tissue extracts; e) Gamma globulin by modified zinc turbidity test. Range of normal values was considered to be between 24 to 41 turbidity units. This range was estimated from the frequency curve of all determinations made in this laboratory on 323 individuals other than patients; f) The presence of antinuclear factor was determined in 29 first degree relatives of propositi with Sjögren's syndrome and 14 relatives of control propositi. The immuno-fluorescent procedure of Coons and Kaplan was used, employing intact nuclei in mouse liver sections.

Results: In comparing groups I, II, and III a significant difference was found in respondents below age 45 in the five parameters listed below. It is noteworthy that a consistent frequency relationship was maintained between group I (highest prevalence), group II (intermediate) and group III (lowest) in each of these five parameters.

Prevalence of Clinical and Serological Abnormalities in Sjögren Relatives

Parameters	Group I (1° relatives), percent	Group II (other rel.), percent	Group III (controls), percent	P value [1]
2 or more diagnostic criteria (ARA) for R.A	17. 0	5. 5	2. 5	<. 01
Reduced lacrimal secretory rate	29. 8	15. 1	5. 0	<. 01
Abnormal circulating tissue antibodies	36. 2	13. 7	12. 5	<. 01
Hypergammaglobulinemia	20. 9	11. 3	2. 6	<. 05
3 or more "Sjögren" signs	21. 3	6. 8	0	<. 01

[1] The method of Mantel and Haenszel was used to obtain a chi square with two degrees of freedom for evaluating simultaneously the significance of the departure from expectation, sex adjusted at the 3 levels of relationship.

These results demonstrate a definite familial aggregation of "possible" or "probable" rheumatoid arthritis, decreased lacrimal secretion, abnormal circulating antibodies to tissue constituents and increased gammaglobulin. The evidence cited above suggests that this is due to heredity rather

than environment, but the exact genetic mechanism remains to be elucidated. (Drs. Burch, Bloch and Bunim)

Program Planning for 1962 to 1964

The most promising approaches to a better understanding of etiology and pathogenesis of rheumatoid arthritis and connective tissue diseases consist of the employment of immunologic and human genetic disciplines. Well-trained, promising, young immunologists have been recruited; an immuno-chemist, an experimental immuno-pathologist and one scientist who has been exploring the genetic (type-specific globulin groups) and immunological aspects of the rheumatoid factors. These men will join the staff of the Arthritis and Rheumatism Branch in 1963.

The role of genetic factors in susceptibility to development of rheumatoid arthritis and other connective tissue diseases may be determined by appropriately designed family studies of propositi encountered in the course of our population surveys. These propositi are especially suitable since family units among the Blackfeet and Pima tribes live close together and on the same reservation for many generations. The prevalence of rheumatoid factor, clinical rheumatoid arthritis, ankylosing spondylitis and systemic lupus erythematosus, as determined to date by our unit, seems to be greater in the Blackfeet Indians than in the general population of the United States.

It is also planned to study, in adequate numbers, identical and fraternal adult twins, one or both of whom has or had rheumatoid arthritis, ankylosing spondylitis, osteorarthrosis or gout.

Effects of Steroids, Essential Amino Acids, Pyridine Nucleotides and ADP on Structure and Catalytic Activity of Glutamic Dehydrogenase (GDH)

As reported in the 1960 Annual Report, in collaboration with Dr. Gordon M. Tomkins of the Section on Metabolic Enzymes, it has been observed that various steroid hormones and diethylstilbestrol inhibit the glutamic dehydrogenase reaction by promoting the dissociation of the enzyme molecule into 4 subunits. We observed further that these subunits possessed alanine dehydrogenase activity. Thus, by altering the physical state of the enzyme molecule, both the catalytic activity and substrate specificity of the enzyme were changed. Both these effects were antagonized by ADP and DPN. This general problem has now been extended along several lines in order to confirm the basic observations, and to increase our knowledge of the nature and the consequence of the steroid-GDH interaction.

Evidence for Change in Physical State of Enzyme

Dr. Tomkins initiated a study of the light scattering properties of GDH which we have continued in this laboratory. By this means, we have been able to confirm that the enzyme molecule is indeed disaggregated into subunits by diethylstilbestrol and various steroids, and that this disaggregation is antagonized by ADP and L-leucine (see below). It has been repeatedly borne out that a low concentration of DPNH, TPNH or DPN must be present in order for the steroids or steroid analogs to disrupt the enzyme molecule. This requirement for DPNH is distinctly different from that required for the catalysis of the chemical transformation as shown by distinctly different dissociation constants. It is interesting that the value of this constant is of the same order as that for the DPNH enzyme complex demonstrated by a study of DPNH fluorescence enhancement by GDH (as shown by Dr. Tomkins). Studying the kinetics of the disruption by diethylstilbestrol with this technique, it is apparent that only when the enzyme is completely dissociated does alanine dehydrogenase activity appear, while GDH activity is lost much more rapidly; i.e. only the most associated form of the enzyme functions as GDH, while the most dissociated form functions as alanine dehydrogenase. Other pertinent observations made with light scattering include the fact that all of the substrates, with the exception of ammonia, appear to have some effect on the physical properties of the enzyme.

Effects of L-leucine and Other Essential Amino Acids on Structure and Function of GDH

It has been observed that the essential amino acid, L-leucine (but not D-leucine) stimulates the rate of oxidation of glutamate catalyzed by either the crystalline enzyme from beef liver or a crude preparation of rat liver mitochondria by as much

as 100%. In so doing, it protects the enzyme from inhibition by steroids, phenanthridine and DPNH. Sedimentation experiments have revealed that leucine functions to maintain the enzyme in the aggregated state. The essential amino acids, isoleucine and methionine, and the non-physiologic amino acid, norvaline, are also effective though somewhat less so than L-leucine. Leucinamide, leucylleucine, and leucylglycine were inactive. The amino acids, glycine, alanine, valine, lysine, serine, threonine, norleucine, arginine, ornithine, tryptophan, phenylalanine, histidine, tryosine and α-aminobutyrate were all ineffective, as were the α-keto analogs of leucine and isoleucine. Thus a high degree of specificity was observed for the leucine effect. These findings may represent a mechanism by which essential amino acids can regulate general amino acid meatbolism.

Dissociation by Steroids and Other Agents

To gain some knowledge about the structural requirements for the steroid inhibition of the GDH reaction, the effects of a large number of analogs of progesterone, estradiol and androstene, 3,17-dione have been examined. It was found that even minor alterations of the steroid nucleus, particularly those which disturb either the planarity of the molecule or increase its polarity can drastically reduce inhibitory potency. It is also interesting that the introduction of a double bond in the 16 position in progesterone enhances its activity three-fold. Thus, the inhibitory potency of the types of steroid tested roughly correlated with the degree of polarity; those compounds that are least polar are most inhibitory.

As mentioned, the presence of pyridine nucleotide is required for disaggregation of GDH to proceed. It has also been observed that both the catalytic activity of the enzyme and its responsiveness to steroid inhibitors are markedly affected by salt concentration. The fact that certain SH groups in the enzyme are important in determining whether or not steroid can control the reaction was reported in the 1960 Annual Report. This observation has now been confirmed, and the additional observation made that parachloromercuribenzoate not only can modify the inhibitory effect of the steroid hormones, but also can prevent stimulation of the enzyme by ADP and leucine; thus this SH reagent appears to interfere with the control of the enzyme both as regards inhibition and stimulation.

Previous workers have shown that o-phenanthroline (op), a potent zinc-binding reagent, can also dissociate GDH into subunits. This led to the proposal that zinc was responsible for maintaining the aggregated state of the protein. Our findings with the steroid hormones led us to question whether "op" might work by some other mechanism. Accordingly, we have found that phenanthridine, m-phenanthroline, and phenanthrene-9-aldehyde, all non-chelating analogs of "op", also dissociate the enzyme and thus inhibit the GDH reaction. ADP antagonizes the action of these compounds also. A study of the properties of these molecules may lead to a better understanding of the mechanisms by which the enzyme is disaggregated.

A number of other amino and keto acids have been examined as substrates for glutamic dehydrogenase, and evidence has been obtained suggesting that norvaline, α-ketovalerate, α-aminobutyrate, α-ketobutyrate, α-ketocaproate and α-ketoisocaproate serve as substrates for a form of the enzyme intermediate between the fully associated and the fully dissociated state. Thus, with maximum dissociation of the molecule (diethylstilbestrol at pH 8 and above) the reactions involving these substrates are inhibited, while partial dissociation (diethylstilbestrol at pH 7) results in stimulation. Both of these effects are again reversed by ADP. Thus, glutamic dehydrogenase appears to have a spectrum of activities depending on its state of aggregation.

In summary, we have been able to confirm the relationship between physical structure and catalytic activity of GDH by independent means of measurement. From our studies, it becomes apparent that control of this enzyme is a rather specific process and apparently involves specific sites on the enzyme molecule which are concerned with control. This is borne out by the fact that there appears to be a specific binding site for pyridine nucleotide, which is concerned with control, as well as the site for steroid, ADP and leucine. Whether or not some of these sites are identical has not as yet been determined.

The question of the intimate mechanism of the steroid effect has not been elucidated. The fact that the enzyme molecule can come apart rather

easily on dilution suggested that the attractive forces which maintain the enzyme in the aggregated state are some sort of weak, non-covalent interaction. (Dr. Yielding)

Effect of Steroids on Phosphopyruvic Kinase (PEP Kinase)

The fact that PEP kinase is so sensitive to salt, plus the reported observation that urea dissociates it into subunits, prompted us to test the effects of steroids. It was observed that DES and estradiol were moderately good inhibitors of the reaction and, interestingly, that this effect could be antagonized by high concentrations of DPN. Urea, deoxycholate and sodium dodecyl sulfate also inhibit the reaction. Examination of the crystalline enzyme in the analytic ultracentrifuge, as well as in sucrose gradient density centrifugation, failed to reveal any evidence of dissociation of the enzyme molecule. Similarly, experiments with light scattering showed no gross change in the weight average molecular weight under the influence of diethylstilbestrol. Agar-gel electrophoresis has now revealed that the inhibitory steroids cause a drastic change in the electrophoretic properties of the enzyme, and that these changes are well correlated with the kinetic findings. (Drss. Yielding and Kimberg)

Metabolism of Aromatic Amino Acids and Homogentisic Acid in Phenylketonuria, Alcaptonuria and Ochronosis

Objectives in studying patients with diseases associated with abnormal metabolism of the aromatic amino acids, namely, alcaptonuria, phenylketonuria, tyrosinosis and albinism, have been several: 1) to determine the exact nature of the metabolic defect in these conditions; 2) to study the hereditary pattern of "these diseases and, if possible, to develop tests which will detect the heterozygous state in relatives carrying the trait; 3) to study the formation and deposition of the pigment derived from homogentisic acid and to determine how it produces the pathological changes in the connective tissues, particularly the joints (ochronotic arthritis); 4) to study the cause of ochronotic arthritis, nearly always associated with alcaptonuria, and 5) to attempt various means of treatment of these metabolic diseases.

Phenylketonuria

Our method for blood phenylalanine has been modified so that it can be used with 0.1 ml. of whole blood. This permits quantitative determination of phenylalanine in the small samples of blood obtained by heel prick from newborn babies. The practical usefulness of the method has been tested by obtaining blood samples in duplicate from approximately 40 babies at the D.C. General Hospital. The analyses were made without technical difficulties, indicating that the method is applicable for such a determination, but an unexpected finding was that even though none had phenylketonuria, approximately 10 percent of the babies had significantly elevated levels of blood phenylalanine or tyrosine, and two had elevated values of both amino acids. Nearly all of the babies with elevated levels had the so-called "physiological jaundice of the newborn" and we suspect that the elevated levels were due to the immaturity of the liver and the delayed development of the liver enzymes concerned with the metabolism of these amino acids. Further analyses are being made in premature infants and in older white and negro babies.

Evidence of elevated phenylalanine levels in non-phenylketonuric babies is of considerable clinical importance, since it has been generally assumed that this laboratory finding alone is enough evidence to make the diagnosis. Obviously other data are necessary, even in families with known phenylketonuria.

Tyrosine and ascorbic acid

It has been known for many years that guinea pigs and man deficient in ascorbic acid metabolize tyrosine incompletely when given extra amounts of this amino acid. Studies in our laboratory over the past few years have shown that vitamin C acts in only one of the enzymatic steps in tyrosine metabolism—the oxidation of p-hydroxy-phenylpyruvic acid to homogentisic acid. In this reaction, ascorbic acid does not function as a specific cofactor of the enzyme, but as a nonspecific reducing agent which protects p-hydroxyphenylpyruvic acid oxidase from inhibition by its substrate. However, a number of other reducing agents can also protect the oxidase from substrate inhibition, such as analogues of ascorbic acid, some quinones and a variety of dyes, such as 2,6-dichlorophenol-

indophenol. Furthermore, studies with purified enzyme preparations have indicated that ascorbic acid protects the oxidase indirectly by reducing another liver component. Evidence that ascorbic acid is acting indirectly in this reaction is that with each stage of purification of the enzyme, ascorbic acid becomes less and less effective in protecting the oxidase from inhibition.

Recently we have attempted to determine the chemical structure which is shared in common by the compounds able to protect the oxidase. These studies have been undertaken to learn more about the mechanism by which the enzyme is protected, and to give us an idea of the compound through which ascorbic acid acts to protect the liver enzyme *in vivo*.

In vitro experiments have shown that coenzyme Q_{10} is the only naturally occurring compound which has both the proper structural requirement to protect the oxidase and much greater effectiveness in the presence of ascorbic acid. With purified oxidase preparations, the combination of Q_{10} and ascorbic acid is at least 1000 times more effective than either ascorbic acid or Q_{10} alone. *In vivo* evidence that coenzyme Q_{10} participates in tyrosine metabolism has also been obtained. Frankly scorbutic guinea pigs (completely depleted of ascorbic acid) did not have their p-hydroxyphenylpyruvic acid oxidase protected When Q_{10} was injected intraperitoneally. However, moderately vitamin C-deficient guinea pigs (liver concentrations of ascorbic acid reduced to approximately 6 mg%) had complete protection of their liver pHPP oxidase when Q_{10} was injected. These animals moderately deficient in Vitamin C would have had complete inhibition of their oxidase if not given Q_{10}. These *in vivo* suggest that the liver component through which ascorbic acid acts is coenzyme Q_{10} or a closely related quinone. Further studies on the role of coenzyme Q_{10} in the metabolism of tyrosine are under investigation.

Connective tissue and ochronosis

Our studies are completed on the distribution of homogentisic acid in the tissues of guinea pigs at various times after injection of the acid intraperitoneally. *Young* animals were found to have the same unusual distribution pattern of relatively large concentrations in skin and cartilage and very little in muscle and brain, as we found earlier in older animals. We can therefore eliminate the possibility that aging of the collagen explains the high affinity of the connective tissues for homogentisic acid. Binding experiments to determine the affinity for homogentisic acid of homogenates of skin, cartilage and serum albumin have shown that there is no significant binding under equilibrium dialysis conditions for skin or cartilage, but homogentisic acid is bound to the extent of 40 percent by serum albumin. The behavior of homogentisic acid is thus very similar in its binding and distribution properties to gentisic acid.

We have also developed a new specific method which allows us to determine small amounts of benzquinone acetic acid, the oxidized form of homogentisic acid, in blood and tissues. Since this quinone is the likely first intermediary product in the formation of the ochronotic pigment in the connective tissues, this method will be of value in studying the distribution and fate of homogentisic acid via this oxidative pathway.

Alcaptonuria

The renal clearance of homogentisic acid in an alcaptonuric patient has been studied. Gentisic acid, a close analogue of homogentisic acid which is also actively secreted by the kidney tubles, does not alter the renal secretion of homogentisic acid, nor does it cause an elevation in the plasma level of homogentisic acid in the alcaptonuric patient.

We suspect that the remarkable ability of the kidney to secrete homogentisic acid is an important protective mechanism to delay the development of ochronosis in alcaptonuric subjects and that this ability may gradually become less effective with age. Attempts to measure the capacity of the kidney to secrete homogentisic acid have been made by oral loading tests with L-tyrosine. Even though these resulted in establishing very high plasma levels of HGA, essentially complete renal clearance was found, even at the elevated levels. Whether the capacity is less in older alcaptonuric patients is not known and should be determined. Neither sulfinpyrazone nor probenecide had any effect on the plasma level of homogentisic acid, nor the renal clearance of HGA.

Attempts to inhibit the formation of homogentisic acid with Antabuse (the disulfide form of diethyldithiocarbamate which is a very potent

inhibitor *in vitro* of the enzyme which forms homogentisic acid) did not inhibit this pathway in the alcaptonuric patient. This method of treatment is of theoretical interest and we will continue to search for an effective agent to inhibit p-hydroxyphenylpyruvic acid oxidase *in vivo*.

An alcaptonuric patient was also given a few grams of shikimic acid to see whether this compound could be metabolized to homogentisic acid. Shikimic acid is an intermediate in the synthesis of the aromatic amino acids in bacteria and in other organisms able to make their own tyrosine and phenylalanine. Man cannot make phenylalanine (and tyrosine) directly, presumably because of a metabolic defect somewhere along this pathway, although exactly where has not been determined. Alcaptonuric patients represent ideal subjects to study for the location of this defect, because nearly all of any phenylalanine formed would be changed to homogentisic acid. Shikimic acid did not yield homogentisic acid but further studies with other intermediate compounds are planned. (Drs. LaDu, Seegmiller, Zannoni, Malawista and Jacoby)

Gout

Effect of Certain Uricosuric Agents on Renal Clearance of Uric Acid

In an effort to gain a better understanding of the mechanism involved in the renal excretion of uric acid, we have continued studies of the effects of various drugs on uric acid excretion. The uricosuria noted in last year's report to accompany the administration of azauridine to cancer patients has been shown to be the result of two actions of the drug. The first seems to be a direct effect of azauridine on the kidney. The second action is an indirect one mediated by orotic acid which accumulates in cells as a consequence of the metabolic block in pyrimidine metabolism and is excreted in substantial amounts in the kidney. The intravenous infusion of orotic acid as the sodium salt also produced a uricosuria. Certain similarities of chemical structure shared by these three compounds suggest that these compounds may be utilizing a common transport mechanism in the kidney. The necessity of administering these compounds intravenously limits their clinical usefulness. An enzymatic method has been developed for determining microgram quantities of orotic acid in biological fluids which will permit further studies of the mechanism involved in the renal excretion of orotic acid.

The marked antagonism of uricosuric doses of sulfinpyrazone and of uricosuric doses of salicylate has been investigated by determining simultaneously the renal clearance of uric acid and of inulin. In a gouty patient receiving sulfinpyrazone the urate clearance was reduced to 5% of the initial value by the intravenous administration of sodium salicylate without a significant change in glomerular filtration rate as revealed by inulin clearances. After the infusion was discontinued the urate clearance was further reduced to 0.5% of the initial value. These results suggest that addition of salicylates dissociates two aspects of the renal handling of uric acid in almost completely reversing the block in tubular reabsorption of filtered urate induced by sulfinpyrazone and at the same time blocking the tubular secretion of urate. The net result is an almost complete suppression of uric acid excretion. (Drs. Seegmiller and Howell)

Acute Arthritis Induced in Gouty Subjects by Intra-articular Injection of Microcrystalline Urates

The way by which the hyperuricemia of gouty patients may give rise to the acute attack of arthritis has been under investigation. We have been able to show that the introduction of a suspension microcrystalline sodium urate crystals into the knee joint of volunteer gouty subjects gives rise to an inflammatory reaction, while similar injection of amorphous suspensions or solutions of sodium urate gave rise to little or no response. The pain, warmth, swelling and effusion resulting from the injections usually subsided spontaneously within 24 hours, but in some patients was accompanied by typical acute gout involving joints in addition to those injected. Phagocytosis of urate crystals was observed in both the induced inflammatory response and spontaneous acute attacks of gout. The fact that urate crystals are observed in joint fluid of some gouty patients between attacks without phagocytosis suggests that factors in addition to the simple presence of urate crystals may be involved in the genesis and propagation of the

acute attack of gout. (Drs. Seegmiller and Howell)

Cystinosis

Studies have been made of the presumed abnormality of cystine metabolism which results in the deposition of cystine crystals in the tissues of patients with Lignac-Fanconi syndrome with cystinosis. The incorporation of cystine–S^{35} into urinary sulfur compounds has been studied in three cystinosis patients and in three non-cystinosis patients. Data now available show a substantially smaller amount of S^{35} in total sulfates in the two cystinosis patients than in the one normal subject whose studies are now completed. Studies have also been made of the rate of oxidation of cysteine to cystine by plasma of normal and cystinosis patients without revealing a significant difference. Likewise no substantial difference in activity of enzyme systems concerned with reduction of disulfide groups has been demonstrable in either erythrocytes or in liver obtained post mortem. These include cystine reductase which catalyses the reduction of cystine with DPNH as the cofactor, cystine transhydrogenase, which reduces cystine in the presence of reduced glutathione, and homocystine transhydrogenase, an enzyme which reduces homocystine and which has previously been described in other tissue by Racker. (Drs. Seegmiller and Howell)

Demonstration of Enzymatic Defect in Acatalasia in Human Cell Lines (in vitro)

It has been demonstrated that the metabolic defect of acatalasia persists in tissue culture obtained from not only the homozygous carriers of the abnormal gene but also from persons who are heterozygous for the abnormal gene. In the latter, no catalase activity was demonstrable in cell lines propagated *in vitro* as long as three months. Cell-free enzyme preparations from normal cells showed no inhibition of catalase activity upon addition of similar cell-free enzyme prepared from acatalasic cells. (Drs. Howell and Krooth, NIAID)

Clinical Trial of Anti-rheumatic Drugs in Rheumatoid Arthritis, Psoriatic Arthritis and Lupus Nephritis

Anti-folic Acid Compounds in Psoriatic Arthritis

This study is being conducted in collaboration with Dr. Eugene Van Scott and Dr. Robert Auerbach of the National Cancer Institute. As of this date 13 patients have been or are currently being studied. Eleven of the group have completed the protocol. The clinical response to amethopterin, administered in a series of three injections of 1 to 3 mg/kg at 10-day intervals, is being evaluated. Parameters of measurement have included sedimentation rate, changes in area and degree of skin involvement, the joint index reflecting the tenderness, pain on motion, and swelling of the joints, and morning stiffness. The results of the first 11 patients, although not statistically significant in this low number, have been encouraging. It is anticipated that between 20 and 30 more patients will be required for this study.

Side effects observed have been mild. The most prevalent complaint has been nausea and anorexia for the first 2–4 days following injection of the anti-folic compound. One patient developed oral ulceration and another alopecia. One death has occurred in this group two months following the final dose of the anti-folic compound. This death was due to cerebral thrombosis and was believed unrelated to the medication. Seven patients have shown a recurrence of psoriasis and arthritic symptoms between one and three months following the last injection. Four of these 7 have received a second course and have shown satisfactory improvement a second time.

Hydroxychloroquine

A study of the efficacy of hydroxychloroquine in treatment of rheumatoid arthritis has continued under the guidance of the American Rheumatism Association's Committee on Cooperating Clinics. The work on this project has been carried out at the D.C. General Hospital in the Georgetown Rheumatology Service Clinic. The current trial (trial II) has been completed and our Institute's participation was to the extent of 14 patients. The analysis of this double-blind study is as yet not complete.

Dexamethasone and Prednisone

The long-term followup study of these compounds has continued with the re-evaluation of the patients receiving this medication. Posterior subcapsular cataracts (PSC), reported last year, have been found in additional steroid-treated patients. In all, 95 rheumatic disease patients have been evaluated. Of these, 72 patients have received corticosteroid therapy. Thirty of these 72 had PSC on examination. This evaluation, an extension of the study reported one year ago, continued to show the same relationship of the development of cataracts to both the dosage and duration of corticosteroid administration.

6α Fluorotriamcinolone

Metabolic balance studies of a total of 3 patients have been completed, employing 20 mgm. of this corticosteroid daily (Drs. Whedon and Lutwak). Based on the observations of the first 2 patients there is some suggestion that this material is capable of causing a positive calcium balance. The result of the third study is as yet undetermined. Five patients have been previously studied in our Institute, receiving short-term course of 6α fluorotriamcinolone. The antirheumatic potency of this compound was found to be midway between that of dexamethasone and prednisone—⅓ to ½ the potency of dexamethasone and 1½ to 3 times the potency of prednisone. A study of the pituitary inhibiting potency was made on one patient, and it was found that 4 mgm. of 6α fluorotriamcinolone daily was inadequate to completely suppress the pituitary as measured by plasma hydrocortisone levels. This would suggest that the compound is somewhat less potent as a pituitary suppressant than is dexamethasone.

A long-term trial of the anti-inflammatory effect of this steroid has been instituted at the Rheumatology Clinic at the D. C. General Hospital. It is planned that 10 patients will participate in this trial, 3 of whom have already received the compound from 4–5 months each. The dosage employed has been from 6–10 mgm. daily. As of this date no significant side effects have been observed. These patients are receiving periodic radiologic examination to assess the degree of osteoporosis while receiving this drug.

High Dosage Corticosteroid Therapy in Lupus Nephritis

The efficacy of high doses of corticosteroids (equivalent to 50 mgm. of prednisone) in the treatment of the renal lesion of lupus has been reported by Robert Kark and co-workers at the University of Illinois. Their data show the significant reduction in mortality among patients treated in this fashion as compared with the 100% mortality in their control series. Their data also show regression of the renal lesions, as observed by serial biopsies, during high dosage corticosteroid treatment. We have observed at the Clinical Center four patients with systemic lupus erythematosus and biopsy evidence of nephritis. One patient was treated *without* corticosteroid therapy and two other patients have been treated with prednisone, 50 mgm. daily. In all three of these a repeat biopsy, performed three to four months later, showed regression of the renal lesion. The fourth patient has not as yet been treated, although the high dose regimen will be employed in this case. It is important that no effects have been observed in these patients at high dosage, other than severe Cushingoid facies and marked weight gain have been noted. (Drs. Black, Bunim, Kimberg, Wohl, Bitensky and Howell)

GASTRO-ENTEROLOGY UNIT

Whipple's Disease

Therapy

In all three patients with Whipple's disease studied at NIAMD it has been observed, as was previously reported by others, that patients may fail to respond satisfactorily to treatment with corticosteroids but respond dramatically when antibiotics are added. A remission of the disease is thus induced.

Hypoalbuminemia

With Dr. Thomas Waldmann of NCI we have demonstrated for the first time that the hypoalbuminemia of this disease is attributable, at least in some instances, to enteric leakage of albumin

and that this exudative enteropathy reverses itself when therapy induces a remission.

Electron Microscopic Studies

With Dr. William G. Banfield (NCI) we have studied the histological changes in intestinal biopsy specimens from patients with Whipple's disease using the electron microscope. We have found, as have two other groups recently, that the tissues contain unidentified bodies the size of small bacteria or large viruses. We have extended the observations made by others to show that the number of these bodies in the tissue appears to parallel the patient's clinical state. Attempts to culture these bodies using methods suitable for detecting bacteria or viruses have been unsuccessful to the present time. We have not yet had the opportunity to obtain tissue from a lymph node of a patient with Whipple's disease. Such tissue should offer greater chance to culture an organism if that is what these bodies are.

PAS Stained Bodies in Macrophages

With Dr. Samuel Spicer of NIAMD we have studied the histochemical appearance of the material that is present in tissue macrophages of patients with Whipple's disease and is detectable by use of the periodic acid—Schiff stain. Dr. Spicer believes that the PAS positive material is different from normal intestinal mucus, that it is not a sialomucin and that it is not a sulfated mucopolysaccharide. (Dr. Laster)

Hypogammaglobulinemia

Together with Dr. Waldmann of NCI we have completed a study that has demonstrated the following: Patients that present with hypogammaglobulinemia as their major disturbance have, in a high percentage of instances, an associated hypoalbuminemia. This has not been appreciated heretofore. We have studied six such patients, many of them having concomitant intestinal diseases, and have found that the hypoalbuminemia can be due to 1) impaired synthesis, 2) abnormal loss of albumin through the gastrointestinal tract, and 3) a combination of these two mechanisms. We have also found that when therapy is capable of improving the patient's condition to the extent of bringing the albumin levels back to normal in those patients with excessive enteric leakage of albumin, the gammaglobulin remains abnormally low and does not return to normal in parallel with the albumin. (Dr. Laster)

Hypolipidemia

In the last Annual Report we described a condition known as A-beta-lipoproteinemia. We have since acquired a second patient with this extremely rare disorder and, together with Dr. John Bieri of NIAMD, have shown that in both patients there is a striking deficiency of vitamin E.

We are also studying the genetics of this disorder and have found that although one of our patients has no beta-lipoprotein, his mother, father and sister have normal levels of this serum constituent. This finding contrasts with that reported from England in which each of the parents of a propositus had half normal levels of serum beta-lipoprotein. These observations leave the question of the genetic aspects of the disorder somewhat confused at the present time. (Dr. Laster)

Inborn Errors of Metabolism

In our previous annual report we indicated our intention to study the biochemistry of the human intestinal mucosa and to utilize this readily available tissue to study derangements of biochemistry in man. In collaboration with Drs. Topper and Segal of NIAMD, we have shown that intestinal mucosa from a patient with galactosemia has an extremely low capacity to metabolize galactose 1-C^{14} in contrast to mucosa from non-galactosemic patients. Thus, the intestinal mucosa is an excellent tissue for studying other inborn errors of metabolism, not only because of its availability by biopsy methods (and we have done about 250 intestinal biopsies since our unit has been started), but also because a wide variety of pathways of metabolism can be detected in this extremely active tissue.

Extending this further in collaboration with Drs. Field and Williams of NIAMD, we have studied a sibling and the two parents of a patient with von Gierke's disease and have found that the brother and mother had levels of intestinal

glucose-6-phosphotase that were comparable to those obtained in studies of mucosa from control subjects, but that the activity of the mucosa from the patient's father was half normal. We chose not to biopsy the patient himself because of a bleeding tendency (not uncommon in this disease). Since the intestinal biopsy is much safer than a liver biopsy, this observation extends our diagnostic armamentarium in relation to glycogen storage diseases.

We are in the process of developing methods to utilize this tissue to study other inborn errors of metabolism involving pentoses, purines and pyrimidines. (Dr. Laster)

Biliverdin Reductase

We found that crystalline albumin added to a pure preparation of this enzyme caused a marked stimulation of biliverdin reduction by DPNH but not by TPNH. This observation is being investigated further. The only incomplete area in this study is our inability to obtain satisfactory values for the stoichiometry of the reaction. This may be due to the fact that we do not have sufficiently pure biliverdin. This compound is difficult to prepare and we have recently come upon a new synthesis which may allow us to bring these studies to a satisfactory conclusion. (Drs. Singleton and Laster)

CLINICAL ENDOCRINOLOGY BRANCH

Carbohydrate Metabolism

Glucose

Last year a method was devised for analyzing the extent of glucose metabolism by the hexose monophosphate oxidative pathway (HMP) in intact human subjects. Detailed kinetic analyses for normal man were presented. The work has now been extended to patients with hypo- and hyperthyroidism. Interestingly enough, the proportion of the overall glucose metabolism attributable to this "shunt" pathway was decreased in both types of patients. The absolute quantity of glucose traversing the HMP pathway, however, was increased in hyperthyroidism and decreased in hypothyroidism due to the marked corresponding changes in overall glucose oxidation. As a part of these studies, a new and simplified method was developed for determining the specific activity of C^{14} gluconate by employing direct liquid scintillation counting of filter paper segments. (Dr. Segal)

The study of glucose metabolism in isolated tissues—particularly the endocrine glands—has continued. The *in vitro* effect of thyroid stimulating hormone (TSH) on thyroid slices has been examined further. A more sensitive assay for TPN and TPNH based on oxidation of 6-phosphogluconate-1-C^{14} was developed, and provided confirmation of the previous finding that TSH causes an increase in TPN. TPNH fell somewhat, but not enough to account for the rise in TPN. Attempts to obtain similar effects with thyroid homogenates were again unsuccessful. Epinephrine and serotonin, on the other hand, stimulate glucose-1-C^{14} oxidation in thyroid mitochondria and microsomes, apparently through increasing TPNH oxidation. Whereas epinephrine acts catalytically on thyroid slices, serotonin does not.

The effect of TSH on glucose oxidation in bovine thyroid slices has been employed as an assay for TSH in human plasma. The method is sensitive to .014 milliunits of TSH, and gives a linear response over the range .014 to 0.5 milliunits. Normal human plasma contains 1–10 milliunits/100 ml. (Drs. Field & Pastan)

In bovine anterior pituitary slices, the aldehydes corresponding to epinephrine and serotonin were as active as the amines in stimulating glucose oxidation, but the acid and alcohol analogues were without effect. The catechol amines and their aldehydes were effective in homogenates fortified) with TPNH, suggesting an action on TPNH oxidation, and this effect was localized to the supernatant fraction. A number of tryptamine and phenylethylamine derivatives related to epinephrine and serotonin also increased oxidation of glucose-1-C^{14} by pituitary slices and the effect could be blocked by monoamine oxidase inhibitors. (Drs. Field & Barondes)

The various zones of the bovine adrenal cortex were examined. Glucose-1-C^{14} oxidation and glucose uptake were higher in the zona glomerulosa compared to the zona fasciculata, whereas phosphorylase was higher in the zona fasciculata.

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ACTH was found to stimulate glucose-1-C^{14} and glucose-6-C^{14} oxidation in adrenals taken from hypophysectomized rats, but was ineffective in normal rat or bovine adrenal slices. Epinephrine and serotonin, however, stimulated glucose oxidation in normal adrenals. (Drs. Field & Williams)

Phosphorylase activity was also studied in the corpora lutea of bovine ovaries. This activity was stimulated by chorionic gonadotrophin as well as by growth hormone, but pituitary FSH, LH, TSH and ACTH were without effect. Thyroid and testicular phosphorylase were not affected by the corresponding trophic hormones, but chorionic gonadotrophin did increase phosphorylase activity in dog liver slices. Corpus luteum phosphorylase could also be increased by epinephrine, but was decreased glucagon.

Glucose oxidation was studied in rat salivary glands. Although this was stimulated by acetylcholine, epinephrine, serotonin and histamine, atropine inhibited only the acetylcholine effect, antihistamines only that of histamine, and monoamine oxidase inhibitor affected only the catechol amines. TSH and NaI had no effect on glucose oxidation—an interesting observation since salivary tissue actively concentrates iodide. (Field)

It is hoped that these studies will lead to a better understanding of the functioning of the endocrine glands.

Hypoglycemia

Hypoglycemia has been induced in normal human subjects by the administration of alcohol following a 48 hour fast. This effect, which had been noted clinically, is apparently not due to increased insulin release since there is no concomitant increase in plasma unesterified fatty acids.

The hypoglycemic effect of L-leucine in patients with islet cell tumors has been under investigation. Studies with isolated rat diaphragm showed no effect on glucose uptake, with or without added insulin.

In patients with leukemia, an artifactual hypoglycemia has been shown to arise from glycolysis by leukocytes after blood withdrawal. Methods have been devised to overcome this artifact. (Dr. Field)

Insulin and insulin antagonists

The effect of insulin in preventing K^+ and water loss from isolated perfused rat liver has received further study. A more closely controlled, paired perfusion technique has been developed, leading to the new observation that inhibition of K^+ transfer is accompanied by inhibition of glucose production. The time courses of both effects were similar, with a maximum at 90 minutes. Urea production was also reduced, but the time course was different. Gluconeogenesis was studied in livers depleted of glycogen, and it was shown to be increased by glucagon and decreased by insulin. This effect of insulin, however, contributed only 15% of the total insulin effect on glycogen-laden liver. It appears, therefore, that the major effect of insulin in the perfused liver is on inhibition of glycogenolysis. The relationship of this effect to that on K^+ and water are under investigation. (Drs. Mortimore & Wetzel)

Further studies on the metabolism of I^{131}-labeled insulin by the isolated perfused rat liver have shown that neither ACTH nor glucagon affect insulin degradation. This is taken to indicate that the degradation of insulin is specific and not shared by other proteins. As part of this study, it has been shown that labeled insulin is heterogeneous with respect to rate of degradation. A fraction high in diiodotyrosine content is more slowly degraded, and is presumed to be less active metabolically. (Drs. Mortimore & Tietze)

The plasma insulin antagonists obtained during different clinical situations have been shown to alter the insulin effect on adipose tissue and muscle similarly. In a study of further cases of chronic insulin resistance treated with adrenal steroids, there was a reduction of required insulin dosage as well as insulin antagonist in all. Plasma insulin binding capacity was also reduced in 4 of 5 cases, and insulin-I^{131} disappeared more rapidly than normal from the plasma of 1 of 2 cases. Plasma from uncontrolled diabetic patients was found to inhibit glucose uptake by dog retina. Although this effect was shared by plasma from non-diabetics with renal failure, control of the diabetes resulted in a disappearance of the inhibitory factor. This factor was shown to be non-dialyzable.

The studies indicate the presence of a varity of inhibitory agents which are variously affected by steroids or other treatment. (Drs. Field & Keen)

Glycogen storage disease

The assay of phosphorylase in leukocytes has been applied to several types of glycogen storage disease. It is low only in cases with deficient hepatic phosphorylase. In one family study, the patient's mother had a leukocyte phosphorylase level about 50% of normal, while the father's was normal.

In patients with glucose-6-phosphate deficiencies, jejunal mucosa obtained by biopsy has been studied. This enzyme was normal in the mother and in a sibling of the patient, but the father had a 50% reduction from the normal. The assay in intestinal mucosa was found to be relatively specific since only a small amount of phosphate was released from β-glycerol phosphate. The increased glucose-6-phosphate concentrations reported in erythrocytes and plasma of heterozygotes could not be confirmed. (Drs. Field & Williams)

Galactose and galactosemia

Metabolism of C^{14}-labeled galactose was studied in 8 galactosemic patients ranging in age from 6 to 30 years. All had the typical clinical disease in infancy and all had absent galactose-1-phosphate uridyl transferase in their erythrocytes. Nevertheless, 2 patients were able to metabolize galactose to CO_2 in nearly normal fashion. Thus, a subgroup of the disease has been discovered in which pathways of galactose metabolism exist in tissues other than red blood cells.

Factors regulating galactose metabolism have also been examined in erythrocytes from normal and heterozygous individuals, and in mixtures of normal and galactosemic erythrocytes. It was found that even in the presence of a profound depression in galactose oxidation, the stimulation of UDP galactose epimerization by a variety of methods could result in a considerable stimulation of galactose metabolism. Thus it appears that the transferase enzyme does not function maximally even when its level imposes a profound limitation on galactose oxidation. (Dr. Segal)

Amino Acid Transport and Protein Synthesis

The occurrence of aminoaciduria in galactosemia stimulated an interest in the mechanism of aminoaciduria, and studies with rat kidney cortex was undertaken. An energy dependent concentration of aminoisobutyric acid and several C^{14}-labeled amino acids was demonstrated in tubular cells. The energy source has not been identified, but is not glucose. Kinetic analysis indicated that the data best fit a 2 compartment system, and the rate constants for influx and efflux have been calculated.

This technique has been applied to several experimental situations. Maleic acid, which induces aminoaciduria in animals, has been shown to inhibit amino acid accumulation by rat kidney cortex slices. This results from an increased rate of efflux, whereas influx is unaltered. Phlorizin, which inhibits penetration of glucose and other sugars into certain cells, was found paradoxically to increase amino acid accumulation by rat kidney cortex. This was due to a decrease in efflux, the rate of influx again remaining unaffected. Growth hormone, which increases accumulation of α-aminoisobutyric acid in rat diaphragm, had no effect on the rat kidney preparations. In rats harboring pituitary tumors producing growth hormone, prolactin and ACTH, however, amino acid accumulation by kidney slices was increased. The effect was shown to be dependent on adrenal secretion. Other studies have demonstrated competitive inhibition of lysine accumulation in the presence of ornithine and arginine.

Similar work has been begun utilizing thyroid slices. Preliminary observations indicate that TSH stimulates amino acid transport into this gland. (Drs. Segal & Rosenberg)

The effect of growth hormone on incorporation of C^{14}-leucine into protein of rat xyphoid process was studied. No *in vitro* effect of growth hormone was detected, but prior administration of the hormone to hypophysectomized animals had a stimulatory effect. Oxidation of glucose-1-C^{14} in cartilage was, however, stimulated by *in vitro* growth hormone. (Drs. Williams & Keen)

Biochemistry of the Thyroid

Iodide transport

Studies of the iodide concentrating mechanism of the thyroid gland has continued with a detailed comparison of univalent anions other than iodide which are concentrated by thyroid tissue or which inhibit iodide "trapping". The concentration of TcO_4^- and ReO_4^- require cellular integrity, metabolic energy and K^+, as in the case of iodide, but the K_m values are 3×10^{-7}M and 1×10^{-6}M, respectively, as compared to 3×10^{-5}M for iodide. Their transport is inhibited by ClO_4^-, SCN^- and BF_4^-, which also inhibit iodide. The K_m values, or K_I values for a series of inhibitors, were shown to bear an inverse linear relationship to the partial molal ionic volume;—i.e., the larger the ionic volume, the greater the "affinity" for the thyroid transport mechanism. Such a correlation did not exist with crystal radii or limiting ionic conductances.

Preliminary studies have shown the presence of a ouabain-sensitive K^+-dependent ATPase in thyroid tissue. Correlation of this with iodide transport is under study. (Dr. Wolff)

Iodoproteins

The subfractions of beef thyroglobulin obtained by DEAE-cellulose chromatography have been the subject of continued study. They were found to be antigenically identical and to have the same neutral sugar content. They differed strikingly, however, in sensitivity to disaggregation by heat at pH 9.5. This correlated also with a difference in iodoamino acid composition, determined by spectrophotometric titration. The more stable fraction, which was retained more strongly on DEAE cellulose, contained 2.3 times as much iodine as the more labile fraction. The difference was mainly in diiodotyrosine, and some in thyroxine, but the monoiodotyrosine content did not vary. The differences probably reflect thyroglobulin synthesized at different periods of time and stored in the thyroid follicles. (Dr. Robbins)

The spectrophotometric procedure for iodoamino acid analysis employed above can be expected to have wide utility. It enables their determination in as little as 3 mg of intact protein. It has also been used in the study of thyroglobulin iodinated *in vitro* in various media. Iodine introduced in the absence of urea is found mainly in diiodotyrosine, whereas in 8M urea, a rise in monoiodotyrosine at low levels of iodination is observed. The formation of thyroxine is the same under both conditions, but no thyroxine is formed by the iodination of a trypsin digest of thyroglobulin. It appears that the secondary and tertiary structure of thyroglobulin influence the pattern of iodination, and that the primary structure is required for thyroxine synthesis. (Dr. Edelhoch)

Study of the antigenic (i.e., antibody-combining) properties of thyroglobulin has continued. Short-time tryptic digestion produces several components with precipitating activity, but these disappear after prolonged digestion. These digests, however, still contain antigen as shown by inhibition of precipitation and passive cutaneous anaphylaxis. The active fragments vary in size from an average molecular weight of 700 to 8000, but the former give only limited inhibition of precipitation. Physicochemical studies suggest that the antigenic activity is related only to the primary structure of the peptides. (Drs. Edelhoch & Metzger)

Thyroxine and iodotyrosine synthesis

The study of the *in vitro* thyroxine (T_4) synthesis in which 3,5- diiodophenylpyruvic acid (DIHPPA) is coupled to diiodotyrosine (DIT) has been continued. In an attempt to further the analogy to biological thyroxine formation, the DIT peptides glycyl-L-DIT and L-DIT-glycine were prepared. Both reacted with DIHPPA to form the corresponding peptides of T_4. Other DIT analogues also were effective. For example, diiodophloretic acid (desamino-DIT) plus DIHPPA gave tetraiodothyropropionic acid, and monoiodotyrosine (MIT) gave 3,3′,5′-triiodothyronine. In all cases, the yields were from 10 to 18%.

A simple method for preparing DIHPPA was found to be by the use of L-amino acid oxidase (in rattlesnake venom) and DIT. Conditions were worked out whereby thyroxine in 10–15% yield could be synthesized in a one step procedure by the use of snake venom and DIT. This represents a great simplification over the usual methods of thyroxine synthesis.

The foregoing reactions, after modifications allowing for the use of microgram or milligram quantities of reactants, were applied to synthesize various types of labeled thyroxine. Since DIT forms the inner ring and side chain of T_4, and DIHPPA forms the phenolic ring, the use of appropriately labeled starting material has led to the preparation of T_4-I^{131} labeled in the 3,5 or 3′,5′ positions and T_4-C^{14} labeled in the phenolic ring, or in the inner ring and side chain. By using the L-amino acid oxidase procedure with DIT-I^{131} or DIT-C^{14}, the formation of uniformly labeled T_4 has been obtained. These compounds will be extremely valuable for studies of thyroxine metabolism. (Drs. Cahnmann & Shiba)

Thyroxine transport in blood

A comparative study of thyroxine-protein interaction in the sera of 31 animal species, including mammals, reptiles, amphibians, birds and fish, was completed in collaboration with Drs. Farer and Blumberg (Epidemiology and Biometry Branch, NIAMD). Only primates had thyroxine-binding protein patterns closely resembling those in man, a finding of significance in studies of thyroxine metabolism in animals. These findings also may be useful in taxonomic studies. (Dr. Robbins)

In some animals with very low serum binding of thyroxine, as well as in certain human diseases, one zone of thyroxine in electrophoretic patterns was shown to be an artifact arising from dissociation of thyroxine and subsequent chromatography of the free thyroxine resulting from fluid flow on the paper strip.

An investigation of thyroxine-binding proteins in early human fetuses was carried out with sera collected while Dr. Robbins was a visiting scientist in Copenhagen, Denmark. Unlike the rabbit, the human fetus had thyroxine-binding proteins qualitatively identical to the adult, although differing in quantity. An interesting incidental finding was the presence in early fetal life of a post albumin protein in large quantity which is not present in adult serum or even in more mature fetuses. Further study of this protein awaits procurement of more fetal serum. (Drs. Robbins & Andreoli)

A study of the kinetics of thyroxine-binding by serum proteins was undertaken in collaboration with Dr. M. Berman (Biomathematics Office). In the initial studies, a rapid dialysis technique was developed based on dialysis from a thin layer. Free thyroxine dialyzed with a half time of 5 minutes and meaningful measurements could be made at 2 minutes after adding thyroxine to serum albumin or whole human serum. At 25°C, pH 7.4, and very low protein concentrations, the reaction was complete in this interval. More rapid measurements have now been obtained with a spectrophotofluorimetric technique, and preliminary results are encouraging. The procedure is of intrinsic interest since rates of this type have not heretofore been measured. In addition, the rates have a bearing on problems of thyroxine metabolism. (Drs. Robbins & Rall)

Previous studies of the kinetics of iodine metabolism in man have revealed discrepancies between assumed synthetic and metabolic mechanisms and the observed kinetics. Preparatory to an investigation of these matters, methods are under development for the detection of small quantities of labeled iodoamino acids in blood. Chromatography on a strong anion exchange resin has led to satisfactory separation of MIT, DIT, iodide and the thyronines from as much as 25 ml of blood. Methods for separating T_4 and T_3 are under study. (Dr. Lewallen)

Action of thyroxine on isolated systems

The inhibitory effect of thyroxine on crystalline glutamic dehydrogenase (GDH), which apparently results from dissociation of the enzyme into subunits, has been investigated further. Dissociation and enzyme inhibition was also produced by SCN, but this action was not reversed by ADP, in contrast to the finding with T_4. The molecular sites of the various actions have been under study. Since T_4 inhibits noncompetitively with respect to TPN and competitively with respect to ADP, it was concluded that T_4 inhibition is due to interaction at the "activating" site, not at the "active" site. T_3 interferes equally with fluorescence enhancement of DPNH and TPNH suggesting a third site on the enzyme that is kinetically inactive but which binds these cofactors. T_4 was found to inhibit metabolism of norvaline, α-ketovalerate, α-ketoisovalerate and α-ketobutyrate by GDH. In contrast, pyruvate reduction was first stimulated by T_4, but was inhibited by higher levels. ADP inhibited the metabolism of these keto acids, in

contrast to its stimulatory effect on glutamate and α-ketoglutarate metabolism. (Dr. Wolff)

The effect of thyroxine on the swelling of isolated mitochondria has been under study in collaboration with Dr. R. Michel and O. Michel at the College de France. The swelling produced by 10^{-7}M T_4 is accompanied by negligible (<2%) metabolism of T_4, indicating a lack of correlation between these phenomena. Although ATP inhibits the swelling effect of T_4, it has no effect on binding of T_4 by mitochondria, so binding and swelling also are without correlation. Iodine (I_2) causes mitochondrial swelling at a dose of ~5 x 10^{-6}M, and this effect is similar to that of T_4 in a number of ways. (Dr. Rall)

Dosimetry of I^{131} radiation

A general mathematical approach to the problem of radiation dosimetry to the bone marrow by I^{131} was developed. Calculated doses in a patient with metastatic thyroid carcinoma compared to reported cases of exposure to whole body x-ray in single exposures indicated that comparable hematologic effects were produced by larger calculated doses in the former. This discrepancy was attributed to: (1) recovery from radiation effect during the relatively prolonged exposure to I^{131} radiation, (2) absorption of β-ray energy by bone trabeculae and (3) inequality of blood and bone marrow isotope concentration. (Dr. Lewallen)

Physical Chemistry of Proteins

A study of the molecular properties of serum gamma globulin was pursued in collaboration with Dr. R. Steiner, Naval Medical Research Institute. Detergents and urea produced more profound structural modifications than did acid or base. The effect of alkali was seen even after extensive unfolding by detergent or urea. Fluorescence polarization was used as an indicator of configurational changes in addition to the more conventional methods, and was found to be a more sensitive indicator of such changes. A study of the denaturation kinetics indicated heterogeneity in γ-globulin, and it was interesting that a highly purified antithyroglobulin antibody showed a degree of heterogeneity similar to that of unfractioned γ-globulin. (Drs. Edelhock & Metzger)

METABOLIC DISEASES BRANCH

Mineral Metabolism

Osteoporosis

Metabolic studies from this Branch continue to produce evidence of the important relationship of dietary calcium intake to the pathogenesis and possible therapy of post-menopausal and senile osteoporosis, a disorder of thinning bones which by recent surveys has been shown to affect 26% of males and 29% of females over the age of 50 years. As the result of the work of this laboratory in conjunction with two English investigators, it is gradually being realized that the long-held previous concept of the pathogenesis of osteoporosis (solely impaired matrix formation resulting from hormonal imbalance) is inadequate. The idea of multiple etiologic factors affecting mineral utilization is gradually being more widely appreciated.

A. Effect of increased dietary intake of calcium on calcium balance. To date eleven patients with post-menopausal or idiopathic osteoporosis have been studied at several different calcium intake levels, balance studies being carried out for at least 30 days at each level. Ten of these patients showed increased retention of calcium up to an intake of about 800 mgm. per day. In six patients, the dietary calcium has been subsequently increased to as high as 2400 mgm. a day; three continued to show increasing retention of calcium at each higher intake level; one patient of the six showed improved calcium retention at an intake of 1600 mgm. a day but not above this level; and two actually showed more negative balance with increasing dietary calcium greater than 800 mgm. a day. The last two have been shown to have intermittent steatorrhea, and the intestinal absorptive defect for calcium is under further study. Two patients who showed retention of calcium at high intake levels have been re-examined after several years at the increased level and continue to demonstrate positive balances.

B. Effect of dietary phosphate on mineral retention—preliminary results. Since the molar ratio in which calcium and phosphorus are retained in bone is well known (1.5), the relationships between the relative amounts of these ele-

ments actually retained in the balance studies to the amounts presented in the diet was considered to be of interest and possible importance. This analysis was made possible by the fact that in most of the studies the phosphorus intake was changed relatively little as the calcium intake was successively raised. At a dietary molar ratio of 0.4, the retention ratio (calculated from calcium and phosphrous balances) was to the order of 0.8 (with a correlation coefficient, r, of 0.67); at an intake ratio of 0.8, retention ratio was 1.5 (r, 0.98); at intake ratio of 1.2, retention ratio was 7.8 (r, 0.18). The intake ratio of 0.8 was found in our studies at 1600 mgm. per day of calcium intake. These results suggest that (a) the calcium being retained *is*, in fact, in bone; (b), an optimal dietary ratio of calcium to phosphorus may be essential for best absorption and utilization of these two elements.

C. Effect of vitamin D on calcium balance. Moderate doses of vitamin D have been added to the high dietary calcium regimen in five studies on four patients with osteoporosis. In four of these studies, much more positive calcium balance was achieved. The full significance of these results is not clear, but they suggest that vitamin D, although hitherto believed to be closely associated only with osteomalacia among diseases of demineralization, may be a significant influence or factor in some cases of osteoporosis.

Bone Metabolism

Radioisoptic measurements of calcium kinetics in association with metabolic balance studies have been in use in this laboratory over the past five years as indicators of calcium metabolism and of the mechanism of influence thereon of various physiological factors. In addition to measurements of readily miscible pool size, a mathematical term, Ca_B, has been calculated, representing the rate of disappearance of tracer from the circulating pool after correction for urinary and fecal excretions. This value is assumed to represent the rate of incorporation of isotope into bone by the processes of new bone formation, of exchange with crystal surface calcium, and of exchange with more unreactive crystal calcium. Undoubtedly, this number may be slightly in excess of the "true" value for rate of calcium incorporation into bone, since no corrections are being made at present for losses in sweat, but it does provide a function for comparison.

A. Mechanism of effect of elevated dietary calcium intake in osteoporosis. The results of twenty-one radiocalcium studies in five patients with osteoporosis have thus far been analyzed. As has already been reported by this laboratory, Ca_B values in osteoporosis under control conditions were not significantly different from those in normal subjects. This finding has suggested that if the rate of incorporation of calcium into bone in this disease is normal, the process by which demineralization comes about over several years time may well be increased resorption, a suggestion recently supported in part by microradiographic studies. With respect to the influence of increased dietary calcium intake in osteoporosis, analysis of combined isotope and balance data have suggested the following conclusions: 1) increased dietary intake of calcium over several weeks led to increased absorption and retention of calcium; 2) since the miscible calcium pool did not increase, the additional calcium absorbed was deposited in the body outside of this readily available pool, most probably into bone, in view of the extent and duration of the calcium retention; 3) the gradual decrease in rate of incorporation of calcium into bone, Ca_B, with successive isotopic studies on increased calcium intake may be explained by the filling or saturation of osteones (bone units at the microscopic level) with mineral, others having shown that in "untreated" osteoporosis osteones are relatively unsaturated with mineral; and 4) since increased retention of calcium on increased calcium intake was not associated with an elevated Ca_B, the retention can probably be best accounted for on the basis of decreased bone resorption.

B. Mechanism of effects of various factors on bone metabolism in osteitis deformans (Paget's disease of bone)—preliminary results. Osteitis deformans is characterized clinically by very rapid bone destruction and reformation, the rate and extent of which is roughly indexed by a highly elevated serum alkaline phosphatase. Calcium–45 kinetics studies in six patients readily confirm this rapid turnover, indicating a large pool of readily exchangeable calcium, a high Ca_B and retention of a large fraction of administered isotope. Alterations in these indices upon administration of

various factors suggest that corticosteroids decrease the excessive rate of bone formation and that androgenic-anabolic steroids have the reverse effect. Increasing the dietary calcium intake resulted in no change in serum alkaline phosphatase, an increase in the exchangeable pool and a decrease in Ca_B and in the fraction of isotope retained; it is not clear as yet whether these latter findings indicate that increased calcium intake decreases bone formation or merely tends to saturate with mineral steones which ordinarily form so rapidly that they take up mineral poorly.

C. Electrolyte and mineral metabolic effects of synthetic adrenal cortical steroids. To date thirteen metabolic balance studies of the effects of five adrenocorticosteroids have been carried out in eleven individuals, including normal subjects, patients with rheumatoid arthritis and patients with generalized osteoporosis accompanying rheumatoid arthritis. These studies have each necessarily required several weeks, including an average of 33 days on the steroid plus lengthy pre- and post-drug control phases, because of the sluggish shifts of calcium as compared with the response of other elements to hormonal action. In summary, although the majority of the patients developed increased urinary calcium on the corticoids, the effects of fecal calcium were quite varied. In the normal subjects and in the patients with rheumatoid arthritis alone, fecal calcium usually increased. resulting in negative calcium balance. On the other hand, in patients with rheumatoid arthritis and osteoporosis, fecal calcium was decreased resulting in no change in calcium balance or in more positive balance. The locus of this difference in effect, whether at the bone or intestinal mucosa, is as yet undetermined. In addition to the above reported studies, during the past year a third 90 day metabolic study has been completed of the effects of a new synthetic, 6-alpha-fluorinated corticosteroid, but analyses are not yet complete.

D. Citrate metabolism studies in various bone diseases. Kinetic studies of citrate metabolism were undertaken because of the known high citrate content of bone and the likelihood that citrate may be importantly involved in the processes of bone resorption. Data obtained utilizing a constant infusion technique in 15 patients with bone disease and 8 normal subjects were analyzed with respect to a two compartment model. Computation was made of four major variables: apparent volume of distribution, primary mixing pool size, net fractional rate loss from this pool, and turnover of the pool. The following significant deviations from normal in citrate kinetics were noted: in osteoporosis, an increase in fractional rate loss; in severe Paget's disease, a three-fold increase in pool size and turnover; upon administration of adrenal corticosteroid, an increase in fractional loss rate; in hypoparathyroidism, a decreased turnover; and in idiopathic hypercalciuria, a decreased pool size and turnover. Certain of these findings, particularly those in Paget's disease, coincide with what would be anticipated from previous knowledge of bone metabolism in these disorders, but assessment of significance must await more detailed analysis.

Energy Metabolism

Studies of energy metabolism involving use of the Metabolic Chamber for continuous analysis of respiratory gas exchange over extended periods of time continue to be involved in characterization and analysis of obesity from both the physiological and metabolic point of view. Certain factors have become evident which contribute to a small but significant extent to the maintenance of obesity once established, notably pronounced physical inactivity under most circumstances and less increase in energy expenditure during exposure to cold than normal individuals exhibit. Energy balance observations, involving precise measurement of caloric intake and energy expenditure, have not as yet substantiated the idea of a "hypophagic obesity" type, as described by others, but patterns of energy expenditure analyses suggest the possibility of significant metabolic differences among obese individuals and between certain obese individuals and normal subjects. Studies also continue of important temperature regulation processes, including the interrelationships between metabolic response to cold, body insulation and body fat content, and the mechanism of response to cold exposure in patients with various types of fever.

Obesity

A. Energy balance during weight reduction. During the course of weight reduction studies in

obese patients it has become clear that total energy expenditure is reduced dramatically as weight is lost. Reductions of energy expenditure as large as 1200 Kcal per day have been observed. Each kilogram of body weight lost in the various subjects has been associated with a reduction in energy expenditure of from 30 to 70 Kcal per day. This pronounced decrease in energy output with weight loss is probably associated with two factors: 1) reduction in physical work required to move the body mass, and 2) loss of metabolically active tissue. The contribution of both factors is currently being assessed.

Not only was total energy expenditure over the whole day reduced upon weight reduction consequent to caloric restriction, but resting metabolism both awake and asleep was also reduced. In most patients studied the reduction in resting metabolism occurred, as one would expect, in proportion to the decrease in surface area or in other units of reference of body tissue in common use. In certain obese individuals, however, the reduction in resting metabolism was greater than would be expected. Since a similar observation had been noted previously with semi-starvation at similar caloric levels (600–1000 Kcal/day) in individuals of normal body composition who had minimal or no fat stores available for energy (U. of Minnesota), it is suggested that some obese individuals respond metabolically to diet restriction as though semi-starved even though obviously literally surrounded by abundant energy-loaded fat. Impaired fat mobilization and utilization may thus be one of the metabolic problems in certain types of obese individuals.

B. Metabolic characteristics of obese individuals. A limited number of biochemical analyses made during the course of weight reduction studies have shown a fall in total serum cholesterol, and increases in serum and urinary ketones, the ketone changes being accentuated during phases in which exercise was added to the regimen. Inter-individual variation was noted in other responses, some subjects showing a fall in serum phospholipids and rise in fatty acids. Responses to the administration of glucose also varied considerably.

C. Contribution of physical activity to weight reduction during caloric restriction. The contribution of physical exercise to negative caloric balance has been assessed at several levels of caloric intake. Interpretation of these results has been complicated by the fact that caloric compensation for imposed physical activity (daily walks) occurred during the remaining or non-walking hours of the day. This compensatory reduction in caloric expenditure was observed even when activities for the remaining hours were controlled. Compensation was never quite complete, however, and regular exercise still contributed significantly to negative caloric balance. The data suggested that the less the dietary caloric restriction, the greater the relative contribution of exercise to negative caloric balance and hence to weight loss.

The obese patients observed to date (both by visual observation and by scrutiny of continuous records of oxygen and carbon dioxide exchange) appear to lead distinctly hypokinetic lives. This hypokinesis apparently may be induced by the obese state and subside with the latter's removal, as suggested by one successfully reduced male and one female who appeared to increase their spontaneous physical activity in proportion to the extent of their weight loss. On the other hand, most patients thus far observed have not changed their physical activity habits as a result of weight loss. Two groups are suggested: 1) Inactive—a state perpetuated by obesity and lessened by weight reduction; enhanced mobility through extensive weight loss permits these individuals to perform activities they were formerly unable to do, for example, such simple things as bending over and tying shoelaces; and 2) Hypokinetic—lack of normal stimulus to move or be moved, or a lack of "appetite" for physical activity of any kind.

The relatively small daily energy expenditure associated with the hypokinetic state which tends to maintain overweight is fostered by the following impressive behavior; inactivity compensatory to imposed physical activity, use of body support whenever possible (e.g., when walking on the treadmill, a firm grip on the attached upright support was found to be necessary) and routine assumption of energetically economic body positions. In addition to these physical means, the saving of calories by the obese is enhanced by a metabolic device not frequently called into play:

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a metabolic response to cold which is minimal or absent.

Cold and Body Fat Content

It was demonstrated previously that an inverse relationship existed between body insulation and the metabolic response of near nude subjects to cold air. Insulation, however, was not completely identifiable with either total body fat or subcutaneous body fat. Subsequent investigation has revealed that obese subjects who lose considerable amounts of obesity tissue on caloric restriction follow the formerly established prediction equation for increasing metabolic response to cold as they lost weight and insulation. Therefore, no distinctive metabolic irregularities in obese subjects have been identified which suggest abnormal hypothalamic regulation of body temperature or heat production with implications for associated changes in hunger or satiety mechanisms.

Periodic Fever and Energy Metabolism in the Cold

Investigations of the metabolic and vasomotor response to cold air exposure have been carried out in patients with periodic fevers of natural (periodic Mediterranean fever) and experimental (malaria) origin, since both groups symptomatically suggest poor cold tolerance, the question arose as to whether temperature regulation against cold would be modified either transiently or permanently in these disease states. If modification of regulation should be seen, what inferences could be made concerning the mechanism of the aberration? The following is tentatively suggested by the limited data in hand: a) experimental malaria decreases the metabolic response to cold but recovery of a more normal response occurs within 3 weeks following the febrile period; b) one bizarre case of periodic Mediterranean fever failed to respond metabolically to cold while his body cooling patterns were remarkably a typical and highly variable, indicating either a lesion in or a lack of sensitivity of the central temperature regulating mechanism; and c) two periodic fever patients have responded to cold within normal limits when afebrile. There has been the suggestion in one patient that exposure to cold stimulated the occurrence of a febrile attack when the exposure to cold occurred in the "sensitive" period before an expected febrile period.

HEMATOLOGY UNIT

Blood Diseases

Neonatal thrombocytopenic purpura

Using the sensitive techniques developed for measuring immunoreactions involved in post-transfusion purpura (see publication), the syndrome of neonatal thrombocytopenic purpura was studied in six children born of four normal mothers. For the first time, it was clearly demonstrated that maternal immunization against antigens on fetal platelets was responsible for thrombocytopenia in the infant. Neonatal thrombocytopenic purpura was found to be in every way analogous to erythroblastosis fetalis, in that mothers who themselves lacked an antigen which was present on fetal platelets by inheritance from the father, developed either blocking or complement-fixing antibodies against the platelet antigen. The antibody, transmitted through the placenta, was capable of destroying platelets in the fetus and newborn. The appropriate form of therapy to be used in cases of isoimmune neonatal purpura is not clear as yet, for effects of steroids, splenectomy and other forms of treatment on the natural course of the disease cannot be evaluated without regard to the nature and level of the responsible antibody. With the recognition of different specific antibodies and means of measuring different types of antibody activity, evaluation of therapy will be simplified and is at present under study.

Platelet-antigen systems

Over the years there have been numerous attempts to establish platelet groups in the same manner that red cell groups have been established, but none of the platelet antigen systems reported so far have withstood the test of time. The isoantibodies found in this laboratory to be the cause of post-transfusion purpura and neonatal purpura, as well as isoantibodies which have developed in individuals receiving multiple transfusions, have permitted these NIAMD investigators to identify

and characterize clearly the nature and inheritance of at least *seven* platelet antigens. All of the antigens identified by these antibodies are inherited as codominant alleles; all of the antigens have differed from known erythrocyte antigens, and so far there has been no indication of linkage between these antigens and erythrocyte antigens or other inherited characteristics. By using quantitative complement fixation procedures and quantitative adsorption techniques, this laboratory has shown that the amount of antigen per platelet is directly proportional to gene dose. Thus they have been able to translate directly genetic phenotypes into genotypes and identify *three genotypes* on the basis of reactions with each antibody. The ability to genotype individuals directly has simplified considerably the determination of gene frequency as well as the differentiation of the various platelet groups. As a result of finding so many different platelet-antigen groups, these workers have proposed a systematic nomenclature (see publication) which we hope will prevent the confusion which has arisen in erythrocyte-antigen notations.

Platelet transfusions

During the past seven years there has been much interest in the possibility of replacing platelets in individuals who have thrombocytopenic purpura, due either to bone marrow failure, as in aplastic anemia, or to suppression of megakaryocyte activity by malignant cells, as in leukemia. Recent studies elsewhere have suggested that on a statistical basis, thrombocytopenic patients transfused with large amounts of platelet concentrates tend to bleed less frequently than untreated patients. In none of these studies, however, has an attempt been made to assess the function of transfused platelets by such tests for platelet function as clot retraction activity and correction of the bleeding time. In a series of patients, including leukemics and individuals with aplastic anemia, this laboratory has determined the survival of transfused platelets as well as the effects of transfused platelets on the bleeding time and clot retraction activity. In four of these patients platelets survived at what appeared to be therapeutically effective levels by platelet count, and the clot retraction of the patients' blood was corrected, but their bleeding time was not corrected. Since the bleeding time is perhaps the best test for the physiologic effectiveness of platelets, it appears that more is to be gained by evaluating the physiologic effectiveness of transfused platelets, than simply by a statistical analysis of bleeding tendency in large groups of patients who may or may not bleed regardless of therapy. These studies also indicate that packing of platelets, which is necessary when numerous normal donors are used to provide platelet concentrates, markedly decreases the effectiveness of transfusion primarily by diminishing the yield of circulating platelets. The haphazard response to packed platelet transfusions reported by other investigators appears to be due simply to the unpredictable effects of concentrating platelets, rather than to antibody formation or to changes in responsiveness of the patient.

The mosaic pattern of sites for attachment of antibodies on cell surfaces

A proposed model for antibody attachment and complement fixation on cell surfaces, described by equations derived by T. Hill (collaborator at University of Oregon, see previous publication) was found to be applicable to the problem of determining the precise geometric pattern of antibodies attached to cell membranes. From data obtained on the amount of complement fixed when relative concentrations of antibodies and cells were varied, and from calculations made using various proximity factors in Hill's equations, it has been possible to show that drug antibodies attach to cell surface sites which are arranged like the four corners of a quadrangle, that cells which contain one gene-dose of antigen for isoantibodies have antigenic sites arranged in a linear fashion, and that cells with two gene-doses of antigen have antigenic sites arranged as two parallel lines in close proximity. The ability to determine the spacial arrangement of antigenic sites on cell surfaces may have fundamental implications concerning the structure of cell membranes and the manner in which different types of antibodies cause cellular injury.

Anti-hemophilic factors

The thromboplastin generation test, which is the best technique so far devised for measuring anti-hemophilic factors (AHF), does not permit measurement of these factors when they are present *in vivo* in concentrations of less than 5% of normal.

Therefore, measurement of the *in vivo* half-life of anti-hemophilic factors has been possible only after relatively large amounts of these factors have been infused *in vivo*. The half-life of AHF appears to be in the order of 6 to 10 hours and of plasma thrombophastin component (PTC) in the order of 12 hours by this method. From numerous measurements of changes in the clotting time and prothrombin consumption produced by infusing minute amounts of anti-hemophilic factors in five patients with AHF deficiency and two patients with PTC deficiency, it was found that amounts of these factors in the range of .02 to 1% of normal produced correction of clotting time and prothrombin consumption which was proportional to the concentration of the factors. Standard curves relating the degree of correction of clotting time and prothrombin consumption to the *in vivo* concentrations of anti-hemophilic factors in the range of 0.02 to 1% permitted a simple assay for determining *in vivo* survival of these factors after infusing amounts equivalent to 0.25 to 1% normal concentration in hemophiliacs. The intravascular "half-life" of AHF by this method was found to be 8 hours, the "half-life" of PTC, 24 hours. After infusing large amounts of anti-hemophilic factors, the initial decay could be measured by the thromboplastin genertion test, and the subsequent decay from 1% to 0.02% by correction of prothrombin consumption. It was found that the initial decay of AHF and PTC was more rapid the larger the amount infused, but that subsequent decay of very low levels of the factor was greatly prolonged. Thus, it appears that once the extravascular compartments are saturated with anti-hemophilic factors, the decay of AHF is in the order of 12 hours instead of 6 to 8 hours, and the decay of PTC is in the order of 3 to 4 days, instead of 1 day. This information concerning the nature of extravascular stores of anti-hemophilic factors and the true biological "half-life" of the factors provides a basis for developing better therapeutic regimens with anti-hemophilic agents currently available, and the new method of assay will permit evaluation of therapeutic agents which may be developed.

Gamma-globulin

Studies of the nature of the reaction of a gamma-globulin which arises in otherwise normal individuals and appears to react stoichiometrically with AHF to inactivate this factor completely (see 1960 report) have been directed at measuring the survival of the gamma-globulin *in vivo* and the molar concentrations of AHF-anti-AHF involved in *in vivo* and *in vitro* reactions. The minimum concentration of anti-AHF required to inactivate all circulating AHF in a normal individual has been determined by *in vivo* infusions of the inhibitor, and the survival of the inhibitor *in vivo* has been determined. These studies not only provide information concerning the molar concentrations of a clotting factor which has been too labile to purify, and the production rate of the factor, but also indicate the intensity and degree of plasma relacement therapy necessary to treat the disease caused by the inhibitor.

Refractory anemias

Lack of production of erythropoietine does not appear to be an etiologic factor in "refractory" anemias. High concentrations of erythropoietine have been demonstrated in 23 of 25 patients with "refractory" anemia; in two patients the results of the bioassay were borderline. There also appears to be a correlation between the hemoglobin level, functional capacity of the stem cell and erythropoietine concentration. In general, a patient with a functionally intact stem cell compartment has a lower erythropoietine level at a given hemoglobin concentration than does a patient with refractory anemia, where the stem cell compartment is affected. Presumably this difference reflects utilization of erythropoietine.

Only one of nine patients treated with testosterone has responded with increased red cell production; this patient had an acquired hypoplastic anemia and the white cells and platelets were unaffected. The difference between the results with testosterone in this laboratory and those reported in the literature may reflect a difference in the etiology of anemias which morphologically and chemically may appear similar.

PEDIATRIC METABOLISM BRANCH

During the year 1961 problems selected for investigation in the Pediatric Metabolism Branch were cystic fibrosis of the pancreas, other diseases

leading to malabsorption in children and disorders due to glycogen storage.

Cystic fibrosis of the pancreas has constituted the objectives of many of the studies and much of the effort conducted in this Branch. During the past year a total of 110 patients with fibrocystic disease have been studied at the NIAMD, many of them referred from faraway States or even from abroad for further investigation of the diagnosis and treatment of this condition. Of this number 62 were hospitalized on the Pediatric Metabolism service and the remainder followed in the outpatient department of the Clinical Center.

Cystic Fibrosis of the Pancreas

Cystic fibrosis of the pancreas is a generalized disorder in which there is a widespread dysfunction of exocrine glands. Abnormalities in exocrine secretions include such varied findings as an abnormal physicochemical behavior and chemical structure of mucus and electrolyte abnormality in the composition of eccrine sweat and saliva. The common denominator responsible for the widespread dysfunction of so many and perhaps all exocrine glands and therefore the pathogenesis of cystic fibrosis has thus far escaped detection. It is generally agreed that the basic defect in this disorder whatever its nature is genetically transmitted probably as a recessive trait.

The investigations on this disease in the PMB have been conducted with the ultimate aim of elucidating the basic defect and thus establishing a better and more logical basis and approach to treatment of this disease.

Genetics and Electrolyte Abnormality

It is generally agreed that the basic defect in cystic fibrosis is transmitted as an inherited character. There is no general agreement, however, about the mechanism of transmission thought by most investigators to be a recessive trait but envisioned by others as an autosomal dominant character.

Studies have been pursued on evidence which has been gathered in the previous year in this Branch on the effects of varying genetic endowment on the diverse manifestations of the disorder itself. It has been thought that eccrine sweat defect in patients homozygotic for the gene of cystic fibrosis consists of two separate features: *one*, a high level of sweat electroyltes and, *two*, lack of the normal decrease in sweat chlorides and sodium concentrations in response to stress (e.g.: dietary salt restriction, administration of steroids, environmental heat). Evidence has been uncovered by us that a proportion of the heterozygotes for the gene of cystic fibrosis may show abnormally high levels of sweat electrolytes, but are capable of decreasing the sweat electrolyte concentration in response to stress situations.

It is becoming clear, therefore, that the unknown metabolic or enzymatic defect which may be the common denominator of the generalized exocrine dysfunction in cystic fibrosis may be absent in varying degrees in homozygotes, heterozygotes, and possibly intermediary cases according to the genetic endowment. This is true of other inherited errors of metabolism (e.g.: sickle cell anemia, galactosemia and others). If a similar mechanism obtains in cystic fibrosis it may account for some of the many variations in the degree of involvement and in the severity of the clinical picture.

The "sweat test" is used throughout the world in the diagnosis of cystic fibrosis of the pancreas. In an effort to determine further the values indicative of this disease, sweat levels determined in more than 1,000 control children and 550 patients with cystic fibrosis were analyzed for various parameters, such as effect of various types of stimulation and many others. A better and more precise definition of the values considered to be pathologic and the ones which are within normal limits were thus obtained greatly contributing to the diagnostic usefulness of these determinations and enhancing the value of this test as a tool for genetic and clinical investigation. (Gibson, di Sant'Agnese, Jones and Powell)

The possible relation of cystic fibrosis in the pediatric age group to many of the common gastrointestinal and pulmonary disorders of adults has been pursued. This is one of the challenging problems at the present time and is actively under study in this Branch in collaboration and in correspondence with many of the principal investigators in this country and abroad.

Mucus and Mucopolysaccharide

During the year of 1961 studies have been continued to elucidate and if possible to uncover the basic defect in mucopolysaccharide metabolism presumably responsible for many of the manifestations of the generalized disease. Investigations have been pursued by analytical methods in various body secretions. Leads uncovered previously as to the occurrence of distinctive mucopolysaccharide fractions have been pursued and further definition of their abnormal composition has been sought. In addition, studies have been continued to try to clarify the factors involved in the unusual physicochemical behavior of mucopolysaccharides, previously described. Investigations in the biosynthesis of mucopolysaccharides by studying the nucleotides involved have also been actively continued.

Previously it was shown by our group that in the duodenal contents of fibrocystic patients there is a mucopolysaccharide fraction which is easily denatured and rendered insoluble in water. Whereas this substance is present in almost all samples from patients, it does not obtain in any of the samples from controls. In collaboration with Dr. Dische, Department of Biochemistry, Columbia University, further investigations along these lines have been continued and work is proceeding on isolation and further analyses of these mucopolysaccharide components by a variety of different methods including continuous-flow electrophoresis, chromatography, fractionation with organic solvents. Factors which may explain this abnormal behavior are being further defined.

In collaboration with Dr. G. Ashwell, Laboratory of Biochemistry and Metabolism, NIAMD, studies on the biosynthesis of mucopolysaccharides have been actively continued. A number of compounds have been isolated and the structure and composition of some sugar nucleotides not isolated heretofore have been described.

A program has been initiated to determine the distribution of phosphorylated metabolites in the blood of normal controls and patients with cystic fibrosis. It is anticipated that information concerning the levels of organic, inorganic, and acid-labeled phosphates will provide meaningful insight into the problem of energy balance in cystic fibrosis. A defect in available energy might account for the abnormal sweat and secretory defects in other exocrine glands. (Gabriel, Pallavicini and di Sant' Agnese)

Pulmonary Involvement and Intestinal Malabsorption

From the clinical standpoint pulmonary involvement is the most important feature of cystic fibrosis and progressive lung disease accounts for more than 90% of deaths in this highly fatal condition. Further definition of the mechanisms involved are being actively pursued and therapeutic trials of various newer antibiotic drugs and physical agents are being investigated. Encouraging results have been obtained with the use of some of the new antiStaph synthetic penicillins. Other products of this and of another nature are being studied at the present time.

Investigations are also being continued in the intestinal malabsorption seen in cystic fibrosis by means of fat and nitrogen balance studies and other methods. Investigations have been performed on some patients with either normal pancreatic function or partial pancreatic involvement and interesting results have been obtained. Such studies have not been performed in any laboratory or hospital setting heretofore. The effects of various types of pancreatic extracts and antibiotic agents in the treatment of this disease are also being actively pursued. (di Sant' Agnese, Jones and Powell)

Glycogen Storage Disease

Congenital and usually familial errors of carbohydrate metabolism lead to a group of disorders characterized by accumulation of glycogen in various tissues and organs leading to enlargement and dysfunction of the structure involved, and frequently to death.

In this past year a number of studies have been performed in an attempt to provide an explanation for the surprising ability of some of the patients suffering from glycogen storage disease to sustain abnormally low blood glucose levels without undue difficulty. Contrary to presently accepted opinion, some of the intermediate compounds of carbohydrate metabolism were not found to be increased in various blood fractions. They can, therefore, be excluded as a primary

source of energy in the patients with this disorder suffering from hypoglycemia.

A new type of glycogen storage disease was uncovered and studies of a highly specialized chemical nature were performed in order to define further the basic metabolic abnormality. Investigations have also been carried out to clarify further the clinical picture and chemical findings in other types of glycogen storage diseases under observation. (Pallavicini, Gabriel, di Sant' Agnese and Powell)

Intestinal Malabsorption

Investigations have been continued on intestinal malabsorption in the pediatric age group other than cystic fibrosis of the pancreas. The recently developed fat absorption tests by means of fats tagged with I^{131}, the oral small intestinal biopsy, and the recognition of wheat and rye gluten as a noxious factor in the diet of many pediatric patients with malabsorption offer new tools for investigation of this very confused field. Several children with a variety of conditions leading to this clinical manifestation have been studied in the past year.

One of the major difficulties in the evaluation, diagnosis and investigation of patients with intestinal malabsorption has been the lack of simple and accurate methods for determination of steatorrhea and the impaired absorption of other substances. Work has been expended in the past year in the elaboration of simple methods that could be used widely by general practitioners and hospitals without elaborate laboratory and clinical facilities. In particular, tests using lipiodol, a commonly available substance, have been perfected and a xylose tolerance test adapted to the use of pediatric patients. (di Sant' Agnese, Powell and Jones)

The National Institute of Dental Research

INTRODUCTION

Summary statements by the Dental Institute's program leaders, introductory to the project reports of their principal investigators, provide in varying degrees of adequacy, a highlight account of research activities by organizational categories. While such reports understandably emphasize an integrated project effort within the respective segments of the Institute and make prideful reference to the more noteworthy evidences of productivity, the actual daily experience of research operations reflects an optimum orientation of the laboratory and branch chiefs toward programs of research rather than scientific disciplines.

Appreciably influencing the daily conduct of research activities throughout the Institute is a continuing series of regularly scheduled scientific sessions where program leaders share responsibility for making critical appraisals of programs and discuss implications for future planning. At these weekly luncheon meetings, investigators occupying positions of team leadership present detailed accounts of their programs. Thus, all echelons of research administration are kept informed of the total Institute effort; a forum is provided for the discussion and assessment of new and continuing programs; ways and means are sought to further program productivity; and relationships of the various programs to each other are considered in terms of actual and potentially fruitful collaborations with other NIH scientists and outside groups.

It has been said that groups conducting research are confronted with two tasks: to give relative weight to the goals of its individual members, and to establish priorities for the utilization of its total resources. Inasmuch as research programs are ideally characterized by forward movement with, however, an always existing prospect of reversal or stasis, some yardstick of measurement is desirable to assess the differential of productivity or diminishing returns. Recognizing that a judgment of accomplishment must frequently await the test of time, and that a presumably important contribution today may become the insignificant event of tomorrow, any inventory of research accomplished should be retrospective.

In attempting to evaluate our current program, a valuable take-off point is the analysis prepared five years ago for the HEW Secretary's Consultant Group on Medical Research and Education (Bayne-Jones Committee). This report, covering calendar year 1957, described the Institute's over-all program under three general categories: areas receiving major emphasis and warranting future expansion; areas presenting promising leads for future expansion; and minor project areas receiving evaluation for evidences of productivity and justification for continuation. The following comparative program descriptions, covering a five year span, hopefully will provide an objective view of the patterns of change, the successes and failures in terms of the foreseen and the unforeseen, and implications for the future.

[Note: Pages 228–233 are arranged in coordinate columns, *i.e.*, the column on the right is explanatory of material in the left-hand column.]

Program Activities for Calendar Year 1957

I. Major Program Areas—

A. Human Genetics:

Studies during this year were limited largely to an inbred population group in Southern Maryland (Brandywine Program). Among the more significant findings was an incidence of hereditary dental defects 300 times greater than previously reported for any other population group in the U.S. A correspondingly high incidence of other genetically determined conditions (sickle cell anemia, albinism, etc.) attracted the interest of other categorical Institutes of NIH as well as a number of investigators from outside institutions. While productivity was reflected in the publication of three papers, the major effort during the year was devoted to problems of program organization and planning for a considerably broadened base of operations to include other isolate population groups in the Eastern U.S. as well as an emphasis on the biochemical aspects of hereditary disease.

B. Periodontal Disease:

1. Epidemiological studies. Following the development and testing of a field method for the mensuration of periodontal disease, efforts were directed toward the compilation of a descriptive epidemiology. In the first of these studies, conducted in India (in cooperation with the WHO, the Indian Council on Medical Research, and the University of Michigan), data were assembled which showed a prevalence of advanced periodontal disease at earlier ages than ever previously recorded for comparable population groups in the U.S. While oral hygiene status undoubtedly contributed to this incidence, nutritional factors were an additional consideration. Another study in Alabama called attention to the importance of socio-economic factors in explaining the marked racial (negro vs. white) differences in prevalence.

(*Continued on page 229, column 1*)

Course of Program and Current Status

A considerable expansion during the past four years included (a) cytological studies of a newly described oral disease in an isolate population in North Carolina; (b) a study of hereditary patterns of dental development in the offspring of consanguineous marriages in Japan; (c) a study of human chromasomal aberrations in relation to growth defects such as clefts of the lip and palate, micrognathia and other oral and facial deformities; (d) a study of the genetics of secretor factor in saliva and its relationship to other genetic markers; and (e) the identification and study of additional isolate population groups in Eastern U.S. These activities led to the publication of fourteen reports during this period. Among the more significant contributions were the description of an heretofore unrecognized hereditary oral disease (benign intraepithelial dyskeratosis); the clarification of mechanisms of abnormal oral development based on longitudinal twin studies and analysis of family data; the identification of a rare gene mutation defect resembling Albers-Schönberg disease; and the collection of histochemical, genetic, social, physical, dental, and laboratory data from the Brandywine group. Major emphasis in the future will be given to the programming of this data for machine analysis.

A considerable expansion of epidemiological studies occurred during the past four years. Particular emphasis was given to surveys conducted in cooperation with the Interdepartmental Committee on Nutrition for the National Defense. Findings of significance were reported in a series of twelve publications. While it may be concluded from the narrative report of the Epidemiology and Biometry Branch that considerable progress was made, a major problem was encountered in the recruitment of professional staff. Because of the critical shortage of qualified dental epidemiologists in this country, much of the total program effort in epidemiology was directed toward preceptorship training of promising young investigators. Plans for the future include an increased attention to this inservice training pro-

(*Continued on page 229, column 2*)

A later attempt to implicate various chronic systemic illnesses was unsuccessful. The total year's effort led to the publication of five papers. Plans for the future were to collaborate with the ICNND in its international surveys.

2. Laboratory studies. Of particular interest during this year was a study to compare the biochemical pattern of inflammatory response in normal periodontal tissues with comparable structures in animals subjected to various systemic disturbances such as alloxan diabetes. The observed alterations in glycogen, DNA, and soluble organic phosphorous led to speculation regarding the factors that control resistance and susceptibility to inflammation. Other areas of laboratory research were concerned with the identification of microorganisms associated with periodontal disease, and the histo-chemistry of the attacked connective tissue. These studies were moderately productive and were reported in eight publications. Plans for the following years were to broaden the base of activity to include germfree studies and an emphasis on a better understanding of the composition and structure of collagen.

Broad expansion of laboratories activities included analytical studies of collagen and other proteins in relation to function and disease, and the utilization of germ-free technics to clarify the role of bacteria and calculus formation in periodontitis. Inasmuch as collagen is a major structural protein and appears to play a specific role in certain metabolic processes, an understanding of its molecular structure was considered essential to an understanding of pathologic processes in periodontal disease. The recent elucidation of the crosslinking mechanism between subunits of the collagen molecule may provide one explanation of the changes that occur in connective tissue with age and thus account for the greater prevalence of periodontal disease in elderly persons. However, the continuing complexity of the problem of periodontal disease may be illustrated by the further finding that calculus, a provocant of periodontitis, can form in the germ-free mouse, whereas, in the rat, there is evidence to suggest that it may be induced by inoculation of pure cultures of microorganisms recovered from diseased animals. The contributions from these and other laboratory studies of periodontal disease have been reported in over sixty papers during the past four years. While plans for the future are to continue an emphasis on the laboratory approach to this complex problem, a considerable effort will be made to develop a more meaningful and productive clinical program.

C. Nutritional Aspects of Oral Disease:

Major attention during 1957 was directed to the further demonstration of the role of nutrition in dental caries. Following the evidence that lysine supplementation in lysine deficient rats appreciably reduced the incidence and severity of caries, studies were initiated to evaluate the possible anti-caries effect of phosphate compounds. A substantial body of evidence was accumulated to show that a significant relationship exists and that the effect is presumably mediated through some local activity on the tooth surface. This

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Particular attention was directed during the past two years to the initiation of a caries study in American Indian children who were given $CaHPO_4$ in the form of a two per cent additive to the daily bread consumption. This study was planned in collaboration with the NIAMD, the Division of Indian Health, and the Bureau of Indian Affairs. Data accumulated after a one year period does not support the early laboratory evidence of a relationship between dibasic calcium phosphate and caries. However, a longer period

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hypothesis was supported, to some extent, by the evidence that mineral ash, calcium, and phosphorous content of enamel and dentin were not altered following extreme deficiencies or variations in the Ca and P of the diet.

The investigations pursued during this year were reported in three publications. Plans for the future included a clinical trial of the phosphate compound.

of observation will be required before a reliable judgment can be made. In another recently initiated study on the same population group, an effort is being made to evaluate the feasibility of using a dietary calcium additive as a means of reducing the skeletal burden of strontium-90. This project, in collaboration with the NIAMD, is formulated on the premise that the dilution of strontium-90 (ubiquitously present in dairy, grain, and other foodstuffs) with dietary calcium free of this nucleide, will bring about a reduction in strontium deposition in skeletal tissue.

The degree of activity in the nutritional program during the past four years is reflected by the publication of approximately twenty papers. While the dollar support has substantially declined since FY 1958, this field will continue to receive attention. Plans for the future include a detailed strontium and calcium balance study on Indian children brought to the Clinical Center from the caries testing program in South Dakota.

D. Bacteriological Aspects of Oral Disease:

1. Germfree studies. This program emphasized two major problems; technical operations and diet preparations. In the latter category, although the process of autoclaving was found to cause a considerable reduction in nutritional value of caries test diets, success was achieved in the maintenance of a caries-potentiating effect. In other studies designed to test microbial relationships, an unavoidable delay was experienced because of the limited capacity of the two available tanks. Although progress during the year was slow, a considerable expansion of activities was envisioned for the early future.

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Due to the considerable requirements for space, significant expansion of the germfree program continued to be delayed until facilities became available in the new laboratories of the Dental Institute in July 1961. Nevertheless, the previous years of limited activity were quite productive. The several contributions of note were related principally to studies of periodontal disease, dental caries, calculus formation, and the interaction of microbial and nutritional factors as they affect the oral and systemic welfare. Most significant, perhaps, was the finding that caries in the rat and hamster is a transmissible, infectious disease and that a single, host-specific microorganism can be implicated. Although the causative organism in each animal species is of similar type (anaerobic streptococcus), neither strain is cariogenic for the heterologous species. A further observation of significance is that since both organisms are potent producers of lactic acid, and neither produces extracellular proteolytic enzymes, acidogenesis is obviously not the only factor involved in caries initiation. An important direction of research during the next year will be the application of these findings to studies in man.

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2. Other microbiological studies. Major attention during this year was given to a study of bacteremia following dental treatment. Conducted in cooperation with the NHI, this investigation provided the important information that bacterial invasion of the blood stream, following routine operative and oral surgical procedures, was considerably higher (82–88 percent) than previously reported. The obvious implications were for more effective prophylactic procedures to remove risk to patients with rheumatic heart disease and other cardiac lesions. No plans were made to extend this study into succeeding years. The remaining activities in the area of microbiological research were devoted in large part to studies of the nutritional requirements and metabolism of oral bacteria, and the isolation, cultivation, and identification of oral spirochetes.

Personnel previously occupied with the bacteremia studies were able in the past several years to direct their attention to other program areas. In addition to the expansion of the gnotobiotic program, areas of emphasis during the past year have included viral infections of oral tissues; relationships between tissue enzymes and experimental infections; and the nutrition and metabolism of oral bacteria. Productivity of this over-all, expanded operation in microbiology is reflected by the publication of over forty papers (exclusive of those previously mentioned in connection with periodontal disease research) in the course of the past four years as compared with two in 1957. Plans for the future are to establish an Immunology Section for the study of cellular and humoral mechanisms of resistance to the oral microbiota; and a further exploration of tissue hypersensitivity to antigens of the oral microbiota in relation to periodontal inflammation.

E. Electron and X-ray Microscopy:

For the greater part, this program was directed toward (a) the determination of the crystal structure of calcified tissues by electron diffraction; (b) the investigation of the reaction of various chemical agents, such as fluoride compounds, on tooth surfaces, with emphasis on the mechanisms by which acid-solubility of enamel is altered; and (c) the development of technics for the application of contact and projection x-ray microscopy to studies of hard and soft tissues. While these objectives were, in great part, successfully met, considerably more was learned about the submicroscopic structure of organic matrix of calcified tissues than about the mineral content. These and other findings, were reported in four publications during the year. Plans for the future were to increase the scope of studies related to mechanisms of calcification, crystallographic approaches to a better understanding of the structure and composition of mineralized tissue, and further development of technics of x-ray microscopy for application to problems of interest in dental pathology.

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While earlier efforts emphasized the development of new and improved biophysical methods for studying calcified dental tissues, the current program has been characterized by a considerably broader area of activity. Receiving major attention have been studies to (a) characterize, in detail, the inorganic components of mature and developmental calcified tissues (including composition, atomic structure and dimensions of the fundamental apatite crystals in tooth and bone), and (b) describe the crystallization processes involved in normal and abnormal calcification (using both vital and model systems), and the chemical reactivity of the mineral phases.

Among the more challenging concepts that emerged from these studies were (a) that the major phase of all biological apatites is some calcium deficient hydroxyapatite with compensatory hydrogen bonding, and (b) that the higher chemical reactivity of young calcified tissue compared to older tissue may be a result of the gradual perfection of this calcium deficiency. The degree of program productivity during the past four years is demonstrated by the publication of over thirty papers. Plans for the immediate future will be to continue an emphasis on crystallographic studies (in parallel with studies of the organic

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II. Program Areas With Future Promise

A number of activities, although receiving modest levels of support during the year, gave promise of significant accomplishments in the early future. These included:

A. The development of new histochemical procedures for the demonstration and precise localization of enzyme activity in teeth and supporting connective tissues.

B. Studies of the physiological response of ambulatory patients to various general anesthetic agents.

C. Studies of prosthetic reconstruction procedures for maxillofacial defects and the formulation of principles of design that could contribute to improved functions of mastication.

D. Studies of the chemical composition of saliva in caries-free and caries-active individuals.

E. The development of caries-susceptible and caries-resistant strains of animals as an approach to the further study of genetic factors involved in the caries process.

The productivity of this heterogenous group of project activities was evidenced by the collective publication of ten papers.

III. Minor Program Areas

A number of research projects were pursued during the year with minimal support. Although designated as minor, each was evaluated carefully as a prospective area for future emphasis. Included among the studies were:

A. Biological effects of certain instrumentation procedures on dental pulps and surrounding tissues.

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matrix) in order to provide a better understanding of the structural alterations involved in malformation and caries.

While some portions of the program effort considered promising five years ago did not later justify significant increases in dollar support, their degree of productivity was sufficient to warrant continuation. These included the assembling of baseline physiological data for evaluating patient response to newer drugs used in general dental anesthesia, and the design and construction of obturators and other prosthetic devices used in cleft palate and post-surgical cases involving extensive loss of tissue.

Areas of current major emphasis that evolved from the earlier, modestly supported projects were: (a) an expanded program effort in histopathology and histochemistry that led to the development of new histochemical staining technics and ultimately to the discovery of a new connective tissue fiber (oxytalan), and (b) an expanded animal caries program that led to the discovery that caries resistance and caries-susceptibility in different strains of hamsters is governed by the absence or presence of a specific microorganism and not by genetic constitution alone. This major contribution to our knowledge of dental caries is now being further augmented by large scale studies to identify and classify the causative microorganisms in other animal species. It is hoped that the early future will see an application of these studies to a better understanding of the disease process in man.

The degree of productivity represented in the three continuing projects and the two major program areas is reflected by the publication of almost forty papers during the past four years (exclusive of those previously mentioned in the microbiology program).

While moderately increased levels of support were provided during the past four years for most of the project areas, particular attention was given to studies of the biological effects of operative dental procedures on pulpal tissue, and the effectiveness of certain chemical agents in the control of dental caries, and the pathogenicity of oral spirochetes. The following are some of the more

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B. The biological effects of fluorides.

C. The effectiveness of chemical agents in the control of dental caries.

D. Factors which influence the pathogenicity of oral spirochetes.

E. Diseases produced by fungi in the oral cavity.

F. Differentiation of strains of oral viruses.

Although varying degrees of progress were made, all the listed studies were considered sufficiently promising to warrant continued support in the next year. Accomplishments were reflected by the publication of seven papers in three of the study areas; pulp response to operative dental procedures, fluorides, and oral spirochetes.

significant accomplishments which have been reported during the past year in a series of eight publications.

1. Pulpal response to the newly introduced high rotary speed instruments, and to restorative filling materials was shown to vary directly with the speed and pressure used in instrumentation, the thickness of dentin overlying the pulp, the type of coolant used, and the size of the rotary cutting tool.

2. A pronounced caries-inhibiting effect was demonstrated in rats when the carbonyl-binding compound, sodium metabisulfite, was added to the diet. The degree of effectiveness approached 83 per cent reduction in caries when the chemical was made available on an uninterrupted basis for 90 days. Intermittent feeding of the compound for lesser periods continued to show a beneficial effect, although to a lesser degree. Parallel studies have been in progress to evaluate the effectiveness of other carbonyl-binding compounds.

Efforts in the next year will be directed to a continuation of these programs with the primary objective of evaluating newer dental restorative materials and rotary instruments; and testing a variety of chemicals for their anti-caries properties. Continued attention also will be given to the study of viral infections of the oral tissues, the role of bacteria in the formation of dental calculus, and methods of cultivating oral spirochetes.

While this review of intramural activities is necessarily deficient in its coverage of the Institute's over-all program activity, its purpose has been to deal in generalities so as to provide a readily assimilated digest of the relative productivity of different program efforts; the manner in which changes and trends in the recent past have brought us to the present; and the implications of these events for the future.

LABORATORY OF MICROBIOLOGY

Occupancy of the new dental research building in 1961 made possible for the first time geographical consolidation of the laboratory's programs, concomitant intensification of research in microbial physiology, and initiation of concerted effort in immunology. For convenience, the year's advances are summarized in the categories of dental caries, periodontal disease, microbial physiology, immunology, and virus research, although the research of most of the staff overlaps into several of these areas.

Our gnotobiotic studies of the etiology and pathogenesis of experimental dental caries in rats and hamsters are now extensive enough to permit some generalizations and to demonstrate the great ecological subtlety of this problem. To date, only acidogenic, nonproteolytic streptococci, of a kind differing from all recognized species, have been found to serve as the specific transmissible microbial factor in rodent caries. Well-known acidogenic, nonproteolytic and proteolytic streptococci, and a number of species from other genera are not cariogenic. Furthermore, cariogenic streptococci

from rats are not cariogenic in hamsters, and vice versa. Explanation of these specific host-parasite associations should be very significant for human dental caries. This should be greatly facilitated by the recent development of a much more nearly defined cariogenic diet than hitherto available, which enables better control of this important determinant of caries. A logical sequel to the discovery of a specific etiological agent of caries would be development of a protective vaccine. To date vaccines of the cariogenic streptococci have engendered no detectable circulating antibody in tests with hamsters. To be sure, the absence of such antibody does not preclude active resistance to infection. However, vaccinated hamsters have as yet evidenced no increased resistance to caries. In another kind of attack on the caries problem, continuing studies of the inhibition of experimental caries by sodium metabisulfide have shown that certain addition complexes of this salt with aldehydes and ketones were also effective caries preventives; these derivatives might offer practical advantages. Also, even intermittent administration of the anticaries agent, e.g., for one day each week, reduced the caries scores by nearly half. It is felt that these experimental studies offer a sound basis for serious consideration of clinical trials of these anticaries agents, as in a dentifrice.

Formation of dental calculus in the gingival area is recognized as the most important immediate provocant of periodontal disease. Systematic studies of the bacterial deposits that initiate calculus deposition are therefore essential. Contrary to the general impression that filamentous forms predominate, our studies show that various species of streptococci outnumber all other types at all stages of calculus formation, and in both supra- and subgingival locations. Large numbers of *Neisseria* species, gram-negative bacilli, diphtheroids, and fusobacteria are present during the first two weeks (starting with clean teeth), but then decrease. Filamentous organisms such as *Actinomyces* and *Nocardia* species, *Leptotrichia buccalis*, and *Bacterionema matruchotii* were rare during the first two weeks but increased steadily thereafter. The over-all predominance of streptococci alters sharply the focus of study of the etiology and prevention of dental calculus. Accordingly, the calcification of various oral bacteria has been studied in a model system *in vivo*, namely, within cellophane sacs implanted intra-abdominally in rats. Under these conditions the bacteria receive a constantly renewed supply of nutrients and calcifying fluid without interference of body cells, mucus, and the like. These seems to be little specificity at the calcification stage, for all of a variety of oral bacteria tested have developed calcifications, both extra- and intracellularly, strikingly similar to those seen in human dental calculus, as judged by optical and electron microscopy, and X-ray diffraction.

In the field of microbial physiology, continued study of the enzymatic synthesis of folic acid by lactobacilli elucidated the role of phosphorylation of a reduced pteridine as an intermediate step. In another study, metabolism of galactosamine by a lactobacillus was shown to proceed via galactosamine-6-phosphate, which is deaminated to yield ammonia and tagatose-6-phosphate. A third study concerned the role of lipoic acid in the dissimiliation of lactic acid. The evidence indicates that lipoic acid functions as an electron acceptor to convert lactic acid to pyruvic acid as the first step in dissimilation. A fourth project concerned the biochemistry of growth and development, as investigated through the biochemical factors initiating and controlling morphogenesis of *Dictyostelium discoideum*. Stimulation of differentiation is ion-specific (potassium, magnesium, and phosphate stimulate, but sodium chloride and sulfate do not) but not critically dependent on ionic strength. Hydrogen-ion concentration likewise is not critical throughout the range pH 5 to 7. There is no correlation between ability of a substance to chelate and its ability to stimulate differentiation. Histidine accelerates morphogenesis during the early stages, but apparently not by stimulating protein synthesis. Rather surprisingly, highly effective stimulators of differentiation do not increase respiration of *D. discoideum*.

Continuing studies of the pathogenic potentialities of gingival bacteria in relation to periodontal disease developed on the immunological side. It was shown, contrary to all expectation from the literature, that vaccination of mice with killed or living veillonellae engendered significant resistance specifically against the lethal effect of glucolipid endotoxin from the homologous bacteria. On the other hand, guinea pigs and rabbits vaccinated with oral viridans streptococci developed

typical allergic inflammatory reactions to intracutaneous injections of streptococcal protein. In a separate immuno-chemical study, use was made of the ability of endotoxins to coat erythrocytes and make them agglutinable by homologous anti-endotoxin serum (indirect hemmagglutination); in this way, five well-distinguished serotypes of *Veillonella* species were discerned, although chemical analyses revealed no significant difference in chemical composition of the respective endotoxins. Another aspect of endotoxic activity relates to nonspecific changes in host resistance. It was found that certain doses of glucolipid produce in mice a marked elevation of the plasma level of lactic dehydrogenase. However, this phenomenon is not a useable or specific measure of endotoxic action but (as in the generality of cases) an indication of massive trauma, for it develops only with nearly lethal doses of endotoxin.

As an outgrowth of the foregoing experiments, collaborative studies with the National Cancer Institute revealed a contaminating virus in several supposedly purified virus strains, namely, a hitherto unsuspected virus whose only known manifestation is a many-fold increase in plasma lactic dehydrogenase. The specificity of this manifestation, in contrast to the increase of plasma lactic dehydrogenase owing to trauma, is indicated by the lack of a concomitant increase of plasma glutamic-oxalacetic transaminase. Two other virus studies were brought to fruition during the year. Five serogroups of herpes simplex virus have been defined by kinetic analysis of the neutralization reaction between the respective strains of virus and homologous and heterologous antisera. Finally, definitive evidence has established, by serial transmission in tissue culture, the occurrence of a presumably new virus in the salivary glands of germfree rats but not in any of the many other organs tested. It is believed that this is the first viral agent isolated from germfree animals. It has been found also in nearly all ordinary laboratory rats of the same strain.

LABORATORY OF BIOCHEMISTRY

Enzyme Chemistry

The major accomplishments of the Section on Enzyme Chemistry concern the increased understanding of the chemical nature of two enzymes: carboxypeptidase B and aldolase. At this time the mechanism of action of no enzyme can be explained in terms of the chemical structure of the protein; therefore, the progress made in describing the catalytic properties of these enzymes is of significance in leading to an understanding of the mechanism of enzyme catalysis in general.

The activity of carboxypeptidase B as studied by Dr. Folk and Dr. Wolff was found to depend upon the single atom of divalent metal incorporated in each molecule of enzyme. A metal (cadmium) that produces a very active catalyst for one type of substrate (an ester) is completely inactive with another type of substrate (a peptide) that is split rapidly by enzyme containing another metal (zinc or cobalt). Further analysis of the reactions catalyzed by these enzyme variants with several substrates and inhibitors has permitted the estimation of rates of the partial reactions that compose the over-all reaction. The determination of the effect of each metal on the separate processes of substrate binding, product binding, and substrate splitting is becoming possible through these kinetic analyses, and there is now a prospect for defining the role of the metal in this type of enzyme.

The principal developments in the studies of aldolase conducted by Dr. Mehler are: the evidence supporting the identity of the enzyme-substrate combination that is measured spectrophotometrically with the catalytically active Michaelis compound, the appreciation of the role of electrostatic forces in binding the enzyme and substrate through analysis of the effect of ionic strength on the kinetic and spectrophotometric phenomena, and the alteration of the enzyme by proteolytic degradation to give a new enzyme species with altered catalytic properties. These findings have permitted the elaboration of a model of aldolase that is being tested and refined through current studies.

Prenatal and Dietary Factors in Experimental Dental Caries

In continuation studies by Dr. C. T. G. King the influence of prenatal factors on dental caries and development of oral structures was investigated. This year's results confirmed previous

evidence that acidosis increases the susceptibility to dental caries in the progeny of white rats. Since non-specific stressor agents, corticosterone, tremorine, electroshock, and induced anemia, did not increase caries susceptibility, it appears that acidosis is acting as a rather specific agent. In additional studies on the relation of variations in amniotic pressure to the development of the foetus, agar was introduced into the amniotic sac to produce 50 percent resorption. Of the remaining viable embryos, 37 percent had cleft palate, 29 percent had limb deformities and 6 percent had a variety of deformities including umbilical hernia. Upwards of 50 percent of the viable embryos which appeared grossly normal demonstrated skeletal malformations.

In other studies by Dr. F. J. McClure on the effect of phosphates on experimental rat caries, results demonstrated interesting variability in the cariostatic effect of different phosphate minerals. Added to phosphorus deficient cariogenic diets the soluble phosphates, i.e., ammonium phosphate, and sodium metaphosphate were very cariostatic. A complex commercial insoluble phosphate, victamide was not cariostatic. With respect to organic phosphorus compounds, sodium phytate was again demonstrated to be highly cariostatic, and in addition phytin, a calcium and magnesium phytate, as well as betaglycerol phosphate proved to be highly cariostatic. The results with the organic phosphorus compounds are most provocative particularly as regards the availability of their phosphorus within the oral cavity. The results pose interesting speculation as regards a significant role of phosphorus in the control of dental caries.

Saliva Chemistry

Under the direction of Dr. I. Zipkin research has continued to perfect analytical procedures for the spectrophotometric determination of tyrosine and tryptophan in parotid saliva. Correlation with chemical procedures is satisfactory particularly with respect to tyrosine. A good correlation was found also for tryptophan but the presence of a constant non-tryptophan ultra-violet absorbing material was also indicated.

In collaboration with Dr. L. Avioli (NCI), a study was made of the transport of calcium by the parotid gland. Following injection of Ca^{47}, the salivary Ca^{47} showed a maximum specific activity between one fourth and one half hours. Ca^{47} was always lower in the saliva than in the blood and paralled the blood Ca^{47} after the first 2-4 hours. The difference in specific activity between blood and saliva may reflect a delay mechanism from blood to saliva, or may represent a relatively non-measurable pool of salivary calcium.

Protein Chemistry

The major problem under investigation by Dr. K. A. Piez was the structure of collagen and its role in metabolic processes. Several important advances were made during the past year. Specifically it was shown that the collagen molecule contains subunits which probably correspond to single chains. There appear to be three such chains in the molecule of which one is different in composition and properties from the other two. The two types can be divided and studied separately. Further studies are in progress to more completely characterize these subunits. Dr. Piez has also shown, in collaboration with Dr. J. Gross of Massachusetts General Hospital, that as collagen matures *in vivo*, intramolecular crosslinks form. This results in the formation of chain pairs which can be separated from the single chain subunits. The process of crosslinking continues intra- and intermolecularly and appears to be intimately involved in the maturation of the connective tissue and the development of the properties essential to the normal function of the tissue. For example in lathyrism, a toxic condition in which connective tissue has a drastically lowered tensile strength and the collagen is easily soluble in neutral salt solution, crosslinking between chains occurs at a greatly diminished rate. This maturation process is amenable to further study since it also occurs *in vitro* in purified collagen stored at 37°.

Dr George Martin studied mineralization in the aorta as isolated from rats. Incubated in serum, the aortas fix calcium and phosphate as hydroxyapatite. Since elastase, but not collagenase or other proteases, prevents the mineralization, it appears that in this system elastin rather than collagen may be active in the initiation of a process related to calcification. This recent observation

indicates that this system may provide a new approach to the complex problem of calcification.

In a collaborative study with Dr. Davie of Western Reserve University, Dr. Folk and Dr. Gladner (NIAMD) have shown the β-lactoglobulins A and B, which are genetic variants, have different amino acid residues in two positions in the polypeptide chain. The amino acid differences are sufficient to explain the different properties. These results have important genetic implications, with regard to the molecular events responsible for the expression of a mutation.

An important part of the program in protein chemistry is the learning and development of new procedures. In this area, Dr. Lewis has become proficient in the use of the ultracentrifuge and associated equipment and devised new methods for the determination of molecular weights and other properties of macromolecules. In parallel technical studies, Dr. George Martin has put into operation a gas chromatogram which can be used for the analysis of many classes of compounds. This relatively new tool will permit many studies not previously possible. Equipment for monitoring radioactivity in a flowing stream has also been acquired and tested. Methods have been devised which will permit simultaneous determination of concentration and radioactivity in the effluent from columns used to separate amino acids and proteins.

Calcification and Strontium Studies

In cooperation with Dr. S. Natelson, The Roosevelt Hospital, New York, Dr. R. C. Likins is exploring the feasibility of using a dietary calcium additive as a means of reducing the skeletal burden of strontium-90 in children. The study was made possible through the current study by the NIDR and the NIAMD to determine the cariostatic effect of a calcium phosphate additive to bread.

The special study group consists of 500 children equally divided into a test group receiving bread supplemented with $CaHPO_4$ and a control group receiving unsupplemented bread. Children in both groups were given 5.0 mg. of strontium as the chloride twice daily for ten days. At the end of this period, 20 ml. of blood were obtained from each of the subjects. Studies are currently in progress to ascertain whether or not the calcium supplement has significantly reduced the serum Strontium: Calcium ratio. Strontum: Calcium ratios in urine samples are also being determined in order to provide information on the absorption and excretion of strontium.

In an additional project, Dr. Likins in cooperation with Dr. Jacob Menczel (NIH Foreign Fellow from Hadassah University Medical School), has initiated studies on osteoporosis as induced in white rats. Specifically, one objective is to evaluate the relation of fluoride to bone resorption. Thus far the technique for the total immobilization of the rat for periods up to two weeks by means of a whole body cast has been perfected. During this period of induced immobilization, bone resorption occurs, and may be studied by means of the calcium balance and subsequent tissue analysis. In due time the investigation will be extended to include a study of the effect of orally administered strontium, as well as fluoride, on osteoporosis.

LABORATORY OF HISTOLOGY AND PATHOLOGY

The program of the Laboratory of Histology and Pathology is broadly subdivided into three categories: biophysical studies of the mineralized tissues, histochemical investigations of connective tissues, and experimental pathological studies of dental caries.

The present year has seen both a marked improvement in the armamentarium of equipment available for the biophysical studies, and the addition of a number of essential personnel. Although it is not generally realized, enamel and dentin were by several years the first of the organized tissues to be studied by electron microscopy and diffraction, and it was in this laboratory that the earliest beginnings were made. Over the ensuing years, rapid technical advances occurred in this area, and in general it was possible to keep abreast of the times. However, much of the equipment was rapidly becoming obsolescent, particularly the available electron microscopes, and the acquisition of a modern high

resolution instrument this year was of vital importance. In addition, other newly-developed related instruments, such as those involved in contact and projection microradiography, have recently come to the forefront as valuable tools for both structural and analytical studies of the mineralized tissues. The laboratory is now well equipped for these applications of X-ray techniques. Further, it is hardly possible to operate a well-rounded program in biophysics without the utilization of X-ray diffraction and spectrographic methods. This year we have added to our staff two physical chemists, experienced in crystallography, and have equipped a modern crystallographic laboratory.

The basic ultrastructural studies of the crystalline component of dental enamel have been continued, with an eye toward defining more accurately the dimensions of the crystals and their orientation within the prismatic units of the enamel. This work has been carried on mainly by electron microscopy and diffraction of mature and developing material, and it presently appears as if the crystals are considerably longer than previously thought. Determination of crystal arrangement, which is quite intricate, has been largely a matter of morphological definition. A clear understanding of enamel crystallinity is essential for interpretation of the structural alterations involved in malformation and caries.

During the latter half of the year, with the opening of the crystallographic laboratory, investigations of the crystal chemistry of the calcium phosphates (apatites) involved in mineralized tissues were initiated. These parallel the ultrastructural studies, and are concerned with such matters as the atomic arrangements within crystals, crystal texture and perfection, and the chemical properties of the mineral. The concept of "defect apatite," characterized by missing calcium atoms in the crystal lattice, as the crystalline substance really present in calcified tissues, is being pursued, both by X-ray diffraction and infrared spectrophotometry. The latter technique provides a means of determining the hydrogen bond content of the apatite, which is thought to compensate for the missing calcium atoms. There have already been numerous ramifications of the work on crystal chemistry, the principal one being a study of the effect of fluoride on crystallinity in bone. The results indicate the presence of improved, or more perfect, somewhat larger crystals in the bone of animals which have been exposed to fluoride-containing drinking water. It has also been shown both *in vitro* and *in vivo* that Sr^{89} is rejected to a greater extent by larger apatite crystals than by smaller. This has led to the initiation of a study of the reaction to Sr^{89} of bone apatite in animals exposed to fluoride for periods of up to a year, on the assumption that larger, more perfect crystals should be present than in non-fluoride animals. All of this latter work has a bearing both on the basic biological mechanisms in water fluoridation, and on the relation of fluoride content of hard tissues to retention of ingested strontium (fallout).

The problem of mineralization itself has for some time been of prime interest in this laboratory. Inasmuch as numerous workers in biochemistry and microbiology have similar interests, it is natural that in this area our personnel should be involved in considerable collaborative, as well as individual effort. The primary objective, from the biophysical and histological standpoints, is to determine the relation between inorganic crystals and organic matrix, the mechanisms and sites of crystal nucleation, the sequence in which mineralization occurs, and the cytological basis for calcification. All of these facets are being approached by electron microscopy, microradiography and related techniques. Several projects within the laboratory concern normal mineralization of various substances. One, a microradiographic study of the mineralization sequence in rodent enamel, is of particular interest, because it has been found that the pattern is not the same as in other species, including the human. This is especially important, because the rodent is the most commonly used of all experimental animals. Another study deals with the development and mineralization of cementum, a tissue which has been largely neglected, and yet is of great importance as a dental constituent involved in periodontal disease. Mineralization is also being studied in ectopic calcifications such as calculus; inasmuch as this substance forms externally on teeth, it can be handled experimentally in the form of deposits laid down on plastic strips fastened to the teeth of human subjects. A series of studies has been completed in which a first, membranous structure has

been identified, on which a microbial and fibrillar organic layer is subsequently laid down. Deposition of mineral (apatite) within this matrix has been followed, as well as the crystal growth which takes place. Various collaborative studies on the mechanisms of calcification have also been continued; these include such systems as intraperitoneal implants of collagen, bacteria, and other organic matrices. It is of interest that both native and certain reconstituted collagens, as well as living or dead cultures of micro-organisms will mineralize when implanted in dialysis bags. These findings suggest that the factors heretofore thought to be involved in promoting or inhibiting calcification need further investigation. A beginning has been made over the last two years in the study of abnormal mineralization. For this first work at the ultrastructural level it was felt that a test system should be employed which was fairly well defined physiologically as well as histologically at the optical level. The pathological condition selected was avitaminosis D, established by a rachitogenic diet. Attention was this year directed toward a comparison between dentin formation in normal and in rachitic rats. The electron microscopic observations indicate that in tooth formation in rachitic animals dentin development seems to begin normally, and then suddenly becomes atypical. The odontoblasts associated with formation of the tissue become distorted, and there is a marked delay in the production of collagenous matrix fibrils, and an even greater lag in mineralization. Once under way, matrix formation and calcification seem to progress normally, so far as microstructure is concerned. Beyond the direct data obtained, studies such as this are of some importance in several other respects. First, they provide a basis in experience for forming some impressions on the degree to which ultrastructures in the developing tooth germ may reflect very acute generalized pathosis in bone formation and calcification. These ideas will serve as a guide in planning future experiments in pathological mineralization. Second, they give new information on the possible interrelationships between cells and the extracellular production of organic matrices and their mineralization. This in turn bears on the common present-day problem in cytology, the relation between structure and function.

Turning to the histochemical program, emphasis has for the past few years been placed upon determination of the chemical composition and reactive groups in normal and diseased connective tissues. These investigations are not only contributing to our general knowledge of the connective tissues, but also have a direct bearing on the structure and function of the supporting tissues about the teeth. A new type of fibers, named oxytalan, was discovered in this laboratory several years ago. These fibers have now been shown as a normal constituent in various specialized connective tissues, such as periodontal membranes and ligaments. In addition they have been found to develop in the reparative tissue following damage in periodontal disease, radicular cysts and granulomas. Current comparative embryologic and histochemical studies have indicated that the fibers probably should be classified in the elastic group, most likely being pre-elastic in nature.

Further histochemical investigation of a large series of human specimens obtained at autopsy has led to the finding that bone and periodontal tissues also undergo degradation at sites remote from the inflammatory foci in periodontal disease, and without concomitant localized inflammation. Observation of this phenomenon leads to some questions regarding the role of inflammation in periodontal disease, and future work will be directed toward clarification of this point.

Various other collaborative histochemical studies have been continued, including work on the mechanisms of vitamin C action, the dermal connective tissue changes associated with amyotrophic lateral sclerosis, and the development of selective stains for nucleoprotein and collagen.

The remaining group of studies have been concerned with dental caries, a long range project on the etiology and characteristics of the disease as investigated in carefully controlled animal experiments. Various factors in the host-parasite-diet complex have been under study, and this year emphasis has been placed on the microbial and dietary factors affecting the course of the carious process. The use of tracer strains of microorganisms, primarily pure cultures of streptococci rendered identifiable through induced antibiotic resistance, has been continued. Such labelled organisms have been recovered from carious lesions

or plaques both in animals inoculated directly and in animals which have received the organisms by natural transmission from a previously inoculated mother. This technique has made it possible to study both the transmission of caries activity into animals previously uninfected, and to make some estimations of the degree of caries activity. A new strain of streptomycin-resistant streptococci has been found which seems to establish itself more effectively than the strains used earlier. As an extension of this work, an attempt was made to produce an active immunity in animals by injection of a vaccine prepared from known cariogenic streptococci. Results were negative. Another promising technique for the induction of caries has been transfer of plaque material from animals with the disease. An interesting sidelight in development of the method is the preliminary observation that periodontal disease also seems to arise in animals subjected to transfer of suitable plaque material. The discovery of the infectious nature of plaque substance recovered from the periodontium may well lead to a profitable series of investigations on experimental periodontal disease. The microbial characteristics of these plaques are now under investigation in collaborative efforts with the Laboratory of Microbiology, NIDR, and the possibility of inducing peiodontal disease with pure cultures of organisms is being studied.

EPIDEMIOLOGY AND BIOMETRY BRANCH

In the past, most studies in oral epidemiology have been concentrated upon dental caries. In the United States this disease strikes early in life and can destroy the dentition before adulthood unless checked by preventive or treatment measures. Principally because of earlier and more adequate dental care, tooth mortality from this cause has been reduced to a point where more teeth are actually being lost to the periodontal diseases than to caries. In some other areas of the world, caries is low in prevalence but periodontal disease is widespread and so severe that many young adults are virtually edentulous from this cause. For these reasons periodontal diseases were studied as closely as dental caries during the past year of activity in the Epidemiology and Biometry Branch.

During the calendar year 1961, principal investigators of the Branch examined about 24,000 persons, most of them adults. These persons were participants in a series of broad programs, including (a) nutrition surveys in cooperation with the Interdepartmental Committee on Nutrition for National Defense, in Lebanon, Trinidad, Burma, and with American Indians in Montana; (b) school children in Montgomery and Prince Georges counties, Maryland, where the domestic water has been fluoridated since January, 1952; (c) clinical trials of phosphates as caries inhibitors, added to food in a study with Dakota Indian children, and in a dentifrice tested with children in Pennsylvania; (d) tests of the Keyes-Fitzgerald hypothesis of the communicability of dental caries in animals, to determine whether it is applicable in human caries; and (e) a survey of mottled enamel in Grand Rapids, Michigan, as part of that fluoridation study.

Nutrition Surveys

Investigators from the Branch participated as dental examiners in nutrition survey teams of the ICNND sent to Lebanon, Trinidad, Burma, and to Montana to study American Indians. This Interdepartmental Committee was constituted in 1955 to coordinate nutrition surveys of armed forces in friendly countries. Its field survey teams are made up of specialists in nutrition, medicine and dentistry, biochemistry, food technology, agriculture, and other disciplines according to the opportunity presented by the specific situation. Within this framework observes from the Branch have been encouraged to carry out specific and general research relating nutritional states to the oral diseases.

Data from the 1961 surveys are being added to data previously gathered in similar fashion in Alaska, Ethiopia, Ecuador, Chile, Colombia, South Vietnam, and Thailand. These studies have been analyzed as individual units and as components of a whole program, on the general hypothesis that populations depleted in any nutrient will exhibit more or more severe signs of disease if such deficiency is etiologic for that disease.

The prevalence of dental caries has been shown to vary widely; from levels as high as or higher than those seen in adults of continental United

States to virtual absence of disease. High prevalences were reported from the larger villages of Alaska, throughout Trinidad, and in some areas in South America. Caries was virtually absent in Ethiopia, affecting an average of less than one tooth per person at all ages up through 40 years, and nearly as rare in Eskimos of some remote Alaska villages, in South Vietnam, and in Palestinian refugees in Lebanon. Preliminary analyses have elicited no consistent relation between dietary or nutrition findings and dental caries, except a very general tendency for low caries prevalence to occur in areas where calorie intake is marginal or low. Further evidence that dental caries is inhibited by flouride ingestion was adduced in Thailand, Ecuador, Colombia, and Chile.

Taken as a whole, periodontal disease constituted a much greater problem than caries in these national groups. Disease levels lower than those observed in the United States were seen only in remote areas of Alaska and in primitive Jivaros of Ecuador. Gingival disease with relatively little tooth loss from this cause was reported from Ethiopia. Elsewhere the onset of periodontal disease was early, prevalence was virtually universal, and severity was extreme. Despite their favorable experience with dental caries, as many Lebanese as United States citizens were edentulous in middle life, due to tooth loss from destruction of supportive tissues. Equally high levels of disease were seen in Trinidad and the three countries in Southeast Asia. There was a general tendency for periodontal disease scores to be higher in populations in which vitamin A intakes, or vitamin A levels in serum, were low. Persons within a population, however, who were subsisting on inadequate intakes of vitamin A were no more apt to exhibit disease than persons whose intake was adequate or high.

One known etiologic factor in periodontal disease is local irritation, which is usually the result of subgingival deposits of calculus. The influence of calculus deposits, for example, accounted for nearly 90 per cent of the variance in group periodontal scores in Lebanon. But it seems obvious that calculus is much more damaging in the mouths of Lebanese or Vietnamese civilians than in the mouths of Ethiopians or Eskimos. The proper analysis for these additional factors is one which cancels out the effects of such known factors as calculus. This can be done by multiple correlation-multiple regression technics. These are simple in principle but entail enormous numbers of arithmetic calculations. Multiple correlation analysis of the 20 items of information considered in a study of Alaskan Eskimos, for example, would require that each value be used as a multiplier about 250 times. A superficial analysis already carried out, in which each items was used as a multiplier about 40 times, required several months of hand computation. Such problems as these are readily amenable to solution by electronic computer methods. Computer programs for simultaneous consideration of factors which may affect peridontal disease or dental caries as elicited in these ICNND studies are now under preparation.

Several leads for further study have been uncovered. Some populations seem to be free of signs of oral malignancies. In some (notably the Thai) the excretion of relatively large amounts of fluorides in urine by persons using a domestic water very low in fluorides suggests that there may be unknown sources of fluoride in Thai foods, or that fluoride metabolism may be influenced by unknown factors not normally operating in North America. A lacquer believed by the Vietnamese to protect teeth from disease was found actually to be associated with lesser caries attack and better periodontal tissues. Christian women in Lebanon were notably more susceptible to dental caries than Moslem women living in the same areas. Periodontal disease is ordinarily more destructive in males than in females but in a matriarchal society of hill tribesmen in Vietnam, in which women carried the dominant roles in social and other activities outside the home, disease was more destructive in females than in males.

Fluorine and Dental Caries

The pioneer study of the practicality of domestic water fluoridation was carried out in Grand Rapids, Michigan, where fluorides were added to the community water beginning in January, 1945. The final field examination required by design of this study was carried out in November, 1961, with a survey of dental fluorosis in children aged 12–14 years, with maximum usage of this community water since birth. Less fluorosis was found than had been predicted on the basis of the observations

on children in 21 cities with varying levels of fluoride in the water, a series of studies which had given rise to the hypothesis tested in Grand Rapids. This lessened degree of fluorosis may have been due to rigid control of fluoride levels in the fluoridation plant. Most "natural" fluoride waters fluctuate in fluoride content, with peak levels much higher in many instances than the average levels over the course of months or years. The finding in this survey supports the original estimate of the safety of controlled fluoridation at the recommended level, and suggests, in fact, that safety-factor calculations were conservative.

The Grand Rapids study was intended to determine whether fluoridation of water would induce the same inhibition of caries seen with naturally-fluoridated waters, and was designed on the assumption that first effects would be seen in children born after fluoridation was begun. For this reason examinations during the first few years of study were limited mainly to children aged 5, 6, 9, or 13 years. Efficacy of fluoridation was obvious after four years of observation. A corollary study was then begun to determine how and in what manner the inhibition of caries was effected. This was designed on a longitudinal plan, to run for 10 years following fluoridation of water in Prince Georges and Montgomery counties, Maryland, in January of 1952. Examinations of children aged 5–15 years were carried out in 1961, the ninth year of study. Repeated observations of the same children indicated that most of the assumptions accepted in conventional horizontal ("one-shot") field study are valid. Teeth in eruption at the time of fluoridation continue to decay at essentially the same rates as before. Caries was arrested on smooth surfaces of teeth fully calcified, but not in eruption, at the time of fluoridation; there was little effect on pit-and-fissure caries in these teeth. Caries on all tooth surfaces was inhibited in teeth still undergoing calcification at the time of fluoridation. There was a general decrease in the prevalence of early periodontal disease in older children as the study progressed. Final analysis after the tenth year is expected to yield a family of findings ranging from actuarial-type tables which will permit estimation of the impact of fluoridation upon a public health dental program for children, to inferences with the sequence and mechanics of development and calcification of the teeth.

Other Studies

The Keyes-Fitzgerald hypothesis that dental caries is a specific, infectuous and transmissible disease in laboratory animals raises obvious questions about dental caries in man which can be answered only through the study of human populations. Organisms which induce caries in rats are benign in the mouths of hamsters, and organisms which induce caries in hamsters do not affect rats. Both are streptococci which can be eradicated completely by penicillin in adequate oral concentration. Assuming a parallel process in humans, it was postulated that young rheumatic fever patients who had been receiving oral penicillin daily, beginning at a time prior to the eruption of permanent teeth, would show no lesions of caries in those teeth after some years at risk. In a pilot field study such children were examined in Pennsylvania. Lesions of caries were demonstrated in their permanent teeth, indicating that the original hypothesis was false or that the actual dosages of penicillin had been inadequate to suppress the cariogenic flora *in toto*. However, the affected numbers of teeth and tooth surfaces were less than in the mouths of randomly-matched children in the public schools of the same community who had not received penicillin. Once an organism specific for man is identified its role can be verified by demonstrations of absence in such caries-free populations as those in Alaska and Ethiopia (described earlier in this narrative) and uniform presence in caries-susceptible groups.

Findings in some animal studies have suggested that phosphate may be an effective inhibitor of dental caries under certain conditions. A clinical trial of the effect of phosphates added to flour used in baking was carried through its second year in Indian school children in South Dakota. This is a double-blind study, with the clinical observations being made by an independent examiner. Data are collated and analyzed in the Branch. This study has an additional year to run. Besides analysis of the dental caries data, statisticians of the Branch have helped tabulate and analyze collateral data on growth in height and weight, and

skeletal calcification in the same children during the term of trial.

A clinical trial of phosphates added to a dentifrice was begun with institutionalized children in Pennsylvania. This study is designed for a term of two years, involving a minimum of three clinical and x-ray examinations.

In addition to direct research activities, a considerable amount of time was spent in consultation on the design and conduct of field studies being made by others, particularly NIDR research grantees, officers of the Navy Dental Corps, and Public Health Service regional dental officers. Biometrics services were furnished in about 20 studies involving NIDR investigators from other laboratories.

CLINICAL INVESTIGATIONS BRANCH

INTRODUCTION

The scope of research activities in the Clinical Investigations Branch, National Institute of Dental Research, while originally centered around oral disease problems, has become more extensive and now includes problems of speech, hearing, swallowing, and respiration, as well as a continuously developing program on a variety of inherited disease conditions and abnormalities of genetic origin. For purposes of this review, we may describe the various research projects under four headings or sections: (1) Studies on oral diseases, (2) Medical investigations, (3) Studies on oral pharyngeal development and function, and (4) Familial and isolate population studies in human genetics.

The opening of the new building for the National Institute of Dental Research furnished an opportunity for considerable growth in the Clinical Investigations staff, even though most of the staff have their laboratories in the Clinical Center. Thus, the professional staff increased from 20 to 28, the supporting staff from 17 to 26, for a total staff increase from 37 to 54 persons. This increase was chiefly in the beginning of a new section on "Oral Pharyngeal Development and Function," headed by Dr. James F. Bosma, with 6 professional and 5 subprofessional staff members, and an increase in the Genetics Section, headed by Dr. Carl Witkop, from 6 to 8 professional and from 4 to 7 subprofessional staff members. These changes have increased the broad range of projects in the Clinical Investigations area, even though the National Institute of Dental Research staff is small, compared with the staffs of the other National Institutes of Health institutes. While some of the research projects are definitely aimed at helping the dental profession solve some of its more difficult clinical problems, the general aim of most of these projects has been to develop new and more adequate research methods and data to elucidate the underlying mechanisms of the normal and abnormal biological processes as they are related to health and disease.

Oral Diseases

Research projects on oral diseases include those concerned with the teeth, such as caries, erosion, periodontal disease, and pulpitis, and those concerned with pathological conditions of the mucous membranes and underlying oral structures, such as aphthous stomatitis, leukoplakia, infections and painful temporomandibular joints. It is recognized that one cannot dissociate the health of the oral structures from the health of the entire body, and that one cannot dissociate the health of the individual patient from that of the family and the social genetic community from which he has come. It is also recognized that clinical investigations should include related laboratory studies on physiological and biochemical mechanisms, as well as studies of similar disease conditions in model animal experiments.

(a) Dental Caries: Two research projects are being conducted in this area: The first is a comprehensive clinical and laboratory study of the multiple factors involved in the etiology of dental caries by Dr. Stephan. This has included the use of advanced instrumentation for making intraoral observations and measurements on the caries process, the evaluation of predisposing systemic and familial factors in children with rampant caries, and multifactorial experiments in laboratory animals to determine the effects of strain of animal, the cariogenic properties of various dietary materials, and the cariogenic and infections potentialities of specific types of oral bacteria derived from patients with rampant caries, using the fluorescent antibody technique to trace specific microorganisms. Although this study deals with the

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etiology of dental caries in all of its complexities, it is leading to a clarification of the relative importance of different basic factors involved in the caries process, and particularly to the host-parasite relationship as affected by diet. As more knowledge is gained on the parasitic and cariogenic properties of different microorganisms, particularly in tracing specific organisms using the fluorescent antibody method, a more specific control of potentially cariogenic microorganisms may be developed. From a practical standpoint, the dental profession has been very happy to refer patients with rampant caries for this study, since it represents one of the their most difficult and expensive treatment problems. From the results achieved thus far by prescribing new dietary habits for patients, it appears that caries activity has been greatly reduced in patients who have followed this control program.

The second caries research project, by Dr. Ship, Dr. Mickelsen (NIAMD) and others, is on the "Effect of Dietary Phosphates on Dental Caries in Children." This study is a field study testing a single dietary factor, calcium phosphate, for its effects when added to bread on the dental caries incidence in Indian school children in South Dakota. It was based on earlier laboratory studies by Dr. McClure, in which the addition of certain phosphates to the diet of rats decreased the development of caries in these animals when they were fed cariogenic diets. The plans for this study have been widely discussed in previous reports, and the present report gives the results of examinations to the spring of 1961. No difference in the increments of either decayed, missing and filled teeth or tooth surfaces was found between the children in the control and the children in the phosphate-supplemented groups. Since the data so far suggests that dietary phosphate supplements are inactive in preventing dental caries in children, the major findings of the project will be on the effects of the supplements on growth and development of Indian children. This study will be terminated at the end of the present school year.

(b) Dental Erosion: The study on the Etiology and Control of Dental Erosion, which was begun this year by Dr. Stephan, is developing a comprehensive picture concerning the mechanisms operating in the production of this little understood condition. It appears that the local effects of specific foods, physical as well as chemical, have been operative in some cases.

(c) Periodontal Disease: Three project reports have been made in this area. The first is a study by Dr. Baer on the therapeutic evaluation of a periodontal dressing used following surgical gingivectomy in the treatment of advanced periodontal disease. It was found that a periodontal dressing containing eugenol produced a greater inflammatory response in healing tissues than did a dressing without eugenol, and that a hydrogenated fat zinc bacitracin dressing was the most useful of those tested for postoperative healing. This work was published in the Journal of Dental Research.

The second report by Dr. Baer is on the effect of the milk factor during nursing on the development of periodontal disease in two different strains of mice, one of which is "susceptible" and the other "resistant" to periodontal disease. Experiments in which young mice of the "susceptible" strain were nursed by a mother of the "resistant" strain, and vice versa, failed to change the subsequent susceptibility of the young mice to periodontal disease. It was concluded that a "milk factor" did not appear to affect the susceptibility of mice to periodontal disease.

The third report on periodontal disease is a morphohistological study of changes in the periodontium in human autopsy material by Dr. Stanley. In this study, an attempt has been made to reconstruct in three dimensions the microscopical picture of pathological processes of the periodontium. The picture developed indicates that a single histological section may not furnish an adequate description of the periodontal process for a given specimen, and that step-serial sections are needed for a suitable diagnosis.

Dr. Wertheimer has also reported studies related to periodontal disease, chiefly histochemical staining of the secondary dental cuticle, and a field study on periodontal disease in the West Indies.

(d) Pulpitis: Another project by Dr. Stanley is concerned with the histopathology of the human dental pulp, particularly in studying the pathological changes induced by dental drilling procedures and the placement of filling materials. This study has furnished the dental profession some very practical information on operative procedures, particularly in regard to optimal cutting

speeds, the use of coolants and methods for the placement of malgam and certain temporary filling materials. An interesting observation has been the finding of occasional mitotic figures in cells approximating the predentin.

Dr. Archard and Dr. Stanley report a current study on the histopathology of the buccal membranes in normal individuals and in patients undergoing cancer chemotherapy and in patients with multiple myeloma. Dr. Kakehashi and Dr. Baer have reported a study on the Influence of Several Dietary Materials on the Deposition of Calculus in the Sprague-Dawley rat. Dr. Baer has also reported a "positive pressure" appliance for the treatment of gingival hemangiomas and hyperplastic gingival tissue in pateints treated with dilantin.

The project on recurrent aphthous stomatitis, which was formerly conducted by Dr. Ship, has been renewed by Dr. Graykowski. This is a fairly common and painful disease of unknown etiology, and the current project seeks to determine if any relationship exists between the presence of recurrent aphthous oral lesions and the metabolism of individuals in which they occur. There is suggestive evidence that the various mucosal changes associated with iron deficiency anemia may in some ways be correlated with aphthous stomatitis.

(e) Anesthesia: A study of general anesthesia in ambulatory dental patients by Dr. Driscoll, with Dr. Christenson and Dr. Hebert (CC), is developing important information concerning the physiological effects of anesthesia and oral surgical procedures. This includes continuous data on pulse, blood pressure, arterial O_2 saturation, respiratory phenomenon, cortical brain activity and the electrical activity of the heart EKG. The accumulated data from this study has been and will continue to be used as a baseline of comparison for new anesthetic drugs which are being proposed for use in oral surgery. Since in some areas there are almost as many general anesthetics administered in dental offices as in the local hospitals, the basic physiological data from this study will prove very important for the dental profession.

A new study was initiated on the physiological responses of the dental patient under hypnosis by Dr. Drury and Dr. Driscoll. Although the use of hypnosis in dental practice has become more widespread in recent years, particularly in the treatment of apprehensive and fearful dental patients, no formal research has been done on the physiological responses of the dental patient to this procedure. This study will be similar to the anesthesia study just described, and will include measurements of blood pressure, respiration, oxygen saturation of the blood, EKG, EEG, body temperature, galvanic skin response, and psychological data recorded in the Minnesota Multiphasic Personalty Inventory Test. The findings in this study will be compared with those in the general anesthesia study for an objective evaluation of those procedures in oral surgery and will furnish much needed basic data, which should prove of great value to the dental profession.

Another new study initiated in 1961 by Dr. Gamble, Dr. Driscoll, Dr. Swerdlow and Dr. Lloyd, with the cooperation of the Psychological Testing Center at NIH, is on the use of subperiosteal implants for stabilization of artificial dentures. It is estimated that one out of five edentulous patients is unable to wear conventional mandibular dentures, generally due to advanced resorption of the alveolar ridge. A thorough psychological evaluation of patients who are unable to wear conventional dentures will be made. In these cases, the use of subperiosteal implants offers the possibility of securing greater stability for artificial dentures. This study is aimed at finding a satisfactory solution for a very difficult prosthetic problem, which becomes particularly important for people as they advance in age.

Oral Pharyngeal Development and Function

As was mentioned earlier, a new Section on Oral Pharyngeal Development and Function was organized this year, with Dr. James Bosma as Chief. He reports that the activities of this group have been devoted principally to its own orientation and to the introduction and adaption of investigative methods. A major experimentation is in the interaction of the different professional specialists whose interests converge in the "portal area" of the mouth, pharynx, nose and larynx. In the first nine months of the Section's existence, research investigators in the dental specialties of orthodontics and prosthodontics and the medical specialties

of pediatrics and otolaryngology have been brought into close working relationship with specialists in speech therapy and physiology. The most significant interactions thus far have been between speech and orthodontics. Further efforts will be made toward the introduction of methods and personnel of the basic scientific disciplines, particularly anatomy, physiology and anthropology.

These three major disciplines also afford appropriate categorization of the efforts in this Section since its inception.

(a) Anatomy: Initial efforts of this area have been in anatomical dissection. On the basis of comparative studies in infant and adult humans and in four other species, Y. Takagi, J. Waters and J. Bosma have developed a concept of how the muscles of the cervical spine interact with the muscles at the upper end of the pharynx. The particular effects of this interaction upon the cavity of the pharynx in the palate area is now under study in normal humans and in those having impairment in this area by reason of neurologic disease or malformation.

The anatomical growth of the pharynx and face in the human is also under continuing study. Dr. Peter Coccaro has submitted reports on two items of this study: (1) growth of the soft palate in cleft palate persons, and (2) growth in height of the face in impaired children wearing dentures. Dr. Richard Grossman has developed implementation for comparing the physical softness or elasticity of tongue, lip and face tissues at different stages of development, and in pathologic conditions.

(b) Physiology: Neurophysiological studies of the pharynx region in respiration and in feeding have been initiated. The implementation of this now includes an 8-channel ink-on-paper recorder and/or a 7-channel tape recorder, with each system capable of combining information from several muscles and from sensors of the muscular effects, including motions or pressures or sounds accomplished. With this apparatus, Drs. Bosma, Irwin, Takagi and Lifschiz are engaged in study of sensations from the pharynx which influence its own actions and also those of the larynx and the trunk in respiration.

The muscular actions of the mouth area of the human are also under study in normal and in impaired subjects by Dr. Richard Grossman, employing observation techniques of very small strain gauges and soft tissue displacement indicators. This instrumentation is designed to provide information about the physical forces accomplished by the tongue and face muscles. In impaired persons, these muscles are capable of accomplishing marked deformity with displacement of teeth and incapacity of oral actions in eating and in speaking. Identification of these abnormal muscle actions is essential for proper definition of causes of oral deformity in individual subjects and for the design of their appropriate therapy.

Studies on speech adaptation of respiration have also been initiated. A remarkably sensitive and informative method of airflow perception, developed by Dr. Svend Smith, a ten-week guest from Denmark, has been introduced—the first such instrument in America. By this instrumentation, the minute air pulses of speech are recorded, along with the sounds. This highly sensitive and discriminate recording makes possible the study of the fine articulations of human speech. Currently, Dr. Smith is preparing an adaptation for simultaneous and differential recording of airflow from the nose and from the mouth under a developmental contract from this Section. This dual instrument will aid in demonstration of the actions of the soft palate in speech.

As a separate speech physiological observation, the influence of obturators upon palatal patencies have been observed by Drs. Ralph L. Shelton and Ralph Lloyd. This observation has been made by speech recording on tape and also by cinema observation of facial movements. The effect of surgical removal of parts of the soft or of the hard palate are highly similar, and these nasality distortions are abruptly improved by plastic obturators. The nasality of congenitally cleft persons is acoustically similar to that of those having surgical removals, but the placement of an obturator does not afford abrupt and uniform improvement in nasality. In palatal deficiency of either kind, the subjects variably employed similar patterns of movement distortions in the face of exaggerations of closing actions about the mouth and the nose. This observation is being prepared for cinema publication.

Drs. Shelton and Bosma also employed cinema and tape recording to study the actions of the soft palate, tongue and pharynx in twenty subjects who had surgical removals of parts of the face by reason of local cancer. These patients, most of whom were at the Sloan Memorial Hospital in New York, afforded unique opportunity for study of these motions, which are otherwise not accessible to observation. Particular details of related motions of palato-pharyngeal wall and tongue have been discussed for the first time. This material is in preparation as an extensive documentation cinema.

(c) Anthropology: We shall add an anthropologist, Dr. Melvyn Baer, to this group in July 1962, on a Visiting Scientist appointment for a period of one year. In anticipation of his work with us, preliminary and methodological studies are in progress in which immature experimental animals are being injected with alizarin red-S, which stains the portions of skeleton currently being calcified. By appropriately scheduled studies of these animals in litters, it is possible to define the portions of facial and cranial skeleton currently growing. The general schedules of growth in these areas can thus be described in normal animals and in those impaired by local surgery or imposed neurological disease.

Medical Investigations

At the beginning of the year, Dr. A. D. Merritt, who had been Chief of the Medical Investigations Section, left NIDR to become associate Professor of Medicine at Indiana University. His project report "Genetic Studies in a Population Isolate (Brandywine) with Particular Reference to Hemoglobin and Haptoglobin Patterns" covers work done with Dr. Witkop. Sickle cell hemoglobin has been found in approximately 20% of the tri-racial isolate population (approximately 10% of the national Negro population), and a 2:1 "modified" haptoglobin pattern was found in a correspondingly increased percentage. These findings will serve as a correlation point for other disease states in the tri-racial isolate study.

Other studies include "Familial Neonatal Hepatitis—Study of a Large Family," and "Hereditary Renal Dysfunction with Associated Anomalies," by Dr. Cassady. These include genetic, clinical and laboratory studies on a family of 35 members with a high frequency of "giant cell hepatitis of the newborn" and a study of six families with hereditary renal dysfunction. In addition, studies have been begun at Gallaudet College for the Deaf on a number of individuals with hearing impairment and abnormal urinalysis. These studies will be further described in the part listed under the Human Genetics Section.

Human Genetics

During the last year, the Human Genetics Section directed considerable attention to program expansion and the recruitment of qualified personnel to round out a program in human genetics.

Two new program areas have been developed in the Human Genetics Section through recruitment of new personnel. This includes activities in cytogenetics and statistics. Dr. Herbert Cooper came on duty on December 1 to take charge of the cytogenetics unit, where he will primarily deal with defects of chromosomes, translocations and the genetics of tissue culture. In the area of statistics and research in genetic analyses, we have been able to recruit Dr. Bertram Hanna and Dr. C. Chung. These men are developing high-speed computer programs of analysis of various types of genetic data, especially those involving large population studies.

Brief descriptions of the primary projects of this Section are given below.

(a) Hereditary Defects in Enamel and Dentin: This project was initiated by a survey of 100,000 school children in the state of Michigan for the prevalence of these defects. It was determined that dentinogenesis imperfecta occurs about once in 8,000 individuals and that amelogenesis imperfecta occurs about once in 16,000 in the general population of the state of Michigan. At least two distinct genetic diseases of dentin exist, dentin dysplasia and opalescent dentin. At least five distinct diseases of enamel formation exist: hypoplasia of enamel, inherited as a sex-linked dominant trait; hypocalcification, as an autosomal dominant trait; hypomaturation, as a sex-linked recessive trait; pigmented hypomaturation, as a recessive trait; and local hypoplasia, as an autosomal dominant trait. Methods of restoration

and treatment have been compared in a large series of patients by Dr. Lloyd and Dr. Driscoll.

(b) The Brandywine Study: The Brandywine Study was the first large population study of the Human Genetics Section. This is a study of 5,000 individuals of Caucasian, Amerindian and Negro ancestry, who reside in Southern Maryland and who have had their marriage patterns restricted to this small isolate for several hundred years. To date, information has been collected on the approximately 5,000 living individuals, as well as some 12,000 deceased predecessors. This group essentially furnishes us with a genetic population laboratory. At the present time, most of the historical, genetic, social, physical, dental and laboratory data have been accumulated and are now being processed for machine analysis. A complete genetic, medical and dental history was obtained from each individual. Each individual was given a physical and dental examination. Laboratory procedures included a complete genotyping, abnormal hemoglobins, serum electrophoresis and saliva studies.

To give some idea of the mutational load present in this population, 20.7% of its members are sicklers, the highest known rate outside of Africa; 1.6% are albinos, the highest known rate of albinism in man; 3.7% have opalescent dentin, the highest known rate of this condition in man; as well as 23 other well-defined, simply inherited conditions. A study of the biochemical defect in albinism by Dr. C. Witkop, NIDR; Dr. E. Van Scott, NCI; and Dr. G. Jacoby, NIAMD, showed that the most common type of this disease is not due to a lack of the enzyme tyrosinase, but appears to be a defect in a tyrosine transport system. These findings strongly suggest that this disease can be treated—in fact, these workers have induced local pigmentation in these subjects.

(c) A study of Tri-racial Isolates in Eastern United States: This project was developed from the Brandywine project when the question was asked, whether or not other inbred populations of a similar nature existed in the United States.

Through the cooperation of Dr. Calvin Beale of the Department of Agriculture, formerly of the Bureau of the Census, it was determined that approximately 100,000 people are members of such isolates residing along the eastern seaboard. These extend from New York to Louisiana, and we have identified 26 such populations, all of whom show some type of hereditary abnormality. Investigations of these isolates and their genetic diseases led to the next study of hereditary dyskeratoses.

(d) Cytological Investigation of Hereditary Dyskeratoses and the Effects of Cancer Chemotherapeutic Agents on Cell Division: In the Haliwar isolate of North Carolina, a unique disease leading to blindness, associated with a lesion of the bulbar conjunctiva and a white lesion of the oral mucosa, was studied to determine whether or not this was a genetic disease or an environmentally induced condition. A cytological investigation of this disease and other hereditary oral dyskeratoses revealed a new method of diagnosis by exfoliative cytology. The cell defect in two of these diseases appeared to arise from abnormal cell division. A study of epithelial cell division found that this cell change could be experimentally induced in humans by certain cancer chemotherapeutic drugs. Through this work a method of predicting toxicity with those drugs and a better methtod of drug selection shows hope of reducing drug trial times from three months to three days. Results of these investigations led not only to the description of a new hereditary disease of mucosa and conjunctiva, but also to investigations of a new neurological condition which is inherited as a recessive trait, causing mental deficiency, spasticity, and ichthyosis. This latter condition is of unusual interest to the dentist because of the oral neurological abnormalities. These have been recorded in motion picture form. It was determined that loss of sensation in the oral cavity resulted in abnormal motor function. This concept of sensory loss has led us to reevaluate other apparent oral motor disturbances and may offer a solution to some of these defects and the reevaluation of certain types of speech therapy. This disease appears to be a biochemical error involving amino acid metabolism—the aminoaciduria is being investigated.

(e) A Familial Study of Kidney Disease: Three observations concerning certain oral diseases led to a study of a familial form of kidney disease: (1) The oral-neurological ichthyosis disease was known to have an associated kidney defect with aminoaciduria; (2) pedigrees of certain tooth defects indicated peculiar genetic ratios that ap-

peared to violate Mendelian segregation; and (3) certain oral clefts with ear defects were associated with an abnormal number of chromosomes. Dr. George Cassady, Mr. Maimon Cohen, Dr. Bertram Hanna and Mr. Ronald Robinette found indications that a type of hereditary renal dysfunction involved similar mechanisms. Because sufficient patient material appeared available, a study of this disease was undertaken to define this peculiar type of inheritance in man. At first this kidney disease appeared to be a rare condition which was manifested by hematuria, beginning in childhood; a peculiar loss of hearing, which was evident early only on audiograms in the range of 4,000 cycles; associated defects of the eye, such as astigmatism and myopia; a possible increase in the frequency of middle ear infections; and an abnormality of the external ear. The first point of attack on this illness was the epidemiological aspect. Closely associated with this were the clinical aspects. As a result, seven large kindreds from the general population were obtained, and field studies were set up to investigate all known relatives. Patients were admitted to the Clinical Center for detailed study of the clinical aspects of the condition. These studies have shown the following: (1) The condition is not uncommon. For example, routine examination of the out-patients admitted to the Dental Clinic revealed five new propositi within one month; (2) additional families were accumulated by contacting local physicians; (3) Because loss of hearing was one of the outstanding manifestations of the illness, children attending Gallaudet School and Gallaudet College were examined, and additional cases were found in this population; (4) foam cells are found in the kidneys of affected persons; (5) thus far, lipo protein electrophoresis patterns appear to be abnormal in affected persons; (6) for the first time a "new" type of inheritance has been reported in man, involving preferential segregation of the autosome carrying the defective gene with the X-chromosome.

While we have no direct estimate of the frequency of this disease, it becomes obvious, when compared to other fairly rare conditions, such as opalescent dentin, which occurs about once in 8,000 individuals, that this kidney disease is much more frequent—perhaps in the order of one in 400 individuals.

(f) A Study of Consanguineous Marriages in Nagasaki and Hiroshima: This study was initiated by Dr. James Neel of the University of Michigan, and Dr. Jerry Niswander from our Section was invited to do the oral aspects of the study. This involved examination of the offspring of 5,000 consanguineous matings who were unirradiated, and a comparable control group to determine the effects of consanguinity. One of the important pieces of information that this study attempted to determine was the average number of deleterious recessive genes carried by man. This figure is important in determining safe levels of total lifetime radiation exposure.

The data for this study have now been collected and are being processed at the University of Michigan, where Dr. Niswander is obtaining a Ph. D. degree in genetics. One of the important observations that he made was that developmental time, as indicated by tooth eruption time, can be changed markedly in the same population by environmental factors.

(g) A Nutritional Survey of the Chilean Population: In 1960 a nutritional survey of the Chilean population was conducted under the sponsorship of the ICNND. Dr. Witkop of this Section participated as the American representative, and Dr. Luis Barros of the Chilean Army participated as the Chilean dental representative. Dr. Barros is at present a Visiting Fellow in the Human Genetics Section; his primary duty is to process the data obtained in this study. The objective of this study was to determine the nutritional status of ¾ of 1% of the entire civilian population and 10% of the military population. Every fifth person in this original sample was given a dental examination.

Every other person who obtained a dental examination had a blood sample drawn for nutritional determinations, hemogram, genetic abnormalities of the erythrocyte and hemoglobin, and a genotyping. In addition to information collected on caries and periodontal disease, prevalence of 14 oral anomalies was determined on this sample. This sample was selected to represent a low and medium socioeconomic, geographic and age distribution equivalent to the entire Chilean population. Three significant findings can be reported at this time. These have been described in detail in the recent publication, "Nutritional Survey of

Chile, March–June, 1960, Interdepartmental Committee on Nutrition for National Defense, August, 1961."

(h) A Study of Genetic Factors in Saliva: This project is being conducted by Dr. Wolf of the biochemistry unit and consists of an electrophoretic and immunochemical study of salivary proteins. The initial approach in this study has been to determine the occurrence and factors that govern the genetics of secretor factor in saliva, localization of the secretor factor in the various glands concerned, and the relationship of secretor factor titrations to other genetic markers and diseases. The second aspect of this study concerns the use of electrophoretic techniques to separate from the saliva the various constituent protein elements and to determine if these are under genetic control. At the present time, Dr. Wolf has developed a knowledge of the various factors that cause variation between individuals.

(i) A Study of Hemoglobinopathies: This study was conducted by Dr. Rucknagel and Dr. Schneiderman. It is now nearly complete, as far as the field and laboratory data are concerned. These data are now being analyzed for selective factors in the populations, which apparently are such as to maintain a very high frequency of this gene in the Brandywine population. In addition to this, several new hemoglobinopathies have been under investigation. These apparently do not coincide with any of the known defects reported to date.

The above include the major studies of the Section. In addition to these, numerous families and patients are being followed for what appear to be genetic defects, such as familial neutropenia, causing a severe periodontal condition in the patients in whom it appears; various disorders of connective tissue, such as osteogenesis imperfecta, where enzyme studies have shown a phosphatase abnormality in developing teeth and bone; a familial form of neonatal hepatitis; and a hereditary form of osteoarthritis, associated with extensive degenerative disc disease. This latter disease is common in a large Caucasian population residing in Southern Maryland.

NATIONAL INSTITUTE OF NEUROLOGICAL DISEASES AND BLINDNESS

INTRODUCTION: A DECADE OF RESEARCH PROGRESS

Action Potential Transmission Within Excitable Cells

If one had to select from the past ten years the most important single advance in basic knowledge of the nervous system, one would place high on the list the ionic theory of nerve transmission and conduction. This theory is dependent upon the concept that across the nerve membrane there is a free diffusion of small ions such as potassium and chloride but a lower order of permeability of sodium ions. The availability of the giant squid axon, the intracellular microelectrode, and the voltage clamp technique as developed by Dr. Cole and colleagues, combined with an analysis of a massive amount of mathematical data now possible with computer technology has demonstrated that the ionic theory can explain such things as propagation, velocity, saltatory propagation, the all or none law, and the phenomena of accommodation. In the past decade, the Laboratory of Biophysics with Dr. Cole and his colleagues in combination with the Laboratories of Physiology at Cambridge and Plymouth, England, have brought forth an imposing amount of data to confirm the ionic theory of neural transmission. Simultaneously, Dr. Tasaki and his colleagues in the past two years through the thermodynamic treatment of radiotracer movements across biological membranes have suggested possible exciting modifications of the ionic theory. Using similar techniques, Dr. Karl Frank and his colleagues have in the same period of time devoted their interest to the study of the soma of the neuron. Such studies were started initially with bridge circuits and microelectrodes and have subsequently included voltage clamping of neurons utilizing double-barreled microelectrodes. Doctors Frank and Fuortes demonstrated that the membrane of the postsynaptic cell becomes selectively permeable to inorganic ions after presynaptic secretion of an excitor or inhibitory substance resulting in depolarization or hyperpolarization of the cell soma. They have demonstrated that this somatic component spreads electronically with decrement to a sensitive neural target area. This target area is in the region of the axon hillock and has a threshold approximately one-third that of the soma and dendrites. They have also demonstrated that although this synaptic potential is larger in the soma and dendrites that, due to lower threshold, firing would initiate at the axon hillock. Apparently, the soma and dendrite have longer refractory periods than the axon hillock; the repetitive firing of the latter cannot invade the former. Dr. Del Castillo in his stay in the National Institutes of Health demonstrated a similar type of neural transmission in medullated nerves using the voltage clamp apparatus of Dr. Cole.

One of the main problems in the past in the transfer of this thesis to the muscle cell has been to learn how such an electrical excitation was transmitted to the internal part of the cell. The work of Taylor and Huxley seems to have demonstrated conclusively that this is through the endoplasmic reticulum by utilization of micropipette stimulations at the level of the Z band. Utilization of giant photoreceptor cells of the *Limulus* allowed the first comparison of a physiological stimulus, i.e. light, as compared to the artificial depolarization by electrical currents. Dr. Fuortes has used microelectrodes in such cells and has demonstrated that the impulses generated after illumination are preceded by depolarization of the membrane and that the frequency of the firing is a linear function of the amplitude of the depolarization. Depolarization by electrical currents results in firing that has a frequency which is a function of intensity of the current. In both cases, depolarization appeared to be consequent to changes of mem-

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brane permeability. Thus, the photoreceptor cell appeared to act not unlike the motor neuron of the spinal cord when stimulated by a physiological event.

Molecular Structures and Synthesis

Again, new instrumentation techniques have made possible studies of the molecular level cellular constituents. In particular, X-ray crystallography, ultracentrifugation, the Archibald equations, and computers have made possible studies at a molecular level. Thus Dr. Davies, who originally was in the combined program, along with Pundenz and Kendrew at Cambridge was able to crystallize and give a diagrammatic structure of the protein myoglobin. In the meanwhile at Cambridge University, Watson and Cricks working on the X-ray crystallographic studies of Wilkins were able to give the probable structure of DNA (desoxyribonucleic acid) and to advance the theory that this structure indeed was the template for genetic characteristics. This opened the entire field for the chemical understanding of heredity. Hersey in the meantime was able to demonstrate that viruses infected bacteria by injection of pure DNA. These two discoveries by English and American investigators thus initiated the field of molecular biology. Since many neurological disorders are genetically determined, the Institute has initiated studies in this area of research. Thus, the study of the even-numbered phages is an admirable working tool for the study of chemistry of genetics. The formation of protein by DNA could best be studied after the release of DNA virus from the infected cell. The protein coat of the T2 phage has been studied by Dr. Cummings in the past year in an attempt to construct by means of electron microscopy and the ultracentrifuge the molecular structure of the subunits of the coat of the T2 phage. The ability to substitute alternate purine (transition) or pyramidine (transversion) linkages into the DNA molecule has resulted in mutations. Dr. Ernst Freese is the recognized authority in this particular field and will be joining the Institute in April to take over the new Laboratory of Molecular Biology.

The structure and synthesis of various lipids carry an importance also to neurology in that the cerebrum and other parts of the central nervous system contain vast amounts of such lipids. Some years ago, Dr. Roscoe Brady was able to synthesize sphingosine; and he subsequently followed this with the now verified theory of fatty acid synthesis in which he has demonstrated two reductive steps. In the first step, β ketoacyl-coenzyme A is reduced to β hydroxy-acyl-coenzyme A. In the second step, unsaturated acyl-coenzyme A is reduced. In both these steps, Dr. Brady was the first to point out that the source of hydrogen was TPNH dependent. He also demonstrated the importance of malonyl-coenzyme A in the biosynthesis of fatty acids with chain lengths from C4–C18. More recently, Dr. Brady has demonstrated the action of the coenzyme of vitamin B12 and has shown that an unusual carbon-cobalt linkage must exist.

Enzymes and Intermediary Metabolism

In this field, the role of the isotope reigns supreme. The utilization of the unstable isotope with highly sensitive detection equipment, such as liquid scintillation counters, as well as stable isotopes utilizing mass spectrometry has been extremely productive in research techniques. Utilizing such techniques, Dr. Tower has during this period, done much to demonstrate the interaction of glutamine, asparagin, glutamic acid, and gamma aminobutyric acid.

Studies of the central nervous system have been done utilizing tissue slice technique and the Warburg apparatus. The normal and alternate metabolic pathways have been mapped, and the enzymes necessary in such systems have been elicidated by Dr. Tower in the case of pyridoxine and by Dr. Albers in a case of specific γ aminobutyrate-α ketoglutarate transaminase. Dr. Horvath has continued his studies in the subfractionation of the various proteins of contractile nature in muscle as well as the proteins in the muscle aqueous phase. In this, the techniques of salt extraction, ultracentrifugation, starch electrophoresis, and moving boundary electrophoresis have all been utilized. Similar studies have been carried out by Dr. Resnik in the crystalline proteins of the lens, and the physical characteristics of such proteins have been clarified. More recently, Dr. Trams and Dr. Resnik have been studying the physical nature of gangliosides and along with Dr. Irwin have demonstrated their property of

sequestering quaternary ammonium compounds. The pharmacological importance of the latter observation cannot be understated. In the study of the electroplaque of the eel, Dr. Trams and Dr. Irwin have demonstrated that the ability of a reactor substance to be removed by curare is due to its enzymatic nature and it is not, as has been suggested in other centers, the receptor protein. When one turns one's attention to ocular tissues, the enzymatic contents must then be determined by the microtechniques made available recently by Lowry of quartz microbalances as well as the cryostat for sectioning frozen tissues. Dr. Bonting and his staff have been able to demonstrate in the past two years both a sodium and a potassium ATPase which may in fact underlie the phenomena of the so-called "sodium pump".

Ultrastructure

During the past decade, the resolution of the electron microscope has now approached the level at which the chemist and the microscopist may join in so-called "molecular anatomy". The argument of the existence and nonexistence of the cell membrane has thus been resolved. The electron microscope clearly shows it to be present. Actually, much of the initial studies of the neuron has been in the Section of Ultrastructure by Dr. Palay. It was Dr. Palay who was among the first to demonstrate that the Nissl substance of the neuron was a form of ergastoplasm. It was within the latter that the endoplasmic reticulum of the nerve cell was found and is presumably the site of synthesis of RNA. The first subcellular description of neural secretion came from this Section in which Dr. Palay showed two types of neural secretory vacuoles, one of which presumably arose from the Golgi complex of the neuron and the other from the multivesicular substances of the neuron. The ability to differentiate the axon from dendrites was also described in this Section. Dr. Wanko was able to demonstrate the beautiful geometric anatomy of the lens during this period of time. In his Section, the sliding hypothesis theory of muscle action presented by Huxley in England has been confirmed here; and more recently, as may be noted in the last Annual Report, the fine structure of the toxoplasma organism has been elucidated; and at least four types of methods of reproduction seen. Keith Richardson, in the few months he has been here, has already demonstrated vacuoles in the neurites of the autonomic nervous system at the myoneural synapsis of the vas deferens. These contain material not unlike that seen in cells of the adrenal medulla. If this material is a catechal amine, then this finding would give much substantiation to the theory that synaptic vesicles of the motor neurites of striated muscle may indeed contain a humoral substance such as acetylcholine. Perhaps the best correlation of chemistry and electron microscopy is the combined studies of Dr. Cummings and Dr. Wanko which confirmed Dr. Cummings' mathematical model of the subunit of the protein head of the T2 phage.

Basic Studies in Audition

It is of interest that two discoveries which have been universally accepted as important landmarks in the field of hearing have originated from this Institute. Thus in 1959, Dr. Tasaki and his colleagues were able to show that the positive endolymphatic potential of the internal ear originated from the stria vascularis. This discovery of the postive endocochlear potential is a major physiological landmark in auditory physiology. At approximately the same time, Dr. Rasmussen in the Neuroanatomical Laboratory was able to demonstrate the so-called olivary-cochlear bundle which now bears his name. This is a descending tract going from the brain to the receptor organ, the first such tract demonstrated in the nervous system. Subsequent physiological work both here and in other laboratories has demonstrated that this tract exerts a modulating influence on the auditory receptor cells. This was the first demonstration of a servo mechanism within the central nervous system. There are now indications that such descending pathways which have modulating influences exist in all sensory systems of the body. Dr. Ajmone-Marsan has recently demonstrated by physiological techniques such a system descending from the calcarine cortex to the geniculate body. Thus, it appears that the brain may modulate its own perception of external stimuli.

Anatomical Studies

It is clear from the above that, as one pushes research more and more to the cellular level, the past techniques of embedding, fixation, and sectioning are not critical enough to allow an interpretation of normal structures. In the work of Dr. Cammermeyer and Dr. Palay, this Institute has led the work directed toward the reduction of artifact to a minimum. Until the perfusion technique of Palay using osmium oxide, it has been difficult indeed to study the central nervous system of any animal by electron microscopy. Dr. Palay's technique is now widely utilized in all electron microscopic laboratories. Similarly, Dr. Cammermeyer has devoted his energy to the reduction of artifactual changes in the nervous system by perfusion techniques and is now initiating studies of the reconstruction of the nervous system in many species of animals. This has given much insight into the interrelation of the various cells of the nervous system and to the vascular structures of the nervous system; this was not previously possible. The importance of such lines of endeavor cannot be underestimated, as interpretations of abnormalities are highly dependent upon absence of artifacts.

Pathology of the Nervous System

The Laboratory of Neuropathology today can hardly be compared with that of a decade ago. The modern neuropathologist now uses labelled antibodies and fluorescent microscopy. He uses electron microscopy, tissue culture, and histochemistry and tends to correlate the latter with electron microscopy, as will be seen later in the clinical discussion. The response of neural tissue to radiation of both gamma waves and heavy particles occupies part of the time of the neuropathologist now. The response of cerebral structures to radiofrequency waves and to hypothermia now must be recognized by the pathologist.

By utilizing fluorescent antibodies, Dr. Klatzo and colleagues have been able to demonstrate the phenomena of pinocytosis in neuroglial cells of the brain. The use of immunofluorescence has given much insight to the mechanism of the blood-brain-barrier breakdown. Using such techniques, the lesions may be seen to extend far beyond the site of destruction into the white matter. Accompanying such lesions are abnormalities of glycogen metabolism. It appears from tissue radiated with heavy ion accelerators that neuronal destruction as well as blood-brain-barrier changes occur. The utilization of fluorescent antibodies and tissue culture has allowed the localization of specific muscle proteins in both normal and diseased states. Using tissue culture, Dr. Engel was able to localize glycogen within the I-band of the myofibril as well as in the sarcoplasm. In growing cultures of the muscle, cholinesterase was found by Doctors Engel and Klatzo to be diffusely present throughout the muscle fiber and, in particular, at the level of the Z-band. In a further extension of this study, Dr. Engle and Dr. Mumenthaler found that such localization of this enzyme remains in this position until innervation has been accomplished *in vivo* at which time the localization was found only under the end-plate. Histochemical techniques have become invaluable in the study of pathology of both brain and muscle as has now electron microscopy. Clinical studies to be related further in this report are hence a combination of findings of many of the previously listed investigators utilizing their specific techniques.

Physiology of Cell Aggregations

The fundamental physiological studies of the past decade preceding the 1950's have continued to be fruitful. Such studies utilize the conventional electrical stimulation and ablation with placement of lesions and electrodes by means of stereotaxis. In the past decade, this has been expanded by the utilization of intracellular microelectrodes and extracellular small tungsten electrodes. Utilizing such techniques, Dr. Ajmone-Marsan and his colleagues have extended our knowledge of those structures of the thalamus which relate to the nonspecific thalamic projecting system. They pointed out the relationship of spontaneous cortical electrical activity to areas in which cellular aggregate after discharge are commonly found. Probably the most fundamental study using such techniques was that of Dr. Wade Marshall in his studies of spreading depression. Microelectrode techniques utilizing stereotaxis have been carried out by Dr. Choh Luh Li who has studied the interrelationship of the thalamus

and the pyramidal tract and by Dr. Nelson who has studied the effects of binaural stimulation on single cells of the medial geniculate body. Using microelectrodes, Dr. Li and his colleagues were able in denervated lower vertebrates to demonstrate the origin of the fibrillation potential within the muscle fiber. Such techniques are now being expanded and utilized in tissue cultures. Isolated cells may be more easily penetrated by micropipettes. A study of the unitary analysis of the response elicited in the visual cortex has been done in the cat by Dr. Widen and Dr. Ajmone-Marsan.

Embryology

The addition of Dr. Alfred Coulombre to the Intramural Program is filling a recognized gap. However, before this addition, the Section of Pediatric Neurology under Dr. Dekaban had accomplished an Atlas of the development of the human brain using the Carnegie Institute collection as well as the collections from certain centers in Europe. Dr. Coulombre has initiated two productive studies in the six months he has been here. The introduction of pharmacological denervation in the early stages of the chick embryo by microinfusion of pharmacological agents appears to be a productive technique indeed. In addition, microdissection of ocular structures in the area of the limbus in the early embryo of the chick, followed by challenging with different types of tissues, will give insight into what structures are responsible for the shape and curvature of the eye.

Regeneration

The regeneration of tissues in the adult animal has been in the past a diffuse and often conflicting and frustrating study. In the past decade, however, the availability of H^3 (tritium) and its subsequent labelling to thymidine has given a powerful tool in the study of mitosis of cells of various systems. Thymidine is subsequently incorporated into DNA and, due to its low energy, remains localized in the site of the original DNA synthesis in the chromosome. Thus, Dr. von Sallmann and his colleagues have studied during the past year the corneal endothelium, the choroid coat of the eye, and other ocular structures. They have demonstrated without doubt a consistent replacement of corneal epithelial cells. Similar techniques in the study of muscle in young rats have been frustrating. To date, no unequivocal labelling of muscle cells has been obtained from young rats. Labelling of the nuclei of muscle cells has been successfully done in tissue culture. It would appear that later in life, at least, muscle grows without obvious mitoses. Similar studies using tritiated thymidine and autoradiography are being conducted by Dr. Guth and Dr. Feringa. Autoradiography has been utilized in the past to study the formation of the various cells of the cerebellum with considerable success.

Pharmacological Studies

The past decade again has seen marked changes in the basic tools used by the pharmacologist. The hydraulic kymograph has given way to electronic recording by utilization of transducers, strain gauges, and D. C. pen recorders. Using such methods, Dr. Irwin and his colleagues have demonstrated the interrelationship of blood and tissue cholinesterase systems, their substrata, and other enzyme systems working upon such substrata. Dr. Irwin and his group are one of the first to demonstrate that the competitive block of D-tubocurarine is reduced or prevented by inhibition of muscle cholinesterase. On the other hand, the block of depolarizing drugs is prolonged by inhibition of plasma cholinesterase or muscle cholinesterase. In the case of decamethonium, this could not be due to destruction by cholinesterase as decamethonium has no ester group and hence could not be destroyed. Succinylcholine, on the other hand, has such an ester group and thus could be destroyed by cholinesteras. Dr. Irwin was one of the first to demonstrate, however, that the prolongation of the blockade by inhibition of plasma cholinesterase was identical in the two substances; and thus this inhibition prolongation is not due to destruction of the depolarizing compound. Thus, muscle cholinesterase has but a minor role in relation to the total block. These investigators subsequently pointed out that muscle cholinesterase is low in quantity and is not uniform in various species or organs and, hence, has both a species and organ specificity. It is thus dependent upon both substrate

and enzyme activity. More recently, these investigators have obtained derivatives of Galanthamine which is an alkaloid first isolated in the Union of Soviet Socialist Republics. Galanthamine is also a phenanthrene derivative. Dr. Irwin and his colleagues have formed several derivatives of lycoramine, in particular, the carbamate form and demonstrated such to have potent anticholinesterase properties. These compounds have subsequently been used in the clinic.

Applied Research

All the techniques described above have been utilized in attempts to induce pathological lesions in animals which resemble those seen in man. Thus, Dr. von Sallmann and his colleagues were the first to demonstrate that tryptophane deficiency may be represented purely by cataract formation before any other signs. X radiation, proton radiation, mimosine, and other substances which induce cataracts have all been investigated over the past decade in this Branch and compared histologically and under the electron microscope. Differentiation of the various types of cataracts can be frequently noted by the absence or presence of the proliferation of the Bow nuclei.

Using sensitive transducers and pen writing D. C. amplifiers, Dr. Macri has carried out a systematic study of the effect of the vascular supply of the eye in reference to glaucoma in animals. Dr. van Alphen was the first to demonstrate that the inherent elasticity of the choroid coat of the eye may have importance in refractile abnormalities. Basic studies in electroretinography were carried on in this laboratory using the diurnal squirrel which has a pure cone retina.

The relationship of diencephalic structures to intraocular pressure was systematically studied by Dr. von Sallmann in attempts to induce glaucoma in lower animals. Dr. Windle and his group in the Laboratory of Neuroanatomical Sciences did a systematic study of the neuroanatomical changes subsequent to neonatal asphyxia in primates in attempt to clarify the various problems underlying cerebral palsy. These same investigators carried on a neuroanatomical study as to the neuroanatomical substrate of the toxic manifestations of the ataratic drugs in attempt to produce Parkinson-like syndrome in animals. Doctors Guth and Frank first demonstrated that a vagophrenic anastomosis could in fact carry afferent volleys back to the brain stem and initiate diaphragmatic movement by way of regenerating vagal fibers through the distal phrenic nerve.

Dr. Alvord and Dr. Kies isolated the water soluble fraction necessary to induce allergic encephalitis in animals. Dr. Baldwin and his colleagues have carried on a prolonged systematic study of the various cortical and nuclear structures of the temporal lobe in their relation to behavior and seizures in higher primates.

The projects listed above are but a few carried out by the Institute in an endeavor to induce in lower vertebrates lesions not dissimilar to that seen in disease in man. The rest of this report will deal with the application of the above research techniques to man.

Applied Research in Man

During this decade, almost all of the techniques described above had been utilized in investigations of disease in man. In the early days of the Institute, it became apparent that to adequately control studies in neuromuscular disease an over-all survey of the response of striated skeletal muscle to disease must be made. A double-blind study which established the basic reactions of muscle to disease and formed a pattern which is shown to be valid through over 1000 muscle biopsies was subsequently published in monograph form. The utilization of radioisotopes and newer techniques of cationic analysis early established that in the residual muscle fibers there was a decrease of potassium and an increase in sodium. However, such potassium as was present turned over with the same rate as potassium did in normal controls. Baseline electrical recording of the action potentials of striated muscle and its variations in the many neuromuscular diseases was also carried out and completed in over 400 muscle cases. The interaction of the various endocrine glands occupied the Institute for over a year in a study of their relationship to disorders of striated muscle. The early studies in pathology allowed for the first time a separation of the various disorders which result in the hypotonic infant. Intracellular recordings in single muscle fibers in man have been accomplished. It was demonstrated that hyper-

polarization was not responsible for the paresis of familial periodic paralysis. On the other hand, electron microscopic studies demonstrated a marked swelling of the endoplasmic reticulin which could in fact result in the paresis from the basic studies listed above. More recently, similar studies have been completed in the myotonias which are accompanied by periodic paralysis. Here again, no change in the resting potential of the muscle fiber was found. Here again, an increase in the diameter in the endoplasmic reticulin was found. Abnormal aldosterone secretion was found not to be the cause of the paralytic attacks. It was in this Institute that hyperkalemia was first associated with the disorder paramyotonia congenita. Systematic investigations of both the contractile and noncontractile proteins in a variety of neuromuscular diseases were carried out over the past decade by Dr. Horvath. The pathology of striated muscle has been recently extended by the work of Dr. Engel, utilizing cytochemical procedures. He has described the "muscle target fiber" which is characteristic of the neuropathies. It was in this Institute that the first association of a myopathy with Sjogren's syndrome was established; and more recently, a study has been completed in which the various causes of late onset sporadic myopathy have been separated. Studies of the excretion of pentoses in myopathic disorders were completed early by Dr. Tower; studies of exchangeable potassium using radioisotope tracers were completed which demonstrated that the decreased exchangeable potassium was in all probability merely a function of residual muscle mass. Studies in myasthenia gravis have been largely pharmacological in nature, and it was in this Institute that the first lycoramine derivatives have been utilized with some success in this disease.

In disorders of the visual system, Dr. von Sallmann's unit has led the way with collaboration of a group of distinguished visiting scientists, such as Dr. Bornschein, Dr. Dodt, and Dr. Tansley in the understanding and development of electroretinogram. The first electroretinograms on patients with familial night blindness and on achromates were done here. Such studies were later used to separae the infantile from the juvenile forms of cerebromacular lipidosis. At this time in Cambridge, England, Dr. William Rushton had perfected a technique of the measurement of rhodopsin in the living animal. Subsequently, such studies were utilized in the measurement of the regeneration of visual pigment in patients with primary cone or rod defects here at this Institute by Dr. Rushton. It had long been recognized in the so-called pigmentary degenerations of the retina that the electroretinogram was extinguished. Through the use of a maze computer, Dr. Gouras has been able to retrieve the signals of the extinguished electroretinogram from background noise.

In the past two years, the extraction of cataracts has been made easier by so-called chemical or enzymatic zonulysis. The substance utilized was alpha-chymotrypsin. Dr. von Sallmann was able, by use of his flat mount preparations combined with tritiated thymidine, autoradiography, to demonstrate that this substance is comparatively safe to use in man. It was in this laboratory that the first electron microscopic studies in human cataracts have been carried out and different types separated. In studying an extremely large series of choreoretinitis patients, this laboratory established that toxoplasmosis is a common etiological agent. An effective treatment was established for the young patient of short duration history. Here, also, was reported the first association of cataracts with long-term steroid therapy. A theoretical presentation of the refraction theories of emmetropia and ametropia in which the elasticity of the choroid was first shown to be of importance was established by Dr. van Alphen. The response of the eye to systematic diseases was also further clarified. The description of retinopathy and angioid streaks was described in patients with sickle cell anemia. The vitreous opacities in familial amyloidosis were also described which have subsequently became of much diagnostic importance. Cataract changes in dystrophia myotonica were further clarified, and it appeared that those patients which had visual impairment stood a much stronger likelihood of also being diabetic. Dr. Van Buren has carried out an extensive topographical analysis of the retinal ganglion cells in man and higher primates and has correlated these with visual fields and has demonstrated the retrograde degeneration of such cells, transynaptically, after lesions as far away as the occipital cortex.

In studies of the brain, Dr. Baldwin and his colleagues have continued their detailed meticu-

lous studies of the functional anatomy of the temporal lobes and the deep nuclei of the brain in relation to function and spontaneous discharge, i.e. seizures. In this study, subdural electrodes, electrodes planted in depth within nuclei and chronically left in place, combined with electrocorticography and electroencephalography were used with the aid of Dr. Ajomne-Marsan. Dr. Van Buren has studied the autonomic reaction by using various transducers and D. C. pen writing amplifiers after stimulation of the orbital surface of the frontal lobe and of the temporal lobe in man. Such studies were carried out also in higher primates by Dr. Baldwin and Dr. Van Buren.

The first attempt at high resolution detection of brain tumors by utilization of dense, metalic, multichanneled collimators was successfully carried out within the Institute in now over nine hundred patients. Such studies were correlated with normal and abnormal contrast radiological studies by Dr. Di Chiro. Intensive studies of the effects of hypothermia on the brain have been carried out by Dr. Baldwin and his associates. A new stereotaxic instrument was devised by Dr. Van Buren and has been used successfully in the treatment of involuntary movements. Pharmacological treatment of seizures by utilization of glutamine, asparagin, and gamma aminobutyric acid in man has been carried out by Dr. Tower and his group. Such normal metabolites are effective in the treatment of seizures but do not exceed the effectiveness of empirical medication now available. The effects of monamine oxidase inhibitors on patients with seizures were studied by Dr. Bushnell Smith and shown to be without effect unlike lower vertebrates. The study of the effects of pyridoxine in the precipitation of seizures was also studied by Dr. Tower and Dr. McKhan in a patient with pyridoxine dependency.

In the early days of the Institute, fractionation of proteins of the spinal fluid in patients with various neurological disorders was studied. It was found that the gamma globulin increase seen in multiple sclerosis was nonspecific. This Institute carried out the first double-blind pharmacological study of isonicotinic acid hydrazide treatment of multiple sclerosis and demonstrated it to be without effect. Finer radiological techniques have been developed by Dr. Di Chiro in the study of normal and abnormal structures of the brain. The technique of fine pneumoencephalography resulted in the last year in a beautifully documented Atlas demonstrating many structures not previously seen. The technique utilized was that of fractional encephalography in combination with laminagraphy. Dr. Di Chiro has also demonstrated that the volume of sella is more important than its sagittal measurements for the diagnosis of pituitary tumors.

Studies in the developing nervous system have been carried out by Dr. Dekaban in a correlative program covering over four thousand one hundred fifty-six pregnancies in which neural fetal wastage was for the first time statistically documented. It was in this unit that familial idiopathic hyoglycemia was shown to be a cause of mental retardation and seizures.

New Diseases and New Concepts

It is inevitable in such a program that new disorders will become apparent. In 1956, the Institute described a new disorder of muscle in which a central core of abnormal myofibrils was found. Subsequent chemical, electron microscopic, and cytochemical studies demonstrated an abnormality of the myofibrils in the center of muscle fiber, the absence of interfibrillary material, the absence of mitochondria in the middle of the fiber, the absence of phosphorylase A, and almost a complete absence of phosphorylase B in the center of such fibers. These findings have been verified in England and Holland. This Institute gave the first complete description of the neuropathology of a new disorder, Kuru, found in New Guinea. Dr. Eyerman and Dr. Irwin demonstrated in fibrocystic disease that there was an excess of acetylcholine in the sweat, thus linking abnormal neurohumoral agents to this disorder. Dr. Krooth was able to grow galactosemic cells in tissue culture and thus establish a tissue bank of a relatively rare disorder. Dr. Rowley described a new disorder of dwarfism, muscular hypoplasia, pulmonary hypertension, cor pulmonale, and aminoaciduria as a new syndrome. Dr. Altrocchi joined with Dr. Frederickson of the Heart Institute in the description of Tangier disease, a new lipid storage disease, in which there is a familial cholesterolosis with a deficiency of high density lipoproteins. Dr. von Sallmann described in an iso-

lated ethnic group a new familial dyskeratosis of the parilimbal conjunctiva.

Instrumentation

As can be seen from the above descriptions, many of the advances have been made by the availability of new techniques and new instrumentation. The Institute also has developed instrumentation of its own which is now used in many centers. Dr. Frank developed electronic microelectrode pullers as well as micromanipulators with less than eight microns backlash. The voltage clamp technique was largely developed and utilized by Dr. Cole and is now used in almost every physiological laboratory. The Institute developed a new rectilinear scanning apparatus to carry heavy density multichanneled collimators. The unity gain cathode follower that is now used in most physiological laboratories was developed by Mr. Bak in the Laboratory of Neurophysiology. Simplified relay computer networks were developed by Dr. Gouras in his work on the extinguished electroretinogram. Dr. Van Buren has developed a new highly versatile stereotaxic apparatus for use in man. Dr. Baldwin and Dr. Bach developed a radio wave apparatus transmitter with resinating receiving chambers to make minute lesions in animals and were the first to demonstrate such lesions were not heat dependent. Dr. Baldwin and his colleagues have made advances in isolated head hypothermia. The Institute has developed its own recording ergometers. Dr. Gunkel of the Ophthalmological Branch has perfected self-recording tangent screens and a variable illuminating dark adaptation apparatus. He also aided in the development of the rhodopsin regeneration technique utilized by Dr. Rushton.

Summary

The Intramural Research Program has been active for eight and one-half years of the decade of which the Institute has been in existence. The research listed above is meant to give but an indication of the type of research productivity which has occurred in these eight and one-half years. It is drawn from over six hundred technical reports and twenty-four monographs. One cannot in this space do justice to all the research of the Intramural Program, and this is an attempt to describe some of such research progress as has occurred. Each Branch and Laboratory Chief has been requested in this current reporting year to give a summary of his own unit's accomplishments during the past reporting year. These reports as presented by the Laboratory and Branch Chiefs comprise Part II of the Scientific Director's Report.

LABORATORY OF NEUROANATOMICAL SCIENCES

Introduction

The past year has seen a completion of the reorganization of this Laboratory. Dr. Sanford Palay has left to accept professorship of anatomy at Harvard University. This was a commitment made approximately three years ago. In compliance with the recommendations of the last annual report after consultation with the Scientific Counselors, the Section on Experimental Embryology was established within the Laboratory which in July was filled by Doctors Alfred and Jane Coulombre. These investigators have initiated within this six-month period a program which shows much promise. The Laboratory Chiefs felt that the importance of electron microscopy to the future of the Institute could not be underestimated. As such, they advised that Dr. Keith Richardson, Associate Professor of Anatomy at Washington University, St. Louis, be selected to head the Section of Neurocytology. At the request of the Laboratory Chiefs, the Scientific Director of the Institute is temporarily acting as Chief of the Laboratory. With the establishment of the Section of Embryology, the Section on Development and Regeneration which was Dr. Windle's old section was renamed the Section of Experimental Neurology which was more in line with its actual function; and Dr. Lloyd Guth was made the section head. Thus, the Laboratory now consists of four sections: the Section of Functional Neuroanatomy under Dr. Grant Rasmussen, the Section of Experimental Neurology under Dr. Lloyd Guth, the Section of Neurocytology under Dr. Keith Richardson, and the Section of Experimental Embryology under Dr. Alfred Coulombre. The Section of Experimental Neuro-

pathology under Dr. Jan Cammermeyer has been administratively removed from the Laboratory. This Section will continue its investigations within the space of the Laboratory but will form the nucleus for a new laboratory of neuropathology upon completion of the new research facilities of the Institute.

Eighth Cranial Nerve

The initial discovery by Dr. Grant Rasmussen of the olivary cochlear bundle is a major milestone in knowledge of the functional anatomy of the central nervous system. This was the first demonstration that afferent receptors to central nervous system structures were controlled by way of a servo feed-back efferent system. Similar systems have now been found in other sensory modalities of the nervous system. Vestibular and auditory components of the nervous system, however, have formed a prototype of such studies. It is with these particular structures that this Section has continued the majorty of its efforts. Such studies have been greatly facilitated by the acetylcholine stains of Koelle in which efferent systems within the 8th cranial nerve connections have a greater affinity for such stains due to a much higher concentration of acetylcholinesterase. Such studies also utilized the gold impregnation stains of Golgi, the retrograde cellular changes of Brodal, and an analysis of changes in fiber tracts and neurons after sectioning at various levels as in the brain stem and the cortex. Working with Dr. Rasmussen have been Professor Gosta Dohlman, retired professor from Lund, Sweden, and Dr. Robert L. Boord. The primary interests of this group of investigators have been to determine the ultimate destination of the efferent fibers of both cochlear and vestibular nerves, to study the morphology and function of cupulae of the vestibular apparatus in order to understand the mechanism of hair cell stimulation, and to establish, at synaptic levels, the interconnections between the cochlear nuclei and higher medullary centers. In this study, Dr. Dohlman using a Sudan black staining technique initiated by Dr. Rasmussen and microdissection of the ear has demonstrated that the vast majority of the hair bearing receptor cells and their associated innervation were located on the side parts of the ampulla next to the planum semilunatum. Such cells previously were believed to lie on the transverse ridge surmounted by the cupula. The fact that they are on the side has provoked new speculations as to how they are indeed stimulated. It is now difficult indeed to make the hair cell the origin of the microphonic response. Dr. Dohlman raises the interesting speculation that a chemico-electrical influence rather than a mechanical one initiates neuroactivity from the hair cells.

Using the methods described above, Dr. Boord and Dr. Rasmussen continued their study of a comparative anatomy of such connections in submammalian vertebrates using predominantly the pigeon in order to determine the termination of efferent cochlear fibers and the projection of afferent cochlear tracts to primary auditory centers. They have found that the efferent fibers terminate in the organ of Corti in a manner similar to mammals. The afferent fibers and the associated lagenar nerve from the cochlea of birds appear more simply constructed than that of mammals. Unlike the cochlear nucleus of mammals, this nucleus is uncoiled and hence more convenient to work out the connections of the various parts of the cochlea. These investigators found a tonotopic organization in such a nucleus preceding successively from the apical to the basal parts of the cochlea. All fibers of the nerve apparently branched upon entering the medulla and the lateral ascending fibers terminated in the angular nucleus, the medial in the nucleus magnocellularis. In both cases, a precise localization was found within the nucleus, in the angular nucleus from ventral to dorsal, and in the case of the magnocellularis nucleus from medial to lateral. The lagenar nerve was also precisely tonographically represented in these nuclei. A precise point-to-point relationship in a relatively simple nucleus in the submammalian species will form the ground work of physiological studies concerned with tone representation. Studies are also under way by this group of investigators oriented to localizing areas of the cortex of the cat that received direct connections from the medial geniculate bodies and to establish which of the five cortical auditory fields have efferent connnections with the medial geniculate. A combined ablation study in which all lesions will be placed in the various parts of medial geniculate body and in the auditory corti-

cal areas A–1, A–2, and AP as well as the insular and temporal gyri has been initiated. Additional studies are under way to determine what fiber systems other than auditory project to the medial geniculate body as well as what intrinsic interneuronal connections exist within the geniculate body.

Physiologists have long reported that taste may well be represented in the ventral posteromedial nucleus of the thalamin. Anatomical varification of pathways from the nucleus solitarius which is the main receptive nucleus within the brain stem for taste from the 7th, 9th, and 10th cranial nerves to the ventral thalamus and, subsequently, to the cortex, has as yet not been delineated. Dr. Morest has initiated such a study in which he has placed small lesions within nucleus solitarius and will be studying such axonal interconnections, the knowledge of which is sadly deficient.

Ultrastructure Studies in the Nervous System

Perhaps the greatest legacy left by Dr. Palay to the Laboratory of Neuroanatomical Sciences was his method of vascular profusion of buffered osmium oxide which has minimized artifactual distortion for electron microscopic studies. Almost all the investigations listed by the Section of Neurocytology indicate that such investigations would have been impossible without such techniques. The new Section Chief, Keith C. Richardson, joined the unit in September of the reporting year and in this three-month period is already well into his studies concerning the chromaffin cells in the dermis, the innervation of smooth muscle in the vas deferens, and ultrastructure changes in the fasciculus gracilis and its nucleus after cord hemisection. This unit has already confirmed the rich innervation of the outer longitudinal muscle coat of the vas deferens. Such endings apparently exist in the form of single neurites lying in grooves upon the surface of some of the muscle fibers. Such neurites contain granules surrounded by membranes similar to those found in the adrenal medulla, carotid body, and some terminals in the central nervous system which are associated with the presence of catechol amines. The study of the degenerating myelinated fibers of posterior columns after transection of the cord is difficult owing to the oedema in the region of the degenerating fasciculus. Due to such oedema, the perfusion fixative is less successful here than in other studies.

Dr. Brightman has continued his studies on the ciliated ependyma of the cerebral ventricles also using the perfusion fixed technique. Dr. Brightman has found no basement membrane, and such cells abut directly against numerous glial and neuronal processes. He indicates that large molecules would have to pass through the ependymal cells and not between them presumably by pinocytosis. This latter conclusion was arrived at by utilizing dialyzed ferritin injected directly into the ventricles. Dr. Naumann has initiated an electron microscopic study of the interaction of fibers of the ascending reticular activating system and descending cortical modulating system.

Dr. Wolfe has continued his studies of the fine structure of the area postrema. He has found an abundance of neurons and nerve fibers within this area, disproving the belief that only glial cells comprise the area postrema. No confirmation that such an area has neurosecretory function could be found.

Regeneration and Reinnervation

Dr. Guth studied reinnervation and regeneration of the cervical sympathetic trunk and found stimulation of T1 and T3 roots elicited pupillary dilatation. Stimulation of T2–T7 roots elicited vasoconstriction, indicating that the preganglionic fibers had regenerated selectively to their appropriate postganglionic cells. One month after crushing T1–T3, electrical stimulation of these roots produced no pupillary dilatation whereas stimulation of the intact T4–T7 did. Six months postoperatively, electrical stimulation of T1–T3 produced pupillary dilatation where stimulation of T4–T7 did not. Thus, although pupillary postganglionic cells accept innervation from a foreign source, they exhibit however a preference for their original innervation. In studies in the sciatic nerve after transection of the cord, the proportion of fibers from the L4 and L5 segments going to the soleus and plantaris muscles was equal. This would indicate that regeneration of the sciatic nerve is probably random rather than

a selective process, and thus showed no evidence of selectivity in nerve regeneration as was demonstrated in the sympathetic system.

It has been shown in the past that mammals are allergic to intraperitoneal injections of their own tissues. It has been thought that the antigen contained in the brain is normally kept from the general circulation by the blood-brain-barrier. Thus, the possibility that a glial scar tends to block axonal regeneration following injury to the nervous system may be, in fact, and auto-immune response to the release of brain antigen due to a breakdown of the blood-brain-barrier. Since injection of central nervous system substances during the neonatal period has been reported to make animals tolerant to the central nervous system substance, newborn rats were thus injected on the day of birth and at regular intervals thereafter with small amounts of fresh, homogenized rat brain. After the animal had reached the adult size, Dr. Feringa transected the facial nerve, and the distal end was inserted into the cerebral cortex. The amount of regeneration of facial nerve and the amount of glial scar formation was then compared to animals who had not received CNS substances. His preliminary findings indicate that rather than producing a state of tolerance to the injection of brain substances, in newborn rats the injections in fact produce an inflammatory response in the cerebrum by itself. The implantation of the peripheral nerve, similarly, seems to produce an increased incidence of inflammatory lesions. Thus, the hypothesis of a blood-brain-barrier preventing an antigen release does appear to be valid.

Dr. Feringa, in addition, carried on pharmacological studies with 6-mercaptopurine and amethopterin to see if glial scars could be significantly altered. He found that even at toxic levels of these compounds, no significant alteration in the amount of scar formation was found. Using tritiated thymidine and autoradiography, Dr. Feringa has continued his studies on the regeneration in the spinal cord in the newt. He has found only ependymal cells labelled to date. Considerable technical difficulties with autoradiography have been encountered in this study.

Dr. Bernstein has carried on his functional studies on color vision and taste. In color vision, the goldfish is utilized using conditioning responses. He has found that whereas normal fish react to the wave length characteristics of the colored stimuli, a fish with a forebrain ablation does not. The neuroanatomical structure of the fish brain allows for the manipulation of certain color visual centers without blinding the animal. Similar conditioning studies are anticipated in the coming year in gustatory discrimination by Dr. Bernstein.

Changing of Anatomical Relationships by Embryonic Manipulation

Dr. Coulombre and Dr. Drachman have initiated a study of injection of neuropharmacological agents in the developing chick embryo. The agent used to date is that of curare to see the effect of the same on the morphology and physiology of the neuromuscular and skeletal systems of the embryo. The technique is of value in that it has been demonstrated by others (see report of Doctors Engel and Mumenthaler) that the myoneural junction is established in the chick during the second week of embryonic life. Present studies are an attempt of "pharmacological dissection" of the neuromuscular apparatus during its formation in order to produce permanent changes as against the transient effects on the adult organism.

Doctors Alfred Coulombre and Jane Coulombre are continuing their studies on the microsurgical removal of transient thickenings in the conjunctival epithelium of chick embryos at different stages of development to see the effects on subsequent development of the ocular skeleton. Chemical dissection of the living epithelium from its underlying mesenchyme followed by a confrontation of the conjunctival epithelium and limbic mesenchyme of different ages both *in vivo* and in organ culture is also utilized. Since the eye must meet stringent optical requirements to function adequately, the present and previous work of these investigators as been directed towards the many factors responsible for the shaping of the developing eye. They point out that events occurring at the limbic area during development have proved to be important in determining the radius of the curvature of the cornea which is one of the most important refracting structures of the eye. Thus, a study of events occurring at the lim-

bus during development extend the understanding of factors which shape or misshape the eye.

Experimental and Comparative Neuropathology

The Section of Experimental Neuropathology will serve as the nucleus for the new Laboratory of Neuropathology upon completion of the research facilities in the mid '60s. Because this Section utilizes facilities of the Laboratory of Nueroanatomical Sciences and has been closely coordinated with them in the past, a report of this Section will be included at this particular site. The over-all endeavors of this particular Section has been to minimize artifactual abnormalities dependent upon fixation of structures of the central nervous system. Having once accomplished this, the normal characteristics of the central nervous system may be redefined. New concepts about the spatial assocation of blood vessels, neurons, and glial cells involved may be thoroughly studied. Thus, Dr. Cammermeyer has found two neuronal types throughout the brain stem and spinal cord of different animal species, one eosinophilic and one basophilic. In order to determine the significance of these two type of neurons and their distribution in several animal species, experimental materials from various sections of the brain stem in various animals such as the sloth, weasel, and otter are now under way. The next objective is that once a preparation is free of artifact it may be used to study the effects of fatigue, muscular activity, rest, and pharmacological agents upon the various neurons of the nervous system. The effects of hypoglycemia and anemia upon the cells of the central nervous system may also be thus studied. The final objective of this Section is to define the stepwise pathological changes in neurons and neuroglial cells as well as the vascular system after total body irradiation or in specific nuclei after section of the appropriate nerve. Dr. Cammermeyer emphasizes the similarities of the oligodendrocyte to the cerebellar granule cell. He points out that both type cells differ upon advancing age from other central nervous system elements by retaining their tinctorial qualities and not accumulating lipid materials. After nerve section, Dr. Cammermeyer finds that in the appropriate nucleous the nuclear eccentricity of the cell is independent of the type of histological technique used. The size of the neuron is apparently unaltered throughout the acute phase of reaction to axonal damage, and the final replacement of basophil material in the cytoplasm occurs first next to the nucleus.

LABORATORY OF BIOPHYSICS

The central objective of the Laboratory of Biophysics is the understanding of the nature and the implications of the ion movements fundamental to the initiation and propagation of the nerve impulse. The work has centered about the voltage clamp, a recent and rewarding approach in which the electrical current flow across the squid axon membranes was first measured after a sudden change of the membrane potential.

In general some of the earlier antagonism to the voltage clamp concept and to the interpretations of its application seems to have abated. The confusion may have been lessened as more results appear for more preparations from more investigations. The language of the specialty that it is has become more familiar as it has also been used by more of the courageous to attempt to interpret phenomena in cells for which clamp measurements are not as yet possible. So the present period is one of widespread but intensive fact-finding, and as the likenesses and contrasts between the treatments by various membranes of various ions grow attention will focus with increasing sharpness on the underlying, universal cell problem as to the fundamental nature of the ion permeability of living membranes. The emphasis at the present and probably for awhile to come is rather on the asking of questions than on the supplying of answers. And there is no way to now do more than blindly guess which questions may lead to the most revealing answers.

The work of the Laboratory during 1961 is the work of the following: John W. Moore, Richard FitzHugh, Robert E. Taylor, William J. Adelman, Jr., W. Knox Chandler, Leonard Binstock, Uichiro Kishimoto and Kenneth S. Cole. At the middle of the year Dr. Moore, an original member of the Laboratory, resigned to become associate professor of the reorganized Department of Physiology at Duke and a fellow of the National Neurological Research Foundation. In Septem-

ber, Dr. Chandler returned to inactive PHS reserve status on fellowship for graduate study of physical chemistry. Also in September at the end of his Visiting Associate appointment, Dr. Kishimoto returned to his post in Japan.

We have also worked in collaboration with Lorin J. Mullins, and R. L. Sjodin of the University of Maryland, David E. Goldman and Fred Julian of NMRI, Alan L. Hodgkin of Cambridge University, John C. Dalton of University of Buffalo, and Fred Diecke of George Washington University.

The dissemination of new scientific knowledge for others—and us—to build upon has continued at a rather high rate. Seven articles have appeared in literature during the year and eight others are in press. Two invited papers were given, one by FitzHugh to the Biophysical Society and the other by Cole to the first International Biophysics Congress while ten papers have been contributed at these and other meetings. The staff has probably even more than in the past participated in local, national and international professional activities.

Among the as yet unpublished work, there have been some developments of technique. Adelman, Binstock and Taylor have been gradually improving and simplifying the squid axon clamp while increasing its reliability and accuracy. Taylor and Chandler have developed a Schering bridge for precision high frequency alternating current measurements of the axon membrance. The Laboratory encouraged and helped Kishimoto to evolve and apply a new voltage clamp to the enigmatic plant cell Nitella while Moore has collaborated with Julian and Goldman at NMRI to achieve an adequate clamp on a lobster axon and so pave the way for work on other and smaller axons. There are however important increases in the resting potential of lobster and squid axons near regions in sucrose solution which may delay or even limit the use of some of these developments. A most spectacular advance was the internal perfusion of squid axons at Plymouth a year ago and it is a great disappointment that Adelman, Dalton, Binstock and Kishimoto were unable to repeat this work at Woods Hole—almost certainly because of the unavailability of sufficiently large animals and axons.

Studies of the properties of analytical models of axons and axon-like systems required considerable mathematical power—from the theory of differential equations to solutions by digital and analog computers. Chandler, FitzHugh and Cole have a paper in press showing the Hodgkin-Huxley squid axon system to have spatial stability only under conditions which may be difficult to meet with some real axons but a published elementary analysis by Cole indicates that adequate measurements can be made in spite of spatial instability.

The digital calculations of the properties of a theoretical medullated axon with Hodgkin-Huxley like nodes and passive internodes were started several years ago by FitzHugh. The development of an action potential near a stimulating electrode and the approach to its propagating velocity agree well with experiment and are in press. Further problems of threshold and blockade will not be investigated because of the time and expense involved.

The principal effort has been on other systems. The analysis of the passivated iron wire in H_2SO_4 was published by Franck and FitzHugh while work on the more complicated HNO_3 system is continuing. FitzHugh has proposed a simple model adapted from Bonhoeffer and van der Pol and shown in print its usefulness as an example of the many excitable-oscillatory models and in providing an easily understandable physiological state diagram. In present work the model is being used to develop matematical methods such as for the analog computation of the velocity and form of the propagated impulse.

The general nature of the electrical capacity of the squid axon membrane, as representing an inactive, "lossy" dielectric, has long been an acceptable description of the experimental findings while studies of the largely independent parallel conductance have been richly rewarding. But the lack of clues as to the mechanism of this conductance, or of the dielectric loss and the increasing electron microscope information have suggested the more precise studies that Taylor and Chandler have undertaken. With bridge measurements from 10 to 70 kilocycles they have confirmed that the capacity is largely independent of the ionic conductance and depolarization during activity as it is for a considerable hyperpolar-

ization. Although the phase angle seems quite constant for changes of frequency and temperature, the capacity was found to increase by about one percent per degree from 5° to 22°C. This suggests a highly coordinated structure by analogy with solid, high polymer dielectrics of similar properties. The capacity usually increases also as the axons deteriorate. As a corollary of this work it is to be hoped that it will lead to determinations of the elusive series resistance associated with the capacity without which the ionic characteristics must remain uncertain to an unknown extent.

The work of Adelman and Taylor on the "leakage" current in a squid axon clamp has appeared in preliminary form and been continued further. Hodgkin and Huxley assumed this current to be a small, linear and time-independent component that might be carried by chloride ions. But it was first found to involve a rectification in less than 100μ sec that was rather insensitive to external sodium, potassium or chloride ions. The more elaborate recent experiments seem now to be interpreted in terms of an additional millisecond rectification for both hyperpolarization and depolarization with yet another rectification operating in seconds for large hyperpolarization. There is no certain indication of any ionic carrier in any of these processes. These effects are of a disturbing magnitude and may lead to a new mechanism of membrane conduction with quite unpredictable consequences as the functional structure of the membrane.

In a pair of papers now in print, Adelman and Moore investigate and use the "sodium potential" of Hodgkin and Huxley at which the early current reverses in a voltage clamp. This potential closely follows the sodium equilibrium potential to be expected across the membrane under various experimental conditions thus supporting the original concept and providing an accurate measure of the internal sodium ion concentration. In this way the sodium accumulation of the squid axon was found to be about doubled in rate by a ten-fold decrease in the external calcium and magnesium concentration.

The investigations of the effects of detergents on the squid axon membrane have been continued by Adelman and Kishimoto. A cationic molecule drastically and irreversibly decreases the sodium and potassium conductances and the resting and action potentials while anionic forms were less effective and gave initial temporary but reversible increases before subsequent irreversible decreases. An uncharged detergent gave but little change for the potassum conductance with reversible decreases of the sodium conductance and action potential.

In 1960, Mullins, Adelman and Sjodin measured the efflux of sodium and potassium tracer ions from the squid axon membrane under several voltage clamps in an initial attempt directly to identify the ionic current components as formulated by Hodgkin and Huxley. Under the simplest of conditions with no external sodium ion concentration the early clamp current is accounted for by tracer sodium efflux to within twenty percent, but the steady state current fails to agree with the potassium efflux into sea water by a factor of two. In other situations the phenomena are in general considerably more confusing and it is still far from clear whether it is the isotope movements or the ionic currents that are the less well understood. In any case it is particularly unfortunate that bad weather and a diversion of Hodgkin's interest prevented further work at Plymouth and that additional data were not obtained at Woods Hole in 1961. The problem has if anything taken on an increased importance and, with Diecke, Adelman is investigating the possibility of such work with the lobster axon.

In fulfillment of the primary objective of his work at the Laboratory, Kishimoto, with Binstock, was able to apply a simple but adequate voltage clamp to the plant cell Nitella. The ionic currents were similar in form to those of the squid and lobster axon membranes and to the frog and toad medullated axon nodes although far slower and smaller. The data, which confirmed the then unknown results obtained in Tasmania, qualitatively account for excitation and propagation in this unusual cell. But agreement as to the identity of the ionic current components has not been reached. If, as seems not unlikely, its nervelike characteristics are to be accounted for by the chloride ion, the membrane of Nitella will be the first and probably a very important example in which a negative ion has such a function.

Julian, Moore and Goldman have created an artificial node in the lobster axon in which flowing sucrose replaces the myelin of a medullated axon.

It is then possible to investigate the active and the clamped characteristics of the membrane without internal electrodes. With sufficient amplification and a "node" less than 100μ long the potential control seems adequate and the ionic currents are closely similar to those of other successfully clamped axon membranes. The negative steady state resistance in iso-osmatic KCl is a noteworthy similarity to the squid axon while the absence of a slow decline in the long time outward current is a striking contrast.

LABORATORY OF NEUROCHEMISTRY

The Laboratory of Neurochemistry has been reorganized. Dr. Tower was appointed Acting Chief of the Laboratory. The Lipid Chemistry Section continues an established program concerned with the structures, biosynthetic pathways, metabolism and functional significance of the complex neural lipids, particularly the glycolipids which are important units of neural cell structure and are involved in a number of diseases of the nervous system, notably some of the lipodystrophies and a variety of demyelinating diseases. The Section on Proteins and Amino Acids derives from the former Section of Clinical Neurochemistry, NINDB, which has been concerned since its inception with the metabolism and functional significance of cerebral amino acids (both in the free and protein pools) and their interrelationships with cerebral oxidative metabolism and cerebral electrolyte metabolism, all with particular reference to seizure states. The Enzyme Chemistry Section derives from a former subsection in the Laboratory of Neuroanatomical Sciences, NINDB, Section on Neurocytology, and is continuing its general interest in enzyme systems which are characteristic of or of particular significance for neural metabolism and function—the methodological approach being primarily at the cellular and subcellular level with ultra-micro techniques of the Lowry-Linderstrøm-Lang type in terms of the chemical anatomy of the nervous system. Finally the Muscle Chemistry Section, previously a subsection of the Section of Clinical Neurochemistry, NINDB, has been devoted since its inception to a study of the contractile and other proteins of skeletal and smooth muscle as part of a multidisciplinary approach in NINDB to the general problem of neuromuscular diseases.

Together with the future Section on Developmental Neurochemistry, these Sections cover the major areas and disciplines which comprise the field of neurochemistry. The personnel of these sections have all been associated with NINDB for a number of years and have collaborated closely with each other during this time, so that the administrative reorganization into a formal Laboratory of Neurochemistry was simple to accomplish with essentially no disruption of continuity of research. More or less coincident with establishment of the Laboratory, it was possible to effect geographical consolidation of the sections from three different areas into one.

The Section of Lipid Chemistry reports concern two major projects: studies on fatty acid synthesis and studies on gangliosides. Contributions from this Section have aided significantly in unravelling the structure and mechanisms of biosynthesis of malonylcoenzyme A, which appears to be the key intermediate at the initial stages of synthesis of long chain fatty acids. Details of the subsequent steps leading to biosynthesis of the full-length chains are now being delineated. In the past relatively little information has been available on the fatty acid moieties of the neural lipids, yet it is clear that these moieties are varied and complex both in chain length and degree of unsaturation, and to some extent peculiar to neural lipids. It seems reasonable to expect that on the one hand some of the specificity of these lipids and on the other hand some of the neural dysfunctions related to abnormalities of such lipids may relate in part to the fatty acid moieties.

In addition fatty acid synthesis represents one of the two known mechanisms for CO_2-fixation in cerebral metabolism. Particular attention in this project is being paid to the mechanisms by which CO_2 is fixed in the biosynthesis of malonylcoenzyme A, and there are already indications of some rather unusual and unexpected reaction sequences in brain related to this phenomenon. The other locus of CO_2-fixation (recently demonstrated in Waelsch's Laboratory) is the fixation of CO_2 by pyruvate to form oxalacetate of the Krebs cycle, this being a reversible reaction as demonstrated in studies by the Protein and Amino Acid Chemistry Section of this Laboratory. It

is becoming increasingly apparent that we are dealing here with a very complex metabolic area, providing multiple pathways for substrate utilization by the feeding in of pyruvate at both ends of the Krebs cycle (as acetyl-CO A and as oxalacetate) plus a diversion to fatty acid and related syntheses at the acetyl-CO-A stage. When these pathways are considered in relation to the production of CO_2 by glucose oxidation, to the recent localization (by Giacobini) of carbonic anhydrase only in gial cells, and to the well-known physiological effects of alterations of CO_2 tension on neuronal function, it is obvious that a very important and fertile field is under study now.

The broad approach to the glycolipids of the ganglioside type represents an equally challenging and important project. Contributions by the Lipid Chemistry Section are noteworthy in virtually all aspects of this subject. Improved methods for isolation of gangliosides from brain have been developed and are being adopted by many other laboratories. Analyses of ganglioside structure have begun to clarify an heretofore very controversial problem. As a result of studies in this project, the polymer type structure may now be supplanted by a micellar aggregation structure and the oversimplified concept of a common basic unit can be discarded in favor of a family of compounds of closely related structure. The importance of these contributions are two-fold, first, in providing a sound basis for biosynthesis studies now in progress, and second, in providing much needed data with which to relate functional attributes of these compounds to their structural make-up.

In concert with a number of other groups elsewhere, Section personnel engaged in these studies have recognized and begun investigations on the functional attributes of these glycolipids. Already McIlwain and his group in England have proposed that gangliosides may function as carriers in mediating cation transport across neural membranes, and Van Heyningen, Gottschalk, Klenk, Bogoch and others have proposed various receptor roles for these same compounds. The studies here carried out in collaboration with Dr. Irwin of the NINDB Section on Applied Pharmacology indicate a sequestering property of gangliosides for a number of compounds of physiological and pharmacological interest, notably curare and chlorpromazine. It is generally agreed that the gangliosides are primarily constituents of cell membrane structure, so that these various observations focus on the very real possibility of identifying and localizing a major class of cell receptors and of delineating their mechanisms of function. The role of the neuraminic acid moieties of the gangliosides is emphasized by the Lipid Chemistry Section studies, and the apparent existence of analogous glycoproteins expands the horizons of this field enormously. Perhaps the most promising development in the functional aspects of these problems has been the immunochemical techniques developed. Not only does the application of such techniques offer the opportunity to study structure, localization and function by very precise means, but such studies also may have a more fundamental significance because of their bearing on some very general problems of membrane functions and immunological responses.

It is obvious to all investigators in neurological research that an understanding of the details of structure and function of the conducting membranes of excitable cells is one of the central problems in neurology. The studies on gangliosides discussed above appear to represent a most promising beginning in the unravelling of this problem. Certainly we may expect that the lines of investigation being pursued and proposed for this project will yield a wealth of information in this context.

Data from three projects underway in the Laboratory illustrate a facet of the neurochemical make-up of the nervous system which poses a special problem in methodology.

Comparative studies on the characteristics of gangliosides from brains of widely different species and on the amide composition of cerebral proteins isolated from brains of a comparable variety of species both indicate a remarkable uniformity of composition. Furthermore comparisons of liver and brain protein amide composition and of liver and brain ribonucleic acid (both nuclear and microsomal) characteristics also demonstrate an essential similarity. Thus, one is faced with the paradoxes of vastly different levels of functional capabilities among species and of very different functional attributes between organs not being reflected in differences of chemical

composition. What is one obvious fault here is the analytical level of study. More precise and more micro techniques are necessary to bring out the subtle differences which must exist in at least some of these aspects.

Hence the program of the Section of Enzyme Chemistry seems particularly appropriate, since it involves primarily the use of many ultramicro techniques and since it is aimed at just this problem of delineating the features of neural chemical architecture, organization and metabolic constitution which are characteristic of and unique to the nervous system. The close collaboration of this Section in the past with the various other groups in the Laboratory, ranging from lipids and amino acids to neurohumoral agents and hormones, continues and may be expected to contribute greatly to the gaining of a proper perspective in these various problems.

In the field of Protein and Amino Acid Chemistry and the metabolic systems related thereto, two aspects of the work in progress deserve emphasis: the study of brain nucleic acids, and the investigations of cerebral protein amide group metabolism. The study on nucleic acids represents the first thorough and detailed investigation of these components to be carried out on the central nervous system. Parenthetically it is worth pointing out that this project has been conceived, planned and implemented in its entirety by two trainees (Drs. Dingman and Sporn) fresh out of their internships but with some impressive prior experience as a post-sophomore USPHS Fellowship during medical school. From this project we are already obtaining refined methods for isolating highly pure subcellular elements (cell nuclei, microsomes, etc.), basic data on the physico-chemical characteristics of nuclear and microsomal ribonucleic acids, and an indication that there are small but significant differences in such characteristics between the microsomal and nuclear RNA of the same organ. The possibility that these differences reflect the presence of so-called "messenger" RNA in nuclear preparations is under investigation and, if substantiated, would represent a major advance, since identification of this form of RNA has so far been limited to micro-organisms. Regardless of the outcome of this particular aspect of the project, the data being obtained bear on a host of important problems, notably the highly active turnover of cerebral proteins, the derangements of neural cells as a result of genetic and of viral factors, and the currently popular theory of the role of neural RNA in information storage(memory and learning).

The studies on cerebral protein amide metabolism have yielded some important specific information on this subject as well as a powerful new methodological tool for the general study of protein amide residues. The methods adapted from a number of existing enzymatic procedures have been validated by application to proteins and peptides of known structure and have proven to be particularly valuable in studies on the structure and on the structure-function relationships of ribonuclease being carried out by Dr. Anfinsen's group in NHI. Collaboration with this group has been most fruitful for us in demonstrating the applicability of the methods to general problems of protein structure analyses and has also been very useful to the NHI group in solving some of their difficult problems in the assignment of amide residues about enzymatically active centers in the ribonuclease molecule. Despite the extensive amount of effort devoted to these somewhat non-neurochemical studies, the results have been most valuable for the cerebral protein problem since the validation of the methods of hydrolysis and analysis has dissipated any lingering doubts about the significance of the cerebral protein data.

In the latter regard it is clear that there exists in brain a system for the amidation of protein-bound glutamic acid to glutamine and for the reverse reaction of deamidation of protein-bound glutamine to glumatic acid. The latter reaction was first demonstrated by Waelsch's group and the responsible enzyme, transglutaminase, isolated and characterized by them. The existence of this reaction has been confirmed in our Laboratory and the reverse or amidation reaction demonstrated for the first time. These latter studies strongly suggest that the amidation reaction proceeds via free glutamine in a transamidation or amide transfer type of reaction, which if substantiated, would be a very novel type of reaction. The studies have several important implications. The reactions are limted to glutamine. They do not occur in liver or in cerebral white matter, and are thus, peculiar so far to cerebral cortex. Other neural and non-neural tissues remain to be examined. They rep-

resent a major mechanism for the "storage" and transfer of nitrogen as amide groups in the brain. They pose interesting implications for fluid and electrolyte balance in neural cells, since the reversible covering or opening of fixed, free carboxyl groups on cerebral proteins will affect the intracellular fluid environment as preliminary studies have already indicated. And finally these reactions participate as Waelsch has shown, in the "fixation" of biologically active amines, which can reveribly attach by substitution to protein-bound glutamine amide groups. Such a system in the central nervous system thus appears to interrelate with a large segment of the metabolic and functional machinery of this organ.

The studies by the Muscle Chemistry Section on muscle proteins represent another facet of the Laboratory's interest in the organization and function of tissue structural elements. In the muscle studies the adoption of the starch-gel electrophoretic technique in conjunction with methods already developed in this Section are beginning to provide extremely interesting data. Since these studies are being run simultaneously on normal and myopathic human muscle biopsy samples, it will inevitably be some time before definite conclusions may be reached—a function primarily of the diversity of material under study. However, it is now possible to achieve a major degree of separation of the individual constituents of the total protein tissue extract and to anticipate the application of immunochemical (e.g. fluorescent antibody) and histochemical (enzyme) methods to the fractions *in situ* on the starch-gel. It is reasonable to presume that such studies now underway will yield a wealth of pertinent information both for the normal constitution of the muscle protein pool and for its distortions in various myopathies.

These then are some of the projects currently in progress in the Laboratory of Neurochemistry and some of the significant features which may be attached to them. In addition to their immediate scientific significance, they illustrate three additional pertinent aspects. One is the attribute of collaboration both within the Laboratory itself and with other groups in NINDB and at the NIH and elsewhere. Collaboration has been active throughout the history of these groups and has been most fruitful for all concerned, as some of the foregoing examples show. A second attribute is that of training young men in neurochemistry, since all the projects discussed embody major contributions by these young men. Their ability to make such contributions surely marks a milestone in their training, and the research experience and maturation of research judgment implicit therein will accrue to their benefit. And finally the emphasis on basic research deserves reiteration.

LABORATORY OF NEUROPATHOLOGY

An objective in experimental neuropathology has been to define the sequential pathologic changes in both neurons, neuroglia cells and vascular system when animals are prepared according to our standards of perfect histological preparations. Two series of experiments are being scrutinized; one is based on severance of the facial nerve in rabbits and mice, and the other on total body irradiation of various animal species. Study of these materials has helped to establish certain standards of methodology. Furthermore, our concepts about the nature of neuronal reaction to axonal damage has been revised; there is neither swelling nor atrophy of neuronal perikarya. The reported specificity of several post-irradiation changes is challenged; three factors complicate an analysis of such material, namely, autopsy and fixation procedures are not performed with due regard to avoid artifactual changes, complexity of tissue organization is ignored, and cytological reaction to the aging process is not recognized.

The senior investigator, Jan Cammermeyer, participated in a Symposium, "The Study of Cell Structure in Nervous Disease—Quantitation in Neuropathology," held at the Massachusetts General Hospital, Boston, Massachusetts, January 31, 1961, under the direction of Dr. Raymond D. Adams, and a seminar, organized by Dr. Adams, at the Warren Museum, Harvard Medical School, Boston, Massachusetts, February 1, 1961, dealing with means of improving neuropathological methodology; his papers were entitled "A Proposal to Revise a Trend of Neuropathological Methodology" and "Prerequisites of Quantitative Neuropathology," respectively.

LABORATORY OF NEUROPHYSIOLOGY

Investigators in the Laboratory of Neurophysiology, NIMH–NINDB are working on a great variety of fundamental problems of the nervous system. Projects range from detailed analysis of muscle membrane to the social behavior of the South American monkey (Saimiri sciureus). Material used ranges from a single muscle fiber of the frog to the whole brain of the monkey.

The Section on Membrane Physiology's program is directed into the general area of the ubiquitous excitable membrane and is currently investigating muscle fibers. The surface membrane of both nerve and muscle fibers is electrically excitable, i.e., a wave of self-propagating electrical activity travels along it. Ionic currents carried by sodium potassium movements are responsible for this activity and the mechanism of electrical excitability has many features common to nerve and muscle. In muscle, however, there is an additional mechanism for the passage of potassium into or out of the cell. This mechanism is not necessary for the production of electrical excitation. An explanation of the additional passage of potassium that evokes the submicroscopic internal tubular structure of muscle is in accord with many of the experimental observations. The tubules could be the pathway for the additional passage for potassium. Such a system could relay the electrical activity from the surface membrane of a muscle fiber to the internal trigger areas for contraction. Some mechanism of this sort is necessary to activate the internal contractile process with only a short delay after the electrical activity at the surface. The one considered here is admirably suited for this purpose. The work of the Section on Membrane Physiology is to investigate these intriguing fundamental speculations. Such work contributes to knowledge of all kinds of physiological membranes.

The Section on General Neurophysiology has several projects underway. The project on intracellular analysis of hippocampal pyramidal cells was terminated for the present and the principal investigators were invited to present their work at the Collogue International du Centre National de la Recherche Scientific, Physiologie de l'Hippocampus, Montpellier, August 24–26, 1961 and the Fifth International Congress of Electroencephalography and Clinical Neurophysiology, Rome, Italy, September 7–13, 1961.

A project on pH measurement of the cortex has been very successful. It has been demonstrated, previous reports to the contrary, that there is no obvious change in pH concomitant with the reaction of spreading cortical depression. If there is a change it is small and will be difficult to separate from various other physiological and electrical masking phenomena. Work on this problem has led to interesting considerations of, and experiments on, important current questions of blood brain barrier and extracellular space.

Another project of this section is concerned with analysis of synaptic transactions in the lateral geniculate nucleus. This is part of the general program on visual system problems which have always been under investigation by this laboratory. This immediate project is an attempt to analyze the mechanisms of the striking phenomenon of second subnormality discovered several years ago by workers in this laboratory.

A new device for retrieving signals from noise has been developed and put into operation. There are several important applications in physiology as well as possible use in other communication problems.

The Section on Limbic Integration continues with its intensive investigation of one of the highly organized but phylogenetically old parts of the brain, the limbic system. These studies range from observational to electrical and anatomical studies of the sexual behavior and physiologic mechanisms of the squirrel monkey. Investigations are under way on the general afferent connections to this area as well as the efferent systems important to preservation and perpetuation of species. For the first time afferent pathways in the brain stem which are involved in sexual arousal have been identified and studied. Another project under way is the study of central control of blood pressure. Currently this study is focused on interaction of various parts of the brain and carotid sinus mechanisms. An important incidental project is the preparation of a stereotaxic atlas of the brain of the squirrel monkey.

Members of this Section have given several invited lectures and the Section Chief was an invited participant for the Collogue International du Centre National de la Recherche Scientific, Phys-

iologie de l'Hippocampus, Montpellier, August 24–26, 1961 and the CIOMS Symposium on Selective Vulnerability in the Central Nervous System in Hypoxemia, Baden (Zurich), August 27–31, 1961.

In the NINDB part of the Laboratory, several complementary projects are under way.

As a logical outgrowth of the intensive work of the past ten years, the Spinal Cord Section is conducting a program on the development of elementary neuronal reflex patterns with the aim of extending these studies into the fundamental aspects of the elementary learning mechanisms. The Section Chief of the Spinal Cord Section is working at Institute Marey, Paris, as a Visiting Scientist. He and his collaborators are working on basic mechanisms of the nervous system. One project involves the use of the Aplysia in which is found a simple ganglion with cells of very large size and large nerve cells which have no dendrites. This presents the rare opportunity to investigate the cell mechanisms with no confusing contribution from dendrites. Work is also being pursued on ionic exchanges and chemical transmitter agents in these ganglia.

Another project consists of an attempt to study processes of development of neuronal activity pattern changes and learning. This study is being initiated at a very elementary level and consists of following changes of reflex magnitude at motor output and at sites of single cells in the cord as a result of prolonged and controled input excitation through afferent nerves. Another project is the study of ventral horn cells during the perinatal period. This study is contributing to further knowledge of the basic neuronal reactions and is also a part of general study on learning process since the beginning of the elementary neuronal reaction patterns are under way in the fetus.

A Visiting Scientist from Brazil is embarking on a general project on the basal ganglia. This work involves further electrophysiological and neuroanatomical analysis of the complex interaction processes of the basal ganglia complex with other ganglia in the brain. Systematic studies are planned on basal ganglia involvement on such behavioral complexes as sleep and arousal in cat and squirrel monkey and this project may extend into certain aspects of conditioning and learning. The study of this part of the phylogenetically old brain complements the important work proceeding on the limbic system in this laboratory and many of the same technics will be used.

A continuing program on fundamental studies of somatic sensory mechanisms is currently specifically investigating differences in thalamic activity patterns associated with stimulation of skin receptors and joint rotation receptors. This study involves unitary analysis of the thalamic ventro-basal nuclear complex. Sensory pattern recognition and pattern discrimination is one of the continuing problems of neurophysiology of the brain.

BRANCH OF ELECTROENCEPHALOGRAPHY AND CLINICAL NEUROPHYSIOLOGY

Routine diagnostic service

As in the past seven years this Branch has continued to provide routine diagnostic service for the various Institutes. A total of 1966 EEG examinations were carried out between the last report (prepared November 1, 1960) and the present one (prepared December 1, 1961). Patient referrals from the different Institutes were distributed as follows:

NCI	273
NHI	93
NIAMD	72
NIAID	147
NIMH	94
NINDB	1287
Total	1966

The record number of the preceding year was closely approached and the load of electroencephalographic examinations per month (average 151) has been the second highest since this Branch has been in function. As in the past, the majority of referrals is still from our Institute (about 67%) while the NCI leads the remaining Institutes with an average of about 21 patients per month.

In addition there have been 23 examinations carried out directly from the exposed cortex during surgery. The number of these interesting though time-consuming procedures has slightly decreased in comparison with those of the preceding years.

Research Activity

Besides the abovementioned diagnostic service, part of the time has been used in the analysis and elaboration of the data collected in the course of previously described research projects and in organizing them into publishable reports. This has resulted in the publication of six papers whose titles are listed in the 4th section of this Summary.

Active research projects, either new or continuation of previously described ones include the following:

A) A major project 4(C), already started in the preceding year and partially outlined in the last report. This project takes advantage of the patient material utilized by the Branch of Neurological Surgery in the treatment of involuntary movements (see project 56(C)). In these patients, primarily admitted for selective coagulation of subcortical structures, a total of 14–21 cortical and depth electrodes are implanted and kept in place for 7–10 days. Our investigation has the following purposes: a) to analyze the spontaneous electrical activity recordable from different subcortical structures in resting conditions, during natural sleep and during drug-induced sleep; b) to study the changes in the cortical activity following selective subcortical lesions; c) by combined electrical stimulation and recording of evoked potentials to investigate the subcortical connections of the frontal lobe and the interrelationship between the various subcortical nuclear masses and fiber tracts in human.

An exceedingly large amount of highly complex results have been to date accumulated from 30 cases in which repeated records were obtained under different conditions and using different stimulation parameters. New cases are continuously studied. Their analysis is quite elaborate and it involves the localizing identification of each of some 600 electrodes, the study of the effects resulting from stimulation through each pair of electrodes in relation to the remaining 300 pairs and a time-consuming, detailed plotting of these results. These are the first necessary steps toward the completion of part of this project. This analysis is still in course at the time this report is prepared and it is expected it will continue for many months before definite conclusions become available. Part of this experimental approach was recently discussed and very preliminary results displayed at two Symposia.

B) Project 1a(C) deals with a series of interesting findings obtained in an epileptic patient in which an extensive study was carried out by means of chronically implanted subdural electrodes. The details of this study appear in the attached description. Briefly it provides for the first time electrophysiological evidence of interhemispheral (callosal) connections in man; it analyzes the relationship between evoked callosal responses and the spontaneous epileptiform discharges; it provides further evidence for the close identity existing between artifically produced epileptogenic foci in the experimental animal and the cortical electrical events in human epilepsy; finally the results of electrical stimulation permit more rational approach to, if not the solution of, the differential diagnostic problem presented by the case upon which this investigation was performed. This project is completed and ready for publication.

C) Project 2a(C) is a purely experimental one. A detailed analysis of the parallel behavior of single unit and gross electrical activity within a local epileptogenic cortical lesion had been carried out in previous years. The present project is closely related to the preceding one, differing only inasmuch as it applies the same experimental approach to cortical regions not primarily involved by the epileptogenic process. It is a common finding in both human and experimental epilepsy that focal abnormal electrical discharges might be "projected" to other regions and that, with gross surface recording these distant abnormalities may not differ significantly from those at the original focus. It would be of both theoretical and practical interest to learn which different mechanisms are involved in the two ("local" and "projected") situations and whether their corresponding electrographic phenomena are actually or only apparently similar. The experimental portion of this study is partially

completed but final results are not yet available.

Other Activities

This Branch has continued to provide training facilities in clinical electroencephalography and, as in the past, the technical personnel has significantly collaborated on this activity.

In 1961 Dr. R. Gumnit, Dr. P. Altrocchi, Dr. C. Payne and Dr. J. Gergen have spent 6 to 10 months in the Branch for a more or less formal training. This period of time is inadequate for a complete, satisfactory training and can be only considered as an acceptable compromise for residents in neurology wishing to acquire more than a superficial knowledge in this sub-specialty.

MEDICAL NEUROLOGY BRANCH

Introduction

The Medical Neurology Branch has continued its efforts largely in the fields of neuromuscular disorders. Toward this end, a variety again of basic techniques has been utilized ranging from histochemical studies for fine intracellular structures, electron microscopic studies of diseased muscle, micro-electrode recordings of intracellular potentials in spontaneously discharging muscle associated with myotonia, studies of regeneration utilizing labelled isotopes, studies of intermediary metabolism, in particular carbohydrate metabolism as applied to neuromuscular disease, and the use of pharmacological agents and their syntheses.

Studies have also continued in the detection of intracranial pathology by utilization of unstable nucleides. Such studies were done in collaboration with the utilization of instrumentation prepared at the Oak Ridge National Laboratory. Studies also were carried on in new neuroradiological diagnostic techniques as applied to brain, spinal cord, and muscle.

For such studies, 400 patients were admitted and 6,942 patient days were recorded. The average patient stay was 17.3 days. Three hundred seventy consultations and 376 out-patients were seen. Two hundred twenty-seven patients suffered from some type of neuromuscular disorder.

Neuromuscular Disorders—Anatomy and Physiology

A follow-up study covering eleven years on the late onset of sporadic myopathy was completed during this reporting period. The group was broken into patients below the age of fifty and in patients over the age of fifty. In those patients below the age of fifty, a so-called "collagen" disorder was by far the most common cause of such a myopathy. Over the age of fifty, sex separation was apparent. Thirteen of seventeen males with sporadic proximal progressive myopathy had cancer. Only eight of thirty-four females in this series had cancer. By far the majority of females over the age of fifty had a proximal progressive myopathy of an obscure etiology. The relationship of Sjogren's syndrome with myopathy was clarified on the basis of four such cases and reported by Dr. Silberberg and Dr. David Drachman. Endocrine studies in relation to muscle disease were continued and, in particular, severe hypothyroidal status was correlated with exacerbations in myasthenia gravis. In the overall study of neuromuscular diseases, two new disorders appeared. One such disorder was found in a child in whom gross distortion of the interfibrillary pattern was noted. Accompanying this can be found areas free of such fibrils. Such areas, histochemically, appeared to be sarcoplasm. Muscle in such a patient showed focal segmental absence of phosphorylase in fibers of high mitochondrial activity only. Other fibers, histochemically, did not appear to be involved.

In 1951, B. McArdle reported a new muscle disorder in which accumulation of glycogen occurred within such muscle and upon exercise, the lactate and pyruvate in the blood fell. This disease was subsequently elaborated by Dr. Rudi Schmid of the National Institute of Arthritis and Metabolic Diseases who demonstrated a complete absence of phosphorylase A and B. Dr. Schmid and Dr. Hammaker subsequently divided this disorder into three stages: the first, in childhood showing easy muscle fatigability and intermittent dark urine; the second, in early adult life with cramping muscle pain on exertion followed by transient myoglobulinuria; and the third, fourth, and fifth decade with persistent and progressive weakness and wasting of muscle. In the past year, one of the patients admitted to the late myopathy pro-

gram demonstrated histochemically, as well as biochemically, absence of phosphorylase A and B. She apparently had missed the first two stages of the disorder described by Doctors Schmid and Hammaker. Dr. Engel and his colleagues, in addition, further clarified the so-called central core disease described originally in this Institute and demonstrated in this disease a complete absence of phosphorylase A and a severe impairment of phosphorylase B. In late myopathy of the McArdle type, there was no increase in glycogen within the muscle fibers as was originally demonstrated by Doctors McArdle and Schmid. There was, however, again a failure of rise of blood lactate with exercise and again there was normal liver phosphorylase. Hence, the only biochemical defect in this patient detected was in skeletal muscle phosphorylase.

Dr. Engel and his associates, Dr. Mumenthaler and Dr. Drews, have continued their studies on cytochemical methods in the study of neuromuscular diseases. Along with Dr. Wanko of the Ophthalmological Branch, an electron microscopic correlation was carried out. Dr. Engel's studies are largely enzymatic methods on frozen tissues. Myofibrillary architecture is studied largely by myosin ATPase. Mitochondrial abnormalities are studied by cytochrome oxidase, succinate dehydrogenase, DPNH dehydrogenase and DPN-linked lactate dehydrogenase. Sarcoplasm was studied by glycogen stains and amylophosphorylase and uridine diphosphate glucose-glycogen transferase. These investigators found in denervation that the cytochemical changes could not be definitely correlated with any given disease causing denervation. However, they did describe a pathognomonic-type of fiber which appears in denervation of any type which they have termed the target fiber. This peculiar fiber has three zones of change which have been considered by these investigators characteristic of denervated muscle. In dystrophia myotonica, these investigators have confirmed previous histology described from this Institute in that the sarcoplasmic masses histochemically are indeed sarcoplasm that the "Ringbinden" are disoriented subunits of muscle fibers with normal enzymatic activity accompanying such myofibrils.

The growth of skeletal muscle has continued to interest the Medical Neurology Branch. Continuing studies using osmotic pump perfusion of tritiated thymidine in young rats were unsuccessful in labelling sarcolemmal nuclei. This would indicate that growth of muscle beyond the early development age is not by mitotic division. Dr. Engel and Dr. Mumenthaler have been interested in the innervation of embryonic muscle. In chick embryos, the correlation of the motor end-plate and the distribution of cholinesterase was studied. Before innervation of the muscle had been accomplished, as checked by silver stains, the cholinesterase was diffusely distributed throughout the muscle fiber and, in particular, at the level of the Z disc. Such a distribution is also seen in the tissue culture. After innervation has been accomplished, however, such a diffuse distribution of cholinesterase disappears and now becomes limited to the motor end-plate and myotendinous junctions.

New techniques are being developed by Dr. Engel and his colleagues in the study of other functions of muscle by use of cytochemical stains. In particular, Dr. Engel and Dr. Drews have developed a method for the localization of myoglobin and have reported such within the current year.

In the electrophysiological studies of diseased muscle, both extracellulary and intracellulary recordings have continued. One hundred and fifty-nine patients were studied by double-blind techniques with extracellular electrodes; multiple electrodes were used. The accuracy of this study demonstrated a correlation with the clinical and pathological material in eighty-six percent. The following findings carried significant correlation. In denervation, an increase in the duration of the action potential was found in seventy some percent of peripheral neuropathies and in ninety percent of disorders affecting the anterior horn cell. The anterior horn cell disease is differentiated from denervation occurring peripherally by the increased amplitude of the action potential and the increased size of the motor unit. In almost all myopathies, a significant decrease in the duration of the action potential was noted. Insertional and spontaneous activity were noted if much inflammation was present. An interference pattern was noted in all myopathies with a graded strength on the Medical Research Council scale of three or above. Single oscillations or mixed pat-

terns were noted in neuropathic lesions with the same strength. In five of nine cases of sarcoidosis, facilitation was noted on repetitive ulnar stimulation. This finding is not unlike that described in other centers in patients with cancer of the lung. Eight out of ten myasthenics showed significant decrease in the amplitude of the action potential on repetitive ulnar nerve stimulation at rates of 40/cps or less. Intracellular studies were continued as well. Such studies were done largely in patients with myotonia. In paramyotonia, in which this Institute showed an abnormal serum potassium at the time of attacks, once again there was no change in the resting potential during periods of weakness. In all myotonics, an unstability of the muscle membrane was noted accompanying spontaneous firing of the muscle fiber in which the fall-back to the base line was never complete, demonstrating partial depolarization. The latter studies were accomplished by Dr. Forbes Norris.

Neuromuscular Disorders—Pharmacology

Studies in neuromuscular and ganglionic blocking agents have also continued. Dr. Irwin has made available for clinical use the tertiary form of the lycoramine derivatives, and this has been used in six myasthenic patients over the past year. Clinically, to date in this short series this compound has not demonstrated the promise originally hoped from animal experimentation. The toxic and therapeutic levels appear to be closer in this compound than in pyridostigmine now in use. The short-acting hydrochloride quaternary form of lycoramine has, however, been extremely effective and may well be a potent diagnostic tool for the disorder of myasthenia gravis. The onset of this drug is approximately 15 seconds and its duration is usually four minutes or less. In the basic part of this study, Dr. Irwin has demonstrated that the dimethyl-carbamate lycoramine derivatives of the quaternary compound were found to be excluded from the brain. Other tertiary analogs entered freely. This study led him to reinvestigate neostigmine which he found had a limited entry into the brain, although it was formerly believed to be excluded because of its quaternary structure. This suggests that the lycoramine derivatives may be safer than neostigmine in that they would not cause central respiratory depression. Both prostigmin and physostigmine are potent cholinesterase inhibitors and both of carbamate compounds. If the carbamate groups are removed from these substances, they are poor inhibitors of cholinesterase. The dimethyl-carbamate of deoxy-demethyl-lycoramine is a potent inhibtor of cholesterase. When the carbamate group on this compound is removed, however, the remaining substance is still a potent cholinesterase inhibitor unlike neostigmine and physostigmine.

Dr. Irwin and Dr. Trams have continued their study on gangliosides which, as noted in the previous annual report, sequester quaternary compounds. They find that the gangliosides themselves do not function as a tissue receptor substance for acethylcholine, but that they may in combination with other macromolecules have a function as a tissue receptor. They have also demonstrated that chlorpromazine interacts with such gangliosides. Since chlorpromazine has an activity on the brain and since such gangliosides are extracted from the brain, this finding is of importance. Attempts were made during the past year to immunize animals to such gangliosides and to use labelled antibodies in the study of myasthenic muscle. Initial studies were unsuccessful in production of antibodies to gangliosides; but more recently, Dr. Trams and his colleagues have succeeded in doing this. The activity of gangliosides appears to be of a different order of magnitude than that previously described for other mucopolysaccharides or proteins in that Dr. Irwin and his group using bio-assay systems demonstrated that heparin, chondroitin sulfate, and hyaluronic acid do not interact with acetylcholine although they have been reported to do such in the literature. Dr. Irwin and Dr. Trams have joined together on the possibility of receptor substance in the electric eel tissue which might react with biologically active compounds as has been reported from other centers. By use of dialysis technique and sequesting with curare combined with biological assays at different temperatures, these investigators have demonstrated that the substance in the eel is a cholinesterase and not a binding substance as has been reported elsewhere.

Dr. Irwin and his group are now turning their attention to differences in pharmacological actions

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of rapidly contracting muscles from muscle which contracts more slowly. They find in the dog that rapidly contracting fibers have a lower threshold and shorter duration for potentiation after certain cholinesterase inhibitors than do the slower reacting muscles. Since this occurs both after inhibitors which depolarize the muscle membrane and those which do not, this difference therefore is thought by Dr. Irwin and his group to be directly related to cholinesterase.

Cystic Fibrosis of the Pancreas

During this reporting year, Dr. Irwin and Dr. Eyerman have completed their study in neurohumoral factors in cystic fibrosis of the pancreas. They found that cholinergic activity due to a choline-ester which was indistinguishable from acetylcholine was found in all sweat samples but one in high amounts in a series of seventeen patients with fibrocystic disease. By using inactivation tests with alkali and specific enzymes on each sample, they demonstrated that the choline-ester rather than the electrolyte content or choline itself accounted for this observed activity. It thus appears that in this disorder, a neurohumoral imbalance of exocrine glands is involved and is of some neurological importance.

Genetics

The injection by the T–2 phage of desoxyribonucleic acid (DNA) into the bacterial cell makes this virus a powerful tool in the study of chemical heredity. The protein coat of the virus is rejected at the time of injection of DNA but is reformed apparently within the cell at the time of cell death and liberation of the intact virus once again. Thus, the relationship of this protein formed by DNA becomes important in the understanding of genetic mechanisms. Dr. Cummings before joining this Institute had demonstrated that the head of the T–2 phage could exist in two forms. The appearance of the protein coats of these forms indicated that the head length changed without any concomitant change in width. Other investigators have demonstrated that the protein subunits of the head protein were of fibrous nature. Dr. Cummings during the past year has been studying the physical structure of the subunits of the protein coat of the head of the T–2 phage. He found that the T–2 phage could infect the host organism (i.e., inject DNA) when the head was in the long or short form. However in transition conditions, it was found that at sometime during the infective process the long head must contract to the short form and the phage already in the short form could not infect the host. Dr. Cummings calculated a model of the protein coat of the head and tested this with Dr. Wanko in the electron microscope. Theoretical size of the model subunits was two hundred seventy by twenty angstroms. The molecular weight of the determined head protein subunits in the ultracentrifuge using the Archibald equations was found to be between forty-two thusand and forty-three thousand daltons. This would give a dimension of two hundred ninety by twenty-eight angstroms. The eight angstroms difference in width is inherent in the resolution of the present electron microscope.

Karyotyping was carried out with the aid of Dr. Tjio in various neuromuscular conditions, predominantly dystrophia myotonica. No abnormal trisomal findings were reported. Studies in karyotyping are continuing in the so-called dysraphic states.

Brain Tumor Studies

Within the past year, Francis, Bell, and Harris of the Oak Ridge National Laboratory have developed a new prefocused collimator made of cast gold with thirty-seven channels placed within a one hundred fifty pound tungsten shield. In collaboration with these investigators, we have placed their collimator on a scanning apparatus developed here at the National Institutes of Health. Since the beginning of this project, over eight hundred fifty scans have now been done. Approximately a hundred fifty were done using the new collimator. Preliminary statistics seem to indicate that there is no increase in the percentage of detection of intracranial tumors utilizing this collimator. However, there is a marked increase in the ability to tell the type of abnormality. The type of abnormality on RISA scanning is dependent upon high resolution, and there is no doubt that the new collimators developed

at Oak Ridge National Laboratory have a higher resolving power. This resolution allows one to see the edge of the abnormality much more clearly. Thus, lesions with discrete edges occurring at the expected sites may indicate to the interpreter of the scan that the lesion is a meningioma. If, on the other hand, such discreteness occurs deep in the hemispheres, this is most likely a metastatic lesion. Gliomas are commonly nondiscrete. The vascular structures of the brain are more easily detectable with the new collimators. The major communicating veins may now be seen. The lateral sinus, the longitudinal sinus, and the torcular Herophili may also be seen. The accuracy of detection of lesions still sits in the eighty-three percent range. There is, however, no doubt that also more false positive interpretations are given by this technique than by contrast radiological techniques. Dr. Di Chiro's publication of the correlation of radioactive scanning with standard contrast neuroradiological techniques has been published in a monograph form.

Perhaps the most important clinical study to be completed in the current year has been the publication of Dr. Di Chiro's Atlas of fine structures based on combined fractional pneumoencephalography combined with laminagraphy. This Atlas, which has been published by Charles C. Thomas, shows many structures not hitherto described with contrast techniques. In addition, Dr. Di Chiro has initiated a new approach in pneumoencephalographic studies by the utilization of the technique of axial transverse laminagraphy which shows much promise. Equipment is being designed and purchased to carry on such studies in the coming year. Dr. Di Chiro is carrying on his studies using the scanner for possible improvement of radio-iodinated antifibrinogen by means of utilizing epsilon aminocaproic acid. In this he has been joined by the investigators of Rochester, New York, Atomic Energy Unit. Dr. Di Chiro previously established that, at least in sarcomas, radio-iodinated antifibrinogen was of perhaps more value than RISA (radio-iodinated serum albumin). Studies with epsilon aminocaproic acid are dependent upon the fact that this is an inhibitor of plasminogen activation which has been shown previously by other investigators to enhance the localization of antifibrinogen in experimental rat tumors.

Although cerebral arteriography is now a well established technique and the majority of anatomical structures are relatively well recognized, the same has not been true for the arterial supply of the orbits. Dr. Di Chiro successfully demonstrated nine intraorbital arteries and four intraocular arteries and has developed as well an angiographic method for measurement of the axial length of the eye. He was able by utilization of hypaque at fifty percent concentration to demonstrate the choroidal plexus in sixty-four percent of orbital arteriograms.

The Branch plans for the coming year to open for the first time since 1954 a limited study in demyelinizing disorders in which the primary approach will be histochemical techniques applied to electrophoretic patterns of spinal fluid and serum of such patients. Studies will also continue in degenerative disorders, particularly those associated with myoclonus.

OPHTHALMOLOGY BRANCH

This has been the first year since the consolidation of the Eye Branch that no outstanding Visiting Scientists have joined in the research efforts of the unit. The stimulation originating from the association with these scientists, and their publications, based on work carried out in this unit, are keenly missed.

The most important and fortunate change in the professional staff was the return of Dr. M. G. F. Fuortes to the Institute as Chief of the Neurophysiology Section of this Branch in July of this year. No one who knows Dr. Fuortes' competence in the field of electrophysiology and the high quality of his work will doubt that his reappointment greatly raises the overall scientific standard of the Branch. Other favorable developments also deserve special mention, i.e., the rapid and amazingly efficient organization of the Section of Cell Biology by Dr. Sjoerd Bonting and the Laboratory for Electroretinography by Dr. Peter Gouras. Their project reports are ample proof that a great deal has been accomplished in a short period of time. It is to our regret that such a capable investigator as Dr. Edward Okun had to terminate his assignment as Clinical Associate to complete his residency in the Eye Department at Washington University, St. Louis, Missouri.

This synopsis of the many projects which have been under study during the past year is divided into two sections covering laboratory investigations and clinical studies. The far greater number of laboratory investigators, as compared to that of ophthalmologists and medical aides, is reflected in the number of contributions in the respective categories.

In a period of only five months Dr. Fuortes has made great strides in several studies on visual receptors in his overall aim to identify the processes which are involved in the transformation of light into nerve signals. Specifically, the responses to changes of illumination were studied by intracellular recording and stimulation of the eccentric cell of the limulus eye. The responses to square pulses of light show a high initial transient which is followed by a lowered steady state. Various experimental procedures were designed to study this phenomenon of sensory adaptation, e.g., by recording the potential changes occurring across the membrane of the eccentric cell. It was shown that illumination rapidly depresses the responses to light and also that recovery in darkness is rapid. The result of these and several other tests suggest as a working hypothesis, an excitatory substance such as a product of the photochemical reaction, not the photopigment itself, decrease in concentration during illumination and quickly recovers in darkness.

Another study of Dr. Fuortes' deals with the generation of impulses following depolarization. Visual sensory cells are depolarized by light. The depolarization causes propagating nerve impulses and the amount of depolarization is signalled by varying impluse frequencies. There is a simple linear relation between these two phenomena, and it is thought that this linear relationship results from a balance between the inactivating effect of depolarization and the restoring action of repolarization.

Recently Dr. Fuortes selected the eye of the dragonfly for studies of the electric changes evoked in the photoreceptors by illumination. The separation of the receptor elements from conductive nerve fibers by a membrane seems to make these eyes particularly suitable for such investigations. The resting potential of 60 mV of the presumable receptor cells decreases during illumination, whereas the electric conductance correspondingly increases. It seems that the first transformation from light to electricity takes place in the photoreceptor cells. This process delivers a signal which leads to nerve cell depolarization and impulse firing.

Dr. Gouras and Dr. Gunkel continued to study the advantages of the use of computer techniques for the detection of evoked potentials in electroretinography. The linear relation of ERG sensitivity to the area of functioning retina was described last year. The close correlation between ERG sensitivity and subjective dark adaptation threshold, in all but the earliest stages of retinitis pigmentosa, is a new finding. The earliest abnormalities of certain forms of retinal degeneration therefore are detected by the ERG and not by elevated light thresholds.

Great efforts were made to use focal chromatic illumination to map the visual field at the retinal and cortical level with computer enhancement of evoked responses. With this technique, spatial differences in the human ERG were demonstrated for the first time allowing identification of the macular area. Macular lesions of varying nature and dimensions are being studied to determine the sensitivity of the method for detecting abnormal function. It appears that central retinochoroidal lesions of about 10 mm^2 retinal area are detectable. Further experience is necessary to delineate the effectiveness of the new method for differentiating degenerative from inflammatory lesions of the macula, and primary retinal lesions from central loss of function due to optic nerve pathology.

In another study the dynamic properties of the ERG have been examined in pure cone and pure rod retinas. These investigations reveal that there is a difference between the resonance frequency and the low frequency characteristics of the ERG of these distinctly different retinas. This indicates that the dynamic properties at the ganglion cell level are determined at a more peripheral site in the visual system. Dr. Gunkel examined the importance of the absorption of blue light by the aging crystalline lens for the evaluation of light adaptation curves. When white lights are used an age dependent elevation of thresholds is slight, whereas this elevation is very great when dark blue light is employed. The marked night blindness as an age change reported

in recent literature was shown by Dr. Gunkel to be explained by the unfortunate use of blue light for such investigation which effectively measures the pigment of the aging lens nucleus rather than a change of retinal adaptation.

Dr. Bonting and Dr. Gouras, in cooperation with Dr. Gunkel, and Mr. Caravaggio continued their multi-disciplinary approach to examine the correlation of biological, morphological and functional events in the developing visual receptor cell of the rat. The results of the prize winning paper confirm the preliminary findings reported last year but are now based on a greater number of experiments and detailed analysis of the results.

Rhodopsin appears in the albino rat seven days after birth; its concentration increases almost linearly for 21 days and then remains constant. In the first two weeks there is a corresponding increase in length of the outer segments of the receptors, but in the following week, rhodopsin concentration increased by 118 per cent and the length of the outer segment by only seven per cent. The tighter packing of the rod sacs in the outer segments and the greater density of the rhodopsin in the rod sac membrane may explain this result. The amplitude of the electroretinogram, which appears first at 11 days after birth, parallels the rhodopsin concentration after 12 days and becomes constant after 21 days.

C3H mice in which the neuroepithelium degenerates at an age of ten to sixteen days show a progressive decrease of rhodopsin concentration which reaches a zero level at the sixteen to nineteen day period. Some electric activity can be demonstrated at ten days, but fails to develop subsequently. Glutamate treated rat sucklings were studied because here the inner layers of the retina are known to be destroyed predominantly, whereas the photoreceptors appear morphologically unchanged when studied with the light microscope. In these animals the electroretinogram is greatly reduced in amplitude with the b-wave more affected than the a-wave. This might suggest that the b-wave is related to the inner retinal layers. The rhodopsin concentration, however, is slightly reduced. It is possible, then, that even small changes in the photoreceptor layer depresses the electric responses or that the neuroepithelium is not the source of the electroretinogram.

Drs. Bonting, Simon and co-workers greatly extended their studies on the membrane ATPase, now called sodium-potassium-activated ATPase. Twenty-nine of 36 tissues of the cat contain this enzyme. The highest activity was demonstrated in the gray matter, and relatively high values were obtained from tissues concerned with secretory function. Among these were homogenates of the ciliary body. Sublethal doses of ouabain inhibited the enzyme, *in vivo*, and in the cat, decreased the aqueous inflow by 70 per cent. The intraocular pressure in the control eye was moderately decreased. Observations on glaucoma patients who received digoxin, at a dose of 0.5 mg. twice daily, were similar to those obtained in the cat experiments. The inflow rate was reduced by 46 per cent and the intraocular pressure by 14 per cent. It seems justified to consider that sodium-potassium-activated ATPase might be operative in sodium transport across the ciliary epithelium and may contribute to aqueous formation. In view of the slight effect of the inhibitors of the enzyme on the intraocular pressure, and the relatively high dose of digoxin necessary to bring about this effect, the use of the steroid in glaucoma therapy is not considered at the present.

Dr. Resnik continued his extensive search for physical and chemical data on the lens proteins. He isolated proteins of the lens cortex by precipitation at the isoelectric point and by salting out with ammonium sulfate. The properties of the fractions were then studied in the ultracentrifuge. Alpha crystallin was separated into four fractions following chromatography on a DEAE column; immunological analysis by agar diffusion confirmed the presence of four proteins in the alpha crystallin fraction. Sedimentation and diffusion coefficients and the molecular weights were determined for these four proteins of alpha crystallin.

In his study on the structure and the physical-chemical properties of gangliosides, Dr. Resnik determined the changes of molecular weight of the glycolipid in different solvents and shows the wide variations in the molecular weight under varying conditions. The molecular weight was found to be greater in salt solutions and decreased in organic solvents as compared to water. This suggests that this glycolipid, under appropriate conditions, will either associate or dissociate.

Dr. Wolff, in the Section of Biochemistry, investigated the chemical and physical properties of lens enzymes with emphasis on peptidases. He found that lactic dehydrogenase and malic dehydro genase are present in extracts of beef lens cortex in a ratio of 2:1. On the other hand the the ratio of lactic dehydrogenase and malic dehydrogenase in extracts of the lens nucleus was 1:2.5. Tripeptidase, dipeptidase and amino peptidase were found in the lens. Pepsin, trypsin or chymotrypsin were absent. The amino peptidases were studied most extensively, especially in relation to various metallic ions. It is apparent that the enzyme requires magnesium ions for stability and cobaltous ions for activity.

Dr. van Alphen searched for common antigen groupings in tissues of the eye. Most of these tissues contained one or more specific antigens. Lens and corneal epithelium, corneal epithelium and corneal stroma, choroid and vitreous, and possibly choroid and retina hold in common antigens which are not present in the blood. It may be conjectured that these immunological retlationships play a part in the simultaneous involvement of several ocular tissues in a disease process.

Dr. van Alphen and Dr. Macri undertook a comprehensive study to evaluate the action of autonomic drugs on the ciliary muscle and the muscles of the iris in various supecies. In the employed bio-assay the tension of isolated tissue strips were measured by a sensitive strain gauge under isometric conditions. The contraction of ciliary muscle—and iris sphincter strips induced by parasympathomimetic agents and the blocking effect of atropine and homatropine confirmed clinical experience but the results obtained with sympathomimetic compounds were surprising. Such agents relaxed the ciliary muscle of the cat and monkey and produced constriction in the rabbit and chicken. Species differences were observed also on iris preparations. Here, the sympathomimetic compounds produced relaxation of the cat and alligator tissues and contraction in preparations from monkey eyes. The rabbit responded irregularly. The use of nicotine resulted in strong contraction of the iris and sphincter preparation in rabbit, chicken, and alligator with the effect blocked by hexamethonium or d-tubocurarine. Finally, the iris dilator of cat, monkey and rabbit responded strongly to sympathomimetic agents. In such preparations acetylcholine produced a relaxation and atropine a contraction. These detailed and well controlled studies of the effect of pharmacological agents on the interior eye muscles of various species are of interest in view of the potential applicability to disease states of the human eye. In addition, the results might influence our concept of the action and interaction of humoral mediators on the internal eye muscles under different conditions.

The perfusion technique for recording pressure changes in small vessels of the anterior uvea, which has been effectively worked out by Dr. Macri, was utilized in studies of the effect of Diamox on the iris artery pressure and flow rates in isolated iris preparations. Dr. Macri observed constriction of the iris artery and concluded that this event influences the venous pressure as well as the intraocular pressure. The radial arteries of the perfused isolated iris exhibited constriction when angiotensin in a dilution of 10^{-10} was introduced. In the isolated arterially perfused eye the effect of this compound on the intraocular pressure varied. When the experiments were carried out on the intact animal under general anesthesia, intraocular pressure changes following the administration of the compound were irregular and difficult to interpret. More information on the specific and non-specific effects of angiotensin administered systematically is necessary to before the use of the drug on patients with glaucoma can be considered.

Dr. Macri elaborated a working hypothesis in which he considers a circulating vasoconstricting hormone to be related to the maintenance of the intraocular pressure. On the basis of dialyzation experiments it was concluded that this substance is not a catecholamine. He undertook preliminary studies to investigate the merits of this concept. Sera of seven glaucoma patients and of five patients with general hypertension were tested on isolated arterially perfused cat eyes. An increase of the flow rate, interpreted as due to a decrease in resistance to perfusate flow, was observed with sera of glaucoma patients and a decrease of flow rate was noticed in the experiments with the sera of hypertensive patients. Dr. Macri plans to extend the series considerably to validate the early results. Confirmation of the reported preliminary results on a large group of patients and normal

subjects would open a wide field for extensive investigation.

In the Section of Dr. Macri, Dr. Luigi Scullica, Guest Worker from Italy, attempted to study the hemodynamics of isolated iris, ciliary body and anterior choroid preparations by perfusing the long posterior ciliary artery. Other experiments were conducted on intact isolated eyes of the rabbit. The results of the perfusion experiments supplement the information obtained by Dr. Scullica in his extensive work on the morphology of the uveal vascular bed, with special reference to the anastomoses between the various compartments of the vasculature in the anterior uvea.

The research activities of Dr. Wanko's Laboratory for Electron Microscopy branched into several areas, in each of which new and valuable information was gained. The formation and fine structure of toxoplasma cysts and the propagation of the parasite by different modes of cell division was documented by excellent electromicrographs. As to multiplication of the parasite, binary fission, schizogony and endodyogeny (filial generation) were observed. Material for study of the encysted forms of the parasite was obtained from the brain cortex of infected white mice at varying periods after infection. The fine structure of the organisms were compared with the parasites contained in the peritoneal exudate six to nine days after inoculation. In the latter case, the organisms were seen in a vacuole of the host cell while in the brain tissue they are surrounded by a cyst wall. This wall contained membranous and vesicular components which seemed to be a part of the endoplasmic reticulum of the host cell. The opaque material seen between individual organisms and which also forms a part of the cyst wall might be a product of the organism.

In continuation of studies on the crystalline lens, a technique is being developed to modify the fixation procedure to allow for a quick penetration of osmium tetroxide to the deep-seated nuclear portion of the lens. Such a technique will allow electronmicroscopic studies of an otherwise inaccessible part of the lens.

Studies of the fine structure of the normal and diseased bulbar conjunctiva have made progress, although final results are not available as yet. Several types of cells were characterized electronmicroscopically as the goblet cells, the basal cells of pyramidal shape and a third type which might correspond to the dendritic cell of the middle layer. It is Dr. Wanko's impression that the cytopathology in the conjunctiva of patients with Sjogren's Syndrome is limited to the superficial cells of the epithelium.

In the Cytology Laboratory, Dr. Hückel completed his study of the influence of alpha-chrymotrypsin on the healing of corneal endothelial wounds. He tried to approximate the conditions present in surgery of senile cataract of the human by investigating the effect of the enzyme on wound healing of old rabbits. In addition, the allegedly deleterious effect of the enzyme on deeply exposed corneal stroma was examined. In neither of the two situations did the enzyme exert an effect which could be considered as hazardous or contra-indicating enzyme-induced zonulysis in cataract surgery.

The cooperative study of the Branch with the Brookhaven National Laboratory on effects of the high energy deuteron microbeam on the lens of the mouse was extended to long-term observations. Such an approach was chosen in view of the long latency period of radiation effects on the lens. Experiments were terminated forty-four weeks after irradiation because of the development of incipient cataractous changes which were considered as age induced. Histological examination carried out at this time did not show late effects when the microbeam was used. In contrast to these negative results, exposure to the 1 mm. beam at a dose of 1040 rad resulted, in four animals, in widespread cortical opacities around the posterior pole. It was concluded that the ionization tract of a cosmic ray primary can scarcely cause more than a few point opacities in the lens which would not interfere with vision.

Autoradiographic studies of cell proliferation in the corneal endothelium continued in the Cytology Section. In view of the successful application of this technique to investigations of the endothelial layer, the study was extended to several other tissues of the eye. Tritiated thymidine was administered intravenously to young rabbits, and the animals were killed at varying time intervals following the injection. Examination of autoradiographs of the corneal endothelium of these animals yielded considerable information about the life cycle of the endothelial cells.

Mitosis is preceded by a DNA synthesizing period of approximately 16 hours. Between the synthesizing phase and mitosis the cell goes through a quiescent period which lasts less than eight hours. The life span of the cell in the young animal is estimated at about 100 days. Cell division occurs in the peripheral portion of the corneal endothelium, and the daughter cells remain in this region for at least two weeks. The autoradiographic results are in agreement with the classification of this cell layer as a slowly expanding population. When the corneal epithelium was studied in the same manner, a relatively rapid cellular migration was observed. H^3 thymidine is incorporated into the nuclei of the basal layer, and following mitosis some of the daughter cells migrate toward the surface of the cornea and are ultimately shed. The life span of the epithelial cells is in the range of 21 days. Incorporation of tritiated thymidine was also observed in the pigment epithelium of the iris, both layers of the ciliary epithelium and the peripapillary portion of the retinal pigment epithelium, as well as in the vasculature and various connective tissues.

Dr. Scullica and Miss Grimes utilized the tritium autoradiography technique in an investigation of the effects of irradiation on DNA synthesis in the rat lens epithelium. The results of this study demonstrate that a dose of 1000 rad, which produces an immediate inhibition of mitosis, causes a gradual decrease in the number of DNA synthesizing cells during the first 24 hours following irradiation. This indicates that synthesis, if already begun at the time of exposure, goes on to completion. No new cells enter the synthesizing phase, however, and it is this block which results in the decline of labelled cells. When cells are irradiated during the period of DNA duplication, they apparently are unable to proceed through mitosis, although they are able to complete the synthesizing phase. It is likely that these cells degenerate in abortive attempts at division following relief of the mitotic arrest.

In many of the laboratory studies, and particularly in the varied experimental approaches of the work on the electroretinogram, new designs and construction of devices to provide means for following through with the studies were required. Dr. Gunkel's never ending cooperation and ingenious grasp of complicated mechanical and optical problems were again greatly appreciated by all who asked for advice and practical help.

Dr. Okun and Mrs. Collins of the Histology Laboratory have completed two histopathologic studies of considerable interest. The enucleated eyes of 50 senile dogs were opened after fixation and the sites of fundus lesions, visible under the biomicroscope, were sectioned serially and subjected to detailed microscopic examination. There was a high incidence of complete and incomplete holes in the peripheral part of the retina. Such defects in human eyes lead often to detachment of the retina. In dog eyes retinal damage was not observed, nor were signs of posterior vitreous detachment discovered. It would seem plausible that the high viscosity of the dog vitreous, as compared to that of man, prevents such vitreaous detachment which in the human eye plays an important part in the development of retinal separation. In the second study of these authors ophthalmoscopic and histologic changes produced by graded photocoagulation were described and photographed. These photographs composed and extremely instructive exhibit which was greatly appreciated by the audiences of two large meetings. The extent of the coagulation necrosis depends on the light intensities used. In severe burns, all layers of the retina succumbed to complete destruction, and the underlying choroid and even the sclera were damaged. Choroidal rupture, extensive hemorrhages or retinal hole formation sometimes complicated the changes produced by severe burns. The vitreous also was affected by the focused light beam and showed condensation of the fibrous framework in front of the retinal lesion.

In the area of coagulation necrosis, circumscribed lesions were observed ophthalmoscopically and histologically which consisted of degenerate axons in the nerve fiber layer resembling cytoid bodies. Therefore, it was the first time that these histopathologic changes, which have often been studied and variously interpreted in different forms of retinitis in man, were produced experimentally. The vascular changes seen in retinal vessels of different size will be described by the authors in the near future. It is obvious that comparing ophthalmoscopically visible effects of the widely used photocoagulation technique with the histologic findings provide guidelines for the clini-

cal use of this new treatment and will allow to distinguish between desirable and undesirable or hazardous effects of photocoagulation at the time of treatment.

The following section deals with projects which are classified as clinical in the strict sense. Most rewarding were the advances made in the glaucoma project. In this year definite and valuable results of specific clinical studies on intraocular pressure became available, thanks to the concentrated efforts of Dr. Okun and co-workers. The question, whether or not tonography is a reliable procedure, is often posed. The reproducibility of the results of repeated measurements under standard conditions indicates the usefulness of the technique. It was shown that irregularities in the tonogram might be produced by blood pressure changes and that continuous plethysmographic recordings of the blood pressure will help to evaluate otherwise confusing tonograms. Studies of effects of local epinephrine therapy are of special value since the patients can be followed in the Institute for long periods of time and the benefit of treatment, tolerance of the drug and unexpected side effects can be recorded. Certainly any proof that this medication can increase the facility of outflow, as claimed in recent literature, is of the greatest importance.

The use of applanation tonography to monitor the recovery of the intraocular pressure after lowering the tension by compression gave results which allowed estimation of the inflow rate of aqueous humor. This information, when integrated with the values of facility of outflow, adds to our knowledge of aqueous humor dynamics and its disturbances.

Dr. Simon evaluated the charts of 38 patients with myotonic dystrophy and compared the incidence of diabetes mellitus in these patients with that of cataract formation. The recorded visual acuities of the patients were taken as an index for the extent of the lenticular changes. On this premise Dr. Simon arrived at a high positive correlation between the occurrence of diabetes and the degree of lens pathology. Diabetes and lens changes were frequently seen in older patients, whereas the duration of the symptoms of the disease did not seem to influence the disturbance of the carbohydrate metabolism or the degree of lens pathology.

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The detection of a dramatic toxic effect of prolonged use of chloroquine on the retina of two patients is bound to be of great interest to the profession. Reports of similar observations are prepared in other medical centers and might restrict the indication for the use of this antimalarial. Dr. Gouras studied these patients electroretinographically and observed that photopic responses in the depressed electroretinogram were affected to a greater extent than the scotopic response. The disproportionality of the effects on the two mechanisms appears reversed when compared with that occuring in retinitis pigmentosa and allied diseases.

The incidence of circumscribed posterior subcapsular cataract in patients with rheumatic diseases who were treated with corticosteroids was studied by our group on a larger series of patients than reported previously. Thirty out of 72 steroid treated patients exhibited the characteristic lens opacities. At high doses (16 mg. of corticosteroids a day) 12 of 15 patients developed the lens opacity. The effects of dosage and duration of treatment appeared to be additive. Sixty-three per cent of all patients were younger than 49 years. There were several children and young individuals who showed the change. Since there is no proof that the lens opacity progresses as time goes on, the importance of this side effect of steroid therapy cannot be assessed. At the present, the usually minimal impairment of vision does not justify any radical change in steroid therapy, but prolonged observations are necessary to provide more definite information.

The syndrome called dysgenesis mesodermalis iritis et cornae was observed in seven patients and tonographic recordings were obtained in all of them. Corresponding to the gonioscopic findings, tonography indicated moderate to marked impairment of outflow facility which did not react satisfactorily to medical therapy. On the other side, the intraocular pressure could be controlled by a combination of powerful parasympathomimetric and sympathomimetic drugs. Since surgery is usually unsuccessful in this disease, such information, if confirmed by prolonged observation, may be helpful in the treatment of such patients.

Two case reports were accepted for publication because the rare disease described provided a

starting point for discussion of the significance of the respective lesion on a broader basis. Dr. Simon presented a patient with conjugate downward gaze palsy following mumps. The defective optokinetics and oculomotor responses were thought to indicate a medical lesion involving the left uncrossed corticopontine gaze tract, rostral and posterior to the oculomotor nuclei. In context with this case, Dr. Simon prepared a movie on supranuclear gaze mechanisms in which he demonstrated the lateralizing value of optokinetic nystagmus in brain stem lesions involving conjugate gaze pathways.

Dr. Okun studied a patient who has been diagnosed to have papilledema caused by hyaline bodies of the optic nerve. On the assumption that the symptomatology could be explained by this local lesion, neurological and radiological follow-up studies were omitted. Later, extensive intracranial pathology was discovered. After surgical removal of widespread multiple meningiomata the papilledema and the hyaline bodies disappeared but vision was not restored. This observation is instructive since the diagnosis of hyaline bodies was misleading in the interpretation of papilledema in a patient who later showed extensive intracranial pathology. Complete neurological and radiological work-up of such patients are necessary to rule out intracranial neoplasms.

The Uveitis Project, including Toxoplasma infections, still poses the greatest number of unanswered questions. It is well known that it is the complex nature of the disease, the impossibility to obtain tissue for microbiological or histological examination, and the non-specificity of the clinical manifestations which prevent the establishment of a diagnosis by etiological identification or define therapy on a rational basis. The continued cooperation of the Microbiological Laboratory of the National Institute of Allergy and Infectious Diseases is greatly appreciated, but not one positive identification of a pathogen bacteria or virus could be made in the material obtained from patients with uveitis. Particularly frustrating were the repeated negative results of patients with Behcet's disease, although a virus has been isolated and cultured by Dr. Sezer in Istanbul, and all the steps specified by him were strictly followed.

Accumulation and analysis of data, particularly with respect to therapeutic effects observed in patients with lesions that could be defined, might be useful to arrive at a conclusion as to the nature of the disease. The studies of children with uveitis should provide information on the course and prognosis of uveitis, although the etiology is obscure in most cases of the young patients.

Despite our ignorance of the disease on so many points, the majority of admitted patients with acute lesions (excluding Behcet's disease) improved greatly in response to specific or nonspecific therapy and hospitalization.

SURGICAL NEUROLOGY BRANCH

Introduction

During the past year, the Branch investigated epilepsy, involuntary movements, cerebral palsy, brain tumors, subdural hematomata, cerebral syphilis, craniostenosis, internal hydrocephalus, neonatal injury, central core disease, arterial aneurysm, and allergic encephalitis. These clinical studies provided opportunities for research on the visual system, basal ganglia, hypophyseal and hypothalamic systems, brain stem, certain limbic structures, and the parietal and temporal cortices. Cerebral edema and the blood-brain barrier became targets of special investigations which were based on protein labeling, acid radical indicator, and hypothermic techniques. Such fundamental and clinical studies stimulated technical development of a monitoring complex for the human nervous system, cortical recording devices, an extracorporeal circulation, stereotaxic instrumentation, drapes, glare-free lighting, as well as various surgical devices.

Two hundred and thirty-two patients participated in the clinical investigations of this Branch. Of these, 126 were admitted to our study on epileptic mechanisms, 25 to the study on involuntary movements, and 24 were admitted as brain tumor suspects. In addition, a miscellaneous group of 57 patients was admitted because of such diagnoses as cerebral palsy, mental retardation, metabolic disorders of the central nervous system, aneurysms, narcolepsy, Crouzon's disease, and craniopagus. There were 105 major operative procedures. 17 reports were prepared for publication.

Several distinguished neurologists and neurosurgeons visited the Branch during this period. 175 came during the time of the World Congress of Neurological Surgery. Among these, the following heads of department brought special greetings from their respective institutes and institutions: Professors Aritunov, Dott, Falconer, Pennybacker, Rasmussen, Yegorov, and Zumer. Our predecessor in title and philosophy, the Department of Surgical Neurology in Edinburgh, sent a team consisting of the Lecturer, Mr. John Gillingham, and Doctors Donaldsen and Brown, who spent a week reviewing our techniques of profound hypothermia. Professor Gilbert of McGill University and Dr. Terry of the Mayo Clinic joined the Section on Neuroanesthesiology for a similar purpose. Similarly, the Branch was fortunate in the appointment of Professors Rubinstein, Seitelberger, Milkovic, and Ommaya as Visiting Scientists or Fellows.

The Branch Chief expresses his gratitude to the staff of Surgical Neurology and the Neurology Nursing Service for this year's work. He notes with regret the resignation of Mrs. Mary Thompson, who left on orders for active duty with the Army. As in the past, it is a pleasure to acknowledge the enthusiastic support of the Director and Associate Director, NINDB, and the Director and Staff of the Clinical Center. Their help makes our work possible.

Epilepsy

Investigations on epilepsy range from the single cell to the intact and complete human individual. Electrical activity of single cell elements in the cerebral cortex was studied in reference to peripheral stimuli, the summation of which led to spike discharges. Simultaneous recordings from cortical surface and cortical neurons show that the surface negative potential following the sensory primary response grew with depolarization of the cell. In addition, the electrical properties of individual glial elements in tissue culture environments were subjected to penicillin and other drugs known to produce epileptogenic changes in aggregates of cells. A cell aggregate with epileptogenic properties was studied by means of topical penicillin. Histological and histochemical analyses showed a dense proliferative process characterized by fibroblastic activity considered typical of the area and lesion. The firing characteristics of such lesions were noted and it was possible to transfer such a lesion which had been firing actively from one primate brain to another with relatively little change in its "firing" characteristics. In man, epileptogenic aggregates or lesions have been subjected to topical application of local anesthetic and cold physiological solutions. Not unexpectedly, the local anesthetic reduced or extinguished firing, while rather unexpectedly the cold physiological solution did the same. Several such lesions in man have been stimulated and their electrographic characteristics recorded during the year. In the problem of bitemporal epilepsy, patient material has been used for bilateral simultaneous recording at bilateral simultaneous craniotomy. This series of new techniques has led to the solution of at least one hitherto insoluble problem in lateralization and localization. It has also led to the uncovering of specific objective lesions such as subdural hematomata and tumors in cases which previously defied analysis because of failures in lateralization of the lesion.

Lateralization is a primary problem not only in surgical treatment, but also in the study of language and other functional representations in temporal lobe epileptics. During the past year, language testing and analysis of other relevant psychometric test data has demonstrated a clear relationship between competency in linguistic tests and left-sided lesions, as well as between efficiency in visual tests and right-sided lesions. Interestingly enough, patients whose speech is in the right hemisphere but whose seizures arise in the left, have test scores which are quite comparable with those of their peers whose seizures originate in the right hemisphere.

Studies of a detailed biochemical nature in children suffering from seizures have revealed a peculiar type of intermittent hypoglycemia which was precipitated by ingestion of large quantities of l-leucine. Other epileptic children responded to particular visual stimuli which seemed to precipitate their seizures.

In another study of the metabolic aspects of epilepsy, 48 patients were selected from a series of 600 for participation in a project on the relationship between electrolyte balance and seizure frequency. There is a clear relationship between sei-

zure frequency and serum sodium. Reduction in sodium seems to be followed by a reduction in seizure frequency.

Involuntary Movements

15 patients with Parkinsonism, 4 cases of dystonia, 1 case of spasmodic torticollis, and 2 cases of choreo athetosis have been studied with stereotaxic techniques. In addition, 1 case of Ramsay Hunt syndrome was investigated in this series. With relatively few complications, the majority of patients have been significantly improved following creation of lesions in depth. As a part of this study, depth stimulation was undertaken. Somatic sensation, visceral sensation, sensation of change in body position, visual phenomena, and changes in surface recordings have been noted. Stimulation of the ventro-oral thalamus in one case produced a most surprising athetosis of the contralateral extremities, while stimulation in the deep white substance anterior to the head of the caudate produced contraversive turning of head and eyes in another. Concomitant with these physiological studies, endocrinological evaluation of the effects of lesions within the basal ganglia was completed. No significant changes were noted in patients ranging from 2–4 weeks after creation of the lesions.

These studies require careful anatomical control. This has been obtained through study of fixed material which has served as the basis for an atlas of the human basal ganglia. With this atlas, it has been possible to transfer ventricular landmarks from contrast studies and through other means provide for a more accurate implantation.

Discrete electrolytic lesions or temporary lesions produced by pressure were created in the superior and medial portion of the substantia nigra of monkeys which then developed a contralateral tremor. The motor cortex, premoter cortex and basal structures were implanted with microelectrodes capable of recording single unit activity. Involuntary movements of the animal were simultaneously recorded. Simultaneously, electrodes designed for stimulation were implanted in the basal structures. These animals were unanesthetized and freely moving within the restraints imposed by specially designed chairs. Interestingly enough, almost all of the structure tested by recording electrodes fired at some phase of the tremor. This study is being pursued with the hope that it will provide some physiological background for the tremor mechanisms.

Prenatal Lesions

Pathological lesions of the central nervous system occuring during prenatal, intranatal and early postnatal life have been investigated. This investigation was based on pathological examination of clinical and acute specimens. In addition, the chromosome patterns of patients with congenital malformation have been investigated and some relevant chromosomal abberrations demonstrated.

Effects of Surgical Lesions

A quantitative study of the effects of hypophyseal stalk section on human hypothalamus and consequently on water balance was undertaken. Cell counts of various nuclear aggregates were made and tabulated. This was done in 8 patients from 1.5 to 32 months after stalk section. At 1.5 months in one case, 39,000 cells were counted in the paraventricular nucleus, but in cases with postoperative survival of 6 to 32 months, the cell counts varied between 14,000 and 23,000. These large cells and the small cell components showed less striking changes than those demonstrated in the supraoptic nuclear count which fell from 37,000 to 4,000 in periods ranging from 1.5 to 32 months. In a separate study, the relationship between cell density and the total number of cells in the supraoptic showed that the loss of cells causes little tissue collapse and the gross outlines of the degenerating nuclei were well maintained. Well defined degenerative changes were also shown in the tuberal nuclei. These were apparently due to changes in the individual cells rather than any numerical loss. The effects on water balance varied. Some patients required practically no hormonal replacement, while others required almost continual replacement. No relationship was found between the length of stalk remaining and the cell count or between either of these features and diabetes insipidus. Thus the relationship between clinical diabetes insipidus and the integrity of the hypothalamic nuclei contributing fibers to the hypophyseal stalk remains in considerable doubt.

The effect of lesions in the primate visual system and other studies of the structure of the primate visual system have been undertaken. A study of the topographical arrangement of the retinal ganglion cells, with particular attention to the correlation between visual acuity, visual field examination, and cell pattern was completed. In the normal eye, the ganglion cell layers constitute an oval pattern if their relative location is plotted as a function of respective distance from the fovea. Despite the round appearance of the optic nerve head, the retinal defect as determined by reconstructions was found to be broad oval in form, with the long axis oriented in the vertical or near vertical plane. This would explain the oval outline of the blind spot as charted on visual field examination. Lesions in the region of the chiasm or optic tract caused a noticeable loss of the ganglion cells after several months. This was well established between 1–2 years. Lesions destroying fibers in the visual radiations were followed by degeneration of the retinal ganglion cells. The resultant areas of retinal degeneration and visual field defect were larger in the eye on the side of the cerebral lesion.

The effects of surgical lesions on brain stem mechanisms have also been observed. In a surgical procedure, the brain of a chimpanzee was split along the midline from the lamina terminalis caudally to the superior colleculi. Thus, the hemispheres of 3 chimpanzees were completely separated save for the chiasmatic connections. Awareness was unchanged by this section and the animals' response to standard test procedures was either unchanged or changed in a trivial manner. In the macaque, 3 Bremer preparations were made and studied. It was possible to develop "sleep tracings" and various polygraph records. The walls of the third ventricle and its floor have been stimulated under direct vision. Such stimuli produced transient drop in blood pressure and acceleration in pulse.

The relationship between temporal ablation, parietal ablation, and the effects of (recently developed) hallucinogenic drugs has received attention. As found previously, hallucinogenic relatives of lysergic acid and psilocin do not affect the chimpanzee whose temporal lobes have been removed. Various effects were observed after parietal ablation and/or frontal ablation.

The effects of frontal, bifrontal, and frontoparietal ablation at brain temperatures in the range of 15–7° C. have been studied. While ablation is considerably easier at these temperatures, recovery thereafter is somewhat prolonged.

Cerebral Edema

Cerebral edema and the blood-brain barrier were studied by various experimental techniques. The fluorescein-labeled protein technique was utilized in cats to show that this method of labeling protein demarcated an area of edema within 24 hours after the relevant injury. As the protein was incorporated in the glial cells, the mottling disappeared and it was eventually seen in the glial cells around the swollen area. Such areas of edema were also studied with a view to further understanding of relevant glial metabolism. The oxidative enzymatic changes are such that there is an increase of glutamic acid dehydrogenase activity in astrocytes within 12 hours after production of the original lesion. Within 24 hours, a typical train of enzymatic events was noted which corresponded with and was preceded by an enlargement of the glial cells. Finally, there was an intense oxidation-reduction activity coincident with the production of microglial elements.

The blood-brain barrier was also investigated by the more diffuse application of cold in the techniques of profound hypothermia. From these investigations, it is clear that there is relative inactivation of barrier when the brain temperature falls below 25° and remains at those levels for at least 30 minutes. This 'temperature-frontier' is reached at the time when 'electrical silence' occurs and the brain waves disappear. Fluorescein thiocyanate, sodium fluorescein, d-tubocurare, penicillin, and staphcillin all pass the barrier so as to depose sizeable quantities in the brain parenchyma when such low temperatures are maintained.

Deep Hypothermia

After 4 years' experience with the techniques of profound hypothermia, an extracorporeal pumping system with heat exchanger has been developed for clinical use. This provides for simple femoral connections to the patient and will cool with relative safety to brain temperatures be-

tween 7° and 25° C. This is an original development, remotely resembling the Gollan technique, and actually derived from practical experience with 150 dogs and 7 chimpanzees. Its prototype has been used in study of effects and craniotomy in these animals under varying condtions of anesthesia, gas-oxygen mixture, blood substitutes, heparinization, catheterization, etc. Although it requires further development, its technical capability is such that it has provided for survival in the chimpanzee subjected to extensive intracerebral excision and kept off the pump without circulation for 70 minutes.

Topical cooling with refrigerated Elliot's solution in a system designed to irrigate specific cortical areas has provided for diffuse brain cooling to temperatures in the range of 22° C. within a matter of 20 minutes. The systemic side effects of such topical cooling are minimal and the animal's temperature remains within the range of 32–35°, and therefore his capability for independent cardiac and respiratory action is unimpaired. Similarly, topical cooling has been achieved through refrigeration of recirculated cerebrospinal fluid instilled through intraventricular and intracisternal connections. This method, while reliable, is considerably slower and seems less practical. Some brain cooling has been achieved by repetition of the Magoun technique which consists of warming the hypothalamus selectively. This method has some experimental value and will be pursued in the future.

In summary, the Branch has approximately 8 years' experience with hypothermia in large primates. This ranges from the selective effects of cooling on the limbic system, orbitofrontal cortex, electrical characteristics of cerebral hemispheres, to various observations on systemic changes such as cardiac function, gaseous exchange, and changes in permeability in body and brain. Surface cooling has proved a reliable technical support in the operative therapy of neoplasms and arteriovenous malformations. It is hoped that the new pumping device will prove considerably more effective as a new technical background for operative therapy.

Effects of Radiant Energy

Effects of various radiant energies on the nervous system have received further study. The effects of alpha particle radiation on the brain glycogen in rats were studied. 90 rats were exposed to alpha particles emanating from the 60-inch cyclotron in Berekeley. A significant increase in the level of total glycogen was found during 8 days following exposure. It is thought that the disruption of tissue due to radiation injury may lead to liberation, uptake, and conversion of substrates to storage glycogen by glial cells.

Gamma radiation has been applied to pregnant mice from a strain which ordinarily shows no significant incidence of spontaneously occurring CNS abnormality. Likewise, such radiation was applied to a strain which shows an abnormally high incidence of spontaneous malformations. 98 litters were obtained from the first strain and 10% of these had major abnormalities. About 25% showed minor abnormalities. 45 litters were obtained in the second strain.

Monkeys' brains were exposed to ultrahigh frequency energy in the range of 388–400 mc., using power limits between 100 and 1000 watts. An extensive and deep anesthetic effect was produced. This was characterized by lack of response to pain, pressure, and temperature, with preservation of awareness. Discernible clinical effects were evident when the peak power of 1 kw. with an average duty cycle of 10% and an average mean power of 20 watts was used. With a capability for pulsation of high energy transmissions and the further capability for separation of "fields" into transverse and horizontal components, it has been possible to demonstrate clinical and electrographic effects despite low average power. Such an array has proven effective in application to Bremer preparations is which electrographic changes were produced without apparent harm, whereas when carrier wave transmission was applied to such a preparation, it was killed within a short period. Ultrahigh frequency energies have also been applied to human serum globulin and phage T3 with production of discernible changes which do not seem relevant to thermal effects.

Technical Development

Various technical developments have been completed or begun during the period of this report. The *extracorporeal pump* described briefly above is one of these. This is the second such pump to

reach developmental stages in this year. The first is being used regularly in the laboratory. In the operating room, a new *head rest* providing capability for bilateral simultaneous craniotomy and a new *drape rack* providing for smoother instrument passage have been developed. In addition, relatively *glare-proof drapes* of light-weight material, impervious to fluid, have been developed. *Color filters* have been designed for operating lights so as to reduce "glare". Likewise, *glasses* for operating room personnel have been tested and are now being used so as to reduce eye accommodation fatigue and enhance acuity. A new *mattress* for the patient has been developed and is being tested. It provides a fluid-bearing system. A *brain suction tip* and *high vacuum pump* system has been devised and used for subpial dissection. A new *cortical electrode mount* and *holder* has been developed. A *constant-current device* for cortical and other cerebral stimulation in the operating room has been designed, tested, and is now in use. A *radio communications system* for operating room personnel was designed and has been submitted for commercial development. A *plastic printed circuit* cortical recording device with 36 imprints or contact points has been developed and is being tested. A *console monitoring system* designed to provide the maximum parameters of observation from a patient undergoing operation has been designed and awaits installation in the new surgical wing. Similarly, an *x-ray system* for stereotaxic roentgenography in the operating room awaits installation in the new wing. A new *instrument handling system* has been designed, based on the new surgical facility. This facility has provided opportunity for a variety of new technical developments and for an extensive program of personnel training in their use. A test device designed to *estimate stereotypy* in temporal lobe patients, two devices for *testing motor function* in involuntary movement patients, and a series of test objects designed for observation of *motor activity* in automatism have been developed.

An effective combination of epidural and/or spinal anesthesia with succinylcholine, fluothane, and/or hypothermia has a major role in *prevention of ventricular fibrillation.* An *inhalation anestheis system* which provides for simultaneous inhalation by two individuals is in use in the primate laboratory.

NATIONAL INSTITUTE OF MENTAL HEALTH

INTRODUCTION

The problems posed by the mental diseases and by the emotional and intellectual impairments of man continued in 1961 to be of major importance to the people of the United States. The research task of the National Institute of Mental Health is to discover the causes of these disorders, to develop effective methods of treatment, and, finally, to develop ways of preventing their occurrence.

The essential characteristic of this group of disorders is that, regardless of their etiology, their symptoms are mental or behavioral. The integrated and effective functioning of a mind and body in a human being is disturbed or distorted. Effective performance on the job, and useful participation in family life and the life of the community are damaged or made impossible. In the most serious of the disorders hospitalization is required, often for long periods because of the inadequacy of present treatment methods. Primary prevention, at present, except for a very limited segment of the total spectrum of disorders, is more a hope than a reality.

In carrying out its research mission the NIMH of necessity has designed its program to include those disciplines and subject matter aras which bear on the wide range of problems involved. Although a rough and empiric distinction between "clinical" and "basic" laboratories has existed for both administrative and intellectual reasons since the start of the program, it is recognized that precise differentiation between the two is neither possible nor desirable. Mental life and behavior, including intellectual functioning, personality, emotion, motivation, communication and interaction with other human beings, learning and perception—some or all of which may be damaged by the mental or psychiatric disorders—are affected by inherited capacity and biological integrity, by developmental processes at biological, psychological and social levels, and by both beneficial and noxious input from the physical and social environments. Accordingly, the intramural research program of the National Institute of Mental Health has established laboratories to investigate the major problems involved in this panorama of the natural and social sciences.

Ten laboratories or branches plus two special units make up the program. Concerned primarily with problems of direct clinical import are the Adult Psychiatry Branch, the Clinical Neuropharmacology Research Center, and the Biosocial Growth Center. Some elements of each of these programs can clearly be regarded as basic research. The Laboratories of Psychology, Socioenvironmental Studies, and Clinical Science are concerned both with clinical problems and with others basic to the disciplines involved and to an understanding of human behavior and biology generally. The Addiction Research Center in Lexington, Kentucky similarly tackles both the specific problems posed by addiction to narcotic and other drugs and basic problems which are relevant thereto. The Laboratories of Neurophysiology, Cellular Pharmacology, Neurobiology, and Neurochemistry are primarily concerned with pushing back the frontiers of knowledge in these biological sciences which hold the key to an understanding of the nervous system. The Section on Technical Development provides instrumentation service to the laboratories and participates in research as needed.

The research programs and achievements of these laboratories are summarized in the following reports by the laboratory chiefs. There will be no attempt to further summarize them here. It is only candor which prompts the further comment that an association with this complex program limited to the past two months is not long enough to enable the writer to add significantly to what the laboratory chiefs have reported. Certain matters of organization and administration are, however, of sufficient interest to warrant reporting.

Since the completion of the Clinical Center laboratories in 1954, intramural research in the NIMH has been conducted through two programs: a program of clinical investigations, and a program of basic research administered jointly with the basic program of the National Institute of Neurological Diseases and Blindness. During 1960, as a part of reorganization of the two Institutes involved, the laboratories in the joint basic program returned administratively to their respective Institutes, and each Institute appointed an associate director to take responsibility for its comprehensive intramural program. The year 1961 is thus the first year during which the NIMH has had a combined clinical-basic research program under one head.

Until mid-September of 1961 the new combined program was administered by Dr. Robert A. Cohen, who served as Acting Associate Director in addition to carrying his responsibilities as Director of Clinical Investigations. The double burden was a heavy one, and the program owes a great deal to Dr. Cohen's diligence, devotion and effective leadership. The writer, who comes to the intramural program after long absence from the NIH and after a number of years in a quiet foundation, is especially indebted to Dr. Cohen for his effective stewardship and for his past and continuing assistance as guide, mentor and friend. It is appropriate at this point also to acknowledge with gratitude the unfailing willingness and generosity of the staff of the intramural program, of the rest of NIMH, and of NIH, in helping a newcomer get a fair start. Though it is not more than the writer expected, it is a welcome verification of impressions long held of the quality and character of the NIH staff.

As forecast in Dr. Cohen's report for 1960, 1961 saw the departure from the NIMH program of Dr. Seymour Kety, Chief of the Laboratory of Clinical Science. He left in April, 1961 to accept the Professorship of Psychiatry at the Johns Hopkins University Medical School. His loss to the program and to the NIH as a whole was a great one, and it is therefore gratifying indeed to report that he plans to return to his old post in the NIMH on July 1, 1962. As the first Scientific Director of the NIMH, and later Director of Basic Research for the NIMH–NINDB, Dr. Kety embodies superbly the qualities of a first-rate scientist, an effortless and effective administrator, a humane spirit, a leader who understands and appreciates the contributions of both the natural and social sciences, and a warm human being who deserves and has won the respect and affection of all who know him.

A second laboratory chief to leave during the year was Dr. David Hamburg of the Adult Psychiatry Branch. He resigned July 14, 1961 to accept the Professorship of Psychiatry at Stanford University Medical School.

An important new appointment during the year was that of Dr. Howard E. Tompkins as Chief of the Section on Technical Development. With graduate work in physics, a doctorate in electrical engineering, a number of research contributions and patents to his credit, and experience both in industry and in university teaching and research, Dr. Tompkins is a fine addition to the research staff. His appointment is the culmination of a long felt need for a man of his stature to head up the developmental work in electronic instrumentation on which so much of the research on the nervous system depends.

Finally, it is fitting to record here, for the writer and for the staff of the basic program, a tribute to the work of Dr. Robert B. Livingston, Director of Basic Research in the NIMH and NINDB from 1956 to 1960. His devoted and untiring efforts to develop these two Institutes and the NIH as a whole into the finest kind of research and educational institutions were oustanding. His impact will long be felt both by those who worked with him and by those of us who came after he stepped down from his administrative post to resume full-time research as Chief of the Laboratory of Neurobiology.

CLINICAL INVESTIGATIONS

The events of the past year have raised questions which call for a re-examination of the basic philosophy of the Clinical Investigations program. On the one hand, as perusal of the Laboratory Chiefs' reports will show, our substantive research efforts have progressed favorably. On the other, we have been faced by the loss of various university centers of a number of senior investigators at the very moment when our intensive efforts to

build up the component research groups seemingly had been successfully concluded and we could look forward to a test of the fruitfulness of the organization we had asked for and developed. Why, at precisely this time, should the architects of the program turn their efforts and interests elsewhere?

In the development of Clinical Investigations, it had been my original hope to bring together a multidisciplinary group of investigators in the social and biological sciences who might constitute what would in effect be a continuing senior seminar concerned with the elaboration of a more powerful theory of behavior. This approach was based on the possibility that there already exist isolated bits of information known to members of the individual disciplines which, if communicated in a setting where all were confronted by the same behavior phenomena, might contribute to a more coherent understanding of important events. It was felt that the mere existence of these bits of information in time and space and the minds of men is not sufficient to bring about their integration unless the setting for their communication is favorable. It is also important to emphasize that I did not mean to imply by this that the research itself should necessarily be multidisciplinary or that group thinking would take the place of individual thinking. Rather, it was felt that in their attempts to grapple with their data—and, of even more importance, in their efforts to identify critical problems, members of each discipline would be usefully influenced by an intimate awareness of the work of the others. If it so happened that this gave rise to some interdisciplinary studies, these would certainly be supported but no premium would be offered for their selection; individual research would still be fostered; and excellence in content rather than adherence to a desired form would continue to be our goal.

Since the realization of these aims depended to so great an extent on a certain mutuality of interests among the Laboratory Chiefs, it would have been desirable if research operations could have been postponed until several of them had been brought together. We might then at the outset have planned certain studies of common interest and chosen other areas for individual exploration. However, there was an unavoidable commitment to admit patients to the Clinical Center wards within five months after the Director of Clinical Investigations assumed his duties. Starting from scratch, with the help of the Director of Psychiatric Nursing, a reasonably complete core staff had to be recruited and brought on duty in a very short time, long before the laboratory chiefs could possibly be appointed. It was into this operating setting that the laboratory chiefs came one by one over a period of five years.

There are manifest problems inherent in this form of development. One of the most serious revolves about the need to preserve some flexibility for a proposed laboratory chief; this means that the junior investigators who commit themselves to the work can be given only limited support for a stated period; at its very best this requirement sets a tone of impermanency. Another has to do with the manner in which the new laboratory chief enters the program when he finally is appointed. Finding certain operations more or less fully formed, he naturally concentrates his attention on the development of his own program, and becomes so deeply involved in this that it may take considerable time for him to become even superficially acquainted with the interests and activities of the entire group. These particular difficulties were anticipated, and an effort was made to meet them. First in appointing psychiatrists, preference was given to those who had also some graduate experience in another discipline. We were fortunate in recruiting men who had training and, in some cases, degrees in social work, sociology, clinical and experimental psychology, internal medicine, physiology, and biochemistry. Second, each laboratory chief participated importantly in the appointment of all those who came after him. It was hoped that these measures would facilitate communication between the clinicians and those who had no knowledge of or experience with the clinical situation, and, on a broader scale, between all the disciplines represented in the program. Finally, it was recognized that this type of program development would take longer to reach fruition, and that we must guard against impatience and disappointment if at first our research failed to utilize to the fullest our relatively unique resources but instead was represented by studies which might just as easily have been carried on in any good clinical or university setting.

By the end of 1957, when the last of our six laboratory chiefs had joined the program and the first phase of its development was completed, the future appeared promising. The laboratory chiefs held positions of eminence in their respective disciplines; they were congenial in both scientific and personal interests which seemed to augur well for a type of interaction which might prove fruitful. We were fortunate also in having attracted a number of younger and several other older investigators of considerable promise. If this group could be held together for a few years, it would provide an effective test of the idea which led to its formation; and in any event, one could feel confident that investigators of such competence would continue to carry on research of excellent caliber even if hopes for a high order of integration proved unattainable.

After a week's visit at this time, a consultant remarked that Clinical Investigations seemed to him to be suspended somewhere between paradise and earth. This did not mean that either he or we were unaware of serpents and of our own untoward impulses in our Garden of Eden. But he did emphasize what is apparent even when immediate difficulties occasionally loom so large as to take up almost the entire field of vision; namely, that remarkable resources are at our disposal. Not only do we have career positions but we also have in effect career support for our research; hence we can turn our attention to long-term, developmental programs as well as to short-term, cross-sectional projects. Contacts with investigators from many disciplines are readily available—and, even more than that, it is relatively easy to arrange a visit to or a visit from other investigators the country and the world over. Clinicians can spend time with normal subjects whom otherwise they would see only rarely and with difficulty; others have access to the clinical situation as a source of hypotheses not so readily suggested in other circumstances. The organization of the laboratories and program direction are essentially completely determined by the investigators themselves. Yet despite these and other indisputable advantages of our setting we have, in these last three years, lost four of the six original laboratory chiefs, one of two appointed to take the places of those who left, the Chief of Clinical Care, and a larger number of younger investigators. Granted that some turnover is to be expected and perhaps even to be desired, it is my feeling that research of the type I had hoped to see, research which would reflect creative use of the unique resources of the Clinical Center, could best arise after a long period of working together. I had thought of laboratory chiefs staying for fifteen to twenty years, long enough to carry a broad program to a definitive phase of development; of section chiefs staying for ten years, long enough to carry a segment of the program to completion, and of a younger group of investigators who might stay from three to five years, working primarily upon individual projects. To date this pattern has not been attained. Is it, in fact, a good or even a reasonable plan?

Before attempting to answer that question, it might be well to consider some of the problems which have faced us. I do not propose to attempt a thorough review of these, but believe that it may be useful to mention some of the important ones. The first is posed by the newness of the institution and the rapidity with which it reached its vast size. An older institution which supports a large number of individuals with clearly defined and traditional roles, which rarely and only with deliberation reaches out in new directions, has built into its structure many formal and informal supports which make possible the functions it carries on. Our new institution had only a limited number of such formal supports, and no informal ones at the start. These are still developing, but since there are limits to our ingenuity and foresight they are identified almost always at the cost of some inconvenience and frustration. Related particularly to this rapid growth has been some falling out of phase of the administrative and decision-making apparatus. Procedures which work well when the group is small become unwieldy as the group grows larger and more complex. Individuals who were once always informed, if not actually consulted, may now only rarely even be seen. Decision-making ideally should occur at that level at which all the information necessary for an effective synthesis is gathered; if a higher level does not contribute new information to this synthesis, it cannot effectively contribute to the decision. On occasion, the decision-making responsibility has travelled upward hooked to an administrative level which no

longer has access to new information, and here again frustrations result. As befits a new and growing institution, there has been a willingness to examine issues of this nature, and to introduce modifications when the problem was recognized as one which had sufficiently broad implications. Many of us have felt satisfied, both with this readiness for and with the rate of change, although there are naturally instances when, from one individual's point of view, these changes could well have been made earlier. Some, comparing NIH with longer-established institutions, have been disappointed and even angered at the fact that more inconveniences had not been foreseen and avoided or, failing that, are not eradicated immediately upon identification. The last general problem I wish to mention is that of the relatively low salaries. Originally only the clinicians came for less than they earned elsewhere; now many of the outstanding university research centers tend to pay more than we in every field except at the juniorl evels of training and experience.

The foregoing considerations have played some part in the decision of each staff member who has left NIH, but it would be naive to assume that all our problems are related to organizational difficulties. One important factor is the state of development of seminal concepts in the field. In the last twenty years there have been many important developments in neurophysiology, biochemistry, and pharmacology. By contrast, significantly new information in the social-psychological fields has been produced at a slower rate. This relative stasis poses a special problem to those who enthusiastically undertake full-time research only to find that intensive efforts all too often result in but slight achievement. In view of the disappoint which often follows the persistent pounding away at these difficult problems, it remains to be seen whether full-time research is the way of life most likely to result in progress; this some of our staff have not remained to see. Differences in the stages of concept development in the various behavioral sciences serve as blocks to communication, and seem to result in a tendency for one to spend less time with investigations in other disciplines than might otherwise be the case.

It is largely to the universities that the investigators who have left our program have been attracted, and the question must be asked as to how a university might offer a better environment than NIH for the type of research in which we wish to engage. In my opinion, the strengths of the university lie in several areas. First, it supplies the investigator-teacher with an endless flow of students. Are students valuable in helping one formulate his ideas more precisely? Do they provide an atmosphere highly favorable to creativity by the stimulus of the challenge they offer? Our experience has shown that some of our staff do very well, or even their best work without any teaching responsibility, while others find it important to engage in a moderate amount. Similarly, many clinical investigators find need for stimulation in the assumption of some clinical service responsibilities.

Second, at the level of prestige, a professorship at a leading university still is more highly regarded by many than a top-level post at NIH. The ideas of stability, of serene contemplation, and of such amenities as a faculty club are mentioned so often that, while they cannot be regarded as of primary importance, they should not be underrated. We tend to be ashamed of placing any value on considerations of this sort; hence we do not pursue them for that degree of value which they properly have. The issue of "boondoggling" at public expense becomes confused with the question of what intangibles might foster commitment to the research life. Not all progress results from the compulsive and furious activity of the dedicated genius who impatiently brushes away material comforts while brilliant ideas burst forth one after another.

I wish to emphasize the fact that I am still referring to individuals who consider themselves to be full-time investigators. The university has other purposes to fulfill, and at one time or another in the course of his development a staff member may decide that teaching with or without part-time research may offer a more attractive and/or productive career than full-time research.

In favor of NIH, the facilities and support it offers to several hundred scientists probably cannot be matched except in a few instances for special individuals. If our continued experience should lend further support to the impression that those assets of the university mentioned above are

indeed important, there is probably enough flexibility in our situation to enable us to make the appropriate changes.

Finally I come back to the organization of Clinical Investigations and to the conceptions on which it is based. Looking back, it appears that the scientific interaction I had hoped for at the laboratory chief level has actually occurred more often at the section chief level. Development of several fifteen-twenty year research programs at NIH seems much more likely to occur at that level also since the majority of the section chiefs have been with us for five or more years while five of the first eight laboratory chiefs have left. I find myself surveying the scene with a mixture of emotions. There is reason for satisfaction with every area of our research—both for what they promise in the future and for what has been accomplished in the past. The work on the family may be taken as one case in point. It started with five discrete projects in five different areas of the program; each of these was pursued individually with varying degrees of success, the most notable external recognition being the Burgess Award to Dr. Melvin Kohn. With minimal externally-fostered support there has been enough exchange of information so that there is gradually emerging a longitudinal, developmental understanding of the manner in which experiences within the family shape the personalities of its members as a special instance of the impact of environmental forces on inherited apparatuses. Even though they were separately conceived and carried out in different laboratories, a series of studies have been linked together by the gradual growth of this concept and they gain in importance thereby. It is my impression that work in this area will expand rapidly in the coming years, and that it will significantly increase our understanding of factors affecting perception, thinking, behavior, and personality development. Members of our staff are in position to play leading roles in this field by virtue of their contributions to date and their current studies.

Despite these evidences of progress, it is impossible not to feel concern about the resignations of so many of our first group of laboratory chiefs. It is tempting to point to personal factors which might have strongly influenced their moves; it is true that each of them had refused many attractive offers, and had finally accepted the one which seemed to afford him many special advantages. It is also true that each had attained a position of scientific eminence just at the time when large funds were being poured into research, and there existed a relative shortage of highly competent investigators; therefore, great pressures were placed upon them. It is even possible that the excitement of pioneering may have a special attraction for some of them; many pioneers continue to push out the frontier and do not settle down to cultivate explored areas. I do not doubt that these and many other personal factors had some importance, but it behooves us to concentrate on the institutional situation since this is the area we can hope to change by our own efforts; its relative weight in any given instance may be large or small.

For that period in one's scientific life when one is fully engaged in research at the bench, it is difficult to imagine a more supportive environment for many areas of study than is provided at NIH. For some this remains the major form of scientific activity throughout their lives, and it satisfies their image of how they as scientists should live. There is another pattern of scientific life—one which is probably largely the result of the growth of NIH itself. There has been a great proliferation of conferences, of panel discussions, of study sections, of planning committees, etc. There is the greatest pressure upon anyone who displays some conceptual ability and a high level of sound scientific judgment to participate in these conferences, and to assume statesmanlike responsibility for promoting the optimum development of science. In addition to their proven abilities as investigators, the laboratory chiefs also possessed in very high degree attributes which made them valuable contributors in these other areas. They were constantly sought after both at home and abroad. In fact, these personal attributes were of some importance in their recruitment by NIH and in the successful development of their individual laboratories. The image of professional growth of an individual in this group is broader than that of the investigator who proposes to spend almost all his efforts at bench research; it includes the assumption of teaching duties, the editorship of scientific journals, participation in conferences concerned with a wide

variety of problems in addition to those related to his own substantive research. Is this a passing phase in the life history of some of these scientists—possibly of many scientists? We have not had an opportunity to learn the answer to this question in this time of rapidly increasing supports for research and training for research. In any event, these laboratory chiefs felt that as their interests turned more in these latter directions, universities offered an environment for their expression more favorable than NIH. Their leaving had a strong impact on the group; we miss them for their wisdom and their resourcefulness and also because of the personal attachments which developed over the years. Will our research be the weaker for their departure? Obviously, the seminal interaction between these particular individuals which I had particularly hoped for never did and never will occur. On the other hand, the majority of the section chiefs who carried out the research which has brought this program whatever reputation it possesses are still here; their work continues to move forward.

What course shall we follow now? Shall we create another group of laboratory chiefs in the same image—promoting section chiefs already in the program, and looking outside for others who may possess special skills? Shall we accept this as a natural growth process which is bound to be repeated—is it a regular progression from investigator to section chief to the broader interests of the laboratory chief and then out to a university post? Should we propose a modification of NIH to include a recognized training program at an appropriate level, post graduate or graduate, which might afford the teaching opportunity many investigators desire? Should we consider an organizational change—moving from the laboratory to the section as the basic unit; then instead of having laboratories and laboratory chiefs, we might have group leaders of one or any number of sections who come together for a specific study, or series of studies, and then regroup at an appropriate time? I do not have the answers to any of these questions. I have raised them because I believe that the events of this year dictate a reexamination of our situation, serious consideration of alternative methods for the strongest development of our program, and the deliberate choice of the one we regard as best at and for this time.

LABORATORY OF CLINICAL SCIENCE

As in the summary of the annual report for the previous year, the present summary will deal with results in certain problem areas of continuing interest in the Laboratory. The names of the particular individuals who carried out the work have been omitted. Moreover, a number of valuable individual projects of considerable interest which did not fall into one of these areas have been entirely omitted. For any detail concerning laboratory activities during the past year, therefore, the reader is referred to the reports of individual projects. The material contained in this summary is based upon the reports prepared by the various section chiefs:

Office of the Chief, Unit on Psychosomatics, Philippe V. Cardon, Jr.
Section on Medicine, Roger K. McDonald
Section on Physiology, Edward V. Evarts
Section on Psychiatry, William Pollin
Section on Biochemistry, Marian W. Kies
Section on Cerebral Metabolism, Louis Sokoloff
Section on Pharmacology, Julius Axelrod

Schizophrenia

Biochemistry

Last year studies of factors affecting mobilization of free fatty acids (FFA) in schizophrenic and normal subjects were begun in this Laboratory. This year these studies were extended. The results made it clear that some schizophrenic patients do not exhibit a normal fall in FFA in response to insulin. The abnormality has been noted both in young acute schizophrenic patients and in patients with character disorders.

In studies which were related to those above, though not carried out in schizophrenic subjects, it was found that a substantial increase in FFA occurred during hypnotic induction of fear, anger, and depression. The magnitude of the increase depended, at least in part, on susceptibility to

hypnosis. In given individuals, the type of feeling state suggested appeared to influence the nature of the FFA response.

Additional studies relevant to the biochemistry of schizophrenia have dealt with (1) the metabolism of norepinephrine and epinephrine in normal man and in patients with mental and other diseases and (2) the excretion patterns of aromatic amino acids in normal and schizophrenic subjects. By the application of enzymatic hydrolysis methods, it has become possible to estimate more conveniently and probably more reliably the rates of excretion of metanephrine and normetanephrine. In the process of development are methods which will permit the accurate quantitative determinations of norepinephrine and epinephrine and all their metabolites in body fluids; it is hoped that these methods may be applied to studies in normal man and in patients with various diseases. Methods have also been developed for studying the metabolites of the aromatic amino acids, and approximately 20 acid metabolites of these amino acids have been extracted from human urine; of these about half have been identified.

Psychopharmacology

During the past year, studies have been devoted to preliminary assessment of the psychiatric effects of a recently developed psychopharmacological agent, WIN–18501. This drug is of interest because, as a substituted tryptamine derivative, it is structurally related to tryptophane. The drug was fouund to have interesting clinical properties and, in conjunction with other changes in the treatment milieu was associated with substantial clinical improvement in a significant number of patients. Of the seven schizophrenic patients who participated in both the clinical trials of this drug, and a prior study of the psychic effects of several amino acids, the five subjects who showed definite clinical response to the drug were the same five who showed clear-cut changes with one or the other of the amino acids previously studied. The significance of this overlap remains to be determined.

Psychophysiology

Extensive work has been devoted to developing a technique to be used in comparing autonomic reactivity in schizophrenic and normal subjects. This technique measures the extent to which unconscious autonomic changes occur in anticipation of effort, and the degree to which these changes are quantitatively related to the intensity of graded efforts which an individual knows he will be required to make. It has been found that, in normal control subjects, such responses do occur and tend to be so related to the intensity of conscious effort, whereas in schizophrenic patients they are much less frequent and do not show such differentiation.

Family Studies

A major continuing project in the area of schizophrenia research is the study of families in which one sibling has schizophrenia and another sibling does not, and the use of such differences within individual families as an approach to a study of the etiology of schizophrenia. During this past year additional families of the type described and control families (one child a juvenile delinquent and another same-sexed child who is not juvenile delinquent) have been located and interviewed. Efforts have been concentrated on improving methodology of such family evaluations, and on planning for extension of this approach into an additional type of family in which it is believed that definitive studies may be carried out. The latter consists of the family in which there are identical twins discordant for schizophrenia. Though extremely rare, such family groupings are potentially an unusually valuable study population since possible hereditary contributions to the illness are kept constant. Contacts have been established with state hospital systems throughout the country looking for the establishment of a multi-state mental hospital multiple-birth registry so that systematic samplings of twins with mental illness may be possible. Additionally, letters have been sent out nationwide requesting referral of such cases to us. It is planned that the admission of first such cases to the Clinical Center will begin early in 1962.

The Biogenic Amines in Mental State

Previous studies in this Laboratory have shown that certain chronic schizophrenic patients, clinically stabilized on a maintenance dose of a monamine oxidase inhibitor (Marsilid) repeatedly demonstrated significant clinical changes follow-

ing the administration of approximately ten times the normal daily dose of two essential amino acids: methionine and tryptophane. Clarification of the significance and mechanisms of these effects has been sought along several lines of inquiry. Members of the Laboratory have recently demonstrated an enzyme which catalysed the N–methylation of tryptamine, leading to the production of two possibly psychotomimetic substances: Dimethyltryptamine and Bufotenine. A closely related drug, diethyltryptamine (DET), and another possible route by which psychomimetic substances might be produced in humans—6 hydroxylation—were also studied. Investigation dealt with the relationship between the psychological effects and the metabolism (especially 6-hydroxylation) of DET in schizophrenic and normal control subjects. It was found that normal controls showed a positive correlation between the psychological effects of DET and the amount of 6 OH–DET in the urine, whereas schizophrenic subjects did not. It was further observed that there was a delayed excretion curve for urinary 6 OH–DET in schizophrenic subjects as compared with normal control volunteers.

A second follow-up study attempted to establish the role played by the monamine oxidase inhibitor in the previously observed psychological effects of tryptophane and methionine in certain schizophrenic subjects. In a small number of normal control subjects and in the available reactor schizophrenic patients, methionine was given in the absence of marsilid and clinical psychiatric and quantitative psychological observations made. Only minimal changes were observed, tending to confirm the previously held impression that the marsilid played an essential role in the clinical changes previously described.

Experimental Allergic Encephalomyelitis

Encephalitogenic Protein

Biochemical studies on the low molecular weight basic protein isolated from myelin have been continued in order to clarify its structure and function. Because of the similarity of the basic protein to nucleohistone, it was important to rule out the possibility that this encephalitogenic protein might have originated in nuclei and become attached to acidic lipid constituents of myelin during homogenization and isolation. It has been demonstrated experimentally that the basic protein associated with myelin differs in its solubility and electrophoretic behavior from a histone fraction prepared from intact nuclei isolated by ultracentrifugation. Furthermore, these nuclei shown to be free of myelin by phase microscopy are inactive in the induction of experimental allergic encephalitis (EAE).

The encephalitogenic protein combines with calf-brain ganglioside (a water-soluble acidic lipid prepared by Dr. E. Trams, NINDB) to form a stable complex with electrophoretic properties differing markedly from those of the free protein. Complex formation with ganglioside appears to interfere with encephalitogenic activity, whereas the protein as it exists in myelin (presumably combined with lipid) is fully active. Studies of model compounds such as these may yield information on the structural relationships of certain constituents of myelin.

Immunologic Studies

Suppression of EAE by a scorbutogenic diet has been studied further. The suppression of the disease reaction is specifically related to lack of vitamin C rather than to weight loss and inanitation. Attempts to differentiate between the effect of the vitamin deficiency on tuberculin sensitivity and on EAE were unsuccessful. In all experimental conditions studied, EAE susceptibility was correlated with sensitivity to tuberculin protein except in the case in which vitamin C was restored to the diet of scorbutic animals after development of EAE had failed to occur. Because of the complicated immunologic mechanisms operating in EAE induction, no clear-cut interpretation could be made regarding this experimental observation.

Thyroxine

A major area of interest within the Laboratory has continued to be the mechanism of action of thyroxine and its relation to cerebral metabolism. It was previously found during the course of this project that L-thyroxine administration *in vivo* or addition *in vitro* results in a stimulation of amino acid incorporation into protein in cell-free rat liver homogenates. Hypothyroidism induced by surgical thyroidectomy results in reduced protein

synthetic activity. The stimulatory effect of thyroxine has been found to have absolute dependencies for both mitochonria and a substrate for exidative phosphorylation. Evidence has been obtained to indicate that some interaction between the respiring mitochondria and thyroxine must occur for a finite time before the stimulation appears, suggesting the production of an active intermediate. Studies are in progress designed to elucidate the nature of the interaction and its product.

The major efforts in this project during the past year have been directed toward the elucidation of the mechanism of the thyroxine stimulation of protein biosynthesis. The effect has now been localized to one of the several steps involved in the overall process of amino acid incorporation into protein. The stimulated step is the transfer of the soluble or transfer ribonucleic acid bound amino acid to the microsomal protein, and the effect is not secondary to an action on the generation of GTP, the only known nucleotide requirement for this step. This finding represents a major step forward toward the elucidation of the mechanism of the thyroxine effect on protein biosynthesis.

The thyroxine project has also been expanded into additional areas. Studies with homogenates of immature and mature rat brains have demonstrated that immature brains have higher rates of protein synthesis and respond to thyroxine in much the same way as liver. In contrast, thyroxine, if it does anything, inhibits protein synthesis in the mature brain.

Salicylate is known to have a number of thyroxine-like actions *in vivo* and *in vitro*. Studies in this Laboratory have demonstrated that it also has a thyroxine-like action on protein synthesis.

Phenobarbital and 3-methylcholanthrene have been reported to stimulate several microsomal enzyme systems involved in drug metabolism. It has been hypothesized that the mechanism of these effects is the stimulation of enzyme synthesis. It has been found that these agents stimulate amino acid incorporation into protein, a finding which offers support for this hypothesis.

Geriatric Psychiatry

The major activity of the continuing studies in Geriatric Psychiatry consisted of a follow-up re-evaluation of the surviving members of the carefully chosen 54 community-resident male volunteers over 65 years of age who were first intensively studied here by a large group of 21 investigators some five years ago. This represents the first known longitudinal study of patients in this age group. The data obtained in this collaborative follow-up re-evaluation are being analyzed and compared to the now completed analyses of the original evaluations. It is of particular interest that a preliminary examination of the follow-up data suggests that certain phychological characteristics may be significantly associated with mortality in this age group; namely, reduced speed of response, manifestations of senility and poor social adaptation.

Catecholamines

Studies in Animals

A new catecholamine, N-methyladrenaline, was found to occur normally in the adrenal gland of a number of mammalian species and has been subsequently found in the urine of patients with phaeochromocytoma by investigators elsewhere.

The disposition of radioactive noradrenaline has been studied and a portion was found to be taken up and bound at or near sympathetic nerve endings. Stimulation of the splenic nerves of cats treated with radioactive norepinephrine resulted in liberation of the radioactive noradrenaline with a portion being discharged into the blood, a portion o-methylated and a portion again taken up at the nerve endings. This indicates that circulating norepinephrine can be inactivated by binding at the nerve endings and released to act again on stimulation of the nerves.

The action of psychotropic drugs on the disposition of noradrenaline has also been investigated. Cocaine, chlorpromazine, and imipramine prevent the uptake of noradrenaline into the storage site, reserpine and amphetamine release the catechol amine, while psychic energizers (monoamine oxidase inhibitors) prevent the release of the hormone.

The metabolism of epinephrine in the rat has been investigated by the technique of using epinephrine-H^3 and metanephrine-C^{14} and it has been estimated that about $\frac{2}{3}$ of an injected dose of epinephrine is o-methylated while the rest is either

excreted unchanged or acted upon by monoamine oxidase. When o-methylation is inhibited the other two pathways predominate. Norepinephrine also undergoes methylation when circulating, but bound, and presumably endogenous norepinephrine is predominantly deaminated.

In order to study the metabolism and binding of norepinephrine in tissues, the isolated perfused rat heart is being studied. This preparation binds norepinephrine, metabolizes the catechol amine as it is released and can be studied in relation to various drugs as well as to functional changes in force and rate of cardiac contraction.

Catechol amines are thought to play a role in the central nervous system as well as the peripheral sympathetic nervous system. Insight into the mechanisms of activation, inactivation, and metabolism contribute to an understanding of the physiological role played by neurohumors as well as to the mechanism of action of various psychotropic and autonomic drugs.

Studies in Man

One of the major metabolic products of epinephrine and norepinephrine is vanillyl mandelic acid (VMA). During the past year, extensive efforts have been devoted to determining factors which alter VMA excretion in man.

Three aspects of VMA excretion are currently under investigation. First, the effect of a single therapeutic dose of the following six psychotropic drugs on VMA excretion has been determined: reserpine, chlorpromazine, morphine sulfate, pentobarbital sodium, meprohamate, and d-amphetamine sulfate. Only reserpine caused an increase in VMA excretion and only chlorpromazine caused an unequivocal decrease. The fall in VMA excretion following pentobarbital sodium was of borderline significance. Morphine administration resulted in decreased VMA excretion which was interpreted to be due predominantly to extrametabolic factors. Meprobamate and amphetamine had no significant effect on urinary VMA. Using increased tryptamine excretion as a criterion of monoamine oxidase inhibition, it has been established that amphetamine in the dose used here does not inhibit monoamine oxidase, an enzyme involved in VMA formation. Follow-up studies on the effect of repeated injections of reserpine indicate that the initial increase in rate of VMA excretion is followed by a decrease to subbaseline levels in spite of continued reserpine administration.

VMA excretion is also being measured in patients with various types of depression. Although definitive results on VMA excretion by patients in different diagnostic categories have not been obtained, it appears that these patients, as a group, have higher than normal urinary values. Preliminary findings on the relationship between a patient's clinical course and VMA excretion indicate that exacerbation of the disease is frequently associated with an increased rate of excretion of this metabolite. The effect of various antidepressant drugs on VMA excretion is under study with special reference to the patient's responsiveness to the drug and his concomitant VMA excretion.

The third VMA project is concerned with the relationship between VMA excretion and the menstrual cycle. All subjects studied to date show a decrease in urinary VMA with the onset of menstruation.

Sleep and Arousal

A number of studies have been devoted to the effects of stress and level of arousal on peripheral circulation. In one study in this general area, EEG, respiration, and finger pulse were studied during the periods of failure to respond to visual stimuli, which occurred after administration of a barbiturate. The time-courses of these variables, and their inter-relations are different from those observed during sleep deprivation or chlorpromazine effect.

Neurophysiological investigations have been carried out in cats in order to determine the patterns of activity if single neurons which underlie sleep and arousal. Particular attention has been devoted to analysis of the activity of single neurons in visual cortex during sleep with low voltage fast EEG activity (S–LVF). This type of sleep is of particular interest in view of the fact that it appears to be an analogue of that type of sleep which, in man, is associated with dreams. It was found that with the onset of S–LVF there is a marked increase in the rate of discharge of most neurons of the visual cortex. Furthermore, it was found that during S–LVF individual neu-

ronal discharge is highly correlated with the occurrence of eye movements. Eye movements are characteristic of S–LVF in both man and cat. If S–LVF is analogous in man and cat, the present observations would indicate that dreaming is associated with a considerable increase of neuronal activity in the visual cortex. A similar increase in neuronal activity during S–LVF was found in this Laboratory last year in single neurons of the brain stem.

LABORATORY OF PSYCHOLOGY

The Laboratory has continued during the present year in its three major areas of research: (1) the nature and development of psychological functions; (2) the neurological correlates of psychological functions, and (3) the effects of disturbing conditions on such functions in man and animal. In each of these areas attention has of course been focused on a limited number of the subsumed fields, those of particular interest to the investigators. Thus, in the first area our work has mainly concentrated on the study of certain aspects of infant development, and perception, social communication, and creativity in the adult. The neuropsychological studies have concerned themselves with perceptual, problem-solving, alerting and emotional-motivational functions. The studies in the area of disturbed function have been directed not only at what these studies might contribute to the understanding of these conditions specifically, but also for the light the findings throw on the understanding of normal function. The three major fields of investigation in this area have been aging, schizophrenia and stress.

The research of the Laboratory's six sections [Basic: Perception and Learning, Neuropsychology, Aging; Clinical Investigations: Early Development, Personality, Office of the Chief] roughly correlate with most of the specific areas outlined. Increasingly, however, the original delineations of areas and fields have been breaking down. Despite the fact that there has been no attempt to establish a unified program in the Laboratory, an increasing amount of overlap of function within the Laboratory has occurred over the years. Common interests have developed from preoccupation with specific problems and has resulted in collaborations across sections, as well as across laboratories and institutes. Paradoxically the latter appears simpler to achieve.

The present report is for the above reasons organized around an outline of research areas which disregards Section lines. It is, however, based on the detailed reports of the various Section Chiefs.

The work in early development offers a natural introduction to the first area of research—the study of normal psychological functioning. It will be followed by a description of the progress that has been made in the studies of selected aspects of perception, social communication, and creative problem-solving in adults.

Early Development

The major research activities in the area of child development have been centered on the behavior of the human infant, and the relationship between parent behavior and child behavior viewed both concurrently and predictively.

One major study has been the revision of the Bayley scales for measuring the mental and motor development of infants. Tentative age norms for the first fifteen moths of life have now been set up; The Infant Behavior Profile, a new instrument for developmental diagnosis, has been constructed; and the manuals and record forms have been revised and enlarged. Standardization testing was begun this year on children aged 18 through 30 months in order to provide a mental scale continuous with the Stanford-Binet and to bring the former motor scales up to date. To date 120 children of these ages have been tested.

To compare the development of infants living in different parts of the world the investigator has trained a number of psychologists from several countries in administering these tests. These studies will not only yield information on the effects of different child-rearing practices upon the early development of behavior but also furnish psychologists in these countries with tools to use in constructing their own diagnostic instruments.

Results from the use of these scales by the NINDB Collaborative Project are beginning to come in. For example, in 1409 tests on infants under 15 months of age there were no differences in the means and variances of the total scores

earned by Negro and white infants at each month, or between male and female infants.

A study has also been completed on the increments in head circumference from birth through 25 years. The course of growth was found to be remarkably smooth with a small but clear spurt of growth at puberty. These findings will add to the norms on the growth of healthy infants and children and will aid in the diagnosis of anomalies in head size frequently accompanying neurologic damage.

Work has continued on the monograph entitled, *Maternal Behavior, Child Behavior, and their Interrelations from Infancy through Adolescence*, which will be published in the spring of 1962. The pattern of correlations, based upon the data of the Berkeley Growth Study, are consistently different for boys and girls. Boys under two years of age have a pattern of correlations with their mothers' behavior that is reversed as they approach three years of age. Their intelligence between 7 and 18 years of age is correlated with their mothers' behavior toward them during their first three years. Infant girls, in contrast, show a distinctly different pattern of mental test correlations with their mothers' behavior, but at school age, correlations with early maternal behavior become insignificant and remain so. Thus, the intelligence test scores of girls tend to be correlated with maternal *intelligence*, while those of boys tend to be correlated with early maternal *behavior*. This finding can now become an hypothesis for specific testing, all the more interesting because unsuspected.

There has been a continuing exploration of the relationships between parental behavior and child personality within the statistical model of radex theory which has been found so fruitful in the past. The major effort this past year has been to assess parental behavior by means of children's reports of that behavior. The assumption underlying the investigation was that what may influence the personality development of the child is not so much what the parent *says* he does, or even what the parent *actually does* in rearing the child, but more how the child *perceives* the parent's behavior. A set of scales for the measurement of parental attitudes and sets of rating scales for the description of maternal behavior from observations and interviews having been already developed, the task was to develop a set of scales with which to collect children's reports of parents' behavior and to discover the factorial structure of their reports.

Twenty-six scales of 10 items each were drawn up to sample all sectors of a two-dimensional model for parent behavior. Intercorrelations were factor analyzed and yielded as a principal component a dimension of love (positive evaluation of the child; sharing activities, plans, and interests; expression of affection, encouraging independent thinking, etc.) v. hostility (irritability, rejection, neglect, and ignoring). The second principal component was identified as parental control, but because of appreciable loadings on subsequent components appeared to reflect *three different kinds* of control: psychological control, physical control, and extreme autonomy. A comparison of the responses of the normal boys and girls revealed a number of differences in how each sex perceived parental behavior and differences in how each parent was perceived. In general, mothers tended to be rated higher on most scales reflecting love and nurturance. Mothers were higher than fathers on all scales measuring psychological control for girls and on many of these same scales for boys. Boys reported fathers as higher on the physical control variables; and there was a clear trend to report the opposite-sex parent as granting more autonomy. In a study of the reports of parental behavior gathered from 154 normal men and 108 male psychiatric patients, a preliminary analysis suggests that if a subject reports that he considers himself maladjusted he also reports that his parents were hostile toward him during his childhood.

The next steps in this program will include attempts to validate these findings by correlating retrospective accounts by young adults with earlier observations and interviews, by relating the child's behavior in the classroom as reported by teachers and peers to the child's own report of parent behavior, and by correlating reports of parent behavior made by trained observers, by children, and by the parents themselves. Planned also are studies of reports made by siblings, fraternal twins, and identical twins. These studies will permit an evaluation of the validity of a child's report of parent behavior, of the relation of his perception of his parents to his perception of self, and of the relationship of his report of parent behavior

to his overt behavior. They will also indicate to what degree siblings, monozygotic, and dizygotic twins perceive parent behaviors differently.

The development of social and exploratory behavior in the young human infant and the conditions under which it is modified by experience continues to be another principal focus of work in the Laboratory.

One of the investigators, while on leave in Israel, has been studying the course of the smiling response in institution infants over the first 18 months of life. By charting the number of smiles given to an observer who only stands and looks during a twelve minute period and who a minute or two later smiles and talks to the infant, a developmental curve of smiling will be obtained. Since the institution provided minimal conditions of environmental stimulation and limited opportunity for the evocation and subsequent conditioning of the smiling response, the results can serve as a basis for evaluating the unconditioned basis of smiling. At the same time it will provide a baseline against which can be compared the smiling behavior of infants reared in environments offering greater amounts of stimulation, together with greater opportunity for social learning and for the reinforcement of smiling. These environments are: day nurseries; collective settlements (Kibbutzim); and two types of middle-class family environments, the single child family and the multiple child family. Related to these studies of smiling in home and institution infants at different ages are attempts to describe in objective terms the elements of caretaking which evoke and reinforce smiling. Here the focus of study are the circumstances under which stimulation is provided the infant. The question being asked is: do certain stimuli, together with the behaviors of the infant, enter into effective contingencies for learning?

The investigations into the nature of the infant's earliest reactions to his environment and their modification by the environment's response to them has been continued with increased vigor during the past year. Current research has attempted to demonstrate that social and exploratory behavior are related response systems in the young infant and, further, that they are progressively differentiated by the differences in sensory feedback provided by objects in the environment. Work has proceeded in three main areas. In the laboratory, efforts to study the effect of visual reinforcement upon exploratory behavior have passed the pilot stage; studies on free exploratory behavior and the conditioning of social vocalizations have been pursued in an Infant Home; and in the area of maternal care, seen as a prime determiner of infant behavior, efforts to delineate its components have ben extended by the beginning of a cross-cultural study.

An apparatus has been designed and built to record the infant's exploratory behavior and changes in it when visual stimulation is made contingent upon that behavior. The design and construction of suitable apparatus required the solution of the following problems: the naturalness of the experimental environment; the position of the infant in that environment; the class of behavior to be measured; the nature of the sensory feedback to result from the infant's behavior; and the special problems presented by experimental work with the human infant. The apparatus has been in use for five months and about 25 infants have so far been studied, some for several sessions. Ten minutes appears to be the best length for an experimental session. Two types of problems have been studied, in both one and one-half seconds of a motion picture showing brightly colored paper figures moving over a black drum were used as the feedback stimulation. In the first, changes in experimental conditions occurred between sessions a week apart; in the second, they occurred within sessions. While the first pilot studies are now complete, a sufficient number of satisfactory records still have not been obtained. To assure a greater number of such records there must be further systematic exploration of the effects of, among other variables, different responses, longer periods of visual stimulation, and different kinds of visual stimulation.

Associated with these laboratory studies have been studies designed to provide basic information on the waxing and waning of exploratory behavior when a set of dangling rings were suspended across the cribs of infants three to five months of age. Measures were made of the number of times (and the duration of time) the rings were touched during a period of 30 minutes. Eighteen of 21 infants were still manipulating the rings at the end of the period, although at a slower rate than at the beginning and during the

middle of the period, suggesting that habituation sets in very slowly.

Cribside studies of the infant's vocalizations were carried out to check on an earlier study in which the rate of vocalizing was increased by an adult's social response. Since the the social stimulation might have served as an arouser or releaser, it was necessary to test the effect of a "reinforcer" administered on some random schedule. For one group of infants the experimenter's response was made contingent upon the infant's vocalization; a second group of infant's was stimulated according to a non-contingent schedule independent of their vocalizations. Data on 12 infants, 6 in each group, showed the greater increase for infants on a contingent reinforcement schedule. A not unimportant result of the investigation was the successful working-out of equipment and methods (progammed tape on an audio-playback apparatus) so that identical schedules can be used with more than one infant or with one infant at different times.

The work on maternal care has developed in two different directions. In one, the data on maternal care in the dog, now nearing final analysis, have revealed interlocking trends in the maternal activities of contact, nursing, licking and punishing and the puppy activities of approaching the mother, vocalizing, and play. The study will be reported in *Maternal Care in Mammals*, a book of contributed chapters to be published by Wiley's. In the other, a member of another laboratory has been trained in our technique of measuring human maternal care for use in a comparative study in Japan.

Perception

During the past several years a number of perceptual size-constancy experiments have been performed in this laboratory with young adult subjects. Size constancy refers to the fact that perceived object-size remains functionally invariant with varying object-distance in spite of inverse variation of retinal image-size with distance. Traditionally this phenomenon has been thought to derive from optical factors, such as binocular parallax, convergence, and accommodation. Some of the experiments referred to involved special conditions, such as drugs or sleep deprivation, and as a secondary matter visual acuity measures were also obtained on the subjects. No clear dependence of size constancy upon visual acuity was evident, however. Consequently an experiment was devised to study this relationship more specifically.

One group of young adults and one group of aging subjects received an instruction to judge *apparent* visual size at the first session of the size-constancy task, while another of each age group received an instruction to judge *objective* size. On a subsequent day the instructions were reversed for the two groups. In addition, the older subjects were required to perform the task both with and without their normal eyeglass correction. A separate determination of visual acuity was made on all subjects.

Thus far two main results have emerged: (a) There is an interaction between the order of receiving instructions in the size- constancy task and the correlation between size-constancy performance and visual acuity. For subjects receiving the apparent-size instruction first, the correlation was negative; for those receiving the objective-size instruction first, the correlation was positive. (b) Size constancy was maintained by the older subjects even when they were not allowed to wear their glasses, a condition in which they exhibited obviously poor visual acuity. The experiment has not been altogether completed, but it appears fairly clear that if there is a relationship between size constancy and visual acuity it is in the nature of a psychological interaction rather than a simple matter of cause and effect in a strictly visual sense.

Another fundamental problem in research in perception is concerned with perceptual adaptation to visual motion. In order to approach this problem more objectively, a special means of measuring perceived stimulus velocity was developed in which response time is used rather than an explicit judgment of velocity. The subject is unaware of the occurrence of any aftereffect following adaptation to a moving pattern. Findings with normal subjects partly agree with classical descriptions of motion aftereffect but differ in certain fundamental respects which suggest a direction-specific mechanism in the visual system.

Social Communication

A series of studies, growing mainly out of psychotherapy process research, have devoted them-

selves to the problem of how social communication takes place, especially how affect is communicated.

One group of these studies is concerned with the formal rather than the contentual aspect of speech. The general aims of these investigations are to: 1) Utilize speech disturbances as a technique for monitoring transitory changes in ego states; 2) Investigate novelty and stereotypy in speech; and 3) Investigate factors relating to rate of speech. In analyzing the nature and function of speech disturbances, three categories of speech disturbances have been defined: a) "Filled pauses," e.g., "ah" and "repetition." Such pauses tend to occur immediately before words which have a higher than average information value; b) Editorial corrections; e.g., "sentence changes," "omissions," and "incompletions." This type of speech disturbance remains fairly constant over considerable periods of time. c) Articulation errors such as stutter, incoherent sounds, and tongue slips represent a very small but fairly stable proportion of the total speech disturbances.

The filled-pauses type of speech disturbance accounts for most of the variance in the speech disturbance ratio. In successful psychotherapy cases studied, the incidence of filled-pauses decreases significantly over the course of treatment. In a study relating five different affects to the frequency of speech disturbances, it was found that the speech disturbance ratio varies with different moods of the patient. This is related to the fact that filled-pauses vary directly with the speed of speech. In attempting to assess the novelty and stereotypy in speech, it was predicted that in successful psychotherapy a patient should increase in the range and differentiation of use of language. This hypothesis has been tested with two cases and tends to be supported. A mathematical model which will provide a more discriminating measure of skewed distributions than is now available is currently being tested out. This will permit a statistical test of significance of the novelty of observed word frequencies. One of the consequences of this research has been to raise serious doubts regarding the current widely accepted hypothesis that speech disturbances are simply a function of "anxiety."

The study of body movement as related to moods was extended this year to include counts of three body areas, feet, head and hands. The results showed significant associations between frequency of movement and body area, and between frequency of movement and mood. There was a significant interaction between body area and mood, with no effects contributed by replication. The relationship between mood and frequency of movement, with body area held constant, accounted for almost a quarter of the total variance.

A study has been carried out this year on the relationship between body movement and speech, the two areas mentioned above. It began when the excerpts of interviews used to study body movements and moods (see above) were examined for a number of concurrent speech variables. A close correspondence between total body movement and total speech output was found. Speech disturbance ratio also correlated with the other two variables. To explore this question, 16 experimental interviews were run in which speech and movement variables were deliberately manipulated and the effects of the manipulation of each on the other variable tested. In the analysis so far, there is no very strong evidence that the movement conditions affected speech variables or that the speech conditions affected the movement variables. The oustanding feature of the experimental subjects is the tremendous range of individually differences in all variables. The movement patterns were especially revealing in this respect. It looks as though investigators will have first to find relevant movement patterns for each subject, then to compare different subjects by the variability across experimental conditions.

Several investigators in one group have been pursuing the problem of the process of judging affect from brief glances of motion pictures. Excerpts have been selected from one interview in which the two channels of expression (Channel 1 is face only, Channel 2 is the rest of the body) are incompatible, and others where they are compatible. The range of affect at this point is along a simple pleasant-unpleasant dimension, since this covers the largest proportion of variance in all studies of affect. Preliminary running of film at low speeds as compared with standard speed has shown this to be a very revealing technique. Instances of incompatible expression, can be "seen" more readily at slow projection speeds than at standard speed. These first trials at low speeds have also shown us some of the possibilities that lie in the new projector which has now after 7

years of developmental work become a reality. It is a sort of "time microscope." One can never see movements in this detail in real life, and the fleeting expressions, body postures, and forms of movement which flickerless projection at slow speeds uncovers is most enlightening. Whether these are "seen" but not fully registered and reported at ordinary speed, but nevertheless play a significant role as a basis for communication, is one of the major aims of this and other planned studies.

Creativity

An important aspect of psychological functioning revolves around the optimum use of abilities. It is this definition of "creativity" which underlies our research in this area, rather than the more esoteric one which ties it to the relatively few persons who fall outside the ordinary range of distribution. Eventually, of course, the two will have to be integrated into a common theory. The general criteria which a solution to a problem must meet to qualify as "creative" are that it be 1) novel and 2) effective.

One of the assumptions on which many investigations are based is that novelty, i.e., originality of solutions, is related to effectiveness. One study was undertaken to investigate the tenability of this assumption. A recent series of studies at UCLA has developed a technique for enhancing the frequency of "original responses" by a form of training. Our study attempted to determine whether originality training does in fact increase the effectiveness of an individual's performance on a verbal task which requires the accessibility of associations which are uncommon as well as relevant to the problem. It was found that individuals who demonstrated the ability to report many and original associations prior to training did in fact do better on the criterion task than those who initially reported more sterotyped associations. It was further found that training could enhance an individual's *report* of uncommon or original associations. However, this increased capacity did not appear to facilitate production of relevant associations on the criterion task.

Perhaps the most popular theory regarding a psychological state conductive to creative expression is that it is necessary, and perhaps sufficient, to induce a temporary suspension of critical judgment. This view is expressed in the retrospective accounts of many creative persons in the arts and sciences. Psychoanalytic theory considers that a temporary suspension of ego control thereby permitting regression in the service of the ego, is a characteristic of creative persons. Such suspension is presumed to increase the likelihood that novel and effective ideas will be generated. Although some of the avenues by which this desired state of release may be achieved include intensive psychotherapy and drugs, it is the "brainstorming" technique that claims to have demonstrated the greatest success in facilitating creative expression. Our study tested the hypothesis that the technique of reducing critical judgment does not alter "generative" creative ability but instead, the suspension of self-imposed standards increases the individual's willingness to report more fully all of the ideas already available to him. These ideas will include some which are little valued by the subject but which may be more valued by the judge. To test this hypothesis, ideas committed to writing and submitted as team solutions were contrasted with ideas generated by team members during dyed discussions. It was found that when the data were restricted to written solutions as is commonly done in the reports of brainstorming research, more ideas and more good ideas were reported under the low critical than the high critical instructions. When analyses were based on the total number of ideas generated, it was found that although a significantly larger number of novel ideas were expressed on low critical instructions, nevertheless, the number of "good solutions," i.e., novel and effective, did not differ under either the low or high critical instructions. The principal effect appears to be due to shifting the judging task from the subject to an external judge. Reducing critical judgment may enhance the "expressed creativity" of subjects without in any way altering the subject's own evaluation of his ideas. In this event, the so-called "creative" idea would not be pursued by their originator and therefore would not be reported.

One of the essential processes in creative thinking is the recognition of new relationships among ideas, concepts, phenomena, etc. These elements may be "remote associates" or familiar associates. One study undertook to study the influence of two types of motivation and degrees of anxiety in ef-

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fecting the generalizability and integration of learned concepts. The specific hypothesis tested was that subjects (high school sophomores) who were grade-motivated during learning would do better on rote questions than they would on comprehension questions whereas subjects of equal intelligence who were "curiosity-motivated" would do the reverse. Pending further analysis, the principal hypothesis appears to have been supported. Grade-motivated subjects appear to score relatively higher on rote than on comprehension, whereas curiosity-motivated subjects tend to score relatively higher on comprehension than rote. High test anxiety appears to have a greater deteriorating effect on comprehension than on rote scores in both test-grade and test-curiosity conditions. Females significantly outperform males on rote scores although males were superior in comprehension. This finding holds for both forms of motivation. To the degree that our educational system stresses the motivation for grade achievement rather than learning on the basis of the individual's curiosity, this study would seem to have important implications.

A second major area of research lies in the *neurological correlates of behavior*—cognitive, affective and conative. These include the perceptual functions of the posterior association cortex, the problem solving functions of the frontal association cortex, the alerting functions of the reticular activating system and the emotional-motivational functions of the limbic system.

Perceptual Functions of Posterior Association Cortex

Initially restricted to research in vision, this aspect of the program has been expanded to include work in olfaction, audition, and somesthesis. Studies completed and in progress deal with the functional localization and analysis of the neural mechanisms serving these sensory modalities.

Of all the modalities under study least is known about the cortical locus of olfactory processes. However, the results of two recently completed experiments lead to the tenative conclusion that olfaction, unlike vision, audition and somesthesis, has little or no isocortical representation in the monkey. Thus, clear-cut deficits in olfactory discrimination have so far been found only after bilateral damage to prepyriform cortex. The possibility that olfactory mechanisms do not extend beyond the primary olfactory projection area (prepyriform cortex) is of particular interest in view of the fact that perceptual functions in this modality appear to be relatively primitive.

In a recent experiment in audition, on the other hand, discrimination deficits were found not only after removal of the primary auditory projection area, but after removal of adjacent association cortex as well. In this respect, the results in audition appear to be similar to those previously obtained in vision and somesthesis. The data suggest that each of these modalities is served both by primary projection areas and by secondary association areas, with much of the evidence favoring the view that the latter areas contribute to the elaboration of complex perceptual processes.

Before this view can be finally accepted, however, alternative interpretations of the functions served by the secondary association areas must be considered. One possibility currently being investigated is that lesions of posterior association cortex impair associative learning rather than modality-specific perceptual processes. In a recently completed series of experiments on visual generalization, it was found that monkeys with inferotemporal lesions were severely impaired in discriminative performance on these tests despite the fact that in the generalization procedure associative learning is only minimally involved. While the observed impairment is thus not readily attributable to a disturbance in associative learning, further investigation is necessary before this possibility can be ruled out completely. To this end, experiments on visual preference and visual incentive are now being planned which will attempt not merely to reduce but to eliminate associative learning as a confounding variable, in order to measure perceptual processes even more directly. Other experiments which may help to distinguish between these two interpretations of the functions of posterior association cortex involve testing for interhemispheric transfer of discrimination habits following selective lesions in the trained hemisphere. The results of these projected studies—one involving somesthetic habits, the other, visual habits—could provide im-

portant information concerning localization of the "engram" as distinct from localization of perceptual mechanisms.

Problem-solving Functions of Frontal Association Cortex

One problem raised by the auditory study described above concerns the corroboration of an earlier finding that performance on auditory discriminations may be impaired by frontal lesions. However, analysis of the more recent data suggests that frontal lesions, unlike posterior lesions, may impair auditory discrimination by interfering with problem-solving functions rather than with auditory functions, per se. To test this hypothesis more directly, a study now in progress will compare the effects of frontal and posterior lesions on auditory discrimination thresholds; these will be determined after the operated animals have been overtrained on the simple auditory discrimination habit on which the threshold testing is to be based. The prediction that frontal lesions will not alter auditory thresholds is based on other evidence from this laboratory indicating that discrimination performance of frontal animals is unimpaired if problem-solving difficulties in the tests are minimized. An example of this was provided by some recent work on vision in which it was found that animals with frontal lesions were severely impaired in the formation of an object discrimination learning set but were subsequently unimpaired in the formation of a more difficult pattern discrimination learning set. Apparently, once the problem-solving requirements of the test were mastered, animals with frontal lesions, unlike those with posterior (inferotemporal) lesions, performed adequately even on difficult visual discriminations. Thus, in contrast to the perceptual deficits noted after lesions of posterior association cortex, deficits of a more complex, problem-solving nature appear to follow lesions of frontal association cortex.

Earlier analyses suggested that this problem-solving deficit was due to preservation of whatever dominant central set was normally elicited in the given testing situation. This hypothesis seemed to account satisfactory for the frontal animal's difficulty with a variety of tests including classical delayed response, discrimination reversal, object quality learning set, etc., since a general requirement in all such tests is the ability to shift readily between different modes of response by suppressing the predominant mode. However, preliminary evidence from on-going experiments suggest that this conception may be in need of revision. Specifically, a comparison between the effects of selective lesions within frontal cortex indicates that performance on some problem-solving tasks (e.g., delayed response) is maximally interfered with by dorsolateral lesions, whereas performance on other tasks (e.g., object quality learning set) is maximally interfered with by orbital lesions. Such double dissociation of deficits argues strongly against the earlier view that disruption of a single mechanism accounts for the frontal animal's impairment in a wide variety of problem-solving situations. Other experiments still in progress comparing the behavioral effects of dorsolateral and orbital frontal lesions may suggest the specific revisions in the theory which it now seems will be required.

Reticular Activating System

The electrographic and physiological accompaniments of impaired attention in man have now been studied with the aid of four different attention-disrupting agents: chlorpromazine, secobarbital, sleep deprivation, and petit mal epilepsy. Although the behavioral loss may be equated for these agents (by proper selection of doses or subjects) the physiological accompaniments of the impairment vary widely from one agent to another, with each agent producing a distinctive physiological profile. Thus, the neural system responsible for the maintenance of attentive behavior cannot be identical to the system or systems which regulate certain physiological indices of arousal, such as the EEG, respiratory rate, and peripheral vascular tone.

That the system responsible for sustained attention is probably not focally represented in the cortex is suggested by studies of epileptic patients: Neither focal cortical epileptogenic processes nor cortical resections have any effect on performance of the Continuous Performance Test of attention. In contrast, recent results have confirmed an

earlier finding that patients with centrencephalic (presumably subcortical) epilepsy are markedly impaired in attentiveness.

A study involving intracerebral stimulation of the waking monkey was recently undertaken in an attempt to map the hypothesized subcortical neural system essential for attentive behavior. Animals were trained to perform on a test of attention analogous to the Continuous Performance Test, and the EEG was recorded simultaneously with performance. Although a number of the drugs known to be effective in disrupting attention in man were also found to be effective in the monkey, direct intracerebral stimulation in monkey has so far yielded inconclusive results with respect to the localization of attention processes.

Comparisons have been made between schizophrenic subjects and closely matched normal control subjects in terms of their response to various attention-disrupting and attention-augmenting agents. Differences have been found between the reactions of the two groups such as to suggest that central arousal mechanisms may be altered in schizophrenia.

The aim of this research has been to relate the various manifestations of impairment of attention in man (i.e., in epilepsy, schizophrenia, etc.) to a systematic account of the specific changes within the central nervous system occurring in each condition. It is hoped that these studies have contributed to this end.

Limbic System

One possible explanation of the orbital animal's impairment on problem-solving tasks is that such impairment is secondary to an altered motivational state. Support for this view has been provided by the results of a recently completed study aimed at mapping those areas in the forebrain from which food ingestion and ejection can be directly elicited by electrical stimulation. A preliminary analysis of the histology indicates that these alimentary responses can be evoked not only from the diencephalon but from orbital frontal, anterior cingulate, and medical temporal areas as well. The data suggest that damage to these telencephalic portions of the limbic system could produce gross disturbances in adaptive behavior by directly altering the motivational state of the animal.

The initial mapping study employing electrical stimulation techniques has recently led to a variety of additional experiments on the functions of the limbic system. An investigation of the autonomic and motivational correlates of evoked behavior patterns is now well under way. Preliminary results indicate that loci from which penile erections are elicited will generally motivate self-stimulation behavior; loci yielding aggressive patterns will motivate escape-from-stimulation; while loci yielding food ingestion frequently produce the paradoxical result of motivating both self-stimulation and escape-from-stimulation at the same levels of current in the same animal. With respect to the autonomic correlates of these effects and contrary to a suggestion in the literature, loci which motivate self-stimulation have frequently been found to produce sympathetic rather than parasympathetic changes.

Studies of the interactions between simultaneously evoked behaviors have been undertaken and a number of consistent modes of interaction have been observed. For example, two ingestion patterns tend to summate; evoked ejection of food cancels the effect of evoked injestion; aggressive patterns invariably suppress penile erections. These interaction studies will be continued, and the technique applied not only to evoked behavior patterns but to the autonomic and motivational effects of stimulation as well.

Some success has been achieved in conditioning the evoked behaviors using classical conditioning techniques. In future experiments in this area attempts will be made to record electrographic activity during the conditioning process with the aim of detecting electrographic changes, particularly at the site of the UCS, during the presentation of the CS.

After many months of development, a remote stimulator has been delivered which vastly increases the potentialities of the electrical stimulation techniques. Initial studies will compare the behavior evoked from an animal restrained in a chair with the behavior evoked from the same animal when it is freely moving. On the basis of preliminary results it is anticipated that the repertoire of behavior in the free situation will be

considerably increased, and that many effects concealed before will now be clearly seen. Subsequent work will involve the study of social interaction between pairs and among groups of animals thereby permitting observation of an entirely new class of behaviors evoked by stimulation. The potentialities of the remote stimulator for the analysis of neural mechanisms in primate behavior appear to be unlimited.

Aside from the brain lesion studies already reported, the Laboratory has been interested in three other areas of *conditions disturbing to normal expression/function*. These are the effects of a normal process such as that of aging, the effects of an experimenter-induced stress, and the effects of a developed condition such as schizophrenia.

Aging

In the aging field the Laboratory has carried out studies both on human and animal (especially rat) subjects. Four major kinds of studies have been pursued with human subjects: problem-solving, perception, reaction time set, and response speed.

A comparison of two contrasting age groups of adults with respect to their performances on the standard problems programmed by the Logical Analysis Device (LAD) revealed a striking impairment of heuristic behavior in the aged. Additional tests with special, simplified LAD problems failed to bring this form of task within the capacities of the older subjects. During the current year a heuristic evaluation and logical problem programming device was constructed and preliminary observations have been made at several levels of difficulty on young and old normal adults, on schizophrenics, and on children. Since the lowest difficulty levels were well within the capacities of all but two of the schizophrenics, it appears likely that further studies with this equipment will indicate that it is within the range of the aged subject.

It was found in an earlier study that elderly subjects manifest deficits in reorganizing percepts. A further investigation of the change of reversal behavior with age was carried out with elderly and young subjects using a series of photographs of drawings of a cat that merges into a dog, and of a circle that merges into a triangle. Data are still being collected, but preliminary results suggest that old and young shift to the new percept at about the same point in the series. These results, if they hold up, would not corroborate the results of a previous study and would require further experimentation.

A study of reaction time set suggests that in addition to overall reaction slowness, there is special difficulty in the aged in the reaction to brief intervals. The older persons also seem to require more time to overcome the effects of inaccurate overestimations of the duration of the perparatory interval preceding the RT stimulus.

A completed analysis of data obtained during the past two years suggests that the age change in slowing of behavior tends to have the attribute of a general psychological process. That is, whereas young adults tend to show task-specific adjustment of response speed, older persons tend to have a speed of psychomotor performance more characteristic of them as individuals. The principal data on which these conclusions are based were obtained from the use of an apparatus designed and constructed at the NIMH, called the Psychomet.

Because of the implications of the findings and their relevance to psychophysiological changes in the normal adult, the Federal Aviation Agency requested and was granted the use of the apparatus in their current research on aging in civil air pilots. That agency has completed the examination of 160 subjects using the apparatus and the results are now being analyzed in cooperation with the Section on Aging and the Biometrics Branch. Preliminary results indicate significant age trends in the psychological measurements. The trends are appearing even in relatively young healthy men. The further analysis of the data as well as new studies will explore possible physiological bases for the changes. Of considerable importance is the suspicion that the observable behavioral changes may antecede later-to-appear physiological changes related to health factors. Thus there is the research problem of determining the extent to which the behavioral changes of advancing age are premonitory and predictive of length of life and disease states.

The animal studies in aging fall into the following categories: learning under light aversion, water maze and the T-maze in rats, and studies

of physiological mechanisms in various animals.

Studies of the albino rat's light aversive behavior have continued. Two investigations of dose tolerance to and withdrawal from morphine have been carried out. In the tolerance study the animals were tested every second day as the dose was increased weekly for 6 months. It was found that the animals at first decreased their escape rate after an increment in the dose and then adapted quite quickly to it and returned to their normal rate of responding. After withdrawal they were tested every day except on weekends. The light avoidance technique has proved to be a sensitive method for measuring the rate of acquisition of tolerance to the narcotic. Spontaneous activity of rats may be increased or remain unaltered during the course of chronic morphine administration. Withdrawal results in a precipitous fall in activity which eventually returns to normal levels. This work will be reported during the coming year.

In another study, the effect of galactose-induced cataracts on escape from illumination was investigated. No decrease in the rate of escape was found to be associated with the growth of the cataracts. At present the galactose has been withdrawn and the cataracts are in process of receding. In addition to these experiments, investigators in the Section on Aging have been engaged in an extensive interdisciplinary investigation with a member of the Laboratory of Cellular Pharmacology on the effects of certain chemical agents on the learning ability of young rats as measured in the water maze.

Two studies were conducted on age differences in learning ability in the albino rat using a water maze in which an escape path had to be learned. Previous studies had shown that young and old rats were highly motivated to escape from a water tank by swimming, suggesting that motivation to escape from water might be useful in studies of age differences in learning. Results of the first study showed that the middle-aged and old animals, in comparison with the young, tended to make somewhat more errors on the first trial and that there was a tendency for errors to persist in the older animals. In the conditions of the experiment the poorer performance of the older animals may not reflect lower learning ability *per se* as it does a greater dependence upon body orientation in behavior as might be influenced by reduced sensory acuity or perhaps by long standing caging arrangements. A second experiment was conducted on age differences in rat learning using a two-choice water maze. The task proved relatively easy and 65 of the total 100 animals reached the learning criterion of four out of five correct trials by day three. There were no age differences in learning.

Age differences in relearning were studied in animals who reached the learning criterion on day three. Although all three age groups showed increased errors in learning the reversed maze, there were no age differences in the size of the effect. No age differences were found in speed of swimming on correct trials. The swimming speed was considerably less than that previously reported on swimming a straight path. No relation was found between swimming speed and learning ability. The results indicate that age differences in learning and negative transfer effects in the rat do not appear in all types of learning tasks. The precise circumstances under which age differences probably vary with the difficulty and nature of the learning task as well as the form of motivation used.

During the last year the study on T–maze habit reversal has been extended by a replication of previous data and by adding a condition of large food reward, and a more difficult task pattern (multiple–T); data are still in process of collection, however, and preliminary analysis is under way. A new study was begun testing a hypothesis which is conceptually similar to habit reversal deficits with age. Specifically, the hypothesis is that there is increased resistance to extinction with advanced age. As in the previous T-maze problem, rats are of 3 ages, 4–6 months, 9–12 months, and about 24 months.

Various studies of physiological mechanisms have been in progress during the year. A study of the factors governing the distirbution of thiocyanate between blood and cerebrospinal fluid was initiated together with the Head, Clinical Pharmacology and Experimental Therapeutics Service, NCI and a member of the Section on Theoretical Statistics and Mathematics. Results to date have clearly demonstrated that the distribution ratio of thiocyanate is a function of the plasma level of the ion. At high blood concentrations, or after

the intracisternal administration of various substances, the influence of the blood-cerebrospinal fluid barrier may be partially or completely eliminated. During the past summer the urinary creatinine excretion of rats of different ages was measured. In general, it was observed that very young and very old rats excrete smaller amounts of creatinine than do mature adults. However, it was also noted that the quantities collected may be significantly decreased during fasting, suggesting that some fraction of the total creatinine metabolism may be labile. A project has been initiated with the Chief, Section on Neuropathology and a member of the Surgical Neurology Branch, NINDB to determine the effect of cerebral edema produced by cold injury on the size of the extracellular compartment of brain. This will complement previous experiments (in press) in which edema was produced by intoxication with triethyltin. The object of the investigation is to determine whether or not certain forms of cerebral edema are primarily intracellular while other types are extracellular. A project has been initiated to determine the mechanism by which vital dyes are excluded from the brain. It appears possible that this occurs independently of the blood-brain barrier, and results primarily because of a "lack of affinity" for various stains by brain constituents.

Schizophrenia

Over the past year the schizophrenia studies have followed four directions: studies of basic responsivity, studies of thought disturbance, studies in heredity/environment, and theoretical summarizing of already collected bodies of data.

In the area of basic responsivity three major kinds of studies have been carried out: psychophysiological responsivity, preparatory set, and the perception of velocity.

Analyses are being made of the skin resistance, heart rate, and respiration records of subjects taken during sessions in which either a light or a tone are briefly presented repetitively at 30 second intervals. Analyses of the skin resistance data on the latest samples—20 schizophrenic patients and 20 normal controls—confirm earlier results in showing more rapid habituation of the orienting reaction, higher basal resistance, and fewer non-specific responses in the control subjects. The results suggest a higher level of "arousal" in the patients and less rapid adaptation to these mild conditions. Evaluation of the heart rate data is continuing in collaboration with the Data Processing Branch.

Reports of several experiments which used simple reaction time to study the establishment and maintenance of preparatory sets in schizophrenic and normal subjects have been prepared for publication. In one study it was shown that the deficit in the reaction time of schizophrenic patients customarily produced by a long preparatory interval is not due merely to the stimulus. In another experiment the effect of the relative frequency of short and long preparatory intervals on the reaction time of normal subjects was studied. In two other studies differential effects of the sequence of preparatory intervals on schizophrenic and normal subjects were found. For schizophrenic patients, if series of trials with long preparatory intervals are followed by a series of trials with shorter preparatory intervals, reaction time in the latter series is impaired. An apparently similar effect is found when a single long preparatory interval precedes a single short preparatory interval in the context of an irregular sequence of preparatory intervals.

The psychological mechanisms which are responsible for these effects of a long preparatory interval on reaction time on subsequent trials are not clear. One hypothesis is that the level activiation or arousal may be changed in schizophrenic subjects by the long period of attempting to maintain a preparatory set. To test this, we have begun a study in which psychophysiological variables are recorded during performance of the reaction time task. During the year equipment was developed and set up in a new laboratory to enable us to record EEG, skin resistance, heart rate, respiration, finger volume and the pressure on the reaction key on a six channel polygraph. The collection of data is in progress.

Another study in progress is a repetition of some of the earlier reaction time studies but using acute schizophrenic patients and non-schizophrenic patient controls from the inpatient services at Walter Reed Army Hospital and the Bethesda Naval Hospital. Preliminary analysis of some of the data suggests that the acute schizophrenics generally fall between normal and chronic schizo-

phrenic subjects on the "set index," a measure which discriminates with high precision between the latter two groups.

During the past year a study has been carried out to evaluate the performance of schizophrenics using the procedure described earlier for determining perceptual adaptation to motion. These patients have shown a tendency to develop extraordinarily long absolute response-times under repeated testing without manifesting a deterioration in relative accuracy. This tendency is independent of simple reaction time but appears to be related to certain aspects of simultaneously recorded EEG activity. At the present time it has not been determined whether the change in response time represents a basic abnormality in perceptual adaptation to stimulus motion or a change in time perception, or both. The data collection has been completed for an experiment investigating time-interval judgment, in which an attempt was made to duplicate the time parameters of the perceptual velocity task as closely as possible but with no stimulus motion present. These results have not as yet been analyzed.

Several studies related to the nature/nurture problem in schizophrenia are in process The mass of material which was collected on the monozygotic quadruplet schizophrenics over several years is being summarized, organized and analyzed. In the next few months a first draft of a fairly sizeable monograph should be completed. When it is done, it will be essentially a case history, but probably one of the most unusual and most extensively and thoroughly studied case histories that psychiatry has known. Although it with not be possible to make any definitive generalizations about the contributions of heredity and environment to schizophrenia from this study, it should give us a disturbingly clear view of the many-sided complexities involved in trying to understand the etiology of schizophrenic disorders, and it should pose a number of tantalizing hypotheses vividly and objectively illustrated.

Over the last several years a searching examination and re-evaluation of the literature on the nature/nurture problem in schizophrenia has been in process. These have been reported in a series of some half-dozen papers where the probable contributions of heredity and environment and their interactions to the development of schizophrenia have been analyzed. Out of these studies has grown a design for a systematic attack on this problem through the use of a sample focused mainly on male and female monozygotic twins discordant as to schizophrenia. To balance these a sample of monozygotic-concordant-as-to-schizophrenia pairs, with dissimilar clinical pictures, and the same with similar pictures are to be used. As further controls there are to be groups of concordant and discordant dizygotic twins as well as non-schizophrenic twins. Both twins and their families will be studied psychiatrically, psychologically, socially and physiologically. From a systematically organized study of this kind there is hope of being able to determine the proportion of total variance contributed by heredity, by environment, and by the interaction of the two.

The year has seen further advances in the effort to organize theoretically the extensive psychological data on schizophrenia collected at this Laboratory and previously. A brief theoretical presentation, growing out of some of this material, was given in the Collier Lecture at the University of Rochester.

Stress

Two studies in the area of stress were carried out in the Laboratory: one as part of a collaborative study with the Adult Psychiatry Branch's Psychosomatic Project, the other studying large colonies of rats at the Rockville Barn.

Since the first study will be reported in detail by the Adult Psychiatry Branch, it will not be considered here.

The second study dealt with the effects of group size, environmental structure and Vitamin A level on the behavior and the physiology of the rat.

Originally the objective was to determine the impact upon the individual of variations in group size and structure of the physical environment. During the first series of studies with large social groups certain reproductive pathology involving extensive uterine hemorrhaging during the latter stages of pregancy suggested some dysfuction due to Vitamin A metabolism. Liver assays demonstrated that the rats did in fact possess ten times the normal storage of Vitamin A, and further inquiry revealed that the feed company was introducing a fairly high level Vitamin A supple-

ment in their commercial diet. Furthermore, there appeared to be an interaction between Vitamin A and social stressors in producing the observed reproductive and associated behavioral anomalies. For this reason Vitamin A was included as a variable in the second series of studies, recently completed.

Taking only the extremes of Vitamin A, 3 or 12 I.U. Vit. A/gm. diet, and two group sizes: (a) Small, 1 male and 2 females, and (b) Large, up to 50 adults of each sex, the following results were obtained, all statistically significant. Members of small social groups are absolutely larger, possess realtively (and usually absolutely) smaller adrenals, ventricles and kidneys. They possess absolutely and relatively larger fat deposits, and exhibit shorter barbiturate sleeping times. Increased dietary Vitamin A is associated in both small and large groups with decreased barbiturate sleeping times, and in large groups also with decreased ventricle size, decreased maternal competence—ability to rear young, decreased fighting and increased "velocity." (Velocity is defined as the relative amount of time a rat is active in that part of the environment exposing it to interaction with other rats.) In both high and low Vitamin A groups the omega rat had the same low velocity indicating a minimum tolerance level. The velocity of the alpha rat in the low Vitamin A was 30% lower than the alpha rat in the high Vitamin A group. Low velocity rats (in contrast to high velocity rats) are like small social group rats in that they have smaller adrenals, smaller ventricles, smaller kidneys, more fat, and shorter barbiturate sleeping time.

A study was also carried out on tryptophan metabolism. Tryptophan loading following monamine oxidase inhibitor does not alter velocity, doubles social interaction (fighting), increases general state of reactivity to stimuli, and nearly eliminates the pan-sexual behavior which frequently characterizes low velocity rats.

LABORATORY OF SOCIO-ENVIRONMENTAL STUDIES

The Laboratory of Socio-environmental Studies, comprised of sociologists, anthropologists, social psychologists, and a human geneticist, has been pursuing a diversified program of studies on the effects of social structure upon personality and behavior. "Social structure," as used here, refers to any predictably patterned set of social relationships, from a large society to a single family. The Laboratory program includes studies in which the relevant social structures are national societies, social classes, institutions and families.

These studies can be conveniently grouped into four content areas: (1) Many of the studies deal with the family—either as a dependent variable, as in cross-national comparisons or studies of the effects of social class upon familial relationships, or as an independent variable, as in studies of the influence of the family upon the child's behavior. This emphasis reflects the importance of the family for the child's personality development. (2) Several other studies deal with the mental hospital as a social institution. This emphasis reflects both an interest in the effective functioning of mental hospitals, as a matter of humanitarian concern, and the opportunity mental hospitals provide for studies of the functioning of large-scale social institutions more generally. (3) A third group of studies seeks to ascertain which social variables affect one or another important behavioral process. Here one would include our studies of the aging process and of the determinants of level of self-esteem in adolescents. (4) Finally, one study deals with the problem of determining the genetic and environmental contributions in the genesis of mental deficiency.

These studies are faced with inumerable methodological problems: social science does not have at its disposal an armament of methods and techniques adequate to its needs. Hence, most studies now underway include major methodological components, and a few are primarily directed at methodological problems.

This report is intended to present the current status of the Laboratory's research in each of the four broad areas noted above, with major emphasis on research findings of the past year. This emphasis, inherent in the very notion of an annual report, results in a somewhat distorted picture of the Laboratory's program. Most of the studies are much longer term than one year; to present their theoretical rationale, their distinctive methods, and the intimate relationship of this year's "find-

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ings" to those of last year, the year before, and perhaps of the year to come, is impossible in such a report—unless this were to be an unconscionably long document. Thus, what is presented to the reader is an array of cold findings, too-largely divorced from their significance to the individual investigators, to the program of the Laboratory, or to the program of the National Institute of Mental Health.

Reading this report in conjunction with those of the past two or three years will provide a clearer picture of how recent research findings add to earlier work. The articles published by individual members of the Laboratory, of course, provide by far the best exposition of theoretical rationale, methods, and implications—without which research findings lose much of their meaning. But even this report, alone, should indicate which problems we have chosen to tackle, and what results have recently emerged from this work.

The Family

Cross-National Comparisons

The Laboratory has for some time devoted considerable attention to studies of the family—both studies of variations in the structure and functioning of the family in various segments of this society, and studies of the impact of the family on the personality development of the child. To broaden our perspective on the family in this society, we have recently become engaged in studies involving cross-national comparisons of familial relationships. One investigation is a comparison of familial relationships in an Israeli collective settlement, an Arab village, and a Nigerian tribe. This analysis is now in process. Another is the application of the detailed observational techniques for the study of mother-infant interaction, developed in the Laboratory of Psychology, NIMH, to Japanese families. This analysis, too, is currently in process. By way of specifying the broad cultural context for the Japanese research, earlier work was devoted to the development and application of methods for the quantitative measurement of the values of Japanese parents and children. One surprising finding is that the younger generation in Japan appears to be moving toward a *less* individualistic, more strongly collateral, orientation than that of their elders.

Further plans are now being developed for more extensive cross-national studies of the family, with special emphasis on systematic comparisons between the United States and other countries.

Social Class and Parent-Child Relationships

The work on variations in familial relationships within the United States has been proceeding far longer. Last year's annual report summarized the principal results of an extended investigation of the effects of social class upon parent-child relationships. This work demonstrated substantial differences between the values of middle- and working-class parents (i.e., non-manual workers and their wives as compared to manual workers and their wives) and traced the ramifications of these differences for parents' disciplinary practices, and for the ways in which responsibilities for supporting and constraining the children are divided between mother and father. Further work this year has concentrated on the differential handling of boys and girls in the two social classes. In both social classes, of course, boys are given different chores and responsibilities from girls, and in both classes the girls are seen as less apt to create serious troubles for their parents. In addition, working-class parents punish daughters for misbehaviors that are not regarded so seriously in boys. Middle-class parents do not seem to handle boys' misbehaviors differently from girls', but they do make other types of sexual differentiations that working-class parents do not make. Our analysis of characteristically middle-class sexual differentiations is as yet incomplete, but they appear to be more a matter of the parents intervening (usually in a supportive rather than a punishing way) in different realms of their sons' and daughters' activities.

Effects of Maternal Employment

In other investigations, the effects upon the quality of the mother-child relationship of the mother's being employed outside the home have been studied. Overall, mother's employment status does not appear to have any important consequences for the quality of her relationships with her children. However, when mothers' motivations regarding working are considered, one finds that the *non*working mothers who would prefer to have

a job outside the home (but do not, because of a sense of "duty") show the greatest problems in child rearing. The nonworking dissatisfied mothers, compared with the working mothers whether or not they want to work) and with the satisfied nonworking mothers, describe more difficulties in the area of control, less emotional satisfaction in relationships with their children, and less confidence in their functioning as mothers.

Mothers who have no more than a high school education make different kinds of intrafamilial adaptations to job-holding than do mothers who have been to college. Among women with high school training, firmer control over children, assignment of greater responsibilities to children, and delegation of the stricter disciplinary role to the father appear more frequently in families of working than nonworking mothers. For the college trained mothers, these differences do not appear or appear in opposite directions from those found in the high school groups. The college working mothers tend to compensate for time away from children by more planned, shared activities with the children than is found in the college nonworking group.

The Child's Development of Conscience

Another set of studies has investigated the development of conscience in children, with special reference to the effects of familial relationships on this development. A series of experimental studies was designed to study children's internalization of rules, standards and values. These investigations show that the same child-rearing techniques have different effects for boys' and for girls' ability to resist temptation: the more controlling the mother, the greater the girl's ability to resist temptation; a more permissive approach to child-rearing is more effective for boys. There is also evidence that direct, physical forms of discipline are most efficient during the child's early years with the more symbolic forms gaining in effectiveness as the child grows older. Results also indicate that "yielders to temptation" seem to have been deterred from yielding because of increased fantasies of punishment during the temptation.

In attempting to assess the importance of various types of motives which are aroused during temptation, we have found that extrinsic rewards matter a great deal for three and four-year-olds, but are superfluous with five-year-olds. This finding suggests that achievement becomes an important motive in the temptation test around the age of five.

Relevant to this experimental work is a reanalysis that has been undertaken of the classic study of Hartshorne and May (1928), *Studies in Deceit*. These authors concluded that dishonesty in children is specific to the situation in which temptation is presented; there is no general trait of honesty. Their data, they argue, indicate that a person is honest only in those situations in which he has learned not to deviate, and that consistency of behavior from one situation to another is due to similarities in the situations rather than to consistency on the part of the person. The reanalysis of these data, using the factor analytic model, shows sufficient evidence to modify the extreme view of complete situational specificity. Hartshorne and May rightfully dismissed the idea that there are only two classes of people—completely honest or completely dishonest. However, the large amount of common variance extracted by the primary factor, with significant loadings for all the tests, suggests there is an underlying, relatively permanent characteristic of honesty. In addition to this common factor, however, the amount of variance not extracted for each of the tests does indicate that much of the individual behavior is determined by the specific situation.

Methodological Investigations

Since investigation of the complex and intimate relationships between child and parents presents many formidable methodological problems that have not been solved, research on methods and techniques is of critical importance in this field. One of these problems concerns the validity and reliability of retrospective data for research purposes. Most often data on child development and child rearing are obtained retrospectively, through interviews conducted with the mother, and data so obtained are usually accepted at face value—this despite much evidence on the vagaries of recall. An extensive research project investigating the nature of retrospective data on child development and rearing has been undertaken by the Laboratory.

The aims of the investigation are to assess the nature of differences between earlier events and

parents' recollections of such events, and to determine how retrospection is influenced by such factors as the time interval between events and recall, intervening events and recall, intervening events, and the current psychological situation. Two hundred and twenty mothers of children on whom data were collected two to twenty-five years ago have been interviewed. The child's sex, current age, and position in birth order are factors on which a systematic sampling of mothers has been based. The interviews obtain mothers' retrospective reports concerning specific aspects of child development and parent-child relationships. Data from these interviews are compared with a baseline consisting of direct observations, interviews, and ratings gathered at the earlier times.

Among suggestive trends in our preliminary analyses of these data is the finding that mothers systematically over-estimate their children's intelligence in their retrospective appraisals. No such directional trend appears on a number of less ego-relevant characterizations of the child. Mothers' retrospective descriptions of their children's aggression, withdrawn behavior, sympathetic and affectionate behavior agree with baseline data in 50% to 75% of the comparisons. The sex of the child appears to be influential in shaping recollections in certain areas. For example, mothers tend to *recall* greater involvement of the father in the rearing of sons than was reported initially; for daughters the shift is to recall *less* than appeared initially.

We have also begun work on the development of observational approaches to the study of family relationships. Arrangements have been made this year for obtaining subjects, children and their parents, who are a "normal" population and who can be studied over a period of several years in both naturalistic and experimental settings. A nursery school serves as a research facility in which it is possible to set up research record systems, to introduce certain controlled experiences, and to carry out coordinated studies using home and laboratory observations of child behavior and of parents in interaction with their children. During the past year the necessary organizational arrangements have been completed, contacts have been made with the parents, and exploratory research has been carried out in the nursery school.

The Mental Hospital

Cross-national Comparisons

In this area, too, we have embarked, in a limited way, upon cross-national comparative studies. The intent here is to be able to see the organization and practices of American mental hospitals in the perspective of the alternative models that have been developed elsewhere. The principal study to date has been a comparison of nurse-patient and doctor-patient relationships in Japanese and American mental hospitals. In Japan one finds, in a role analogous to the nursing assistant in a private U.S. mental hospital, a group of people known as *tsukisoi.* The *tsukisoi* offer a unique pattern of nursing care in private psychiatric hospitals. They are typically middle aged women with a background of marriage disruption or physical illness, who serve the patient as his individual practical nurse and companion on a twenty-four hour a day basis. Her relationship with the patient resembles the modal mother-child relationship in Japanese culture in several ways: close physical proximity, emotional intensity, and the absence of the expression of aggressive and hostile feelings.

The doctor-patient relationship in Japan is also quite different from those found here. This relationship is characterized, first, by considerable passivity and interdependence on the part of both patient and doctor. The patient takes a fateful role in relation to his doctor; the doctor, in turn, is quite willing to act as one who controls the fate of the patient. A second feature of this relationship is the lack of insulation of the therapeutic situation from those more social in nature. In contrast to the doctor-patient relationship here, in Japan the psychiatrist will join with his patients in social and family activities. Our data do not permit assessments of the therapeutic efficacy of either the nurse-patient or doctor-patient relationships as they are found in Japan. They do, however, illustrate the degree to which the larger culture molds even such "professional roles" as these.

The Mental Hospital in the United States

More extensive work has been done on the organization and functioning of mental hospitals in

the U.S. In one set of studies, dealing principally with Saint Elizabeths Hospital in Washington, D.C., and now being extended to a sample of fifteen hospitals throughout the country, the intent has been to trace the determinants of nurses' and physicians' orientations toward patients, treatment modalities, and their own occupational roles.

The research at Saint Elizabeths Hospital is based on a survey of all nursing personnel, together with an inventory of the characteristics of the 156 wards of the hospital. This approach makes possible the investigation of the ways in which the social structure of the hospital and of its constituent wards, in interaction with the individual characteristics of the personnel, affect the theropeutically-relevant orientations of personnel. In earlier work, described in detail in last year's report, the dimensions of the nurse-patient relationship were analyzed. Subsequent work has been focused on orientations toward treatment methods and the job itself. Analysis of orientations toward treatment modalities has been focused on nurses' assessments of the therapeutic efficacy of psychoactive drugs. It was found that enthusiasm over their effects is positively related to concern with the control and management of patient behavior; skepticism about the drugs is associated with an interest in using one's self as a therapeutic resource. Subsequent work revealed that these orientations result in different feelings about the drugs when used in different ward contexts. On wards where there are a number of externally imposed restrictions placed on patient behavior—such as locked wards and the absence of mechanisms providing patients a voice in ward affairs—control-oriented staff are not enthusiastic about therapeutic efficacy of the drugs. The same types of people, however, when assigned to the less restrictive wards, voice considerable enthusiasm. This suggests that for many staff there is an interchangability between external controls and drugs. If further indicates that assessments of this therapy are influenced not so much by patient-centered considerations as by how well it fits the orientations of staff.

A survey of psychiatrists, now being conducted, will extend this inquiry into orientations toward a larger variety of treatment methods. Scales have been developed to measure the extent to which hospital psychiatrists are committed to psychotherapy, somatotherapies, and sociotherapies. Relationships will be sought between characteristics and experiences of psychiatrists and the treatment methods they espouse.

A fundamental aspect of people's views of their work is the extent to which they feel able to exercise control over their own work. Those who feel powerless to exercise such control we call "alienated." The extent to which mental hospital personnel feel alienated from their work was found to be influenced by a number of features of hospital organization. To the extent that the organization limits the ability of subordinates to exercise reciprocal influence on their superordinates, alienation is exacerbated. This is reflected in situations where one's significant superior is positionally remote from him, where such superiors physically absent themselves for considerable lengths of time, and where the superiors exercise their authority in a peremptory fashion. Each of these situations represents a condition limiting the reciprocal influence of subordinates and results in heightened alienation. One's career in the opportunity structure of the institution is also related to alienation. Here we find that the slower one's career pace in the hospital, the more likely he is to be highly alienated. This is particularly the case where occupational mobility is strongly desired and where there is dissatisfaction with work rewards. Finally, it was found that where one's work assignments in the hospital isolate him from his fellow workers, he is prone to feelings of alienation.

In the current survey of psychiatrists, information is being gathered on attitude toward the role of hospital psychiatrist. We are particularly interested in career experiences and professional aspirations as these enter into decisions to remain in hospital work or to leave for private practice or some other setting.

A study now designed and about to get into the field is concerned with the moral basis of authority relations between superordinates and subordinates on the hospital staff. The plan here is to compare nursing units roughly equivalent in other relevant respects but in some of which the head nurse's authority is respected and regarded as legitimate, in others of which it is seriously

questioned. In this study, we hope to ascertain some of the conditions that make for acceptance of authority in such an institution.

The Social Behavior of Patients

Another aspect of the mental hospital research concerns the social relations of patients—with special emphasis on the *lack* of sociability manifested by chronic schizophrenics. Work with chronic schizophrenics has been directed to exploring the motivation behind the chronic schizophrenic's apparent aversion to social interaction. To date, findings seem to indicate that chronic schizophrenics have a capacity for social behavior that is not generally recognized or exploited in treatment; nevertheless, there is a very real fear among chronic schizophrenics of being placed in a position where they might be objects of hostility from others; there is an equal fear of situations where they are tempted to express their own feelings of agression toward others; and, while these predilections for and aversions to social behavior describe most chronic schizophrenics, there are important differences between male and female patients.

Among chronic female schizophrenics social behavior and present intellectual functioning are *relatively* independent factors. A study examining chronic schizophrenics' preferences among persons with whom they will be forced to cooperate indicated that for men, the lower the level at which these men are functioning intellectually, the more threatening are all aspects of social relationships; for women, the only correlate of low intellectual functioning is a fear of having the other express negative feelings.

An attempt to see whether the schizophrenic's usual pattern of minimal interaction with the surrounding environment would be altered in the presence of immediate danger was made by examining patient reaction to hospital fires. The results indicate that both the presence of schizophrenia and a history of long hospitalization tend to depress the extent and effectiveness of mental patients' reaction to serious danger.

Relevant Social Variables

In these studies, the emphasis is on ascertaining what are the social variables that influence one or another important behavioral process.

Self-esteem Among Adolescents

One study is broad-scale investigation of the determinants (and consequences) of level of self-esteem among adolescents. This study is based on a survey of over 5,000 high school students in selected schools throughout New York state. Level of self-esteem has been measured by a series of questions addressed to the student's conscious self-evaluation; these questions prove scalable, and there is evidence of the validity of the measure from a small series of intensive interviews with high- and low-scorers.

The data indicate that the instrument used here to measure self-esteem is related to two indicators of emotional or psychic disturbance: a scale of "depressive affect" and a scale of "psychosomatic symptoms" which in previous research has effectively discriminated between diagnosed neurotics and normals. In addition, a small sample of normal volunteers at the Clinical Center completed the self-esteem scales, while nurses on the wards independently completed Leary scales on these subjects. Subjects with low self-esteem were significantly more likely to be described by nurses as depressed and disappointed. Further analysis of the data indicated that students with low self-esteem tended to have more shifting and unstable self-images, to present a "facade" or "front" in their relationships wtih others, to feel inordinately threatened by the prospect of criticism, hostility, or failure, and to experience psychic isolation. These factors all contribute to the relationship of self-esteem to psychosomatic indicators of anxiety.

Turning to experiences which might influence the development of self-esteem: it was found that parental interest in the child was predictive of subsequent self-esteem level. In order to gain information about the level of parental interest, and recognizing the danger of retrospective bias, we selected certain recurrent but diverse areas of life which represented fairly specific points of contact between parent and child. The results showed that in each of these various areas of life, signs of parental indifference were associated with diminished self-esteem of the child. Indeed, there is evidence that parental indifference may be more deleterious to the child's feeling of self-worth than are punitive or critical reactions by the parents.

Another earlier experience which appears to have relevance for certain indicators of emotional

disturbance is the experience of growing up in a neighborhood where one is in a distinct religious minority. Children who have grown up in such a neighborhood are more likely than are those raised in neighborhoods populated largely by co-religionists, or in mixed neighborhoods, to manifest low self-esteem and symptoms of anxiety and depression. Such children, our data indicate, are more likely to have experienced discrimination in childhood, and such experiences bear a relationship to these symptoms of psychic or emotional disturbance. It further appears—although our data are inadequate to establish this point firmly—that the more culturally dissimilar the neighborhood from the individual, the greater the psychological consequences.

Social group characteristics have also been found to be associated with the individual's level of self-acceptance. Self-esteem was found to be related directly to social class position and to differ among religious groups. The differences among these groups stem from certain differential patterns of child-rearing, distinctive degrees of family stability, and unequal performance in school.

The relationship of the student's self-conception to his participation in the social life of his peers is sharply demonstrated in this study. Students with low self-esteem are less likely to participate in extracurricular activities in high school, to be elected as leaders of such formal organizations, to be chosen as leaders in the classroom, to be informal opinion leaders, and to participate actively and frequently in discussions of matters of general high school interest. It would appear that this social apathy, lack of peer-group success, and low self-esteem tend to reinforce one another.

Finally, the negative self-concept appears to have implications for the adolescent's role as a citizen. The person with low self-esteem, we find, manifests less concern with public affairs (as indicated by exposure to such affairs in the mass media) and less knowledge of national and international affairs (as indicated by an objective measure of knowledge). Beyond this, he participates less in informal discussions of such matters with peers, even if he is interested. Our data suggest that his fear of criticism and public ridicule, his inordinate self-consciousness, and his lack of confidence in his potential contributions to conversations contribute materially to his low level of participation in such discussions.

Social Psychology of Aging

Our staff has collaborated with scientists of other laboratories in an interdisciplinary research project on human aging. A broad and summary conclusion from the study is that the prevalent image of the aged person has been distorted by research based on aged individuals who are sick or institutionalized; that many manifestations heretofore associated with aging, per se, in large measure reflect illness, and environmental and cultural influences. Disases and personal and social events seem to be rate-determining factors, accelerating or slowing deteriorative processes. The medically healthy men studied in the present sample, in general, presented a picture of persons involved in living—constructive and alert. Factors in the individual's immediate environment were found to be closely related to his behavior and attitudes and adaptations to age changes. Where the environment is characterized by deprivations in terms of loss of intimate persons and social displacement, behaviors and attitudes of the aged show more deteriorative qualities.

Of the many biological and psychological processes and conditions of environment that have been tapped in this study, none contributes so strongly in contrast to the others as to be considered the key or vital factor in aging. The picture is, instead, one of factors reinforcing or cancelling out the effects of others in a manner far too complex to be neatly disentangled or to have relative contributions accurately weighted.

There have been few studies in which it has been possible to obtain longitudinal data on human aging, particularly intensive biological and behavioral data on the same group of subjects. A follow-up of the aged men, five years after the original study, has been undertaken, with an interest in examining the stabilities and changes that have occurred with time, and determining the extent to which certain physical and psychological indicators present four years ago have predictive value for current status and functioning. The follow-up study has value, too, in furnishing a replication of measures, and in so doing permitting assessment of the stability of relationships found in the earlier study.

Genetics and Environment

A reporting system set up in 1937 by Dr. Franz Kallmann provided index information on a large number of mentally subnormal twins in New York State, mainly in the State Schools for Mental Defectives. During the four-year period, July 1952 to June 1956, additional information was abstracted from institutional records and obtained on visits to homes and hospitals. Accessible twins were examined, many of them with the aid of X-rays, electroencephalograms, and psychological tests. Pairs studied in detail were classified as to zygosity. Clinical diagnoses were reviewed in the light of all information obtained. The total sample consisted of 592 multiple births, of whom 179 pairs were studied in detail.

The high-grade undifferentiated defectives revealed nothing of major significance, but three observations on the low-grade and/or clinical cases stand out, even after mongolism is excluded. First, among the monozygotic twins, 73% were similarly affected or "concordant," while among the dizygotic twins only 30% were concordant. It thus appears that concordance in the identical twins cannot, in general, be attributed to the fact that they shared one prenatal environment.

Second, patients with cerebral palsy as part or all of their clinical picture stand out as the only group in which identical twins are frequently discordant. In conjunction with the first observation, this finding implies that most low-grade or clinical mental deficiency, if not marked by cerebral palsy, is either dependent largely on heredity, or is due to environmental factors not shared by fraternal twins. The latter explanation is probably inadequate.

Third, patients with cerebral palsy as part or all of their clinical picture stand out as the only group of twins in which a history of trauma could usually be elicited. Such a history was obtained for a few cases in each diagnostic group, but does not assure a traumatic etiology. Neither does absence of such a history rule out a traumatic etiology.

Although these data do not justify categorical or quantitative statements, they indicate that perinatal brain damage usually affects neuromotor function, and that most other types of severe or clinical mental defects have a large dependence on genetic factors. Of course, the latter types may at the same time depend upon environmental factors, but these are not likely to include perinatal brain damage.

ADDICTION RESEARCH CENTER

General

The Addiction Research Center had a productive and richly rewarding year. As stressed in last year's annual report, the study of addiction is not a narrow scientific field but actually is a subject with complex ramifications in all areas of human behavior, requiring an interlocking set of investigations using the techniques of many disciplines for full elucidation. The interaction and teamwork of the various sections make organization of the annual report difficult, but these interactions between sections represent the most unique characteristic of the ARC and are a source of pride and stimulation to the personnel.

The section on clinical studies of opiate addiction has the oldest program in the center—the development of potent analgesic drugs without addictive properties. After many years of frustration the goal now appears attainable. The isoquinoline compound (ARC I–K–1) described in last year's annual report in a nonaddictive material, and levomepromazine, a phenothiazine tranquilizer, has been reported to be an analgesic. Preliminary studies on the addictiveness of levomepromazine are now underway. In addition, interesting compounds in the indane series have become available. These indanes differ from agents which we have previously studied in that while they are analgesic in animals and in man, the pattern of effects in small animals is distinctly different from that of morphine. Analgesic compounds with patterns of effect differing from morphine have long been sought by the ARC so that studies on this particular group of analgesics will be awaited with great interest.

The psychology section continued its collaboration with the clinical section in carrying out development of improved methods for assessing the subjective effects of drugs. The section on barbituates and alcohol has formulated a theory of initial addiction to opiates and alcohol in which social deviance is conceived as being the main factor in producing addiction in the United States.

The theory will be used as a framework for future investigations. In addition, the section on barbituates and alcohol has continued to refine its methods for studying quantitatively the subjective effects induced by these and other classes of drugs.

The section on additions other than analgesics, barbiturates, and alcohol has been carrying on a long range program investigating tolerance and cross tolerance among various types of psychotomimetics. The object of this program is to sort the psychotomimetic drugs into related groups, with the hope that such grouping will provide important clues for elucidating the ways in which these drugs create such striking mental effects. During the year, the section showed that a high grade of cross tolerance could be developed between LSD and mescaline by chronic administration of either of these drugs. Because of the marked difference in the chemical structure of mescaline from that of LSD and psilocybin, cross tolerance appears to be more dependent on similarity of pharmacological effect than on chemical structure. The pattern of effects of dextroamphetamine differed objectively and subjectively from the pattern of effects of LSD. In addition, no cross tolerance developed between LSD and mescaline on chronic administration, although direct tolerance to either was readily demonstrated. Currently the section is studying tolerance and cross tolerance between cholinergic blockers and LSD.

The biochemical section has been in the process of transition from the clinical biochemistry to the experimental neurochemistry of addiction. The section is now investigating the level of catecholamines and serotonin in brain and other tissues of rats following large single doses of morphine and during cycles of addiction to morphine. No striking changes in the amounts of these amines in tissues have been found so far, despite previous reports of marked alterations of epinephrine and norepinephrine levels in brain during addiction to and withdrawal of morphine. When the work with the catecholamines is completed the section will turn its attention to the distribution and metabolism of radioactivity labelled analgesics in brain during cycles of addiction. Despite the shift in major emphasis from clinical biochemistry to experimental neurochemistry, the section has maintained its interest in the development of methods for detection of opiates in body fluids. This project is of pressing clinical importance.

The section on the neurophysiology of chronic barbiturate intoxication has been pursuing two general lines of investigations: (1) an effort to localize the sites in the central nervous system responsible for barbiturate withdrawal seizures to pharmacological analysis, and (2) possible neurohumoral mechanisms of abstinence from barbiturates. The section has shown that decorticate dogs tolerate higher levels of barbiturates than do intact dogs. Furthermore convulsions are not observed in decorticate dogs unless much higher levels of barbiturate intake are used than is the case with normal dogs. These studies are now being continued in a decerebellate preparations. A mixture of *l*-arginine and glutamate may partially suppress or defer barbiturate withdrawal convulsions.

The section on psychology carried out both clinical and animal investigations. Development of inventories for the quantitative assessment of subjective effects caused by drugs has been continued as has work on the mechanisms of relief of pain by morphine and other opiates. It is now certain that suppression of pain-conditioned anxiety is fairly specific to the opiates and is not dependent upon impairment of discrimination by these drugs. A beginning has been made on the psychological study of factors controlling behavior in the character disorders.

The section on neurophysiology of opiate addiction has developed a method for the development of physiological dependence on opiates by administering a continuous infusion of morphine to dogs for an eight-hour period. Comparisons of such short-term dependence with long-term chronic dependence shows both similarities and differences. The method is being applied to fundamental studies of abstinence from opiates, but also may be of practical importance in screening drugs for addictive properties. The section is also developing methods for concomitant study of electroencephalographic and behavioral changes under drugs.

Addictive Properties of New Analgesics

These studies are designed primarily for the purpose of providing information on the human addiction liabilities of new drugs (chiefly potent analgesics) with morphine-like properties for use by authorities responsible for recommending measures for control of such agents at national and international levels. They also assist the medical profession in evaluating the therapeutic and toxic properties of new drugs in clinical use and provide opportunities for basic research on the mechanisms of tolerance, addiction, and habituation.

During the current year a useful "short" 7-day test has been developed for ascertaining the addiction liability of intravenously administered drugs. In the United States abuse of an analgesic is partially dependent upon (a) the feasibility of taking it intravenously, (b) the quality, intensity, speed of onset and duration of the subjective effects, and (c) whether the drug induces tolerance and physical dependence on repeated administration. The procedures used in the new "short" 7-day test may be illustrated by the following experiment: Morphine, codeine, *d*-propoxyphene and a new synthetic isoquinoline derivative were compared in 7 nontolerant addicts who received every third day a sample dose of each of the four drugs intravenously. After taking all samples, the patients rated the compounds in order of preference. Each subject was then advised that he would subsequently receive, in randomized order, each sample drug which he elected to take and that each drug would be administered on an increasing dosage schedule for seven days. Once a subject has started any medication he had the option of discontinuing it at anytime, and after an interval of three days could start the next drug. Each patient received the same reward for participation in the experiment, irrespective of the number of drugs which he elected to take and whether or not drugs were discontinued before seven days of medication had been completed. After receiving drugs chronically, the opiate adicts again rated them in order of preference; and each drug was evaluated for its physical dependence characteristics by nalorphine precipitation and abrupt withdrawal procedures.

Four New Synthetic Compounds

Addiction Research Center numbers (ARC) will be used in the text to designate compounds.

Dextro - 3 - Dimethylamino-1,1-Diphenylbutyl Ethyl Sulfone Hydrochloride (ARC I–C–26). This is a new synthetic antitussive compound which is related to methadone structurally. Single doses of 25 to 70 mg induced morphine-like effects in postaddicts and effectively suppressed abstinence from morphine. ARC I–C–26 has addictiveness comparable to that of morphine. This information has been communicated to the Committee on Drug Addiction and Narcotics, NRC.

Ethyl 1-(2-Carbamethyl)-4-Phenylpiperidine-4-Carboxylate Hydrochloride (ARC I–D–20). This compound is related to meperidine structurally, and is effective in a dose of 50 mg orally as an antitussive. When single doses were given orally or subcutaneously, no morphine-like effects were observed in former addicts unless the dose exceeded 180 mg; even 1000 mg orally induced only an incomplete pattern of morphine-like effects. When substituted for morphine in addicted patients, it partially suppressed symptoms of abstinence, but very large doses (4500 mg daily) were required. It was therefore concluded that the addiction liability of I–D–20 is low. A report to this effect has been submitted to the Committee on Drug Addiction and Narcotics, NRC.

1,2-Dimethyl, 3-Phenyl, 3-Propionoxy Pyrrolidine Hydrochloride (ARC I–O–1). This compound is a meperidine congener which was developed as an analgesic for oral use. Although it is as potent as codeine in relieving pain, several investigators have observed various signs of toxicity.

I–O–1 has been evaluated for addictiveness by (a) administering it in single doses, (b) substitution tests in morphine-dependent patients, and (c) direct addiction assays. I–O–1 has substantially less abuse liability than codeine; its addictiveness is comparable to that of *d*-propoxyphene. These observations have been communicated to the Committee on Drug Addiction and Narcotics, NRC.

2,2-Diphenyl-4-{1-[4-N-Piperidine) - 4 - Carboxamine]-Piperidine}-Butyronitrile (ARC I–D–

21). This is a new derivative of meperidine and was developed as an analgesic. It has been evaluated for addictiveness by (a) effects of single doses, (b) substitution tests, and (c) the short-term, 7-day intravenous test, but these studies are incomplete.

Acute and Chronic Intoxication With Drugs Other Than Analgesics and Barbiturates

Cross Tolerance Between Mescaline and LSD

As was reported in last year's narrative summary, LSD and mescaline induce strikingly similar patterns of subjective and objective effects, suggesting that both drugs act through similar mechanisms or through common final pathways. A study on cross tolerance between mescaline and LSD was completed during the year. Ten subjects were used. On one occasion, these subjects received mescaline in doses increasing to 5 mg/kg, after which they were "tested" with mescaline (test of direct tolerance) and "challenged" with LSD (test of cross tolerance). On another occasion, patients received LSD chronically and were tested with LSD and challenged with mescaline. A high grade of direct tolerance was developed to both drugs. Patients tolerant to one drug were highly cross tolerant to the other. These results strongly reinforce the notion that these compounds act by common mechanisms. Furthermore, LSD, mescaline and psilocybin seem to constitute a group of psychotomimetic drugs with similar modes of action.

Cross Tolerance Between LSD and Amphetamine

This study was undertaken because of hypotheses that have been advanced that LSD creates a psychosis by acting as a "central adrenergic stimulant" or "central adrenergic blocker." Preliminary studies of the pattern of effects showed that the changes produced by dextroamphetamine were distinctly different from those caused by LSD. Objectively, dextroamphetamine caused little pupillary dilatation, marked enhancement of blood pressure and pulse rate, and relatively little change in temperature of kneejerks. Conversely LSD caused marked pupillary dilatation, less marked increases in blood pressure, and marked enhancement of the threshold for the kneejerk. Subjectively, dextroamphetamine caused marked euphoria followed later in the day by dysphoria. Single doses running up to as high as 0.6 mg/kg did not induce the true or pseudo-hallucinations, illusions, abnormal kinds of thinking, etc. that are so characteristically induced by LSD.

On one occasion 10 subjects received one dose of dextroamphetamine daily. The amount was increased over a period of five days to 0.6 mg/kg and continued at that level through the 13th day. On the 14th day patients were tested after administration of the same dose of dextroamphetamine they had been receiving (test of direct tolerance) and on the 14th day they were tested with 0.5 mcg/kg of LSD (test of cross tolerance). On another occasion the same patients were made tolerant to LSD by administering doses increasing to 1.5 mcg/kg once daily for five days and maintaining this dose through the 13th day. They were then tested with LSD and challenged with dextroamphetamine as above. A high degree of direct tolerance developed during chronic intoxication with either LSD or dextroamphetamine. However there was no appreciable degree of cross tolerance between the two drugs. The results do not support the adrenergic hypotheses.

Cholinergic Blockers

Preliminary experiments have been conducted in order to obtain dose-effect curves for intramuscularly administered scopolamine and a piperidyl benzylase, known as JB-318. Both drugs cause cholinergic blockade in man manifest by pupillary dilatation, tachycardia, and reduction in salivation and sweating. In sufficient dose, both cause marked confusion, sensations of drunkenness with slight ataxia, sedation, anxiety, shortening of the attention span, and auditory and visual hallucinations. The pattern of effects is distinctly different from that of LSD. Confusion and disorientation by the cholinergic blockers are quite marked, but are scarcely manifest with ordinary doses of LSD. The florid, entoptic phenomena seen after LSD are also uncommon after the cholinergic blockers. Direct tolerance to and cross tolerance between both cholinergic blockers will be tested during the coming year, as will cross tolerance between both cholinergic blockers and LSD.

Clinical Studies of Intoxication With Alcohol, Barbiturates and Related Drugs

Work was continued on the psychopathology of alcoholism as compared with that of narcotic addition, on the acute effects of alcohol and barbiturates on verbal report, and exploratory work was continued on techniques for uncovering possible correlations of such drug effects and personality characteristics. One study was an MMPI factor analytic comparison of rather large groups of narcotic addicts, alcoholics, and criminals (all being institutionalized subjects). Although some slight but statistically significant differences were found between the groups (the alcoholics were slightly lower on the Psychopathic deviate scale and slightly higher on the Paranoia scale), such differences did not approach the level necessary for diagnostic significance. A second study was theoretical in nature and attempted to integrate availability of drug supplies and sociological factors with factors of personality and learning as they appear to produce initial addiction to narcotics and alcohol. Social deviance, with its concomitant acceptance of unusual forms of behavior (including the misuse of drugs), was postulated as being the main factor in producing initial addiction in the United States.

Further studies of the subjective effects of alcohol and pentobarbital are in progress, using the Addiction Research Center Inventory which was especially developed for testing such effects. During the year additional dose levels of both drugs were investigated with this inventory to elucidate possible dose-effect relationships. Alcohol and pentobarbital appear to possess many actions in common. In the doses used, both produced a considerable degree of confusion as measured by both subjective and objective methods. Even in the standardized testing situation both induced some degree of euphoria which reached a maximum with intermediate doses and decreased as the dose was increased above this level. In the present testing situation, using former addict subjects, alcohol produced a decrease in sensitivity to the opinion of others, loss in the estimate of other people's worth, and a general increase in conversation and actions of a sexual nature. Common and specific actions of these drugs are being investigated further by several methods. Preliminary use of factor analysis suggests that although alcohol, pentobarbital and chlorpromazine produce specific effects, they may be located also on one pole of a bipolar factor of motivation. Thus under present conditions subjects report tiredness, "sluggishness," disinterest in activity, poor coordination and dizziness.

Biochemistry of Addiction

The biochemical section is studying the effects of morphine and cycles of addiction to morphine on the levels of catecholamines in brain and other tissues. These studies were initiated because of reports from Sweden that addiction to and abstinence from morphine in rats caused definite changes in the concentration of these amines in the central nervous system.

Single injections of morphine sulfate or thebaine

Rats received intraperitoneal injections of 15, 30 or 60 mg/kg of morphine sulfate, or 20 mg/kg of thebaine. An equal number of animals received placebo injections of saline or water. Behavioral and temperature observations were made on the individual animals while 4 rats (2 male and 2 female) were pooled for chemical analysis of heart, brain and spleen for catecholamines and serotonin. Assay was by a modification of the fluorometric method of Shore and Olin. Behavioral studies included hourly observations of activity, "hostility," cyanosis and analgesia. Animals were sacrificed four hours after administration of placebo or drug (15 or 30 mg M.S.) and two or four hours after 60-mg doses.

Administration of 15 or 30 mg/kg of morphine caused a transient initial motor depression followed by stimulation associated with modest hyperthermia after 15 mg/kg and; after 30 mg/kg, by a slight fall in temperature before onset of marked hyperthermia. No signs of respiratory depression were observed. Cyanosis was never observed after 15 mg/kg and rarely after 30 mg/kg. A small and statistically non-significant increase in brain and heart catecholamines and in brain serotonin was found. The administration of 60 mg/kg produced a profound depression of activity and body temperature, maximum at two hours and partially subsiding by the fourth hour. Marked signs of respiratory depression were

noted. Cyanosis was noted in roughly 50 percent of the animals. Brain and heart catecholamines were slightly increased after four hours. When the rats were sacrificed at two hours there was a highly significant increase in brain catecholamines, but no statistically significant changes in the heart catecholamines. Increases in brain serotonin were slight at each dose level.

Of 26 rats receiving thebaine, 4 died and 15 exhibited convulsive behavior (7 had major seizures). Interim periods were associated with moderate sedation. Analyses of organs obtained four hours post-thebaine showed a statistically significant decrease in the catecholamines of the heart, a less notable decrease in those of the brain, and no significant change in serotonin of the brain.

Effects of morphine on body temperature confirmed those of Gunne. Such discrepancies concerning brain catecholamines as exist between Gunne's results and ours are unexplainable except on the basis of the different assay methods, chemical by us and bioassay by Gunne.

Excitation following thebaine is expressed as subconvulsive spasms and major seizures, and is thus qualitatively different from excitation observed after morphine injections. Otherwise the effects of thebaine are primarily depressant.

Effect of Withdrawal of Morphine on Rat Tissue Catecholamine and Serotonin Levels

Rats (an equal number of males and females) were made tolerant to morphine by gradually increasing subcutaneous injections of morphine from 5 mg/kg to 80 or 130 mg/kg twice daily over a period of 40 days. Control animals were injected with adjusted (iso-pH) saline. The rats were sacrificed at approximately 24, 48 or 72 hours after the last injection. Catecholamines and serotonin of brain and catecholamines of heart decreased slightly, but not significantly during abstinence. Changes in the weights of the spleen and adrenals occurred during this period, so results were calculated on the basis of content of the whole organ or pair of organs. So calculated, there was no change in the catecholamine content, and an increase in the serotonin content of these organs. Catecholamine content of adrenal glands was markedly and progressively elevated during abstinence. There was a marked decrease of the rat's weight at 48 hours abstinence and a return to control value at 72 hours.

This experiment is incomplete. Further studies are in progress in which the maximum dose of morphine will be much greater and in which analyses will be carried out during the period of addiction. Determinations of 5-hydroxyindoleacetic acid may be added in view of the above noted changes in serotonin of brain and spleen. It has been reported that in dogs intoxicated with 5-hydroxytryptophane, a syndrome appears which resembles abstinence from morphine. If a central serotonergic system is activated neuronally, a fall in serotonin of those neurones containing this substance may occur. Released serotonin may find its way into the systemic circulation where it can be taken up by platelets.

Follow-up Studies

Dr. John A. O'Donnell of the Social Service section of the hospital is making follow-up studies on former patients resident in Kentucky. He has submitted 16 urine specimens which we have analyzed for the presence of morphine, demerol and/or barbiturate.

Chronic Intoxication With Barbiturates and Related Drugs

Elevation of Electrical Seizure Thresholds

Bilateral adrenalectomies were performed in one cat and 2 miniature dogs. The animals were then maintained by daily administration of desoxycorticosterone and cortone acetate. During this period of artificial hormonal regulation, electrical induction of convulsions daily caused a progressive elevation in the threshold for production of convulsions. This finding confirms preliminary work and indicates that "tolerance" to electrically induced convulsions is more likely to be cerebral rather than extracerebral in origin.

Studies on the problem of whether the tolerance effect depends on a local or a generalized cerebral adaptation have been delayed because of the death of a second preparation in which two anterior as well as two posterior electrodes had been placed. It was intended to stimulate only the anterior pair of electrodes until ECT elevation occurred, and then to stimulate posteriorally to determine whether a similar elevation had developed there.

A preliminary result has been obtained on the effect of an alumina cream cicatrix subjacent to each epidural electrode. This procedure seems to cause an elevation of ECT in itself, but prevents the usual tolerance effect obtained in control animals.

The latter two projects will be continued during the coming year.

Effects of Bilateral Decortication on Barbiturate Withdrawal Conculsions

Two of 3 decorticate dogs were re-intoxicated with higher dose levels of sodium barbital than were achieved during the first addiction cycle. In contrast to their first uneventful withdrawal, both dogs developed convulsive phenomena during abstinence from dose levels exceeding 185 mg/kg. It now seems definite that the decorticate dog can develop modified convulsive seizures during withdrawal from sodium barbital if the final dose level exceeds that ordinarily required for intact dogs.

Eventually depth electrodes will be placed in various brainstem structures following bilateral decortication to determine whether fixed or variable loci of seizure origin can be found. An intact dog with depth electrodes is now undergoing chronic intoxication with sodium barbital.

Effect of Decerebellation on Barbiturate Withdrawal Convulsions

Five dogs were decerebellated by suction while they were puppies. After achieving young adulthood they will be intoxicated along with 5 control dogs. Following abrupt withdrawal of the sodium barbital the occurrence and pattern of convulsive seizures (if any) in the two groups will be observed in order to determine whether barbiturate abstinence seizures originate in the cerebellum. In contrast, it is possible that if these convulsions do originate elsewhere, the cerebellum exerts an inhibitory effect on their incidence or duration. In the latter instance the decerebellate dog may require a lower dose of barbiturate in order to develop abstinence seizures.

Effect of Unilateral Cerebral Cortical Lesions on the Occurrence of Convulsions During Withdrawal of Barbiturates From Miniature Dogs

The initial cycle of sodium barbital intoxication and withdrawal indicated that the presence of a corticomeningeal cicatrix did not increase the likelihood of withdrawal convulsions after discontinuation of doses of barbital smaller than those usually required. A second cycle of intoxication and withdrawal was followed by both focal and generalized convulsions in 2 dogs that had alumina hydroxide lesions. Both of these animals died. In contrast, 2 dogs that had an ethyl chloride spray applied to an area of the cerebral cortex did not have convulsions during withdrawal from similar doses of sodium barbital. Both of these latter dogs survived. A histological study of the scarred areas in these 4 dogs is underway. It is possible that the alumina cream scars produced by injection were deep enough to enable more direct subcortical connections than the more superficially placed ethyl chloride spray lesions. This formulation would emphasize the importance of brainstem structures in the genesis of barbiturate withdrawal seizures.

Effect of Dilantin, Scopolamine, and Other Compounds on Barbiturate Withdrawal Convulsions

A pharmacological screening program to find chemical substances that will suppress barbiturate withdrawal convulsions has been initiated. The ultimate goal of this program is to uncover leads to the biochemical mechanisms that may be involved in the genesis of these seizures.

Thus far dilantin sodium, scopolamine, gamma aminobutyric acid, 5-hydroxytryptophane, pyridoxal phosphate, pyridoxine hydrochloride, amino-oxyacetic acid, hydroxylamine, and *l*-arginine have failed to suppress barbiturate withdrawal seizures.

A mixture of *l*-arginine and glutamate seems to have partially suppressed these seizures in the only dog in which it was tried. Thus, three convulsions developed at 82+, 119+ and 124+ hours of withdrawal, respectively. These seizures occurred during a period when 50 mg/kg of the *l*-arginine-glutamate was being administered every four hours. The last dose of this substance was administered exactly seven days after the final dose of barbital sodium (168 hours) was given. Thereafter the dog had 14 more convulsions between 170+ and 203 hours of withdrawal. Thus 82+ percent of the total number of abstinence convulsions occurred after cessation of *l*-arginine-

glutamate. In contrast, a control dog had seven convulsions, 55 percent of which occurred between 171+ and 216+ hours of withdrawal. The apparent suppression (partial) effect obtained will be confirmed or refuted during the coming year, during which other substances will be screened.

Psychological Studies of Addiction

Subsumed under this project title are continuing studies which attempt to (1) provide information on the psychopathology of narcotic addiction, (2) develop methods for measuring specific and common subjective effects of analgesics, hypnotic, tranquilizing, analeptic and psychotomimetic drugs and relate these effects to personality characteristics, (3) develop techniques for analyzing variables which control behavior in the social deviant and relate these to the misuse of opioids, and (4) develop methods, using animals, for investigating anxiety and related phenomena and for screening of analgestic and tranquilizing drugs.

In project IV above it was reported that the only common personality characteristic found in rather large groups of institutionalized narcotic addicts, alcoholics, and criminals within the limits of the Minnesota Multiphasic Personality Inventory was an abnormal elevation on the Psychopathic deviate scale. This profile peak was found within each group and in the combined sample. Such an elevation is strong evidence of social deviance. Thus since this elevation has also been found in all published reports on the groups whether or not they were institutionalized, the conclusion was drawn that in some way social deviance is centrally causal in the addictions. Considerable clinical evidence supports the position taken, but main confirmation or infirmation must await the gathering of many more experimental data.

During the year work was completed in testing for dose-effect relationships of morphine, amphetamine, LSD–25, and a marihuana-like compound (pyrahexl), using the Addiction Research Center Inventory (ARCI). This inventory, which was especially constructed for assessing subjective effects of drugs, has been described in previous reports and was used on parallel investigations of alcohol, pentobarbital and chlorpromazine (see project IV). Standardization of the inventory has been completed on the above drugs and no medication (control) and placebo conditions for addict subjects. Over 500 completed protocols of combined no-drug (control) and placebo conditions were used to obtain stable baseline standardization statistics. Since results on many scales (previously developed for each of the conditions by cross validation) were neither normal in distribution nor homogeneous in variance, Z-score and T-score transformations were applied. The result was a profiling of scales for the no-drug conditions with means of 50 and SDs of 10. The purpose of these procedures was to express all results in standard form so that data from all conditions are directly comparable. The result is a profile of the response of addict subjects to the spectrum of drugs employed when using specific drug scales or specially developed "rational" scales. Originally two types of scales were developed empirically for each drug condition, one supposedly indicating "primary" and the other "secondary" effects. Since investigations of the patterning generated by these two types of scales showed them to be very similar, only one such empirical scale for each drug condition will be used. The more marginal or secondary items, however, will be employed in describing specific drug effects. For purposes of differentiating some types of drug actions other types of scales and analyses appear promising. Thus the use of factor analysis and other correlative methods have proven fruitful in developing scales which are composed of items that indicate similar drug actions. The most promising of these are: the PCAG scale on which pentobarbital, chlorpromazine and alcohol show marked elevation, and on which the other drugs tested do not; the MBG scale which appears to be chiefly euphoric in content, on which morphine and amphetamine produce marked effects; the LG scale which is chiefly composed of items indicating anxiety, is LSD–25 specific; the AG scale, relatively specific to alcohol as are the MG scale to morphine and the BG scale to amphetamine. It is becoming more apparent as this work continues that, except within a very narrow range of specificity, drug effects occur as response patterns in which much overlapping is found. Thus it may be neither valid nor factual to merely state specificity of actions

of a particular compound without also stating (if possible) the actions the drug may possess in common with standard drugs. These investigations are being continued. Several papers are being prepared for publication, and a manual which will contain standardization data is nearing completion.

Experimental studies are being continued on motivation and drug effects as they may differ in the narcotic addict and normal subject. An initial study of visual recognition thresholds for addict argot has been completed. This study showed that hospitalized former narcotic addicts recognize words related to the acquisition, use, and effects of narcotics more readily than words of comparable degrees of familiarity that are not related to the addict argot. A control group of hospital personnel did not show significantly different thresholds between the same two categories of words. This method appears to be a sensitive measure of the meaningfulness of stimuli. These investigations will be continued, using other classes of stimuli which will include stimuli pertaining to possible conflictual areas of behavior such as heterosexuality, homosexuality, dependency, frustration, aggression, etc. The tests will be given under both drug and no-drug conditions. In other studies physiological techniques are being continued together with psychological techniques in studying perception of affective stimuli and the effects of drugs thereon.

In studies using rates, the previously reported work on analgesics and autonomic blocking agents was continued. Publication had been withheld on several aspects of screening for analgesics and theory of their modes of action pending the results of validating experiments. One form of inhibition sometimes referred to as a conditioned emotional response (CER), is produced by suppressing lever-pressing for food in rats for short periods by pairing tone with shock. The hypothesis was that only analgesics would reduce or eliminate this form of inhibition. It had been shown and reported previously that with one exception (and this drug, pentobarbital, has been reported to induce mild analgesia) only the potent opioids in a series of 10 representative drugs produced statistically significant dose-effect reduction in such pain-conditioned anxiety. Since ancillary studies have produced validating data on mechanisms of actions of the analgesics, a series of reports has been prepared in publication form. The ancillary studies indicated that maximally effective analgesics do not produce amelioration of the CER through the induction of either motor or sensory impairment, through intensification of feeding behavior, direct stimulation by the unconditioned stimulus (tone), or through learning that the shock is not aversive under analgesics. Rather, the evidence in conjunction with previous studies on human subjects indicates strongly that a necessary and perhaps sufficient condition for the presence of analgesia is the absence or marked reduction of pain-conditioned anxiety.

Research in the general field of anxiety at this center embraces not only pain and pain-conditioned anxiety and their alteration by drugs, but also involves, as time permits, investigations on other forms of anxieties and the effects of drugs thereon. One such study on rats, mentioned briefly in a previous report, was completed during the present year. The hypothesis tested was that while autonomic effector activity is not essential for the acquisition of a traumatic avoidance response, such activity increases resistance of this response to extinction. The general procedure was to compare rates of acquisition and extinction of a traumatic avoidance response under nonmedicated conditions with such rates after administration of the autonomic blocking agent Ecolid. Twenty animals were trained on each of three electric shock levels (0.5, 1.0, and 3.0 ma), half of each group received no medication while the other half was tested 30 minutes after the subcutaneous injection of 5 mg/kg of Ecolid. Results indicate that this dose of the drug impairs both the acquisition and performance of the avoidance response mildly but significantly. Although no significant differences in acquisition to asymptotic rates were found between the no-drug groups as a function of the various shock levels, the 0.5-ma., no-drug group extinguished readily, while the other two groups showed little or no evidence of cessation of the response in many trials. The autonomic blocking drug did not enhance rate of extinction over that found in the no-drug, 0.5-ma group, but produced very significant enhancement of extinction in the 1.0- and 3.0-ma groups, the 1.0-ma animals showing the greatest effect. It was concluded that activity of the autonomic nervous sys-

tem is an important factor in the acquisition of an avoidance response, and is an especially important mechanism in maintaining strongly conditioned responses of this type when primary reinforcement is discontinued. The results support the previous work of certain other investigators and provide further evidence on the action of the nervous system in behavior. The effect of Ecolid on extinction appears to be a curvilinear function of the degree of autonomic involvement present during acquisition, which in the present studies appears to be directly related to the shock levels used in training.

Central Nervous System Depressants

During the last year research on the mode of actions of central nervous system depressants has proceeded in four areas:

1. the study of physiological mechanisms involved in acute tolerance and physical dependence,
2. the effect of depressant drugs on the EEG of cats in various levels of arousal,
3. a comparision of the action of I–K–1, morphine, and codeine in spinal dogs, and
4. a comparison of graded doses of morphine and pentobarbital in nontolerant post-addicts.

All of the above mentioned projects are currently in progress and only a preliminary discussion of the results can be presented.

Study of Physiological Mechanisms Involved in Acute Tolerance and Physical Dependence

A reproducible abstinence syndrome can be precipitated with nalorphine in animals that have received an infusion of morphine for eight hours. This "acute" abstinence syndrome has been compared with the abstinence syndrome precipitated with nalorphine in both high (C–5 to 6) and low (T–10 to 12) spinal dogs that have been chronically addicted to morphine. Following an infusion of morphine, nalorphine precipitated only a minimal abstinence syndrome below the level of transection in both the low and high spinal dog consisting of fragmentary running movements, an increase in the magnitude of the crossed extensor or ipsilateral flexor reflex above that following either morphine or nalorphine alone, extensor hypertonus and inhibition of the extensor thrust. In contrast, nalorphine induced protracted running movements in all but one spinal dog chronically addicted to morphine. Surprisingly the skin twitch of high spinal dogs chronically addicted to morphine was markedly or completely suppressed during nalorphine-induced abstinence. We have concluded that whereas the most striking signs of acute physical dependence are mediated at supraspinal levels, the signs of chronic physical dependence are mediated at both spinal and supraspinal levels.

Further evidence bearing on this point has been obtained by comparing the signs of acute and chronic physical dependence mediated by the sympathetic nervous system in high and low spinal dogs. The sympathetic outflow is below the level of transection in the high spinal group but above the level of transection in the low spinal dog. In both the acute and chronic abstinence syndromes in the low spinal dog signs of sympathetic hyperactivity such as mydriasis and tachycardia are seen. In contrast, only small degrees of pupillary dilatation and tachycardia were observed during the acute abstinence syndrome in the high spinal dog, whereas marked tachycardia and mydriasis were observed during the chronic abstinence syndrome.

The Effect of Depressant Drugs on the EEG of Cats in Various Levels of Arousal

The behavioral states of cats with chronically implanted cortical and depth electrodes are being classified on the basis of eyelid position, head position, head and eye movements, body posture and certain more complex motor patterns. Concomitant frequency analysis of the electrocorticogram is being obtained during these behavioral states. Electrical activity in the medial reticular formation is recorded as well. Preliminary results indicate that there is a reproducible mean frequency spectrum for different behavioral states. In addition the effects of pentobarbital, chlorpromazine, imipramine, marplan (iso-carboxyazide), atropine, *d*-amphetamine and morphine on behavioral states and their concomitant EEG frequency spectra are being studied. Several findings are of particular interest. Cats that have received pentobarbital (7.5 and 15 mg/kg) demonstrate moderate to marked ataxia and even a transient loss of

the righting reflex. However these cats maintain control of their head and lids at all times, and can demonstrate a variety of behavioral states ranging from attention to their environment to deep somnolence. As the behavioral state changes from somnolence to alertness there is a progressive increase in the frequency of the barbiturate spindles, similar to changes previously described consequent to stimulation of the reticular activating center.

When atropine was administered the cats were restless, constantly moving about the cage. The EEG showed a relatively constant delta pattern. The EEG could be activated by stimulation of the reticular formation, however the behavioral concomitant was violent ipsiversive turning movements.

Comparative Action of Morphine, I–K–1, and Codeine on Hindlimb Reflexes of the Spinal Dog

I–K–1, an analgesic agent that has approximately the same order of effectiveness as codeine and which is apparently devoid of addiction liability when administered orally, is being compared with codeine and morphine in its ability to depress the flexor reflex in chronic spinal dogs. Preliminary results indicate that 0.28 mg/kg of morphine is equivalent to 2.7 mg/kg of I–K–1, and 4.6 mg/kg of codeine when these drugs are administered intravenously. These doses produce a 50 percent depression of the flexor reflex, and in the case of morphine, the dose is of the same order of magnitude as the dose used clinically in man. Preliminary results indicate that I–K–1 is only $\frac{1}{20}$th as effective orally as intravenously. These findings may explain why I–K–1 given orally fails to produce definite opiate-like signs or symptoms in man but does so when given intravenously.

A Comparison of Graded Doses of Morphine and Pentobarbital in Nontolerant Postaddicts.

The physiological and subjective effects of graded doses of morphine and pentobarbital have been studied in nontolerant postaddicts. Several innovations in techniques were introduced during this study. Pupillary diameter has been determined photographically, using a Polaroid 3000 speed film in an adapted Graflex camera with an f2.8 as well as an accessory 3 diopter lens. This apparatus provides photographs in which the pupillary diameter of subjects, even with heavily pigmented irides, can be measured with a precision of from 0.1 to 0.2 millimeters when photographed in 5-foot candles of light. The reproducibility of determinations of pupillary diameter has been evaluated in 18 subjects, and it has been found that data can be replicated within 0.4 mm in 60 percent of duplicate trials, and that in all 222 observations data have replicated within 0.6 mm.

An analysis of the effects of graded doses of pentobarbital on pupillary diameter has shown that pentobarbital consistently produces miosis. This observation may be of some importance clinically in making a diagnosis of barbiturate intoxication.

Conditioning Factors in Addiction and Habituation (Relapse)

The general theory and results of a number of exploratory studies designed to test it have been described in some detail in previous annual reports. Briefly it is hypothesized that, at least in part, relapse to habitual use of addiction drugs (drugs that produce "physical dependence) represents a conditioned response to stimuli (external and internal) that were more or less regularly associated with instrumental drug-acquisitory behavior reinforced by periodic reduction of abstinence distress during previous episodes of addiction. It is hypothesized that all other factors being equal the strength of such conditioning is directly related to the "effort" expended in acquisition of the drug during addiction.

During the past three years the general design of the studies undertaken to test this theory were based on three early findings in this laboratory: (1) that water-deprived rats will drink avidly a 5-mcg/cc aqueous solution of I–G–2 [1-(beta-diethylaminoethyl)-2-(p-ethoxybenzyl)- 5 -nitrobenzimidazole methane sulfonate], (2) that typical morphine-like effects and, in morphine-addicted rats, suppression of acute abstinence phenomena appear within 4–7 minutes after oral ingestion of I–G–2 (5 mcg/cc), and (3) that without discriminatory cues neither saline-injected nor morphine-addicted rats display significant preference for, or aversion to I–G–2, 5 mcg/cc (compared with water). Further studies were then designed to determine whether, with discrim-

inatory training, either control or morphine-addicted rats would develop preference for I–G–2 as a result of close temporal association of the discriminatory stimulus with the effects of the drug. In one study water-deprived rats, both control and addicted (trained and tested daily when about 22 hours abstinent), were permitted to drink from a tube containing water on certain days of each week, and on other days from a tube containing 5 mcg/cc of I–G–2 to which blinking lights and other visual discriminatory stimuli were attached, the sides of presentation of the two tubes being randomized. On one day of each week, both tubes were presented and the comparative amounts of fluid from each tube were measured over a training-testing period of 54 consecutive days, during which single daily subcutaneous injection of saline in the control rats and 200 mg/kg of morphine in the addicted rats were maintained. Started from "neutrality," both groups of rats developed a preference for I–G–2, which was maintained for at least 34 days following termination of all injections. Subsequent extinction tests indicated that the discriminatory stimuli alone had not acquired reinforcing properties. In another study similar groups of rats were trained to press a bar under conditions of water-deprivation and water-satiation for either water or I–G–2, 5 mcg/cc reinforcement, with provision of visual and tactile discriminatory cues. Using bar-pressing rates on water-satiation days as the criterion, it was found that during the last 18 days of the 52 consecutive day training and testing period, bar-pressing rates were higher in the morphine-addicted group than in the control group when I–G–2 was the reinforcing agent and this "reward" was actually consumed. On the other hand, intra-group comparisons failed to reveal significant evidence of successful discrimination between the drug solution and water. Such "preference" of the addicted group for I–G–2, 5 mcg/cc, was maintained through the third day after cessation of injections, but not thereafter.

During calendar year 1961 these studies were re-designed with a view to simplification and provision of greater opportunities for "learning" on the basis of drug effect. In a study analogous to the tube-drinking experiments described above, control rats (morphine-addicted rats to be added later were placed in a "linear maze" (one for each rat) consisting of three compartments, with food *ad libitum* placed in the center compartment and each end compartment provided with a drinking tube. At first, both tubes contained water (available continuously) and daily measurements of 24-hour fluid consumption were made to determine "end-preferences," if any. Then anise-flavored water (anise used as discriminatory stimulus) was presented in one tube and water in the other. Over a period of several days the rats showed evidence of a slight aversion to the anise-flavored water. Much more marked aversion was found when anise-flavored I–G–2, 5 mcg/cc, was substituted for anise-flavored water. At this point both tubes were kept filled with anise-flavored I–G–2, 5 mcg/cc, for 22 days. Thereafter, the following schedule was adopted: From Friday afternoon through Monday afternoon of each week, both tubes contained the anise-flavored I–G–2, 5 mcg/cc; on Tuesday through Friday of each week, the rats had access to the tubes only 8 hours daily (16 hours water deprivation) one of which contained anise-flavored I–G–2, 5 mcg/cc, and the other water. This schedule has been maintained for three months (present date 30 October 1961). Starting with an initial aversion the mean volume of anise-flavored I–G–2 consumed in the free-choice situation (Tuesdays through Fridays) increased progressively until, during the third to fifth weeks, about the same quantities were drunk from both tubes. During the next three weeks the rats displayed a marked preference for the flavored drug solution, but since then the mean amounts consumed in the free-choice situation has fallen somewhat below that of water. Since this "loss of preference" may conceivably be due to the development of tolerance, the concentration of I–G–2 in the flavored solution has been increased to 10 mcg/kg. These experiments are still in progress, as is a control study on another group of rats, to rule out the possibility of acquisition of preference for the flavor alone. The latter is being conducted under analogous conditions and schedules, except that anise-flavored water is used instead of anise-flavored I–G–2, both in the week-end forced drinking and in the free-choice situations. The projected course of this study is similar to that of the tube-drinking experiments completed in a previous year—i.e., comparison of control and morphine-addicted rats for rate of acquisition of "prefer-

ence" and "relapse" to such preference at intervals following withdrawal of I–G–2 (and of morphine in the addicted rats).

Effects of Drugs and Other Variables on "Mental Set"

The purposes and methods of this study, as well as the unanalyzed results obtained, have been described in some detail in previous annual reports. Briefly, the study was undertaken to determine whether the psychotomimetic drug LSD–25 can alter "mental set" in non-psychotic subjects, in the direction of that characteristic of schizophrenia, and, if so, whether such an effect is specific for that drug. The technique employed was basically that described by Huston and Singer (Arch. Neurol. & Psychiat., *53*: 365–369, 1945) in which reaction times are measured on two procedures; first, with "irregular" (randomized) and second, with "regular" variation of "warning" intervals (3.5, 10, 5, 2 and 20 seconds, in that order). In the present study, however, the tests were also carried out in reverse order; i.e., "regular" before "irregular," and the reaction times on the two corresponding halves of the test were combined in order to equalize the effects of the drugs investigated, which might be expected to change in intensity during the period of time required for the test in either order (about one and one-half hours). Such tests with both orders were made on a group of 10 normal subjects, on a group of 13 chronic schizophrenic patients without drugs, and on a group of 10 postaddicts under various drug conditions (placebo; LSD–25, 1.0 and 2–3 mcg/kg; morphine, 15 and 30 mg; pentobarbital, 250 mg; and amphetamine, 20 and 30–50 mg).

Statistical analyses of the data obtained, begun several years ago, were completed during calendar year 1961. After transformation of all reaction times by a complex, empirical logarithmic formula to insure homogeneity of variance, several standard and mixed types of analyses of variance were applied (Lindquist, E. F.: Design and Analysis of Experiments in Psychology and Education. Houghton Mifflin Co., Boston, 1956), and certain of the differences discovered were subjected to the t-test. The most important findings were as follows:

1. As reported earlier by other investigators, "mental set" in chronic schizophrenic subjects could be differentiated from that of personnel in two ways: (1) reaction times were significantly longer, and (2) differences in reaction times on "irregular" and "regular" procedures (I–R) were significantly smaller. In addition, it was found that in the schizophrenic, but not in the personnel group, "order" effects were significant (reaction times were shorter when the "regular" procedure preceded the "irregular," than when the reverse was the case) and that "optimal" (shortest) reaction times tended strongly to be exhibited at the 3.5-second (rather than the 2 second) interval on the "regular" procedure.

2. Under the placebo condition, postaddicts could be differentiated from schizophrenic patients (but not from normal subjects) with regard to mean reaction time and absence of both significant "order" effects and trend toward "optimality" at the 3.5-second interval ("regular"). On the other hand, the procedure effect (I–R) in postaddicts was very much smaller than in normals, and only slightly longer than in the schizophrenic group, perhaps because in postaddicts (placebo) reaction times on the "irregular" procedure were so short (even in comparison with normals) that there was little opportunity for improvement in regularization of the order of intervals.

3. Significant prolongation of reaction times and a strong trend toward "optimality" of reaction times at the 3.5-second interval ("regular") was produced, not only by *high* doses (2–3 mcg/kg) of the psychotomimetic drug LSD–25, but also by the analgesic, morphine (15 and 30 mg), and the hypnotic, pentobarbital (250 mg). In contrast, the smaller dose of LSD–25 altered reaction times and I–R, so that with respect to these major indices of "mental set" postaddicts could not be differentiated from normals. In the doses employed, amphetamine produced no psychotomimetic changes; on reaction time, the main effect of this agent was to shorten responses, so that the prolongation of reaction time by all other drugs was accentuated in comparison.

Conclusions

The answers to the questions that prompted this study are rather complex. Using as criteria the two classical indices of "mental set" (reaction time

and I–R) it may be concluded that in a dose of 1.0 mcg/kg LSD–25 does not produce a schizophrenic type of defect, even though such a dose is usually sufficient to produce a typical "model psychosis" of intermediate grade. Indeed the effects of this dose of LSD–25 were in the direction of "normalizing" the "mental set" of postaddicts. Using reaction time and "optimality" of interval as criteria, it appears that a schizophrenic type of defect was produced, not only by high doses (2–3 mcg/kg) of the psychotomimetic drug LSD–25, but also by the analgesic, morphine, and the hypnotic, pentobarbital. These findings suggest that, at least to some degree, the disturbance in "mental set" characterizing chronic schizophrenia can be produced by diverse etiological factors which, operating alone, are not necessarily psychotogenic. Among such factors may be considered alteration of perception, bodily feelings, motivation and motor efficiency—effects that are common to all the drugs used in this study, in varying degree. Such relative etiological non-specificity is suggested also by the findings of Huston and Senf (Arch. Neurol. & Psychiat., *109:* 131–138, 1952) who reported that depressive and early schizophrenic patients exhibited comparable degrees of impairment of "mental set" on a continuum between those of neurotic (least) and chronic schizophrenic patients (most).

With completion of the statistical analysis of data, the study on the effects of drugs and other variables on "mental set" has been terminated.

LABORATORY OF CELLULAR PHARMACOLOGY

As in earlier years, the work in the laboratory has continued along four main topics: (1) mechanisms and pathways of protein biosynthesis; (2) biological methylation; (3) biological oxygenation; and (4) alkaloid biosynthesis. The protein synthesis project may be regarded as the main project of the laboratory at the present time.

During the past year, the entire effort of the Section on Alkaloid Biosynthesis has centered on a study of various facets of hordenine biosynthesis in plants. This emphasis is based on the conviction that a study in depth of a single, appropriately chosen, model system may cast some light on a whole series of biochemical phenomena of general importance. Included in this phenomena are cellular differentiation and the accompanying striking morphogenetic events, and the understanding of the complex interplay between genetic and biochemical and hormonal factors in the regulation of enzyme formation and activity.

These problems are peculiar to multicellular organisms and while it may be possible to gain some knowledge about some of these factors in simple unicellular organisms, such as yeast and bacteria, it is clear that we will have to move to a higher level of organization in order to pursue a study of cellular differentiation and the resulting tissue organization. Plants appear to offer considerable advantage in the study of some of these phenomena inasmuch as they are higher organisms and yet somewhat less complex anatomically and homeostatically than higher animals.

Three facets of the overall approach have been pursued experimentally. One project has involved a further study of the methionine activating enzyme. This is a continuation of the earlier studies which have for years been one of the main lines of investigation of this laboratory. It has a direct bearing on the problem of alkaloid biosynthesis since, as it is well known, many alkaloids are highly methylated compounds. It is a reasonable extrapolation of presently available data to state that the methionine activating enzyme may be universally present in living organisms. It catalyzes the formation of S-adenosylmethionine, a compound which supplies the driving force for most, perhaps all, transmethylations occurring in nature. It has been clear for some time that this enzyme and the compound it forms may represent a unique solution to a general biochemical problem. The elucidation of the mechanism whereby this enzyme works has been a difficult and at times discouraging task. However, very recent results strongly suggest that the key to the solution of this mechanism is now at hand. Indeed, it is possible that study of the mechanism will be helpful in suggesting means to study the mechanism of action of a variety of other enzymes.

A second project has been the study of the enzymes immediately involved in hordenine synthesis and their regulation. Considerable information has been gathered as to these enzymes and methods have been worked out for their ready and

sensitive assay in crude and purified forms. Methods have been developed also to assay the levels of the alkaloids which these enzymes synthesize. Data is now being systematically gathered in an effort to understand the interplay of physical, nutritional, chemical, and hormonal influences which regulate these enzymes with the hope of gaining insight into cellular regulatory mechanisms which participate in the biological phenomena occurring in this system. It might be pointed out that hordenine biosynthesis involves a pathway which is entirely analogous from a biochemical point of view to the pathway of epinephrine biosynthesis in the mammal. Thus, a study of the regulatory mechanism involved in hordenine biosynthesis may shed some light on one of the important problems of neurophysiology, namely, the regulation of the biosynthesis of the neurohormones of the adrenal medulla.

The third project represents an attempt to develop new tools to take advantage of the special opportunities offered by plant material for the study of regulatory mechanisms in cellular differentiation and morphogenesis. This project is an attempt to develop the technical means to make available a variety of plant tissue systems ranging from isolated embryos and isolated organs to tissue cultures and ultimately and hopefully to single cell cultures. The advantages which would accrue from availability of a series of such increasingly and progressively simpler plant tissues are quite apparent. The development of such a system requires a solution of a number of very general problems in plant physiology which may, however, be of very considerable importance to other phyla as well.

In the Section on Cellular Regulatory Mechanisms during the last year, it was found that the purified phenylalanine hydroxylating system catalyzes a new type of reaction, oxidative dehalogenation. The reaction shows the same enzyme and cofactor requirements as does the conversion of phenylalanine to tyrosine. The reaction may provide a useful handle for studying some details of the mechanism of the hydroxylation reaction.

During the year work continued on studies of the mechanism of norepinephrine synthesis by adrenal enzymes. It has been found that the highly purified enzyme can catalyze the hydroxylation of epinine, tyramine and phenylethylamine. The hydroxylation of epinine may represent a step in a physiologically important alternate pathway for epinephrine biosynthesis.

Attempts to produce mentally retarded rats by feeding phenylalanine and related compounds have continued during the year. The approach appears to be promising and studies are in progress to determine the specificity and duration of the effect.

A major problem in biology today is the mechanism of cellular differentiation; the mechanism by which a single cell can give rise to the diverse cell types which comprise a functioning adult organism. Viewed at one level, the problem of differentiation is a problem of genetics, one that involves the dynamic interaction between gene and and cytoplasm; where the activity of the gene is regulated by cytoplasmic constituents and where, conversely, the composition of the cytoplasm is determined by genic activity. The fruit fly, drosophila melanogaster, in many respects is a very suitable organism for studying the effect of genes on development. Taking advantage of the opportunities provided by the "sabbatical leave" program at NIMH, a study of the enzyme, tryptophane pyrollase in drosophila melanogaster was initiated last year in Professor E. Hadorn's department at the University of Zurich. The reaction catalyzed by this enzyme, the conversion of tryptophane to kynureine, is on the normal pathway for eye-pigment synthesis. When this reaction is blocked, as is believed to be the case in the mutant, vermilion, eye pigment formation is abnormal. However, when this mutant is subjected to partial starvation during a critical period in its development, the metabolic lesion is repaired at least to the extent that the phenotype approaches normal. A widely accepted but completely untested hypothesis explains the "starvation effect" by postulating alternate pathways of pigment formation. Whatever the mechanism, the starvation effect appears to represent an experimentally accessible system where the interaction between genetic and environmental factors in determining phenotype can be studied.

A micro assay for the enzyme was devised sensitive enough to determine activity in a single fly. Using this assay, the enzyme was studied as a function of developmental stage in the normal animal. Activity could be detected several days

before eye pigment synthesis began. The enzyme was shown to be almost completely missing in v mutants, confirming at the enzyme level the site of the metabolic block. Starvation of the mutant was found to lead to about 3–5% of the wild-type enzyme activity. This low activity can apparently lead to an almost normal amount of eye pigment formation. While this finding renders the older alternate pathway theories unlikely, the mechanism by which starvation leads to or uncovers enzyme activity remains to be explored.

The work of the Section on Proteins has been almost exclusively devoted to the protein synthesis project. As was outlined last year, the relationship between the mechanisms of protein synthesis and ribonucleic acids is one of the outstanding problems in biology. At least three types of RNA are known to participate in the complex and incompletely understood series of interactions whereby the genetic information carried by DNA is made available for the determination of the amino acid sequences of proteins. Microsomal particles, which are rich in RNA, have long been known to be the site, or one of the main sites, for the assembly of peptide chains, and it has been thought that microsomal RNA plays a key role in this process. Soluble RNA (S–RNA) is thought to function in the process as the carrier of activated amino acids, and it is known that there is a variety of molecular types of S–RNA, each specific for a particular amino acid. A third type of RNA, messenger RNA, is a metabolically active RNA; its molecular size is larger than that of S–RNA, and it has base composition similar and presumably a base complementary to DNA. The exact nature of the interaction between messenger RNA, ribosomal RNA, and amino acyl S–RNA is largely unknown, but it must depend in part on the base composition or sequence (or both) of the various amino acyl S–RNA molecules. Thus, studies of base sequences in S–RNA may give some indication of how information is encoded in the base sequences of DNA and messenger RNA and how the information is decoded in the process of determining the amino acid sequences of proteins.

Considerable progress toward the difficult problem of the determination of base sequence in S–RNA has been achieved by the development and use of a variety of techniques. In particular, we have explored the specificity of ribonuclease obtained from *Aspergillus oryzae* and utilized this enzyme for a study of the distribution of guanylic acid in S–RNA. It has been found that certain oligonucleotide sequences containing guanylic acid occur in amounts which considerably exceed the theoretical values which could be expected if the bases were arranged in a random sequence. Moreover, it has been shown that non-randomness prevails throughout the base sequence of the average S–RNA molecule. Two important implications follow from this finding, namely: (1) that the average S–RNA species, and hence each individual S–RNA species, must have a completely defined base sequence; and (2) that S–RNA must be replicated during synthesis by some very exact process which preserves this highly non-random structure.

We have continued exploration in the enzymology of nucleic acids and of S–RNA in particular and are making satisfactory progress in the characterization and purification of several enzymes in potatoes and *Aspergillus oryzae* which may be very useful in our sequence work.

It is now well established that most S–RNA preparations are a mixture of molecular species with a varied spectrum of amino acid acceptor activities. Recent work on the fractionation of these molecular species has in fact revealed the extremely heterogeneous behavior of the S–RNA toward a variety of procedures such as countercurrent distribution, displacement chromatography, etc. From earlier work of this laboratory, the conclusion has been reached that the molecular weight of S–RNA is not a significant parameter in determining its biological specificity. We have continued our studies on the physical properties of S–RNA and have investigated in depth the hydrodynamic size and shape of S–RNA molecules. We have explored a variety of techniques for separation of amino acid specific S–RNAs and have achieved some progress in this direction. As indicated in earlier reports, it is not expected that any single project or experimental approach will lead to a breakthrough in this area, but it is hoped that the small progress in a variety of related areas will have a compounding effect and allow us to continue our advances at an accelerated rate.

Although the individual projects can still be classified as belonging to the four main categories

listed in the initial paragraph of this report, and in the reports of the last several years, it is becoming apparent that a somewhat larger and more ambitious design is developing through the spontaneous interaction of the scientists in the laboratory, namely, an interest in explorations in the area of developmental genetics and chemical embryology. This interest is still in the very early formative stages, but it is gratifying that a common interest, a subculture, as it were, exists in the laboratory and it may be expected that considerable benefits may derive from this to all concerned.

In our work we have benefited from the continued association with Dr. Maxine Singer of the National Institute of Arthritis and Metabolic Diseases, Dr. Theodore Goodfriend, formerly with the Endocrinology Branch, NCI, Dr. Edward Jerome of the Laboratory of Psychology, NIMH and of Dr. Kentaro Tanaka, Visiting Scientist from Japan.

LABORATORY OF NEUROBIOLOGY

Scientific Program

Physical Analysis of Excitability

Excitability is one of the most fundamental and general characteristics of living systems. All plant and animal cells share certain features of cell membrane excitablity which control the passage of ions and food and secretory products. The entire neuromuscular and neuroglandular apparatus involved in behavior depends upon mechanisms intrinsic to membrane excitability. Scientists in this laboratory, in collaboration with Visiting Scientists from Sweden and Japan, are studying membrane excitability through the use of the giant axon of the squid, the plant cell Nitella, neurons in amphibia and vertebrates, and are analyzing various physical, chemical and mathematical models as well as the thermodynamics of artificial membranes. Successful perfusion of the squid axon with preservation of its membrane excitability over a period of hours has been achieved. Various means for spreading out the time course of action potentials and for separating portions of the chemical response of normal action potentials have been exploited. Recent developments in the mathematical treatment of irreversible thermodynamics and a convergence of techniques and concepts in physics, physical chemistry and physiology have contributed to the development of greatly improved insight into mechanisms of cellular excitability.

Studies in this laboratory indicate that permeability of the squid axon membrane is not very different for sodium and potassium ions, although current theories assume this cannot be the case. The approximately equal movements of these two cations obtains whether their radioactive isotopes are applied in or outside the axon in measuring permeability. The flows of radioactive sodium and potassium ions are approximately equal also during action potentials. Considerable experimental evidence indicates that the resting potential is not a pure diffusion potential, as presently held, but is mainly a phase-boundary potential. The action potential may then be accounted for by an exchange of cations attracted to fixed negative charges borne within the membrane itself.

Neurophysiology of Glia and Dendrites

Both glia and neurons have been studied electrophysiologically under direct vision in tissue culture in collaboration with the Department of Anatomy of the University of Texas. Previous measurements in this laboratory on glia membrane resistance and slow potential responses following stimulation have been confirmed and extended.

It is possible to stimulate separate parts of the dendrites of neurons under direct visual observation. The dendrites are found to be excitable and will conduct all-or-nothing impulses. The rate of conduction is slightly less than 1 percent of the rate of conduction along large axons. Contemporary concepts that dendrites are not capable of conducting impulses are based on far less direct and explicit evidence.

Brightness Discrimination

Under increasing illumination, bright objects tend to appear increasingly bright, whereas dark objects tend to appear increasingly dark. A formal mathematical analysis of this phenomenon, which has been dealt with until now only in vague and very complicated physiological and psychological terms, has been accomplished. A relatively simple mathematical model will account for this phenomenon. The essential component is a

stimulus averager used for reference purposes. A simple physical apparatus which will reproduce the apparent discrepancies observed in brightness discrimination can be constructed according to this conception. Interestingly enough, the same model, with very minor modifications, will satisfy qualitatively certain other phenomena of visual experience, such as Mach bands, edge effects, and disappearance of a stable retinal image.

The usefulness of mathematical and physical analogues for biological systems is becoming widely recognized. Pioneering and exemplary efforts to demonstrate by means of mathematical and physical models the mechanisms of the cochlea have been recognized by the award of a Nobel Prize this year to Professor Georg von Békésy at Harvard University.

Prolonged Sensory Stimulation

A number of cats having electrodes implanted along the auditory pathway, and some of these with additional specific operative interventions, have been exposed to prolonged loud white noise. It is evident that there are distinct differences in response among various ganglionic stations along the auditory pathway. There are vast differences in these responses according to whether the animal is awake or anesthetized. Anesthesia slightly reduces background activity in the cochlear nucleus, but this reduction is more marked in the superior olive, and especially the inferior colliculus. Anesthesia induces an only moderate reduction of background activity in the medial geniculate body, whereas in cortex there appears to be an increase in background activity when the animal is anesthetized.

There are substantial changes in dynamic response of various stations along the auditory pathway during and following prolonged auditory stimulation. At the round window, cochlear nucleus, superior olive, and inferior colliculus, there is a sharp, large amplitude increase in electrical activity associated with onset and a decrease associated with discontinuation of loud white noise. Following onset, however, there is a gradual slow rise in activity during continuing stimulation. This rise occurs under control of the middle ear muscles, acting through a reflex arc that is made non-functional by anesthesia. Certain other dynamic intracerebral controls are exercised by forebrain mechanisms. Analysis of such control systems adds to our insight into the processes involved in perception, memory and learning. Techniques devised for this investigation can usefully be exploited in the examination of a wide variety of sensory, motor and visceral systems.

Psychological Effects of Prolonged Sensory Stimulation

A beginning has been achieved toward studying stimulus gradient differences in rats exposed to prolonged sound stimulation. The goal is to determine, following such exposure, whether the animals may perceive a persisting stimulus after the stimulus has been discontinued and whether they may perceive sounds less discriminatively than normally after prolonged stimulation.

Sensory and Cerebello-Cerebral Pathways

An experimental means has been established to compare responses conveyed along the classical trigeminal (face area) sensory pathway with responses conveyed along a cerebello-cerebral pathway of comparable length. Both of these circuits relay through the thalamus, by way of different nuclei. Both paths project onto the same general area of frontal cortex. The experiment is arranged so as to provide insight as to whether sensory pathways are modulated independently and differentially from other projections. If the trigeminal sensory is found to be modulable independently from the cerebello-cerebral pathway, inquiry will be directed to learn whether this sensory modulation is dynamically responsive to previous sensory experience along that same and other sensory pathways. If there is interdependence of control exercised over two supposedly distinctly different functional systems, inquiry will be directed to what may be the mechanisms affecting such generalized control.

Sensorimotor Conduction Systems

Although much is known from an electrophysiological point of view about segmental reflexes, much less is known about reflexes which link together segments at different level along the neuraxis. Although a long descending propriospinal pathway was recognized nearly 20 years ago, studies in this laboratory pioneered in demonstrating that there is also an ascending interlimb

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reflex pathway. This is weaker than the descending pathway and requires either strychnine or the facilitative influence of the brainstem reticular formation to be evident. Both the descending and ascending propriospinal systems are rather diffusely projecting, that is, they influence both sides of the cord, with impulses crossing, recrossing, and crossing back again, decussating rather abundantly at each level of the cord.

Another long intersegmental connecting system discovered in this laboratory involves delayed reflex responses. This system is not contained entirely within the spinal cord: it includes a supraspinal loop through the bulbar reticular formation. The conduction pathway for this delayed reflex system projects from each level of the cord to the bulb, whence it returns to the cord and affects all levels of spinal motor outflow. Conduction latency from lumbar regions to upper segments of the spinal cord is thus paradoxically shorter than for the delayed response to return to the lumbar segments whence the reflex originated.

Excitation of cranial sensory nerves gives rise to reflex effects in other cranial nerves, as well as along the entire spinal motor outflow. Similarly, spinal sensory activation yields motor responses from the various cranial nerves. The relationship of these long interconnecting input-output systems with each other and with the brainstem reticular formation has been considerably analyzed.

This study has been carried out in collaboration with another pair of Visiting Scientists from Sweden and Japan and one from Switzerland.

Academic Activities

The laboratory staff has undertaken to study and discuss together the scientific contributions of a number of distinguished physiologists and psychologists interested in brain mechanisms and behavior. Our attempt is to obtain a clear grasp of the individual's major contributions, develop a critique of these contributions in the light of more recent developments, and document a résumé of our discussions as to the significance of the scientist's professional contributions within the general area of the intellectual pursuit of this laboratory.

About 15 individuals from this and some other laboratories and institutions in the Washington area participated in a working seminar once a week in the evening during the spring. The subject undertaken was neurophysiological mechanisms relating to perception. About forty informal papers were prepared, delivered, and discussed in this seminar. Members of this laboratory collaborting with a representative of the Laboratory of Neurophysiology, taught a course during the Fall semester in the NIH Graduate School, entitled Neurophysiological Mechanisms Relating to Problems in Neurology and Psychology. All members of the laboratory participated in national, and a few in international meetings and several have given invitational addresses on behalf of Federal and non-Federal agencies and universities interested in research being conducted at the NIH.

LABORATORY OF NEUROCHEMISTRY

Section on Physical Chemistry

During 1961 our section continued its research on biopolymers. Our ultimate objective is to determine the relationships between the biological functions of these polymers and their physicochemical properties.

Studies were continued on natural and synthetic nucleic acids and proteins, and of aggregates of dyes bound to these polymers. Particular attention was given to the aggregation of acridine orange bound to deoxyribonucleic acid (DNA), the properties and structure of the proteolytic enzymes, Nagarse (Subtilisin C) and gamma-chymotrypsin, the structure of complexes made from polymers of deoxyadenine, deoxythymine, inosine, cytosine, and deoxy 5-bromuracil, and a wide variety of plant and animal acid polysaccharides such as ganglioside, heparin, and chondroitin sulfate. In general, studies were carried out on the isolated polymers, although the structure of DNA inside bacteriophage and of the hypothesized two-dimensional acid polymer in the squid giant axon membrane were studied *in situ*.

A variety of techniques were employed in the course of these investigations: Gels containing polynucleotide complexes were stretched into fibers to align the long-chain molecules parallel to each other in order to determine their structures by X-ray diffraction. Bacteriophage particles were

oriented in a rapidly flowing medium in order to measure the orientation of the enclosed DNA by dichroism measurements. Geometrical asymmetry in the environment of the tyrosine residues in Nagarse was investigated by optical rotation measurements. The structural relationships of neighboring dye-binding sites in the squid axon membrane were studied by measuring spectral shifts in axon-bound dyes. These experimental studies were complemented by theoretical investigations of the quantum and statistical mechanical bases of the observed phenomena. In certain cases all of the above methods were applied to a single system, such as dye-DNA complexes.

These studies have produced a number of discoveries and new methods during the past year. It was found that the enzyme Nagarse as obtained commercially is contaminated by impurities produced by self-hydrolysis. A method repurifying the enzyme while inhibiting this cannibalistic tendency was developed so that structural studies may be continued with chemically homogeneous material. Conditions were found under which the enzyme can be reproducibly crystallized, making possible X-ray diffraction analyses. Preliminary X-ray diffraction studies indicated the crystal structure of the protein is very complex and therefore work on this problem has been deferred. The specificity of the hydrolytic action of the enzyme on small peptides has been found to be similar to that of chymotrypsin, and the sequence of amino acids surrounding the active site of the enzyme has been found to be the same as in most other proteolytic enzymes. A method for isolating mutant subtilisin was developed which we anticipate will provide a type of subtilisin with an altered amino acid sequence about the active site.

Other X-ray studies in this laboratory have shown that the synthetic polydeoxyribonucleotides containing adenine and thymine and adenine and 5-bromouracil form structures that are the same as natural DNA. Under different conditions new structural forms have been observed and a detailed examination of their molecular configurations is being continued.

Flow studies of bacteriophage have shown that the DNA within the head of the virus is partially oriented. DNA in the head of this virus is injected into the host through a narrow passageway in the virus tail during infection. If the DNA were not at least partially oriented in the virus head, injection would probably be considerably hindered by entanglement, snarling and knotting of the long DNA molecule.

Studies of aggregation of dyes bound to the squid axon membrane indicate that the dye-binding sites are probably less than 10–15 Å apart. It was discovered that whereas the dye-stained axon is stable and active in the dark or in the resting state in the light, it is rapidly inactivated in the light while conducting action potentials. These results provide further experimental evidence for the recently developed theory that axon membranes are perm-selective cation exchangers as well as an indirect photodynamic method for investigating the relation between the membrane binding sites and the biochemical processes responsible for nerve action.

Studies of dye aggregation on natural and synthetic acid polysaccharides have resulted in new methods for determining the concentration of these substances in very dilute solutions, for their identification in unknown mixtures, and for their characterization as to composition and physical structure. Of particular interest is the discovery that brain ganglioside behaves as a simple polyanion under the proper conditions. This work is of considerable importance to carbohydrate chemists working with these, as yet, poorly characterized polymers. From a public health point of view these results are important in providing a method for identifying and characterizing substances such as the plant extractives, the carrageenans, which are widely used as food additives.

Recently it has been proposed that dyes can bind to DNA by intercalation between the base pairs. It has further been proposed that the mechanism of mutagenesis of the acridine dyes involves intercalation. An X-ray diffraction study of oriented fibers of acridine orange-DNA and proflavine-DNA has been carried out in which no direct evidence for intercalation was found. The results are consistent with a model in which the dyes bind on the exterior of the DNA molecule.

Optical rotation studies of acridine orange-DNA complexes led to the discovery of an induced anomalous optical rotation phenomenon called a Cotton effect in this system. By a systematic,

statistical variation of the distance between neighboring dye molecules on the polymer it was possible to deduce the kinds of interactions between two asymmetrically related dyes which cause the induced optical activity. This work provides a new method for varying the distance between mutually interacting units in an asymmetric system in a semicontinuous manner and investigating the effect of such variation on the magnitude, sign and dispersion of optical rotation. By providing an excellent basis for testing current theories of the origin of optical activity this new technique is now providing a better basis for understanding the basis of empirical optical rotation procedures now widely used in the study of the structure and structural changes in biopolymers.

Quantum mechanical studies at various levels of theoretical sophistication on biopolymers and dye-polymer complexes have been carried out. Application of the simple strong-coupling, card-pack stack, exciton band model to the acridine orange-DNA system showed good agreement between the observed and computed spectral shifts. More elaborate theoretical calculations were initiated which cannot be completed until the coordinates of the bound dye have been determined by X-ray diffraction analyses. The basic theory of light absorption and rotation by polymers was extended and applied to the calculation of the optical properties of all types of bio and synthetic polymers. This new theory provides a firmer basis for understanding the reasons why the interaction of light with a given molecule changes when the molecule becomes one unit in a polymer.

Quantum mechanical calculations and experimental data have been brought together by further development of the statistical mechanical theory of the distribution of dyes on a linear array of binding sites.

A new area of interest for the laboratory developed during the year in the initiation of Hückel molecular orbital calculations using high speed computers. In principle this technique permits the calculation of the chemical and optical properties of any molecule in terms of its simple chemical formula. Calculations have been made of transition moment directions in the acridine orange-DNA system and the results were used in conjunction with experimental dichroism data to determine the orientation of the dye on DNA and the magnitude of the interaction between neighboring dyes. With these calculations the laboratory entered a new field of research which has recently produced numerous provocative theories of enzyme action, biological redox catalysis, and carcinogenesis.

LABORATORY OF NEUROPHYSIOLOGY

Investigators in the Laboratory of Neurophysiology, NIMH–NINDB are working on a great variety of fundamental problems of the nervous system. Projects range from detailed analysis of muscle membrane to the behavior of the South American monkey (Saimiri sciureus).

The investigations of the Section on Membrane Physiology into the fundamental processes that control the activity of the membranes of nerve and muscle fibers have been directed towards two objectives. Much data have been accumulated from which it appears likely that there is another pathway for potassium movement across the membrane of muscle fibers, in addition to those pathways that have been studied previously in both nerve and muscle fibers. This pathway is not necessary for the production of electrical excitability and appears to be connected to a space through which only a limited amount of potassium can flow. An intriguing possibility is that these data show the effect of potassium movement across the walls of that internal space in muscle which is the sarcoplasmic reticulum. Some internal conducting structure that leads from the surface of the fiber is necessary to activate the internal contractile molecules after only a short delay following the electrical activity at the surface membrane. The first objective was to incorporate these data into such a model with characteristics determined by data read at the start and at long times after the start of a driving force on potassium that was applied across the membrane. It was possible to obtain an estimate of the change in membane potential for intermediate times by solving equations that were defined by the model. These mathematical solutions agreed well with the data and show that such a model is a physical possibility. The second objective is to see if the model can be related directly to the sarcoplasmic reticulum. It is toward this more difficult objective

that the work of the section on Membrane Physiology is now directed.

The Section on General Neurophysiology has several projects underway. The project on intracellular analysis of hippocampal pyramidal cells was terminated for the present and the principal investigators were invited to present their work at the Collegue International du Centre National de la Recherche Scientific Physiologie de l'Hippocampus, Montpellier, August 24–26, 1961 and the Fifth International Congress of Electroencephalography and Clinical Neurophysiology, Rome, Italy, September 7–13, 1961.

A project on pH measurement of the cortex has been very successful. It has been demonstrated, previous reports to the contrary, that there is no obvious change in pH concomitant with the reaction of spreading cortical depression. It is now proved that such a change, if it exists is so small that separation of that factor from various other electrical masking phenomena is impossible. Work on this problem has led to interesting considerations of, and experiments on, important current questions of blood brain barrier and extracellular space. This devolves on the Gesell phenomenon; the injection of bicarbonate into the blood stream results in an acid shift on the opposite side of the blood brain barrier because the CO_2 diffuses rapidly across the barrier and the bicarbonate remains in the vascular system. The system of measuring the pH change is reliable to 0.05 of a pH unit, hence the 80 second transient of the acid shift can be used to test changes in the blood brain barrier.

It has been shown that the main part of the Gesell shift is not altered by topically applied metabolic inhibitors but is abolished by alcohols of one to five carbon lengths. The results agree well with Broman's work using trypan blue and extends the alcohol series to the limit of five. It has also been shown that the Gesell phenomenon is probably not dependent on the fact that bicarbonate penetrates cells very little, if at all. This follows from consideration of two situations with markedly differing extracellular space. The adult cat has an extracellular space as determined by sodium thiosulphate of 2–4% and the kitten of 16%. The Gesell phenomenon occurs in the kitten also. This lends considerable support to the hypothesis that the blood brain barrier is in the capillary wall.

Another project of this section is concerned with analysis of synaptic transactions in the lateral geniculate nucleus. This is part of the general program on visual system problems which have always been under investigation by this laboratory. This immediate project is an attempt to analyze the mechanisms of the striking phenomenon of second subnormality discovered several years ago by workers in this laboratory. The synaptic transfer reaction is monitored by an electrode in the lateral geniculate nucleus. Application of a tetanus lasting 15 to 20 seconds produces a brief depression followed by an interval of post-tetanic enhancement which is, in turn, followed by a decrease of synaptic transfer reaction to below the normal value. This depression lasts for hours. This phenomenon was previously shown to occur under barbiturate anesthesia and not to occur when the brain stem is sectioned under ether and the reaction is tested after release from anesthesia. When this is done no second subnormality was observed when the original work was done in this laboratory several years ago. The dependence of the state of anesthesia on this reaction has been denied by another laboratory, however. Hence the point has been re-examined using implanted electrodes in chronic cat preparations. Our original observations have been fully confirmed. Further study of this reaction is underway and should contribute useful information about other long lasting changes in the nervous system.

A new device for retrieving signals from noise has been developed and put into trial operation. There are several important applications in physiology as well as possible use in other communication problems.

The Section on Limbic Integration continues with its intensive investigation of one of the highly organized but phylogenetically old parts of the brain, the limbic system. These studies range from observational to electrical and anatomical studies of sexual behavior and physiologic mechanisms of the squirrel monkey. Investigations are under way on the general efferent connections to this area as well as the efferent systems important to preservation and perpetuation of species. For the first time afferent pathways in the brain stem

which are involved in the reproductive process have been identified and extensively studied. Work is continuing on these mechanisms.

The functional relation of the visual system to the limbic system has been a controversial matter for many years. The past two years these workers have demonstrated that impulses from the visual system probably enter the hippocampus by way of the retrosplenial cingulum. Another project under way is the study of central control of blood pressure. Currently this study is focused on interation of various parts of the brain and carotid sinus mechanism. An important incidental project is the preparation of a stereotaxic atlas of the brain of the squirrel monkey.

Members of this Section have given several invited lectures and the Section Chief was an invited participant for the Collogue International du Centre National de la Recherch Scientific, Physilogie de l'Hippocampus, Montpellier, August 24–26, 1961 and the CIOMS Symposium on Selective Vulnerability in the Central Nervous System in Hypoxemia, Baden (Zurich), August 27–31, 1961.

In the NINDB part of the Laboratory, several complementary projects are under way.

As a logical outgrowth of the intensive work of the past ten years, the Spinal Cord Section is conducting a program on the development of elementary neuronal reflex patterns with the aim of extending these studies into the fundamental aspects of the elementary learning mechanisms. The Section Chief of the Spinal Cord Section is working at Institute Marey, Paris, as a Visiting Scientist. He and his collaborators are working on basic mechanisms of the nervous system. One project involves the use of the Aplysia in which is found a simple ganglion with cells of very large size and large nerve cells which have no dendrites. This presents the rare opportunity to investigate the cell mechanisms with no confusing contribution from dendrites. Work is also being pursued on ionic exchanges and chemical transmitter agents in these ganglia.

Another project consists of an attempt to study processes of development of neuronal activity pattern changes and learning. This study is being initiated at a very elementary level and consists of following changes of reflex magnitudes at motor output and at sites of single cells in the cord as a result of prolonged and controlled input excitation through afferent nerves. A project on CNS mateuration is being conducted by electrical examination of the spinal cords of pre and neonatal kittens. Typical excitatory and inhibitory post synaptic potentials are obtained in the 40 day fetus ventral horn cells. The inhibitory post synaptic potentials are very prominent. In the fetal cord the time course of the excitatory post synaptic potential is more slowed than the inhibitory post synaptic potentials.

A Visiting Scientist from Brazil is embarking on a general project on the basal ganglia. This work involves further electrophysiological and neuroanatomical analysis of the complex interaction processes of the basal ganglia complex with other ganglia in the brain. Systematic studies are planned on basal ganglia involvement on such behavioral complexes as sleep and arousal in cat and squirrel monkey and this project may extend into certain aspects of conditioning and learning. The study of this part of the phylogenetically old brain complements the important work proceeding on the limbic system in this laboratory and many of the same technics will be used.

A continuing program on fundamental studies of somatic sensory mechanisms is currently specifically investigating differences in thalamic activity patterns associated with stimulation of skin receptors and joint rotation receptors. This study involves unitary analysis of the thalamic ventro-basal nuclear complex. Sensory pattern recognition and pattern discrimination is one of the continuing problems of neurophysiology of the brain.

ADULT PSYCHIATRY BRANCH

In the Section on Family Studies further progress has been made in delineating connections between individual schizophrenic illness and family patterns of interaction. Perhaps the most significant finding established in this research is that the presence and variety of schizophrenic illness can be predicted from the form of thinking in the rest of the family in which the individual patient has developed. Advances have been made in developing principles for the study and differentiation of the families of psychiatrically disturbed

patients, the program for using systematic research procedures has been broadened in scope, and a deepened understanding of the indications and contraindications for family therapy has been obtained. The foci of study in the program of the Section can conveniently be divided into four major areas: Ongoing relationship patterns within the families of both schizophrenic and nonschizophrenic psychiatric patients; individual cognitive psychology, especially the forms of thinking (thought disorder) of schizophrenics and other members of their families; developmental aspects of individual and familial functioning and pathology; and treatment problems relevant to family patterns.

In order to study the links between individual personality functioning and family patterns, the individual functioning itself has had to be regarded as a legitimate and major area of research concern in the program of the Section. Because personality disturbance in schizophrenia is so diffuse and complicated, research strategy has suggested the desirability of focusing upon the most fundamental features of the disorder. Going back to the original formulation of Bleuler in 1911, clinicians and experimentalists alike have generally agreed that the primary symptom of schizophrenia is a formal or structural disturbance which has been described in various terms such as "fragmentation of the thinking processes" and "looseness of associations." It has been shown by others and confirmed in this program that schizophrenic forms of thinking can be described in terms of levels of organization which are somewhat comparable to the development stages originally described by Heinz Werner. He showed that the development of biological forms is expressed in an increasing differentiation of parts and increasing subordination, or hierarchic integration, of the parts with respect to the whole. Thus, schizophrenics can be characterized in terms of the variety of their thought disorder ranging from highly amorphous, undifferentiated forms of thinking to detailed but highly scattered, disjunctive thinking and through a "normal" range to the excessively fixed, rigidly organized thinking manifest, for example, in certain paranoid states. The varieties of schizophrenic thinking described in these terms have been shown to be highly correlated with clinical ratings of patients along the so-called "process-reactive" continuum in which patients are described in terms of such prognostically significant features as type of onset, defects of interest, and level of premorbid skills and competence.

In the Family Studies program the form of thinking of the individual patients, as well as the other family members, have been studied and rated both clinically and on the basis of projective tests, especially the Rorschach and Thematic Apperception Test. In addition, during the current year more structured tests, especially the Object-Sorting Test, which is widely regarded as particularly useful in studying thought disorder, have been introduced into the program to study the individual aspects of ego functioning. It also seems likely that a number of techniques which have recently been used in the study of cognitive styles and "cognitive controls" will probably be highly useful in characterizing individual ego functioning in terms which are applicable to the problem of schizophrenic thought disorder on the one hand, and to the broader spectrum of cognitive functions on the other hand.

The research focus upon the thought disorder in schizophrenia seems especially strategic when an effort is made to link characteristics of the individual patient with the rest of the family. Although content themes may in some cases be shared by all members of a given family, content has been found to be an erratic approach to patient-family relationships. In contrast, the formal characteristics of thinking seem to be an exceedingly accurate and predictable basis for linking psychiatric patients and the rest of the family in which they have developed.

This conclusion was originally suggested by clinical study of some 35 families of schizophrenic and nonschizophrenic psychiatric patients, and has now been confirmed in "blind" predictive studies using both projective test materials and excerpts from family interaction in conjoint psychotherapy. These tests, particularly the Rorschach and TAT, offer a relatively standard way of sampling attention, thinking-communicating and relating during the transaction between tester and subject. The projective tests seem to be a way of studying the same kinds of stylistic aspects of thinking which have also been observed in the clinical work with the families. Indeed, we have used the same

categories, characterizing patterns of dealing with attention and meaning, for rating both projective test protocols and family therapy excerpts, two distinctly different forms of data. In both approaches judges rate and evaluate the patterns in which the families deal with attention and meaning and estimate the extent to which these varieties of family interaction might lead differentially to particular forms of schizophrenic or nonschizophrenic ego development and thinking.

Thus far, the projective test material has been studied with 22 of the families. On the basis of observations by therapists, psychiatric consultants and ward nursing staff, the hospitalized patient-offspring in 13 of the tested families was, by consensus, frankly schizophrenic; in 5 tested families the offspring was a "borderline" schizophrenic, that is, disputably schizophrenic or nonschizophrenic; and in 4 tested families the offspring was nonschizophrenic but with severe neurotic or character disorder problems. In 12 families the patient, offspring was male and in 10, female. With the exception of two families, the parents were native-born Americans, mostly middle-class, but with a sub-group of 7 lower-class families in which both schizophrenic and nonschizophrenic offspring were represented.

The procedure for the study of the projective tests of the NIMH families was divided into two parts, first, "blind" predictive descriptions and ratings about the patient-offspring on the basis of the tests of the rest of the family, and, second, "blind" matchings of patient and family. The predicting psychologist was first sent the verbatim protocols obtained by another testing psychologist from all family members except the presenting patient. The only other information given to the predicting psychologist was the family role of each family member—father, mother, daughter or son, and his or her approximate age. The task was to study each family's projective tests and to predict, first, if the deleted tests would be from a nonschizophrenic, borderline or schizophrenic young adult, and then to make more detailed descriptive predictions about the deleted offspring.

In the second phase of this study, after the predictive ratings and descriptions had been made, the psychologist was given the tests from the deleted offspring. In each group of families, the patient-offspring were all of the same sex and approximately the same age. The number of families in successive groupings were 2, 2, 5, 4, 5. The test protocols from the patient-offspring were identified by code number only, and clues which might have made matching possible on an extraneous basis were eliminated from the protocols. An attempt was made to match these individual tests with the predictive descriptions based upon the tests of the rest of the family, fitting the offspring with the appropriate family.

On the basis of a three-level diagnostic grouping, frankly schizophrenic, frankly nonschizophrenic and borderline, the predicted characteristics of the offspring compared with the independent clinical consensus as follows: Of 13 patients clinically regarded as frankly schizophrenic, 12 were predicted to be frankly schizophrenic from the test protocols of the family and one was predicted to be a borderline schizophrenic. Of four patients clinically regarded as borderline schizophrenic, all four were predicted to be borderline. Of three patients clinically regarded as psychiatrically disturbed but nonschizophrenic, one was regarded as nonschizophrenic and two as possibly borderline, and none as frankly schizophrenic. On the basis of the predictive descriptions of the offspring a more detailed series of ratings were made about the severity and form of the schizophrenic illness, and once again the predictive ratings were in remarkably accurate agreement with the clinical consensus. In both series of predictions the correlation was statistically highly significant.

In the study in which blind matchings of patients with families were attempted, 16 of the 18 families studied in this part of the project to date were matched correctly with but one reversal. Given groups of the sizes used, the overall probability that this level of predictive accuracy would be achieved by chance is .00004. (In addition, the psychologist correctly matched 11 schizophrenic patients with their families from the protocols made available by the group headed by Dr. Theodore Lidz at Yale.)

The details of the criteria by which the projective test studies were carried out is being reported in a series of publications, and the implications of the findings have been scrutinized. In passing, it should be noted that it is tenable to postulate that thinking disorders which occur in both parents

and offspring could develop in this pattern by virtue of either biologic or social heredity. Certain data in this project are, however, more adequately explained by experiential factors than by genedetermination. For example, we have found that a higher order of predictive accuracy is possible if the family's social organization or constellation is taken into account than if one makes such predictions on the basis of individual parental characteristics, including individual parental thought disorder. It seems that the particular ways in which family members team up together may produce a net transactional pattern that differs from what might be expected from looking at the kind of thinking found in individual family members. Partial or minimal disturbance in one family member seems to be fed in some cases by minimal disturbances in others so that the resultant disturbance may be quite intense and qualitatively differ from that in any individual. In other family constellations the family contribution of one family member seems damped down by the other family members rather than accentuated.

The ways in which the family constellation, or "social system," needs to be understood in order to link individual personality functioning with familial factors has been a consideration of importance not only in the projective test studies but in another variety of predictive studies of families using excerpts from family therapy sessions. In the excerpts studied thus far the presenting patient is nonparticipant with no extraneous clues about his diagnostic characteristics. The working hypothesis has been that the style of thinking and communicating shown by the parents in relation to the therapist will be significantly similar to that which the parent shows with the presenting patient.

The methodological problems in using this kind of material have been examined, and two comparison studies using excerpts are being carried out at present. The data consist of transcripts and tape-recordings of six five-minute excerpts from the conjoint family therapy with each of 14 matched families, seven with a schizophrenic and seven with a neurotic offspring. In one comparison study of this material small details of interaction have been reliably rated from the transcripts; the frequencies with which various interpersonal maneuvers are used can then be compared. In the second study the style of the family transactions is assessed more globally from the tape-recordings of the excerpts and predictions about characteristics of the offspring are made directly. These studies are still in progress, but the preliminary findings in both indicate that predictive differentiations can be made from the patterns of family maneuvers in the conjoint family therapy sessions.

Another study of parental interaction patterns which is in a pilot stage is the use of the structured Color-Matching Test developed in the research with normal marital pairs in the Bio-Social Growth Center of NIMH. The form of interaction and decision-making of the parents of schizophrenic and neurotic patients is being compared with that of the normal pairs.

Another significant problem which involves the concept of the family as a differentiated, organized social system is the comparison of patient and so-called "well sibling" within the same family. A criterion for selection of families intensively studied in this program has been the presence of a sibling of the patient offspring, and a continuing study is being made of the differences between these individuals within the same family, both in surface characteristics and in terms of more underlying characterological features.

The third major research focus in the Section on Family Studies, in addition to individual cognitive psychology and familial relationship patterns, is on the developmental aspects of both individual and family functioning. This research focus has been increasingly in the forefront in the Family Studies program during the year and will be receiving increasingly greater importance in the future. The primary emphasis in the program in the past has been to establish links between the current functioning of patient and family, and with the achievement of positive results on this problem, it is now appropriate to begin to trace backwards and study longitudinally the development of these individual-family patterns.

One phase of this study involves the study of neonatal characteristics and early childhood personality features in both the patient offspring and his siblings. In this study we have been collaborating with the staff members from the Bio-Social Growth Center who have been working on the neonatal characteristics as reported both by par-

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ents and as observed directly. From the retrospective standpoint we are attempting to characterize similarities and differences between patient and siblings going back to the earliest phases of life, looking for differences in such characteristics as sleep patterns, vigor and integration of sucking, tactile and emotional responsiveness, etc. The impression emerging from this clinical material is that the siblings can be differentiated even in their early years in these terms. Such neonatal differences may well contribute to the characteristics of the transactions within the family and help determine the particular place in the family's social system, that is, the role, of one offspring compared to another.

It also seems quite clear that the history of the experience of each offspring growing up with the same family differ in terms of the quality and constancy of the psychological availability of the parents. It appears that the potentially schizophrenic child has characteristically been especially needed and used by the rest of the family as a healer or savior, who spares the rest of the family from confronting certain troublesome difficulties and anxieties.

In addition, it is apparent that there are developmental phases in family life which can be considered in their own right and which affect the quality of the environment to which each offspring is differentially exposed. These are some of the conditions which seem to be significant for ego development and which can be formulated in researchable terms. The research group is presently formulating the intrafamilial conditions at each development phase, especially the kinds of emotional attachments and the kinds of identifications, which lead to successful or unsuccessful ego development, including schizophrenic or nonschizophrenic forms of thinking.

Understanding of these developmental considerations pertaining to family life and psychopathology may be enhanced if given a cross-cultural perspective in which those features of family patterns which seem to have a spurious connection with individual pathology may be sifted out. Currently, consideration is being given to the selection of cross-cultural studies which might make a substantial contribution to understanding of these issues.

The fourth distinct focus in the Family Studies program is the problem of psychotherapeutic technique in working with families. For the past four years conjoint family therapy has been used as a major tool for establishing relationships with families. It is a continuing impression that this approach not only contributes to the research data but also has distinct advantages as a treatment procedure with selected families. Conjoint family therapy is not regarded as panacea which could replace individual psychotherapy or other modes of treatment, but judging from the experience with patients and families who had previously frustrated the efforts of competent therapists, it is felt that this approach can facilitate therapeutic movement in families who are locked in reciprocally stifling or destructive forms of interaction. In the past year reports have been prepared about the varieties of families who seem particularly suited for family therapy, the stages in the therapeutic process during which conjoint sessions may be especially indicated or contraindicated, and the setting and conditions for conjoint family therapy which seem to affect the quality and progress of the therapeutic effort (for example, the effects of concomitant individual therapy, the use of co-therapists, and the varieties of treatment goals that may be adopted). Widespread interest in the technique of family therapy has been apparent at national and international meetings. The experience in the Adult Psychiatry Branch with this approach, which is perhaps as extensive as that had by any group in this country, particularly with the families of schizophrenics, will be reported in a series of publications.

Experience has also been gained on the use of family sessions in which the family participates conjointly in art therapy. The forms of perception and thinking manifest in the art productions of the family members both when they work on separate paintings and when they join together on the same painting have been examined. Both the art productions themselves and the family's interaction about them have contributed to the diagnostic evaluations and treatment program.

The Section on Personality Development has been engaged for the past three years in a study of personality disturbance and identity formation

in middle and late adolescence. Interest has centered on the processes and mechanisms by which the new potentialities which emerge in the individual in early adolescence become relatively stabilized. Basic to this group's empirical research on adolescence has been a conceptualization of the central psychological issues in this developmental phase. Going beyond the formulations of Sigmund and Anna Freud who emphasized the intensified drives and heightened defensive operations occurring in adolescence, Erik Erikson has elaborated a psychosocial viewpoint in which a consolidation of the adolescent's concept of himself occurs through an integration of his altered capacity to conceive of himself, of the new potentialities which have emerged within him, and of the ways in which he is identified by significant others. Other implications for the understanding of this period of identity formation have appeared in the work of Inhelder and Piaget who have demonstrated that the thinking of the adolescent differs radically from the thinking of the child. Logical structures undergo a characteristic cognitive development in this phase, from the concrete operational thought of the child to the formal operational structures and propositional logic of the adolescent.

In this study of personality development in adolescence a situation has been designed which may elucidate these and related issues in identity formation. In the past year systematic investigations had concentrated on the experience of disturbed adolescents with their families. The hypothesis has been studied that a relationship exists between the manner in which parents identify adolescents and the forms in which identity formation take shape in the adolescent. Efforts are being made to test this hypothesis and to elucidate the dimensions and characteristics of this relationship.

Adolescents are studied who have encountered sufficient emotional difficulty in the first year of college to make it necessary that they withdraw from the college situation. The college situation is conceived as one which makes demands on the adaptive capacity of the late adolescent in a number of developmental areas. It makes an increased demand on the adolescent's cognitive abilities. It is a situation which poses a necessity for alterations in emotional investments, with increased reliance on contemporaries of both sexes, because these young people are having their first prolonged separation from their parents.

These adolescents are hospitalized in an open ward of the Clinical Center and, as a part of the therapy program, are seen with their parents in weekly family therapy sessions. Currently, recordings of these sessions are being analyzed in the following way: Areas of discussion are noted and classified which focus on recent or current endeavor on the part of the adolescent. The interaction is then studied between the parents and the adolescent during those discussions which contain delineations of the adolescent by his parents. The term delineation is used to designate parent-adolescent interaction which reveals how the parents feel and think about and identify the adolescent.

By noting the kind of parental delineations which follow the introduction into a family session of a significant current situation in the adolescent's life, a picture is gradually obtained of the dynamics and characteristics of the delineations in which the parents communicate to the adolescent their image of him. As the variety of current situations discussed in each case increases, the parental response to an increasing range of adolescent behavior and endeavor is observed.

Categories of situations and activities in the life of the adolescent are being established which help point to differences in parental delineation from one category to another in the adolescent's behavior. This gives more precision to the questions of what areas of generalization and what areas of specificity can be found in parental delineations around different categories of adolescent endeavor, in which families does a generalized attitude predominate, and in which families do specific categories give rise to a particular kind and style of delineation. It seems possible to compare the relative integration and consistency within delineations from one category to another in the individual case, and relate the nature of these to areas of pathology in the adolescent.

Having established the configuration of parental delineations of the adolescent with reference to a spectrum of current endeavors, the project staff plans to study how this relates to the picture of

adolescent personality functioning obtained in other research interviews and clinical material elucidating the adolescent's college experience.

Thus far, the parents and the adolescent patient of ten families in which the patient was unable to accomplish adequately the developmental tasks inherent in adaptation to the college experience have been observed in regular family sessions. One generalization which can be made about these families is the following: there is in all of them a describable lack of integration in the ways in which the parents delineate the adolescent patient. This lack of integration may be seen in extreme variability in the delineations of the patient as studied over time. Moreover, marked discrepancies appear between conscious attitudes and delineations of the patient and other parental behavior from which contradictory unconscious attitudes and delineations can be inferred.

The project staff is now working with the data in an attempt to elucidate to what extent the quality, as well as the discrepancies and lack of integration in the parents' delineations of the adolescent patient has affected the stability and integration of ego identity in the patient in such a way as to interfere with adaptation and the accomplishment of developmental tasks of adolescence.

The Field Study Group in the Section on Personality Development is now in a period of transition after having completed an initial exploratory pilot study of a group of healthy adolescents in transition from high school to the freshman year of college. Further work is now being planned in the area of personality development during the late adolescent period. In the initial project, students were selected from a group of volunteers at a local high school on the basis of their meeting certain criteria for competence; namely, in their academic work, in their ability to relate closely to a peer, and in their ability to participate in social groups. The sample included 15 students who were regarded as meeting the criteria and an additional five who did not meet the criteria in all areas, who were also studied as a sub-group. The project relied mainly on interview data obtained from the students while they were in high school, during the summer preceding college and spaced throughout the freshman year of college. The parents of the students were also interviewed on two occasions prior to the students' departure and after the Christmas vacation. A final joint interview was conducted with the student and his parents at the completion of the freshman year. During the summer of 1961, a sophomore year interview was conducted with as many of the original subjects as were available.

In organizing the data about how students coped with the anticipation of college while in high school, the following conceptual scheme was utilized: first, a delineation of general personality attributes which had a useful function in dealing with new experiences and change; second, patterns for maintaining a self-image as adequate to the perceived requirements of the new situation; and third, mechanisms for maintaining within manageable limits the distressful effect connected with the transition experience. For these "competent" students, attitudes of pleasure in dealing with newness, effective patterns for matching the self-image with what is anticipated, and mechanisms for mastery of anxiety reinforced one another and provided a general basis for confidence in meeting the situation ahead.

For these students, the decision to attend college represented an internalization of parental and sociocultural expectations. However, they exercised considerable autonomy in the choice of the particular colleges for themselves. The application procedure involved a process of self-assessment, along with an assessment of characteristics attributed to the college. Certain patterns in the choice of college have been delineated.

It has been discovered that a useful approach to the problem of separation from parents was to focus on the separation as an opportunity for the development of autonomy, rather than to delineate specific coping mechanisms for dealing with the separation experience. By including the data from the group of students regarded as less competent in high school, the total sample was divided on the basis of whether or not students were able to utilize the separation from parents as an opportunity for further development of autonomy and also whether or not this coincided with a feeling of continued positive relatedness

with parents. The project staff has been able to generalize these impressions about parent-child interaction among three groups of students.

The pattern of parent-child interaction of the students rated high in autonomy and high in relatedness were revealed as follows: these parents impressed us as distinct people and tended to present greater clarity about their values and standards to the students. Parents valued and fostered the growing autonomy of the child. They were able to tolerate more experimentation on the part of the student within the framework of family standards, making it clear that the child would have to assume responsibility if he moved out of this framework. They regarded the student as ready to take over responsibility for himself. There was a clear definition of boundaries between the parent and child and a value placed on respecting privacy.

The converse of these statements would apply to the parent-student relationships of those students who were regarded as low in autonomy and low in relatedness to parents.

In a third group of students who were rated as high in autonomy but low in relatedness to parents, we felt that the parents were less able to accept the students' departure from previously assigned roles in the family.

At the present time, the Field Study Group is developing a method for utilizing interview data with college students for assessing autonomy as one aspect of ego functioning. The developmental task in the late adolescent period, as defined by Erikson, has to do with the crystallization of a sense of identity. The Field Study Group wishes to study one aspect of this task; namely, the establishment of oneself as an autonomous person, which they regard as having a prominent place in personality development during this life period. They wish to explore how interview material and psychological test data may be used in assessing and following students through this developmental period. Such methods will then be used in studying other variables which may be related to the emergence of increased autonomy, such as patterns of parent-adolescent interaction.

During the spring and summer of 1961, interviews were conducted with nine college students who were at NIH as normal control volunteers or as part of their work program from Antioch College. These interviews are now being reviewed in developing a scale for rating autonomy among college students. Psychological test material obtained from each subject will be explored as additional source material in assessing each subject's capacity for autonomous behavior. During the coming months the focus of the work will be to attempt to develop operational definitions of the concept of autonomy, as well as exploring methods for assessing this through interview and psychological test data.

Another group within the Section on Personality Development has also been studying the transition from high school to college viewed as a research setting for the study of coping behavior in personality development. On the basis of intensive interview data with 14 of the students selected in the Field Study project during their senior year of high school and followed throughout the freshman year of college, this group has found that there is no unitary pattern of coping behavior in the adolescent's strategies for learning to manage various intellectual tasks, either with regard to the total learning situation or to the specific courses to be mastered in his new academic environment. A variety of coping strategies have been shown to operate which are related to the student's academic potential and his personal goals and values. A research aim has been to highlight the range and salience of as many coping behavior characteristics as we can identify in one or more students in our sample. The purpose is heuristic—to search out psychosocial variables in diverse, even if singular, effective strategies of coping behavior in the academic experiences of freshmanhood.

This research group has also formulated a general conception of coping strategies on the assumption that ego-expansion and ego-strengthening processes affect the psychosocial development of adolescents as they work through intellectual tasks during the critical transitional experience of the college freshman year. A coping strategy, in this sense, involves two major processes—maintaining a sense of worth in the new social environment and managing anxiety in the face of complex, accumulating intellectual demands of the new college culture. To maintain a sense of worth in transition experiences involves what has been described as anticipatory mobilization. The indi-

vidual is ready to mobilize inner personal resources to meet the new demands—especially his capacity for effectively doing meaningful work, for actively seeking out problem-solving opportunities, and for working out diverse sources of intellectual gratification outside the strictly formal academic curriculum. By treating academic competence as the independent variable—and various patterns have been identified—the research group can examine the possible developmental consequences for the transition from late adolescence to early adulthood.

To assess more systematically the coping behavior potential of adolescents in transition from high school to college a new psychological instrument, a Student TAT, consisting of ten college situations, has been developed. The Student TAT is a quantitative method for measuring student coping behavior, defined as adolescent competence in problem-solving in specific, potentially stressful and problematic college freshman-life situations. The instrument decisively differentiated between three groups of freshmen—a group of freshmen who were hospitalized for emotional difficulties experienced in their freshman year at college, normal state university freshman, and exceptionally competent freshmen who had been selected independently for behavioral competence on the basis of intensive interview data.

A tentative generalization on the evidence of the quantitative results obtained is as follows: (a) The Field Group of competent, normal freshmen project a view of college culture wherein problematic situations are manageable, and see the college student as coping with these problematic situations through active effort and optimism. (b) The Ward Group of disturbed, hospitalized freshmen show a markedly different pattern in comparison with the Field Group. The Ward Group characteristically project a view of college culture in which problematic situations yield few, if any, solutions and see the college student as characterized by relative passivity and pessimism. (c) It also has been determined which scenes proved to be the biggest discriminators between either of two normal groups and the Ward Group, on each of three categories, Solution, Activity, and Favorableness.

Currently this research group is completing a systematic evaluation of the properties of the Student-TAT instrument—its validity, its reliability, and its predictive and discriminative potential in the assessment of freshmen coping behavior. They are interested in using the Student-TAT as a screening instrument to predict student vulnerability to the stress of transition to college and are conducting such a study with dormitory residents in a local state university. The Student-TAT was administered to 300 freshmen in regular academic standing three weeks before college registration. Then 40 students were selected—all who were rated high and low on the problem-solving categories of competence. This scoring was done blind, with the students' identifying characteristics, except for sex, deleted. These students were interviewed during their freshman college year—once after their mid-semester exams in the Fall term, and once in the Spring term before their final exams. An interview schedule was used which focused on college life situations affecting major areas of academic, social, interpersonal, heterosexual behavior. These interview data are now being coded on similar categories of competence along the relevant dimensions tapped by the Student-TAT. The same range of variables will be scanned in both the Student-TAT ratings and the interview data ratings. Thirty students, seven of whom were among the original sample, were also followed up in similar interviews (modified for practical purposes) to provide natural history data of the student drop-out phenomenon in this college setting.

One purpose of this study is to gain natural history data on various patterns of coping with the transition experience during the freshman year. Another specific objective is to determine the predictive properties of the Student-TAT in assessing coping behavior potential of college-going adolescents.

Another research setting for the study of stressful experience in personality development is the cultural transition of overseas students entering American universities. A pilot survey was undertaken to gain research leads into the emotional problems of African and Asian students attending American colleges, and their adaptive and maladaptive behavior in response to new cultural and academic demands. The goal was to obtain insights into the pertinent factors affecting the African and Asian student's management of

problematic tasks in a new cultural environment. Twenty university health psychiatrists and social scientists were interviewed who were known to have had professional and intimate acquaintance with African and Asian students through the counselling or treatment situation. The interviews were tape-recorded and summarized by the topical areas discussed by this panel of informants.

The major findings of this pilot survey, based on recurrent themes in the observations made by these university health staff members, mostly psychiatrists, and documented by case history examples are: (a) The importance of recognizing certain cultural factors affecting the African and Asian student's perception of professional psychiatric help and affecting his orientation to the use of psychological insights as a way of problem-solving. (b) The importance of making correct diagnosis of crisis reactions which often simulate psychosis—e.g., certain forms of projection are more culturally acceptable in the overseas student's home society than in the U.S. and these must be carefully distinguished from paranoid schizophrenia. (c) The difficulties of making accurate prognoses for young people in university settings generally. (d) The importance of not relying primarily on deep psychotherapy as the method of choice in treating these overseas students.

To test the validity of the Student-TAT cross-culturally, a collaborative study has been started with the Puerto Rico Institute of Psychiatry which, through a special NIMH grant, is replicating our coping behavior study using the same testing and interview procedures and similar criteria for selecting competent adolescents. Twenty Puerto Rico competent adolescents were selected in the senior high school year and have now been followed up throughout their freshman year in the University of Puerto Rico. Six students enrolled in American colleges in continental U.S. The possibilities of a similar cross-cultural replication in India are now being explored.

These Student-TAT and interview data are now being analyzed along the lines of investigations conducted in the pilot study, with the additional purpose of identifying the cultural factors which affect the mode of coping with problematic tasks of college adolescents in other cultural settings, (a) in coping with new and maturing independence drives toward autonomy in decision-making, (b) in coping with tasks of making and maintaining close interpersonal relations in a new setting and (c) in coping with new intellectual demands.

It is expected that the results of this longitudinal short-term developmental study of competent adolescents will clarify significant socio-cultural and personality variables affecting the competence of adolescents in coping with the developmental transition from late adolescence to early adulthood.

In the Psychosomatic Section work has continued upon the investigation of stress and concomitant changes in various hormone levels. With the completion of some of the work on mild stress and endocrine correlates, attention has been directed to a detailed examination of earlier reports of elevated levels of urinary 17-hydroxycorticosteroids in severe retarded depressive reactions. In addition, endocrine responses of normal subjects in severe, prolonged stress situations are being examined in a group of parents whose children have leukemia. An area of work new to the Psychosomatic Section is being developed with the specific focus being neurochemical correlates of behavior. The laboratory for this work has recently been completed and investigative efforts begun.

One of the techniques used for the study of endocrine changes has been the hypnotic induction of various affective states including relaxation and the correlation of these affects with various endocrine responses. The formal aspects of data collection for this work has been completed. Those subjects who experienced sensory distortions during hypnosis were extremely susceptible and reported hypnotically induced affects equal to or surpassing the most intense experiences of that nature in their lives. These same subjects also showed with at least one affect significant changes in their blood level of UFA (unesterified fatty acids) and dramatic falls of 17-hydroxycorticosteroids during induced relaxation. Subjects who were not rated as extremely susceptible to hypnosis did not show intense affect changes nor endocrine responses under hypnosis. In addition to hypnotic susceptibility, other variables related to the intensity and quality of the induced

affect were prior experience with that emotion; mood at the beginning of the hypnotic induction, and feelings towards the hypnotist.

In the study of depressed patients a reliable and valid fifteen-point scale for quantifying nursing observations of behavior has been developed. This has allowed the development of a program for the longitudinal study of depressed patients and concomitant endocrine responses. Confirming earlier findings, it has been found that taken as a whole depressed patients have urinary 17-hyroxycorticosteroid levels quite outside the physiological range with a few patients excreting three to five times the amounts normally seen. In general, it has been found clinically that depressive reactions fall into two subgroups; those who remain in a relatively stable state and those with marked fluctuations in the severity of their illness. In this latter group urinary 17-hydroxycorticosteroid values also fluctuate and seem to correlate positively with changes in specific emotional states, namely depression and anxiety. Data on urinary epinephrine and norepinephrine levels have also been collected but not as yet analyzed.

The study of the parents of children with leukemia has developed rapidly in several directions. The study of endocrine responses in these subjects confirm earlier findings of marked sex differences in leveles of urinary hydroxycorticosteroids. Too, study of this group indicates that a given individual has an idiosyncratic, relatively constricted range of urinary 17-OHCS levels and that superimposed acute stress situations do not seem to greatly shift a subject from this range. Psychological factors which may be associated with persistent high or low steroid levels are being investigated through the use of projective techniques and clinical interviews.

This same group, i.e., the parents of leukemic children, are also being used for the study of psychophysiological (finger pulse volume and palmar conductance) correlates of hopelessness and grief. From clinical observations it has long been suspected that these affected states may be important in the development of disease. Specifically, it has been hypothesized that the physiological concomitants of hopelessness will be opposite to those which have been observed to accompany anxiety and hence render the person less able to biologically defend against a variety of potentially noxious agents. Preliminary findings tend to support this hypothesis.

Since the discovery that the psychological state of dreaming is associated with very distinctive physiological changes during sleep, the conception of this phenomenon has undergone some striking developments. Most surprising in the context of previous assumptions, it now appears that a quite fixed and substantial amount of dreaming is an intrinsic part of normal sleep, occurring every night in every sleeping person, continuing for lengthy periods, and recurring with the regularity of clockwork. Not only is it inexorably regular and predictable in its occurrence, but recent evidence indicates that in some sense it is also a necessary and perhaps vital part of our existence. Over the past few years the Section on Stress has substantiated the observations upon which these conclusions are based, and has explored many of their ramifications.

Although inadequately studied, disturbances of sleep have long been associated with mental illness, particularly at its onset. This naturally raises a question as to whether disturbances of dreaming might also be characteristic of the mentally ill, or possibly implicated in their disturbance. With this in mind a comparison study of the physiological sleep patterns of normal subjects with those of hallucinating and non-hallucinating schizophrenic patients was started. The procedure involved the continued electroencephalographic recording from each of twelve patients over five or more consecutive nights. The intention has been to classify and tabulate the sleep patterns to be found in these records so as to provide data regarding the amount and distribution of dreaming as compared with similar data from normal subjects. This original objective has been thwarted by the fact that the records of the schizophrenic patients are quite difficult to compare with those of the normals. The electroencephalographic recording goes far beyond clinical observation in revealing that their sleep is extremely disturbed, with frequent brief awakenings, and almost unceasing fluctuation and change in the physiological patterns. This means that periods of dreaming, which in the normal are lengthy and continuous, in the schizophrenics are fragmented and interspersed with anomalous patterns. Provocative as these observations are in themselves,

they make it very difficult to arrive at an estimate of "dreaming time" in the schizophrenic patients comparable with that which is easily achieved in the normal. Consequently the aim of data analysis has now become that of simply describing these records in objective, quantitative and reliable form in order to focus upon their detailed differences from the normal. This has necessitated the development of a revised scoring system for the EEG sleep patterns, and continuing efforts to achieve inter-rater reliability in its application.

Such long overdue attention to the pathology of sleep raises many basic questions concerning the descriptive physiology of normal sleep. The concept of sleep depth, for example, is obviously of crucial importance in the assessment of the schizophrenic's sleep, but there is no generally accepted index of this parameter. For this reason the second line of endeavor which we pursue is an attempt to contribute to these basic questions. Over the past year we have adapted existing techniques of physiological recordings to the special requirements of sleep study and have developed equipment for the electronic reduction of the vast quantities of polygraph data obtained in sleep studies. In the near future these technical innovations will be applied to the descriptive study of normal sleep, with the objectives of further characterizing the differences between dreaming and non-dreaming sleep, as well as searching for less ambiguous measures of sleep depth than those currently available.

The experiment in training mental health counselors continued during the year. The object of this project is to test the hypothesis that carefully selected, mature people can be trained in two years to do a limited kind of psychotherapy. Since the existing professions will not be able to fill the need for low-cost psychotherapy in the foreseeable future, other sources of relatively inexpensive manpower must be sought along the lines of the recommendation of the Joint Commission on Mental Illness and Health. One of the greatest unused reservoirs of superior manpower in our population is that large group of intelligent, married women who around age 40 are looking for some constructive activities to take the place of the job of child rearing. Many of these people possess outstanding psychological skill. The need for more low-cost therapy could be alleviated if this gold mine of psychological talent could be utilized.

This project consists of four phases: recruitment; selection; training; evaluation. The first two phases have been completed and were described in last year's report. The training is in process, and evaluation of the first year's work has been completed. The training during the first academic year, 1960–61, which was described in last year's report, consisted of practical training and interviewing with individual and group supervision, observation of interviews conducted by others, lectures and seminar discussions, outside reading and report writing, and placement in community mental health facilities. The program of the second year began with an opportunity for the trainees to become acquainted with a mental hospital by spending ten working days at St. Elizabeths Hospital in a program designed to have them participate fully in the activities of that institution. In addition to a program of seminars on conceptual and technical problems in psychotherapy, each trainee this year will see an average of eight patients for one hour a week, and each will receive individual supervision of her therapy. Also, a group of eight adolescents meet for group therapy once weekly, with a psychiatrist as therapist and one trainee as co-therapist, with the other trainees observing, and the whole process discussed in a supervisory conference. All trainees spend at least one day a week working in a community agency, and six of them spend an additional day in a second agency.

The problem of the evaluation of the first year's work was approached in five different ways. First, in an effort to obtain an objective judgment of the work of the trainees, uncolored by the personal investment of the teachers in the project, the services of four raters from outside the Washington area were obtained. Without knowing anything about the program or the background of the trainees, the raters made blind ratings of tape-recorded therapeutic interviews on a five-point scale on eight criteria, as well as on global impression. After listening and rating each tape, the raters opened a sealed envelope which contained an auto-criticism of the interview by the trainee herself. This was also rated on several criteria as well as on global impression. The average

rating on the global impression of all the interviews was 3.0, that is, in the middle range of the scale. The average rating on the global impression of the auto-criticisms was 3.6. The individual trainee ranged from an average of 2.0 to 3.5 on the ratings assigned to the global impressions of their interviews, and ranged from an average of 2.8 to 4.5 on the ratings of the global impressions of their auto-criticisms. Since no one's average rating was below 2, or passable, and since 3, or the middle range of the scale, represents satisfactory performance, this part of our evaluation procedure has shown positive results.

Second, the changes which took place in the patients seen by the trainees at NIH were assessed, with consideration given to the type of patient and the degree of difficulty of the treatment. There were in all 49 patients, 18 males, 31 females; 21 adolescents, 28 adults. Each trainee saw an average of seven patients on a once-a-week basis. The diagnoses were distributed as follows: 20 personality trait disorder, 12 neurotic reaction, 6 schizoid or borderline schizophrenic, 5 immature or unstable personality, 4 adjustment reaction of adolescence, 2 diagnosis doubtful. None of these patients changed for the worse. In 19 there was no change. Seventeen showed slight improvement; ten showed moderate improvement; three, marked improvement. In evaluating the results it is important to remember that 69% of the patients were considered by experienced therapists to be "difficult or very difficult to treat," and that the length of treatment at the time of our evaluation was in no case more than six months, and on the average, ten weeks. That the patients themselves were favorably impressed is demonstrated by the fact that of those who came asking for help, at most one dropped out of therapy.

Third, supervisors in the community placements were asked to rate the trainees who worked with them. The average rating is "good," and none is lower than "satisfactory."

Fourth, the trainees themselves were asked to evaluate the program. They did this in several hours of discussion with the teachers and in one hour of tape-recorded discussion with no teacher present. Each has written in a personal vein what the program had meant to her with the understanding that none of this would be used without the author's permission.

Fifth, the teachers in the program have reported their impressions.

Final results on this project will not be available until after its completion in June 1962, but it is possible to state at the present time: (1) that the selection procedures were satisfactory in that no candidate has either dropped out or been disqualified by the staff. (2) That after one academic year, the trainees can perform useful therapeutic services if they are carefully and thoroughly supervised and if they have the support of the group. How they will function with less supervision and with little or no support from their own group is as yet unknown.

To the extent that the project is successful it may provide a model for the development of similar training programs in universities or other teaching centers. This would help alleviate the manpower shortage in the mental health professions. It would also suggest lines of development which might be followed by the existing professions in their training of psychotherapists.

CHILD RESEARCH BRANCH

Program Summary

The 1961 annual report of the Child Research Branch covers the second year's operation of our longitudinal research program on the initial stages of family formation, a program which was initiated in 1959. As was described in last year's annual report, the program began with three projects: a study of neonatal patterns of behavior which is a continuation of earlier work in the Laboratory of Psychology, a study of the later expressions of these neonatal behavior trends in the third year of life, and a study of initial marriage. During 1961 these three projects have been gathering data which will be reportable between late 1962 and 1964. As our experience with data has accumulated in all three projects, considerable methodological refinement has taken place throughout the program as well as refinement of concepts and hypotheses. This year's report will describe these methodological refinements and changes in our conceptualization of the research problems.

Early in the year a control study of the seventh month of pregnancy was developed which will

serve to illuminate the meaning of changes encountered in young couples between the period of initial marital adaptation (at four months after the wedding) and the neonatal adaptation by the couple to their first infant. Toward the end of the past year a new study of the neonatal adaptation process between young parents and their first infant was initiated. The project design has been initiated and the project itself should get under way early in 1962. Fortunately sufficient experience has accumulated in the two closely-related projects on the congenital characteristics of neonates and on the husband-wife relationship that it has been feasible to design a study which integrates methods and questions from these two ongoing projects in a meaningful fashion.

For substantive results coming from the program during the past calendar year, we can point to the replication of the earlier finding with neonates that there is a sex difference in muscle strength. On this second sample of infants the pattern of oral integration was also confirmed; this pattern is defined by rate of sucking, amount of negative pressure produced in the nipple, regularity of sucking and intensity of the hunger cry when the feeding is interrupted. It would thus appear that two of the three congenital factors which emerged in the first study of neonatal behavior are worthy of continuing investigation.

Efforts over the past two years to devise a formal model within which to express the complex interactional relationships between developmental variables which are changing through time have resulted in a scheme derived from systems theory. Some effort will be made in this annual report to present this model, although it will not be possible to do more than to suggest the uses to which we put the model in our work.

Behavior Continuities in the Infant and Preschool Child

There seems little doubt now that muscle strength assessed in the first week of life is a sex-linked variable. Exploring its relation to later maturational and family phenomena seems worthwhile theoretically and feasible since with both girls and boys we are now in a position to assess sex-linked and non-sex-linked behavior patterns in the third year of life. The clear elimination of all brain-damaged subjects from the sample and the careful control over level of arousal during the assessment procedure means that this finding is particularly interesting. Indeed our experience with both *muscle strength* and *oral integration* agrees with the experience of others on the importance of level of arousal as a control variable in assessing the rooting reflex; like the rooting reflex, muscle strength and oral integration only become clearly visible when the infant is sufficiently aroused.

It seems conceivable that the level of oral integration in neonates may be an expression, in part, of some persistent aspect of libidinal or aggressive drive intensity. Should this general notion be in some manner borne out by the data, the neonatal mode of initiating and sustaining mouth contact might be related to later stages of pre-ego and early ego development. As pointed out recently by Benjamin, an understanding of the nature of stranger anxiety and separation anxiety in the first year of life depend upon concomitant assessment of libidinal drive intensity, aggressive drive intensity, and the maturation of specific ego apparatuses.

While at this time the data on neonatal skin sensitivity contains a variety of unsolved problems, it is true that shifting the area of assessment from the heel (which was originally selected for reasons of accessibility to the examiner without disturbing the baby) to the abdomen has provided us with a far more sensitive body surface. This increased sensitivity in itself suggests that an anterior body surface relates to the skin-communication situation we are interested in observing with the neonatal family more directly than does the less sensitive locomotor surface of the heel. A newly designed cradle to hold infants during the testing procedure permits us to obtain a clearer baseline level of arousal. Continuous observation has been substituted for time sampling. The addition of the parental questionnaire which formerly was used only in a retrospective fashion with the nursery school, but now is being used in a non-retrospective fashion with the infant sample, represents an important methodological step toward integrating the prospective with the retrospective study.

In the investigation of children in the third year of life, a variety of new observational cate-

gories have been added as a result of experiences with the first year of the project; continuous recording techniques have substituted for time sampling to increase the interpretability of the data. The chronological homogeneity of the sample has been improved since we now limit selection of subjects to a six-month age range (from two-years-and-three-months of age to two-years-and-nine-months). The finding that boys in this age group who were reported by their parents as having been skin-sensitive infants, made more frequent contact with the teacher in the school is provocative. This finding held even after our new *maternal availability index* was applied as an intervening variable. This index is a composite of a number of elements in the family situation which would tend to make a parent more or less available and thus influence the child's need for contact. It includes (1) the total number of children the mother is caring for, (2) the average number of years between each child, (3) the number of years between the subject and his next older sibling and (4) the number of years between the subject and his next younger sibling. This trend in the data implies that neonatal skin sensitivity may be one early developmental contributor to dependency behavior in the preschool child.

An important matter which is of basic concern to the program is the question of exclusion of subjects. From the beginning of our study of preschool children, any children who have a history suggestive of even mild brain damage, or of any developmental disorder, psychological or organic have been excluded from the program. In the beginning, however, we excluded only severely emotionally disturbed children. The first year of the project taught us that two types of children do not show any significant range of behavior on our measures, namely fearfully shy children, and impulsive aggressive children. Therefore, during the past year two teacher rating scales have been filled out twice a week on each of our subjects during their stay in the nursery school laboratory, one rating scale for fearful shyness, and the other for impulsive aggressiveness. In a given week, if a child shows a high score on either one of these rating scales, the child is excluded as a subject that week. Approximately one out of six subjects has been excluded from at least a few observations on this basis. The matter of exclusion of a significant number of subjects from the data on a behavioral basis raises theoretical as well as methodological questions. There is always the possibility that we are eliminating a significant proportion of children with high aggressive drive, for example, and that such elimination of a group which may represent a theoretically salient subgroup will prove unwise. Nevertheless, throughout the program, faced with the choice of whether to study extreme variations in adaptive behavior or midrange patterns of adaptation, we have consistently selected the range of expectable-to-mildly-disordered behavior as being more relevant to our interests in basic personality formation. As Emanuel K. Beller has observed in studying preschool children, the identical variables will show systematic interactional differences depending upon whether the setting contains conflict or is conflict-free. Our assumption in the cases excluded from the data for shyness or impulsiveness is that significant family-generated conflict behavior is being expressed in the nursery school.

The shift to female subjects in the nursery school this year has led to using a more differentiated behavior schedule in order to reflect the greater differentiation of female behavior at this stage. Whereas boys oriented more of their behavior autonomously toward toys and aggressively toward peers, the girls showed greater tendencies toward socialization or shyness, more complex cognitive responses and greater verbal capacity. Does this greater feminine differentiation of behavior in the third year of life partly account for the fact that in the retrospective questionnaire four factors emerge from parental ratings rather than the two factors found with boys? At the moment, it remains a significant problem to the program that a somewhat different set of factors emerge on the retrospective questionnaire of infant behavior on the females as compared to the retrospective reports on the male infants.

To summarize the work of the program aimed at delineating early life maturational channels, we have evidence to suggest that muscle strength is a sex-linked variable in the first week of life. It also appears that the integrative functioning of the oral apparatus is not related to the sex of the child. Current data may provide further clues to the significance—if any—of neonatal skin

sensitivity to early ego development, as well as to the relationship of retrospective parental reports to direct observations of infant behavior.

Interpersonal Patterns of Adaptation

During 1961 exploration of interpersonal adaptations to the initial stages of family formation has proceeded in several directions. The pilot study of the fourth month of marriage has provided an opportunity for exploring our methods. Concepts to describe developmentally-relevant aspects of the marital relationship have been clarified. A study of the first pregnancy has been designed. Planning for the study of initial parenthood during the first neonatal phase has been started. The approximate schedule of program developments is attached (see Appendix A). Appendix B contains a diagram illustrating the model, to be described below, which guides our planning for the analysis of the marital interaction data, and which appears to have heuristic value for the longitudinal program.

Methods

During the past year several methodological refinements have been introduced. Twelve coding categories have been worked out, for the *color matching technique*, some of which have achieved acceptable levels of reliability. Ratings made by the interviewers of general attributes of each marital relationship we assess have been evaluated for reliability by a special study in which both interviewers participated. In a study of six couples, reliability of ratings ranged between r's .58 to .95, with a median of .75. Currently methods are being developed for assessing from the interview material the variable of *cohesion-disruption* and other similar variables of the marital relationship in the seven *areas of interaction* (Food, Housekeeping, Relatives, Friends, Occupation, Affection and, Parenthood.) *Questionnaires* have been scored and data collated for a computer analysis of interrelations among marital role perceptions by husbands and by wives. With regard to the *improvisations*, the conflict-inducing situations in the couples are assigned incompatible intentions, this year two staff workers developed a manual for scoring some forty behavioral items. This manual was developed on the basis of experience with pre-pilot and early pilot couples. Since these items involve highly qualitative interpretations of marital modes of conflict-resolution, certain data will be analyzed by means of consensual group ratings, whereas with other items independent codings and ratings should be feasible.

Marital Relationships

During 1961 some sharpening of hypotheses about the developmental tasks of initial marital relationship has been possible. A limited group of variables have been under consideration and hypotheses formed as to alternative patterns which may exist under various developmental conditions. These variables include *effectiveness of dyadic decision making*, *effectiveness of dyadic conflict resolution*, the seven *areas of marital role interaction*, types of *role participation*, the differential *salience*, or value placed on each marital role by each sponse, and the concept of *cohesive versus disruptive forces* in the marital relationship. Progress has been made toward making operational the concepts of conflict-resolution effectiveness and decision-making effectiveness. As one way of specifying the network of hypotheses related to the variable of conflict-resolution effectiveness, two contrasting couples were selected for clinical study, one couple selected on the basis of observed ineffectiveness in the color-matching data and in the improvisation data and the other selected on the basis of observed effectiveness of conflict resolution. All records on these couples were examined and discussed in order to identify data which was consistent or inconsistent with the judgment of effectiveness of conflict-resolution.

It may be useful by way of illustration to mention some general questions which have high priority for guiding data analysis during the coming year. The project is committed to study several aspects of role participation by husbands and wives in the seven major areas of interaction, and to evaluate stability or instability of role participation in these areas at three points in time: the fourth month of marriage, the seventh month of pregnancy and the neonatal period after the birth of the first child. We distinguish between *segregated marital role functioning* in which only one spouse is active in the area (for example, if the wife does all the purchasing, preparation and serving of food and the husband has very little to do

with food, we would say the food area is segregated). Couples in which both members participate but in which the participation is separate in time, or divided up into unshared activities, exhibit a pattern referred to as *joint-separate*. Areas in which both sponses participate together in the same activities we refer to as a *shared role* pattern. One thesis we plan to examine is whether a higher degree of role-sharing early in marriage permits the couple to adapt more successfully to later role changes at the time of initial parenthood. In other words, does the common middle class stereotype of an initial period with much role-sharing actually have adaptive value in our culture, which lacks the sex role stereotypes of other cultures, and demands a good deal of role selection by couples at the outset of each new developmental stage. Another question we are interested in is the extent to which there are common shifts in participation by husbands and wives in the various areas from the earlier to the later stage, shifts which may correspond to culturally-defined role changes.

The concept of cohesion—disruption in the marriage refers to the interaction of three aspects of the relationship: level of conflict in the marriage, level of satisfaction in the marriage and level of affective involvement. The concept may be applied to the relationship as a whole or only to one area of role participation. Since this dimension may have systematic relationships to effectiveness of conflict resolution in an area, and to salience of the area, it is challenging. For example, a couple who is observed—relative to other couples—to experience a *high degree of disruption in the relationship as a whole* may, quite consistently with this observation, demonstrate *low disruption and high cohesion* in an area which is of *low salience* to the couple. We would also expect that couples with a high overall level of disruption would show ineffectiveness of conflict resolution in areas of high salience.

Pregnancy Study

The seventh month of pregnancy was selected because the experience is no longer one of "being sick" but rather of being in contact with the anticipated third person in the family inside the body.

Informal exploratory interviews by two investigators with a special group of experienced mothers and with another group of pre-test couples, taken together with the research design elements already established in the longitudinal program, served as a basis for planning. In order to control for changes in the marital relationship due simply to the passage of time, and not to the experience of pregnancy, the twenty pregnant couples will be matched with a group of twenty non-pregnant control couples married an equal length of time. The design of the pregnancy study follows the design of the study of newlyweds and includes a joint interview, an individual interview, direct observation of marital interaction and questionnaires. A major difference is that there are no home interviews. The couple's perception of the pregnant state as an influence toward role changes is investigated for each of the seven areas of the relationship. Major changes in feeling about the self as a person are also investigated. Detailed inquiry into planning for the infant is now appropriate. Both pregnant and non-pregnant control couples are questioned concerning the current areas of satisfaction and conflicts, affectional activities within the marriage and the prospective parental image they have for themselves and for the spouse. A supplementary questionnaire designed particularly for the events of pregnancy is included, as well as a short form of Bell and Schaefer's Parental Attitude Research Instrument and Schaefer's new Pregnancy Research Questionnaire. Forms of direct observation of marital conflict-resolution have been devised which follow the design of the newlywed study but are altered to avoid learning effects. With the *improvisations*, a new scene has been devised which involves conflicting parental responsibility for the helplessness of the infant. As an analogue to the color matching technique, there has been devised the *stereognostic conflict technique*. Here conflict concerns touch perceptions. A table with an opening in the top was designed. Subjects are instructed to reach through the hole and feel an object which is placed in a drawer out of sight. The subjects look at a tray of six objects sitting on a table and are asked to match the object felt in the table (but not seen) with one of those on the table. Following the perceptual task, each subject flicks a switch indicating his perceptual choice. The spouse then repeats the procedure and also flicks a switch indicating her choice. Now they begin to discuss the best match, trying to reach an agreement, their

conversation being tape recorded for later analysis in the same manner as with the color matching task. The task situation has been improved in certain ways over the color matching situation. By employing an electric switch, a non verbal signal as the selection indicator, we will know whether failures to state a conflict at the outset are due to misperception or to conscious avoidance of conflict.

Matrix of Variables

One of the objectives of the program during the past year has been to devise a formal model within which diverse relationships between variables could be conceptualized in a consistent fashion. A committee of scientists have begun to devise such a model to serve as a guide for specific data analysis procedures. This model will be presented briefly. The model must account for *state of adaptedness* at the newlywed stage and at the neonatal stage, and for the process of *change in behavior patterns* from one stage to the next. We employ the term *developmental state* to describe any set of variables in equilibrium with one another at a single point in time, or during a single limited time period. Developmental state describes the relationships among any group of variables assessed crossectionally. For example, should a systematic relationship be hypothesized between the dimensions (1) effectiveness of conflict-resolution in an area, (2) salience of an area in the couple's value system, and (3) cohesion—disruption of interactions in the area, this relationship would represent a developmental state. Developmental change results from the influence of a *transition event* on a state. The transition event is a class of experiences which all subjects have in common and which carries common social and biological implications for adaptation. For example, the wedding is a transition event between the engagement stage and the stage of initial marriage; the birth of the child is a transition event between the stage of late pregnancy and the neonatal stage. While the psychological significance of the transition event will vary from one couple to another, there are certain irreducible, common elements inherent in the meaning of a wedding, or of a birth, which may be assumed to have qualitatively a common impact on developmental state. Thus the changes which may be expected in a group of subjects will depend upon the interaction between the transition event and the previous developmental state. We refer to the press to change in a certain direction as a result of this interaction as a *developmental issue.* The developmental issue is the demand, or set of demands, to change at a given stage, acting in a specific direction on a variable, on an individual or on a system of relationships between individuals. The adequacy of coping with a developmental issue at a given stage may be considered in relation to three frames of reference. The couples may be considered as more or less adequate in coping relative to other couples at the same stage of development. The adequacy of coping may be considered in relation to the individual couple's own adaptation at a previous developmental stage. Or, the adequacy of coping may be assessed with relation to an hypothesized type of adaptation, which may be anticipated on the basis of developmental theory.

Comment

At the outset of the present program in 1959 we wrote, "Human development is influenced by a progressively changing interaction of biological and social forces, developmental change being most clearly manifested in a series of transition situations through which nearly all individuals pass. Certain phenomena reflecting this biosocial interaction in early stages of growth are considered to have high predictive power for understanding personality at later phases. The broad objective of this research program is to bring those biosocial interaction situations under direct observation, preferably as they occur in the natural course of events." We believe that during the past two years we have made a substantial beginning in the direction of accomplishing this objective.

The appropriate next steps in research planning to implement the 1959 statement of research aims and principles, will become clearer over the course of the next two years. One possibility is that the program may be in a position, as a result of the analysis of current data, to begin a community study of certain hypotheses to be tested on a larger sample of young families. It may also be possible to initiate more intensive clinical study of a few families who represent theoretically salient development patterns.

It is gratifying to anticipate a new physical facility for our work which will be designed to meet the particular needs of this program, as well as to house certain developmental studies from the Laboratory of Psychology. Planning for the new building is under way, with the hope of moving into the facility in 1963. This will provide considerably improved settings for direct observation of family interaction.

CLINICAL NEUROPHARMACOLOGY RESEARCH CENTER

The past year has seen a steady development of the collaborative program between Saint Elizabeths Hospital and the Clinical Neuropharmacology Research Center (CNRC), CI, IR, NIMH. The Hospital has formally recognized the role it attaches to research on its campus by creating, in parallel with the Clinical Neuropharmacology Research Center, the Behavioral and Clinical Studies Center (BCSC) of Saint Elizabeths Hospital. The Chief, CNRC, has been appointed by the Superintendent as Director of this Center, and is responsible to him for its administration. The Center comprises areas of research not represented within the CNRC; and serves to maximise research resources within the CNRC. Thus, situated in two adjacent buildings there are now on the campus of Saint Elizabeths Hospital basic science laboratories (the so-called Laboratory Studies Group); laboratories for experimental human psychology (the so-called Behavioral Studies Group); and a rapidly developing clinical facility (the so-called Clinical Studies Group). Whereas the Laboratory and the Clinical Studies Groups are principally supported by NIMH, the Behavioral Studies Group is sponsored by the Hospital. The coordination and direction of the overall program rests with the Chief, CNRC, who also serves as Chairman of the Hospital Research Committee.

The Hospital has assigned four residents to the William A. White Service (the so-called research service) on a rotating basis; and, as in previous years, continues to collaborate in the development of a teaching program in Research Method for the benefit of residents and others. The weekly Joint Seminar Programs continue to be extremely well attended; the crowding has, in fact, necessitated the planning of a much larger conference room. Applications continue to be received from investigators abroad for Guest places in the laboratories of CNRC; these appointments are sponsored by grants from parent institutions. At present the Center accommodates three such guest workers from Switzerland, Canada and Belgium respectively. These investigators form a highly selected and well motivated group and are considered an asset to the work of the Center.

Development of an Admission Service

As mentioned in last year's report, the Clinical Studies of CNRC have hitherto centered on problems of the care and the treatment (including the pharmacotherapies) of the chronic schizophrenic patient. This area was chosen not only because of the numerical preponderance of the problem within any mental hospital, but also because a coordinated program of care and rehabilitation was judged a necessary preliminary to the conversion of the William A. White Building (where the CNRC is located) into an active treatment facility. The appointment of the Deputy Chief, in charge of Clinical Studies, CNRC, (who also serves as Director of Clinical Studies, Saint Elizabeths Hospital, and Clinical Director of the William A. White Service) has greatly accelerated these developments. A detailed evaluation program of the resident patient population in the building with regard to clinical and social prognosis has been completed. A number of patients were transferred to other buildings, and a further small number (18) found community placement. The bed capacity of the building was reduced from 136 male and 195 female beds to 100 male and 128 female beds. By August 1st, the Service was ready to receive newly admitted patients, a function it now discharges on one day a week. In keeping with this new policy, the Hospital has agreed to refurbish the wards throughout the William A. White Building. Two wards are being set aside for intensive studies of a pharmacological and psychobiological nature.

In parallel with these developments, a Clinic, known as the William A. White Clinic, has now been established within the William A. White Building. The immediate, and empirical end of this Clinic is to provide an ambulant treatment

Center for patients previously hospitalized in the William A. White Service and now on visit status. The more important objective, however, is to use this therapeutic facility as a research instrument designed to assess clinical and social effectiveness of various therapeutic methods, (including pharmacotherapy, group therapy, family therapy, social and work counselling) in an extramural and after-care setting. The Clinic is particularly fortunate in having associated with it a strong social service staff who already is showing an imaginative approach to problems of routine care, and of research. The service is definitely community centered, a great deal of interviewing being done in the patient's home. Potentially, therefore, it could become a source of epidemiological data of the greatest interest to a program in Clinical Psychiatry.

The addition of these two components—an Admission Service and a Clinic—to existing Inpatient facilities thus marks the beginning of a reorientation of the William A. White Service toward a much needed community oriented treatment facility. The Deputy Chief, in charge of Clinical Studies has organized an excellent conference program, comprising clinical meetings on the ward, and formal case presentations. He has also initiated some preliminary pharmacological studies with new compounds, particularly a metabolite of imipramine. These studies comprise both laboratory and clinical studies; the laboratory work being done by Clinical staff working part-time under the supervision of members of the Section on Neurochemistry.

Despite these encouraging advances, it is only fair to mention some real difficulties which continue to confront the clinical program at the present critical stage of its development. The patients normally referred for admission comprise many chronic patients, who, while, perhaps, admitted for the first time to Saint Elizabeths Hospital, have, in fact, a history of several admissions to other mental institutions. The voluntary admissions remain few and far between; nor are acute admissions at all frequent. Yet, if the William A. White Service is to fulfill its assignment as an advanced and Community oriented treatment facility, much more emphasis will have to be placed on voluntary admissions directly to the William A. White Service. This will require careful education of the public, both medical and lay. The report of the Joint Commission on Mental Health and Mental Illness adds force and urgency to this view.

Furthermore, it is becoming increasingly apparent that the relatively low staff/patient ratio is inadequate to ensure the standards of nursing care, and of treatment which would be regarded as minimal for the Clinical Program of a National Research Center. Because of the constraints imposed upon it, the hospital has not so far, been able to meet the additional staff requirement needed by such a program. It is to be hoped that next year may remedy this deficiency. This will be particularly important, since it is the objective of this Center, and the BCSC, to concentrate on the study of selected patients, rather than to concern itself with large-scale operations; and to emphasize individual differences and patient populations with regard to biological, individual psychopathological, and psychosocial features.

Social Contact in a Psychiatric Ward

The Social Contact Matrix, an instrument to measure the amount and quality of social contact among hospitalized patients, was referred to in the last Annual Report. It has now received further trial, both within the William A. White Service and in a comparative multi-hospital drug study, planned by the Psychopharmacology Service Center, ER, NIMH. In the former setting it was found useful in studying the relation between the presenting symptoms of an individual patient and the social structure of a ward. As an initial step in this study, an individual rating scale, grading manifest symptoms in clearly defined terms, was constructed for each of twenty-five patients. The degree of social interaction between individual patients was assessed concomitantly, and race and level of education also taken into account. An attempt was then made, using a suitable mathematical technique, to correlate blindly symptom fluctuation between patient pairs over time. It was found that, in respect to certain aspects of overt symptomatology, subjects did, in fact, fluctuate in pairs, individual patients apparently influencing other patients in the symptoms which they presented. Social class and level of education appeared factors bearing upon this inter-

action. The study thus represents an initial attempt to measure what has come to be known as the social field in a psychiatric ward; the potential of the method in other settings (particularly in the study of family interaction) has yet to be explored. As mentioned above, the method has also proved useful in comparing social setting in the wards of different hospitals, ranging from public institutions to more exclusive small, private treatment facilities. The data of this particular study must await further analysis; taken together with others they may be of value in quantifying the role of the psychiatric ward as a treatment resource.

Schizophrenia

The problem of so-called thought disorder, be it drug induced or idiopathic, continues to be one of the principal long-term interests of the Center, and forms a natural link between it, and the programs of the Laboratory of Psychology, and the Adult Psychiatry Branch. In preparation of a long-term study of this subject, the Chief of the Section of Psychopathology was assigned for a period of ten months (September 1960 to May 1961) to the Laboratory of Professor Jean Piaget, Geneva, Switzerland, an international authority on the development of thought process in normal children. While on this assignment, he studied the ability of normal children to make use of landmarks in finding hidden objects. Similar tests were applied to schizophrenic populations, and, it is intended to continue cognate studies at Saint Elizabeths Hospital. Another study derives from the numerous reports in the literature—mainly anecdotal in nature—of the distortion of the sense of time in schizophrenic patients. An experiment was therefore initiated in collaboration with the Laboratory of Psychology to investigate the performance of chronic schizophrenic subjects on a complex task requiring integration of judgments of velocity, and of lapsed time. The task consisted in predicting the arrival of a rotating line (which had swept through an aperture of 90° and then disappeared) at target points, marked on its projected trajectory. The patients were tested once weekly. Symptoms ratings were carried out by raters unaware of the patients performance in the test situations. EEG records were also obtained during the course of the test.

A significant number of chronic schizophrenic patients were found to show progressively increasing response times with repeated testing. This change was found independent of reaction time, and found only in those subjects who showed competent performance. The trend was found to be consistent, and will now be examined in relation to other measures of time judgment. Also, in view of the availability of one extremely well studied patient group in the Schizophrenia Research Project at Ypsilanti State Hospital, Michigan, the study is to be extended to this population. It will be of interest to see how sensitive an index this measure will prove to be when compared with other measures in this population.

A further study concerns the estimation dreaming time in non-hallucinating and hallucinating schizophrenic patients. A number of neurophysiological theories concerning hallucinations would predict an increase in the amount of dream activity in the hallucinating patient. The availability of the eye-movement recording technique as an index of dream activity in man makes it possible to estimate the amount of dream time during uninterrupted sleep. Both hallucinating and non-hallucinating patients were tested. Each group of two subjects (hallucinator and control) were studied for five to ten nights; an attempt being made to secure at least six hours' sleeping time each night. While analysis has so far not revealed marked differences in total dream time, both groups were found to show evidence of markedly disturbed sleep patterns. A more rigorous method for classifying E.E.G. protocols was also developed during the course of this study. This method will be useful in future cognate investigations, since it already furnishes a high degree of inter-rater agreement.

The problem of schizophrenia continues to generate empirical attempts to distinguish schizophrenic from normal populations. Two such claims have been examined at the Center during the past year. The first concerns the alleged effects of schizophrenic serum on the concentration of catecholamines in the brain of rabbits; the second, the occurrence of a peculiar body odor in schizophrenia, which has variously been attributed

to an unidentified metabolic defect. Whereas the first day within the purview of the program of the Section of Neurochemistry, the second study arose from the presence of a suitable patient on the wards of the William A. White Building. Both studies are regarded as incidental, but are reported here for the sake of completeness.

It had been claimed (Minz & Walaszek, Fed. Proc. 17, 416, 1958) that the injection of rabbits with serum from schizophrenic patients raised the concentration of epinephrine and norepinephrine in the brain, particularly in the hypothalamus, while the injection of control serum exerted no effect. The serum of 13 schizophrenics and 14 normal non-schizophrenic subjects was therefore tested. The serum was obtained under suitable safeguards and injected into rabbits, the animals being killed 16 hours later. Epinephrine, norepinephrine and dopamine content were determined in the hypothalamus, and other areas of the brain. In 30 such experiments neither normal nor schizophrenic serum was found to exert any effect on the concentration of any of the three catecholamines. The reported results must therefore be questioned.

The other study centered on a schizophrenic patient who emanated a potent odor, which was found to vary inversely with phenothiazine medication. The medication, (trifluoperazine), was discontinued, in order to bring about the development of the odor. Daily records of weight, food intake and odor were kept. Routine blood and urine analysis were also carried out. The odor was found to be associated with the axillae and pubic area, but not with abdomen, palm, areolae, or forehead. It did not emanate from the breath. The odor of the urine was normal over the entire period of observation. All laboratory and clinical determinations were normal except for a slightly increased sedimentation rate which seemed to be at a constant level for the patient. A micro organism, *pseudomonas aeruginosa*, was found to be dominant in cultures obtained from the axilla. This organism was insensitive, *in vitro*, to most antibiotics, commercial deodorants and to pHisohex. It was sensitive to neomycin, and was eliminated after topical application of the drug. Axillary sweat collected by intradermal administration of epinephrine was found to be odorless under aseptic conditions. Considerable odor of a similar quality was produced by incubation of the sterile sweat with a purified culture of *Pseudomonas aeruginosa* taken from the patient. A control sample (without the added organism) developed much less odor. It was therefore concluded that the odor was due to excessive sweating, and bacterial degradation of the sweat; and that, at least in this case, the cause of odor was no different from that of non-schizophrenic individuals.

Psychoactive Tryptamine Derivatives

Because of their relation to naturally occurring indolic compounds, the psychoactive powers of some tryptamine derivatives, (particularly dimethyl and diethyltryptamine) are of great theoretical interest. Studies on these compounds, referred to in the previous annual report, have continued at the biochemical and behavioral level, both in the experimental animal and in man

A number of lower homologues of dialkyl tryptamine (dimethyl-tryptamine, diethyltryptamine and dipropyltryptamine) were found to be good substrates for the miscrosomal hydroxylating enzyme in the liver, being converted to a significant extent to the corresponding 6-hydroxy derivatives. The dibutyl derivative however, was hydroxylated only to a very slight extent, due to inhibition by the substrate itself; and the dihexyl derivative was not hydroxylated at all. At the animal behavioral level, a number of tests, both of aversive and appetitive nature, have shown that both the unchanged compounds, and the 6-hydroxylated metabolites are behaviorally active. It would appear, however, that these two groups of compounds may affect opposing functional modalities. The unchanged compounds were found to produce calming, tranquillizing effects; the 6-hydroxy derivatives tended to lead to behavioral excitation. The final effect of a particular compound may thus depend upon the relative ratio of the unchanged drug to its 6-hydroxy derivatives; lower homologues causing primarily excitation, and higher homologues suppressing spontaneous activity. This work is still in its initial phases; it may, however, shed light on the varying, and at times, contradictory effects of tryptamine derivatives on behavior.

At the human level, a series of double-blind experiments using diethyltryptamine, (DET) was conducted in collaboration with the Labora-

tory of Clinical Science. The drug was administered intramuscularly to 10 normal volunteers and 10 chronic schizophrenic patients. Psychological effects were assessed by interview, suitable questionnaires, and appropriate psychological tests. Urine collections were arranged at 3 hourly intervals, and urinary metabolites determined quantitatively in each sample. A positive correlation was noted between the amount of 6-hydroxydiethyltryptamine (6 HDET) excreted in the urine, and the psychological, autonomic and neurological changes produced in normal volunteers after the administration of 1 mg/kg of DET. Chronic schizophrenic patients excreted only slighly more 6 HDET than the normal controls; the rate of excretion however, was delayed, as compared with normal volunteers It should nevertheless be noted that in man (as compared with the rat) only a small portion (of the order of about 17%) of administered DET can be accounted for as urinary metabolites; possible alternate pathways must therefore be sought. Such a study is now in progress; it is being greatly aided by a program of organic synthesis carried out by a Visiting Scientist from Israel.

Chlorpromazine

The intermediate metabolism of psychoactive drugs, and the individual differences in their handling in the body, represents a principal research interest of the Center. In keeping with this trend, studies are proceeding on the intermediate metabolism of two phenothiazines, chlorpromazine and promazine. Considerable species differences were encountered in the respect to demethylation, hydroxylation, and sulfoxidation of chlorpromazine. For example, phenolic glucuronides were demonstrated in the urine of guinea pigs, rabbits and humans, but not in the case of rats. Demethylation, on the other hand, was found mainly in man. Sulfoxide formation was found in the rat, guinea pig and man, but not in the case of rabbit. Attempts to demonstrate *in vitro* enzymatic hydroxylation of chlorpromazine were unsuccessful. Demethylation and sulfoxidation, reported by others, was confirmed to occur in the presence of liver microsomes.

As an extension of this type of study, the effects of chlorpromazine sulfoxide, monomethylchlorpromazine, chlorpromazine-N-oxide, promazine, 2-hydroxy-promazine and 4-hydropromazine were compared in a number of behavioral tests in the experimental animal. These tests included an estimation of hexobarbital sleeping time, performance on stimulated motor activity (the so-called Rotarod test), and operant conditioning situations, comprising both reward and punishment schedules. In all tests it was found that chlorpromazine sulfoxide was the least active of the compounds. Chlorpromazine-N-oxide, although active, was less active than chlorpromazine or monomethylchlorpromazine. A lag in onset of activity was noted with chlorpromazine-N-oxide in both conditioned response tests. This was not seen with any of the other drugs. Monomethylchlorpromazine was generally only slightly less octive than chlorpromazine.

With regard to the promazine series, 4-hydroxypromazine was about as active as promazine. 2-hydroxypromazine was markedly less active than promazine, except in the sleeping time test. The results are consistent with the view that, at least in the experimental animal, the effects of chlorpromazine and promazine may be due in part to some of the metabolites of the parent compound; and that demethylated, hydroxylated, and demethylated-hydroxylated metabolites may be relatively active members of the series. These studies are now being taken to the clinical level, though here the lack of methods for the estimation of metabolites in body fluid is proving a major handicap. Once these are available, it is hoped to study the individual excretion patterns in man in relation to symptomatology, and individual variation in side effects, both during medication, and following drug withdrawal.

Epinephrine Metabolites

The development of sensitive and quantitative methods for the estimation of catecholamine metabolites in urine and tissues continues to be a central part of the program of the Section on Neurochemistry. The methods for the estimation of metanephrine and normetanephrine described in the previous report have been developed and reevaluated. They appear both specific and sensitive, allowing for the estimation of 0.05 μg of metanephrine and 0.5 μg of normetanephrine.

Since the greater part of these substances is excreted in the form of sulfuric acid conjugates, various methods of hydrolysis were tested and a combination of acid hydrolysis, and incubation with sulfatase, (Glusulase) was adopted as giving the best results. Recoveries of metanephrine and normetanephrine added to urine were found to average 92±11% and 73±8% respectively. Other substances for which methods of estimation are being developed are 3:4-dihydroxyphenylacetic acid (*dopac*) and 3:4-dihydroxymandelic acid (*doma*). These substances which are the unmethylated oxidation products of the catecholamines are estimated fluorimetrically after condensation with ethylenediamine. They can be separated from basic and neutral catechols with the aid of both cation and anion exchange resins. Though the sum of both acids is relatively easy to assay, the separation of the mixture is proving more difficult. Present experiments aim at attempting this separation by high voltage electrophoresis on paper. Other substances being estimated (by the modification of methods of others) include vanillylmandelic acid (VMA) and homovanillic acid. These substances are the 0-methylated derivatives of *doma* and *dopac* and are quantitatively the most important metabolites of the catecholamines in urine. A further substance of interest is 3-methoxytyramine, the 0-methylated derivative of dopamine. It can be readily demethylated by treatment with periodic acid. It is hoped to develop a method of estimation based on this reaction.

At the invitation of the NASA Space Task Group, the above methods were used in the estimation of up to six catecholamine metabolites in the urine of personnel participating in Project Mercury, including suborbital and orbital flight. To obtain valid points of reference, the normal diurnal variation was studied in each individual over four-hourly periods. Over 200 samples of urine were analysed. It was found that the response to stress, though marked in a number of cases, only rarely exceeded the variations shown by the normal diurnal curve. Possible correlations between the excretion of the amines and their metabolites are being studied at present. Other studies on catecholamines excretion patterns during a cycle of drug addiction and withdrawal, are planned in conjunction with the Addiction Research Center, Lexington, Kentucky. They are not advanced enough to be reported at the present stage.

Though not directly related to the above, the Section on Neurochemistry has also continued to examine the effects of drugs on the subcellular distribution of catecholamines. The effects of reserpine, phenylisopropylhydrazine, pyrogallol and dopa, (used alone and in combination), on the distribution of catecholamines in the brain has been previously reported. The studies of these drug effects have now been extended to *in vitro* systems. After some initial difficulties, it was found possible to observe reproducibly, the releasing effects of reserpine on catecholamines in such systems. The epinephrine released from the granular fraction is quantitatively transferred to the soluble fraction. The concentration of reserpine required, however, is high compared with its activity *in vivo;* the minimum dose causing release *in vitro* being of the order of 0.2 mg/10 ml. The effect appears to depend on the composition of the suspension medium. Of particular interest are the conditions necessary for the action of reserpine with respect to ionic and cofactor requirements. The *in vitro* system may thus prove a useful auxiliary model for the study of the mode of action of catecholamines releasers *in vivo.*

Brain Stem Neurones

Work continues to proceed in the Section of Neurophysiology on the organization of neuronal aggregates controlling respiration and vasomotor tone. As mentioned in the previous annual report, these aggregates were chosen for investigation primarily because the functions concerned are relatively well defined, and sensitive to purely psychogenic influences. Three approaches are being employed at present: The first entails the application of a drug to the medulla by arterial injection, and the study of chemosensitive areas in the brain stem by this means; the second, the application of drugs to single cells in the brain stem by means of a multibarelled micropipette; and the third the study of the influence of respiratory reflexes on the discharge of cells during inspiration and expiration.

The first alternative has involved the development of a fine bore cannula containing three sep-

arate channels, and the trial of a number of surgical manoeuvers for the insertion of this cannula into the basilar or vertebral artery of the anaesthetized cat. Localization of injection could be simplified by tying off the basilar artery, and by retrograde injection into the vertebral arteries. Photography of flow during injection was also found a helpful technical device.

Using this technique, central respiratory stimulant effects were observed following appropriate doses of epinephrine and levarterenol. These effects were not blocked by dibenzylene; nor did hypertensin exert any effect when given by this route. Acetylcholine and nicotine both caused respiratory depression. Cyanide caused hypertension and bradycardia. Acetylcholine and nicotine resulted in bradycardia and hypotension; epinephrine and norepinephrine exerted no central cardiovascular effects. Though useful in the broad localization of chemosensitive areas, the inhomogeneity of cell populations in the brain stem left any conclusions in regard to the location of specific chemoreceptors very open. It is hoped that the discrete analysis of the effects of drugs applied to single cells, using a micropipette technique, may assist one in defining the chemical susceptibilities of neurones in the area. However, the technical problems inherent in this approach (particularly those connected with movement artifact), remain considerable.

The third approach stems from the observation, made by an investigator at present holding a Visiting Scientist's appointment at the Center, that different units of the inspiratory group behave in different fashion during swallowing, some increasing and some decreasing their rate of discharge. This finding raises the important issue of specificity of function within an otherwise homogeneous neurone population, or alternatively, of possible differences of synaptic connections within these cell populations. To decide between these alternatives, a systematic study of the behavior of respiratory units durings reflex respiratory responses, brought about by swallowing, over-inflation and over-deflation of the lungs, and stimulation of carotid sinus chemoreceptors and of peripheral nerves is contemplated. In view of the sensitivity of respiration, and, indeed of the respiratory reflexes, to affective stimuli, analysis of the precise mechanisms governing discharge within these cell assemblies should provide a basis for further enquiry of a more psychophysiological nature.

Animal Behavior Studies

The effect of hormone, directly implanted into the hypothalamus, on sexual behavior in the cat has been previously reported. The precise temporal relationships of this form of behavior have been studied further, and individuality of response within a pair accurately assessed. The behavior was found to follow a strict temporal sequence. It is readily reproducible, and therefore presents a pattern particularly suitable for pharmacological enquiry. A further line of evidence is derived from the electrical activity of the brain. This has been recorded in the conscious, unrestrained cat from the amygdala, posterior hypothalamus, pre-optic region, and various areas of the cerebral cortex. Observations were made in a number of behavioral states, including drowsiness, play, alerting, eating, and sexual behavior. These studies are being linked with their pharmacological counterparts.

Work has also continued in the field of stimulus generalization. A prior finding that generalization gradients for punishment-controlled behavior are likely to be flatter than gradients for reward-controlled behavior, has been confirmed in several recent experiments with monkeys and rats. In addition, some drugs (d-amphetamine, diethyltryptamine) have been found to have a marked flattening effect on sharp generalization gradients obtained after animals exhibiting aversive behavior had received discrimination training. In a collaborative program with Stanford University, the effects of lesions in the amygdala on stimulus generalization have also been examined. These studies are still in progress, but so far have suggested that amygdalectomy does not significantly impair operant behavior, controlled by either food reward, or shock avoidance; and that, furthermore, the operation does not affect stimulus generalization after either reward or avoidance training.

A study has also been initiated in an attempt to quantify some of the anti-fatigue properties which

have been variously attributed to aspartic acid salts. Monkeys are at present being tested in prolonged sessions of avoidance behavior, for as long as 5 or 6 consecutive days. The relative effects of placebo, d-amphetamine, and a 10% mixture of the postassium and magnesium salts of aspartic acid in saline are being evaluated. Conclusions must await further trial.

In sum, then, the program of the Clinical Neuropharmacology Research Center has continued to develop along the broad outlines envisioned at its inception. The Clinical activities continue to center on the resolution of problems commonly encountered in mental hospitals, and in mental hospital populations. The laboratory studies, while pursuing theoretical work in their own right, continue to interact with the clinical program to the mutual benefit of both.

DIVISION OF BIOLOGICS STANDARDS

INTRODUCTION

The year 1961 represented the Division of Biologics Standards first full year of operation in its new building. It proved to be a "bitter-sweet" experience in the sense that, while the facilities of Building 29 are excellent and the establishment of most of the Division's activities under one roof has made for better organization, cohesion, and management, space is inadequate for the Division's present and future programs. This was clear in the early construction stages of Building 29. Thus, a great deal of time during the past year has been spent in considering space needs for future DBS programs and in planning for them.

The Division's activities are about equally divided between control and research. The research programs are concerned largely with the basic function of the Division—the control of biological products—although by their very nature, some of these activities could well be classified as basic or fundamental research. However, no programs are undertaken initially unless they have direct bearing on the responsibilities of the Division. The activities of the Division—both control and research—are therefore product-oriented, and their scope, direction, and intensity are dictated by the need to provide essential information for developing requirements and regulations for the licensing and release of biological products.

Not all of the Division's problems are related to new or developing products. Problems often arise concerning established products. The Division must therefore maintain an active interest in areas of research which are no longer of primary concern to the scientific field as a whole, for example, smallpox and the rickettsial diseases.

The newly developed products, however, create the heaviest and most urgent demands. It is in relation to these products that the Division's facilities and resources need to be increased. Of immediate concern are the live poliovirus and measles vaccines. The actual needs in measles prophylaxis are not yet fully discernable, however, it is certain that two forms of vaccine will eventually be made available—live attenuated, and inactivated vaccines, with variations of each. Only time and the verdict of the users will determine which type will be the most suitable for immunization against measles. The Division has been active in the development of these vaccines and has clarified many of the problems. The groundwork for their licensing and release once they are ready for commercial use has been established. In the meantime, the Division is faced with the difficult problem of apportioning its resources, which are still strained by the testing, licensing, and release of live poliovirus vaccines.

Research in other fields of infectious diseases is increasingly active, and viral agents are being isolated at an accelerated rate. It appears probable that there will be additional vaccines for respiratory diseases and arbor virus infections. The possibility also exists that a hepatitis vaccine will be developing in the near future. It is in anticipation of these future needs that the Division is endeavoring to obtain an extension of its facilities.

During the past year, the solution of problems relating to the licensing of live poliovirus vaccine was of pressing urgency. These problems involved the presence of adventitious agents in the vaccine and virus seed used, and the genetic instability of the strains, particularly the Type 3 strain. Now, at the close of the year both of these problems appear to be close to solution. Sensitive methods for detecting SV–40 have been worked out, and the testing methods for determining neurovirulence are sufficiently systematized that the Division can look forward to the licensing of Type 3 live poliovirus vaccine early in 1962. The costly and time-consuming alternative, had the problems not been resolved, would have been to develop new strains.

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During 1961 a serious problem developed in the testing of inactivated poliomyelitis vaccine. Residual live SV–40 was detected in some lots of vaccine after inactivation. Fortunately, only about 25 percent of the vaccine lots were affected, but an exhaustive testing program was required by the Division and, since many laboratories were not yet set up to work with ceropithecus kidney tissue cultures (essential for the detection of SV–40), the Division's testing program had to serve as the determining factor in the release of vaccine during the critical period of spring and early summer.

A third concern of the Division during 1961 was the demonstrated instability of the pertussis component of the multiple antigen preparations containing diphtheria, tetanus, pertussis, and poliomyelitis antigens, which made this product—widely used by pediatricians—virtually unavailable. An extensive testing program was initiated, and much further work will be required to determine the causes of the instability and to work out methods of overcoming it.

One unhappy experience during 1961 was the necessity of investigating alleged violations of the Biologics Law in the sale of plasma and whole blood. The charges involved the updating of whole blood and the processing of plasma for sale without license. These investigations, carried out under direction of the U.S. Department of Justice, consumed a great deal of the time and energy of the Division's Laboratory of Blood and Blood Products.

A continuing difficulty is the recruitment of suitable personnel, particularly senior scientists willing to engage in the type of scientific work presented by the Division and willing to accept the kind of scientific responsibility which is a necessary component of the mission of the Division.

The more detailed activities of the Division are set forth in the following summaries of the programs of each of the Laboratories.

LABORATORY OF CONTROL ACTIVITIES

This Laboratory is responsible for activities dealing directly with licensed establishments in relation to the licensing and control of biological products. It is supported by sections on control tests, pyrogens, and reference standards.

Its activities include:

(a) Determination of eligibility of establishments and of individual biological products for license. This determination is made on the basis of the integrity of management and technical personnel, the physical facilities for manufacturing and testing of products, the scientific and professional qualifications of personnel and the evidence developed by manufacturers and the Division of continued safety, purity, and potency of products, for which an application for license is being evaluated. License applications are reviewed individually when required by an *ad hoc* committee consisting of appropriate members of the staff of the Division.

(b) Supervision of annual and special inspections of licensed establishments and of those for which an application for license has been made.

(c) Releasing of individual lots of biological products for distribution by manufacturers on the basis of review of manufacturer's and of DBS tests and of any other available information relating to the safety, purity, and potency of the lot of the product.

(d) The establishment and distribution of physical biological standards, reference preparations, and control materials. A small culture collection is also maintained mainly for the Division and for licensed manufacturers.

(e) Review of requirements and regulations now in effect for such constructive revision as needed and the development of requirements and regulations for new products.

(f) Maintenance of close working relations with other laboratories of the Division and other agencies to insure continuous knowledge of information needed for the licensing of establishments and new products and for the testing, release, and control of products already licensed.

The scope of activities carried out by this Laboratory is indicated by the fact that during the twelve month period, December 1, 1960–Novem-

ber 30, 1961, a total of 6,375 control tests were carried out to insure the sterility, safety, potency, and purity of licensed biological products as follows:

	Tests
Products for Release	5,680
Inspection Samples	660
Complaint Investigations	35
Total	6,375

The results of those tests served as a basis for the release or rejection of individual lots of products. In addition, 1,586 cooperative service tests were done on biological products not licensed.

During the same period 5,263 lots of biological products were submitted for release by licensed manufacturers. Of these 5,178 lots were released, 23 lots rejected, and 62 lots withdrawn from consideration for lease by manufacturers.

To maintain an adequate supply of physical reference standards for use by the licensed manufacturers in their official control testing, it is necessary to prepare and standardize new liquid lots from the primary dried stocks. The number of lots prepared and standardized during the year were: antitoxins—16, serums—4, vaccines—6, toxoids—6. A total of 333 tests were required to complete a satisfactory standardization of these lots. These tests include flocculation reactions, animal protection tests, animal potency tests, neutralization tests, and a number of specialized tests for specific products.

Standards, reference preparations, and cultures are freeze-dried for greater stability during storage. The following were dried between November 15, 1960 and November 15, 1961:

	Ampules
Cultures	1,497
Serums	35
Vaccines	3,546
Viruses	555
Toxins	969

Official standards, reference, and control preparations currently maintained include 73 items.

Standards, reference preparations, and cultures were distributed to research or control laboratories of licensed and other manufacturers, health departments, and universities in this country and abroad as follows:

Antitoxins	341
Serums	1,475
Vaccines	1,160
Toxins	119
Cultures	475
Viruses	173

LABORATORY OF BACTERIAL PRODUCTS

The research activities of the Laboratory of Bacterial Products proceeded along the lines given in last year's report with the objectives of improving bacterial products and procedures for their standardization. Close association has been maintained with international groups. Mrs. Claire B. Cox, a bacteriologist oriented in biometrics, has strengthened the staff but recruitment of suitably trained personnel for work on bacterial toxins remains a problem.

One of the pressing problems was the conclusion of the investigation, mentioned in last year's report, on the stability of potency of pertussis vaccine component of the multiple antigen vaccine containing diphtheria and tetanus toxoids and pertussis and poliomyelitis vaccines. It was found that the pertussis antigen was unstable with an overall estimated loss of six percent per month. The acceptance of the findings by the manufacturers was complicated by the fact that samples tested by them had been held in the icebox and showed less loss of potency than did market samples tested by the Division. Data submitted by three state laboratories confirmed the findings. To compensate for loss, requirements for initial potency were increased and the dating period was shortened.

A study was initiated to determine the adequacy of the revised requirements for pertussis vaccine in quadruple antigen vaccine. However, the finding of SV–40 contamination in poliomyelitis vaccine of certain manufacturers curtailed further manufacture of the quadruple antigen vaccine which had in a very short time after its introduction largely replaced the use of triple antigens. An investigation of the cause of deterioration of potency is indicated. One valuable outgrowth of the original problem was the demonstration that variations in immune response of strains of mice may significantly affect the measurement of relative potency of an antigen.

The newly developed quantitative mouse protection test for evaluation of cholera vaccine is now ready for trial use by the manufacturers. Studies

on *in vitro* serological measurement of immune response, on laboratory models of cholera infection and rapid tests for identification of bacteria are being continued by Dr. John C. Feeley.

The recent epidemics of "choleriformis enteritis El Tor" in Hong Kong, the Philippines and a few other areas in the Far East has stimulated interest in the study of cholera outside the endemic area of Pakistan where Dr. Feeley last year cooperated in setting up the bacteriology laboratory of the Pakistan-SEATO Cholera Research Laboratory. At the request of Philippine health authorities, Dr. Feeley participated in a field investigation in Manila where improved bacteriological and serological procedures for diagnosis were introduced. Sera and cultures from the epidemic areas will furnish material for evaluating products for which the Division is responsible. The findings of this investigation have implications in international quarantine regulations. The epidemics demonstrate that El Tor vibrios, indistinguishable from "true" cholera vibrios except in capacity to hemolyze red blood cells, are capable of causing epidemics of a disease as severe as and clinically indistinguishable from cholera. This had not been heretofore generally appreciated.

Dr. Michael F. Barile in cooperation with Mr. R. W. Kolb, LCA, is formulating proposed revisions in the regulatory standards for Schick test toxin (diphtheria) based on results of the study of this product.

Improved methods for detection of contaminating PPLO (pleuropneumonialike organisms) and/or L forms in tissue cell cultures have been devised by Dr. Barile. His studies are helpful in the formulation of purity standards for certain viral products and appear to be more applicable to viral than bacterial products.

Dr. Harold Baer and his group (Section on Allergenic Products) are continuing their studies on the specificity of tuberculin. The problem is very complex but results are encouraging. The present tuberculins, O.T. and P.P.D., contain many reactive components some of which cross with heterologous species of *Mycobacterium*. The objective of the study is to provide more purified and specific substances for use as regulatory standards for tuberculin.

The allergen section continues to serve as a depot and distribution center of ragweed pollen for the Committee on Standardization of Allergens (NIAID). This and other liaison activities are helpful in relation to development of regulatory standards for allergenic products.

Studies on sterility testing procedures were resumed with particular emphasis on detection of contaminating fungi and yeast in biologic products and on influence of preservatives.

An analysis of the results of toxicity tests of 595 lots of pertussis vaccine covering a period of 5 years permitted a revision in the test and a more realistic requirement concerning freedom-from-toxicity of the product.

Considerable time was required to select and assay the unitage of a new lot of standard pertussis vaccine to replace the depleted lot which had been in use since 1953 when a unit of potency was adopted. This freeze-dried standard has been widely distributed, on request, to many national laboratories and research workers.

Routine and special assay tests of pertussis vaccine were increased by 37 percent due to the investigation on stability of pertussis vaccine and complications arising out of a product which was more toxic for mice than other pertussis-vaccine-containing products and for which it was claimed to cause fewer reactions in children. The numbers of tests performed are as follows: Pertussis vaccine potency 310; pertussis vaccine toxicity 312; antipertussis serum 18; *Haemophilus influenzae* therapeutic and diagnostic sera 10 (grand total, 650). Other regulatory activities of the Laboratory consisted of participation in (a) the formulation of written standards for allergenic products and pertussis vaccine; (b) the review of license applications for manufacture of bacterial products of which tuberculin was most frequently involved; and (c) liaison activities between the Division and one manufacturer of poliomyelitis vaccines.

LABORATORY OF VIRAL IMMUNOLOGY

An important responsibility during the past year has been the control of oral, live, poliovirus vaccine. With various laboratories submitting samples to the Division for evaluation and eventual action on requests for license, a major portion of the resources of this Laboratory have been assigned to this work. A reference virus, lot NA2, has

been adopted as the NIH Reference Virus for neurovirulence tests and a supply of this virus has been acquired for distribution to interested laboratories. In addition, supplies of each of the three Sabin strains of poliovirus have been stocked for use as reference preparations for the titration of the finished vaccine. One result of this work has been the licensing of Pfizer, Ltd. of Sandwich, England for Type 1, Poliovirus Vaccine, Live, Oral on August 17, 1961, and for Type 2 Vaccine on October 6, 1961. Other laboratories have been working actively in efforts to qualify for license of each of the three types of vaccine.

The impact of the above work has created a heightened index of activity throughout the entire laboratory. In May of this year, Dr. Paul Gerber was assigned the responsibility for studying the tissue culture properties of the candidate live poliovirus vaccines. Training of new personnel was added to the responsibility for studying markers and extraneous viruses in vaccine samples which were submitted for evaluation by prospective manufacturers. In the Section on Pathology, Dr. Ruth Kirschstein was called upon to enlarge the staff of the histology laboratory and of the animal test unit in order to process tissues from the increased number of monkeys which were utilized in the neurovirulence testing program. Among the 56 samples of vaccine which have been subjected to examination to date, tests on approximately one-fifth have been completed with results which have permitted release of the vaccines. Time consuming or critical tests are performed concurrently with manufacturer's tests, where possible, to avoid delay in identification of problems or of release of vaccine.

At about the same time that the need became apparent for increased effort with the live poliovirus vaccine, Dr. Paul Gerber uncovered a disturbing situation with respect to the Salk poliomyelitis vaccine program by a finding that some lots of vaccine contained living vacuolating virus. A detailed investigation showed that this simian virus (SV–40) was somewhat resistant to inactivation by the formaldehyde level which inactivated poliovirus. The release of Salk vaccine was brought to a temporary halt in early May—at a time when vaccination programs were underway in many parts of the country. The manufacturers required some time to develop the procedures for testing for SV–40 because supplies of Cercopithecus monkeys were not immediately available. To avoid needless delays, a program of testing each lot of vaccine for SV–40 was carried out in this laboratory, and until August 1, 1961 when the manufacturers were able to arrange the testing adequately, the test results obtained in this laboratory were used as the basis for release action on both poliomyelitis and adenovirus vaccines.

The control program for poliomyelitis and adenovirus vaccines during the year involved the following examination and release actions:

	Poliomyelitis Vaccines	Adenovirus Vaccines
Samples received	60	20
Vaccines released	59	3
Combined with other antigens	16	15
Vaccines rejected	6	
Vaccines withdrawn by manufacturer	12	2

In the survey of vaccine for SV–40, the following results were obtained:

Samples	Poliomyelitis Vaccine	Adenovirus Vaccine
Negative	41	6
Positive	15	13

(Above figures refer to actions during reporting period only. This accounts for apparent arithmetic discrepancies). These results indicate that problems in removing SV–40 from adenovirus vaccine present greater obstacles than those involved with poliomyelitis vaccine.

The research program carried out by this laboratory has included studies on pathogenesis of a number of infectious agents by Dr. Kirschstein and Dr. Borman; in addition to the emphasis which they have placed on investigating and organizing a clear cut procedure for reporting the results of neurovirulence tests of the attenuated poliovirus vaccines in monkeys.

Dr. Gerber has been active in studies on the biological and biochemical properties of the vacuolating virus. Dr. Van Hoosier has been studying the immunology and biology of the group of viruses which have been associated with monkeys.

Dr. Uhlendorf has studied various aspects of measuring poliovirus antibodies in the serums of a number of animal species. In the latter area Dr. Gerber has been finding that sera from rabbits and goats immunized with poliovirus have contained antibodies of sufficiently high titer to neutralize undiluted poliovirus and provide a means for the search for extraneous viruses in the live, oral, poliovirus vaccine.

The problem of acquiring personnel to carry out our responsibilities has been a most difficult one. The shortage of scientists with training in tissue culture techniques and available for this work is acute. An additional difficulty arose when Dr. Samuel Zaron relinquished his place on our staff in order to work in NIAID at the end of his year of training in London with Andrewes and Isaacs.

LABORATORY OF VIROLOGY AND RICKETTSIOLOGY

The Laboratory of Virology and Rickettsiology, as befits a unit of the Division of Biologics Standards, devoted most of its efforts during calendar year 1961 to problems related to the prevention, by immunologic means, of diseases of man caused by viral and rickettsial agents. The studies ranged from those with clinical overtones, such as infection of volunteers with SV–40 virus and the immunization of children with live attenuated measles vaccine, to purely laboratory procedures such as the development of a continuous tissue culture cell line derived from *Cercopithecus aethiops* (African green monkey).

Simian Virus 40 (Vacuolating Virus)

The very appreciable effort of the laboratory during the year was devoted to work on SV–40. This simian agent, originally described in the summer of 1960, immediately became of great importance in viral vaccines prepared from monkey kidney tissue cultures. In particular, both living and killed polio vaccines and adenovirus vaccine, all of which had been produced on a large scale and employed in human beings, were found to contain living SV–40 on occasion. The occurrence of this adventitious agent in these vaccines was of such import that their availability was threatened.

Little was known about the agent except that it grew in rhesus kidney cell cultures without producing cytopathogenic changes whereas in the cytoplasm of cercopithecus cell cultures (African green monkey) it regularly caused the formation of characteristic vacuoles. Studies on the agent were initiated by several laboratories in the DBS. The efforts of the LVR were concentrated on the following aspects: (1) The development of simpler tools for working with the agent; these were needed to supplement the diagnostic isolation of the agent in primary cercopithecus cells and its subsequent identification through the use of the neutralization test in such tissue cultures. (2) The accumulation of information on the pathogenesis of the virus for man, monkeys, and other laboratory hosts and, finally, the exploration of the ecology of the disease in monkeys, both in the wild and in the laboratory. It was anticipated that studies along these lines would provide information which would help in the elimination of living SV–40 from vaccines and would, incidentally, add to our knowledge regarding this newly recognized viral agent.

Studies of Dr. J. A. Morris and his associates, in the Section on Respiratory Viruses and in the NIAID, revealed that inoculation of volunteers with relatively large amounts of SV–40 (10^4 $TCID_{50}$) by the respiratory route resulted in inapparent infection which was associated with excretion of virus from the upper respiratory tract of some of the volunteers for a short time and was followed by development of specific antibodies. During the course of the acute infection and during the succeeding 9–12 months, no untoward effects attributable to SV–40 have been observed in the 35 volunteers who knowingly received the agent. Sabin's observation that feeding smaller amounts of SV–40 by mouth to human beings did not result in infection was also confirmed during these investigations using materials supplied by him.

Simian virus 40 administered to susceptible cercopithecus monkeys, produced a systemic infection with viremia but little in the way of obvious disease. Observations on monkeys reaching the laboratories indicated that most of the rhesus, few of the cynomolgus, and almost none of the cercopithecus were infected with SV–40. An expedition to India by Dr. Harry Meyer and an associate revealed that SV–40 was a natural dis-

ease of Indian rhesus monkeys inhabiting the jungle. Only about 10 percent of the jungle monkeys were infected, and these were clustered in individual family groups. Continuing observations on the same group of monkeys captured in the jungle, transported to the United States and held under reasonably good isolation conditions in the DBS, demonstrated that SV–40 spread slowly through a group in contrast to measles which spread in monkeys almost as rapidly as among children. Intimate contact or neighborly association seemed to be required for the spread of this simian agent. It was assumed that SV–40 was not indigenous to cynomolgus monkeys of Southeast Asia or to cercopithecus monkeys from Africa, and that when infection did occur in these species it resulted from exposure to infected rhesus monkeys in the course of transportation and holding. Diagnostic tools for isolation and identification of SV–40 have been improved through the application of complement fixing techniques and the employment of a continuous line of cercopithecus cells, developed by Mrs. H. E. Hopps of the LVR, which are fully susceptible to the cytopathogenic effects of SV–40 and unencumbered by any recognizable latent simian agent, including SV–40.

By applying information of the type mentioned above as well as that derived from other sources, it is possible to obtain simian tissue cultures which are free of SV–40 for use in virus research and vaccine production. Dr. B. Eddy of the Section on Experimental Virology, has accumulated information which suggests that simian virus 40 may be associated with the development of sarcomas in experimentally inoculated hamsters.

Measles

During the past several years, progress has been made by many groups in the development of immunizing procedures against measles using both attenuated living and killed vaccines. The DBS has done much to foster and stimulate rapid transition in this field from the basic studies of Enders to the commercial production of such vaccines. In the meantime, Dr. Meyer's Section on General Virology has accumulated experience with live measles vaccine in children and has paid particular attention to the development of standard procedures and reagents which will be needed for the biological control of commercially produced measles vaccines and measles immune gamma globulin. These materials and procedures have been made available to manufacturers and will hasten and simplify the final stages involved in bringing measles vaccine to the physician and his patient.

The degree of attenuation for man of Enders' B level Edmonston strain measles virus is such that it induces a mild but not dangerous form of disease in the vaccinated susceptible child; this is followed by the development of specific antibodies and solid immunity. The somewhat excessive clinical manifestations of the vaccine disease can be essentially eliminated by the concurrent administration of gamma globulin containing an appropriate amount of measles antibody. While such a combined procedure may find common usage in American health practices, it has potential disadvantages when employed in children in developing countries of the world because of the limited supply and relatively high cost of gamma globulin. Although the unmodified vaccine disease is not dangerous in healthy American children, relatively little is known about its severity in children with nutritional deficiencies and heavy parasite loads. In order to determine the severity of the vaccine disease in children in developing areas, a pilot study was begun in Upper Volta, West Africa, in November 1961 by Drs. H. M. Meyer, D. D. Hostetler, and Mrs. B. C. Bernheim of the LVR.

Homologous Serum Jaundice

Dr. J. P. O'Malley and his colleagues published their observations on a newly recognized virus, designated A–1, which appears to bear some relation to the etiological agent of serum hepatitis. The icterogenic pool of human plasma which had induced jaundice in volunteers and which yielded the A–1 agent in tissue culture, elicited antibodies against A–1 in the convalescent volunteers. Perhaps more important was the finding that volunteers infected with different icterogenic pools also developed comparable A–1 antibody response as well as a group of persons who developed jaundice following use of an infected lot of thrombin. The general importance of the A–1 virus in human

jaundice and its relation, if any, to the hepatitis viruses of other investigators who have used different materials and methods are being studied.

Typhus

Studies on immunization against epidemic typhus continued along several lines, all of which were extensions of earlier work of Dr. J. A. Morris and Miss E. B. Jackson, their associates at the DBS and their colleagues in other institutions. One of the efforts was concerned with a better understanding of the factor or factors in the commercially prepared inactivated typhus vaccine and in the experimental live attenuated E strain vaccine which are responsible for inducing resistance to infection with virulent strains of *Rickettsia prowazeki*. Previous work of others had led to acceptance of the idea that resistance of animals to challenge was closely correlated with the presence of circulating toxin neutralizing antibody and roughly correlated with the presence of complement fixing antibodies. Provocative observations mentioned in last year's report, raised the question as to whether resistance unassociated with any detectable antibodies might be induced by vaccine. The laboratory model with which these data were obtained consisted of guinea pigs inoculated with 10 to 100 chick embryo lethal doses of the attenuated E strain. A small proportion of such animals developed antibodies and were resistant but most had no detectable antibodies, yet many of the latter were partially resistant when challenged with a virulent strain (Breinl) of *R. prowazeki*. Extension and confirmation of the earlier observations now suggest that the small dose of E strain organisms induced a state of immunologic preparedness in the guinea pigs which enabled them to respond in a booster fashion when confronted with rickettsial antigen resulting from the challenge. For the time being, then, it is not necessary to postulate the presence of a hitherto unrecognized antibody which is primarily concerned with resistance.

Studies on another aspect of the booster phenomenon in immunization against typhus employed killed vaccine. Here the idea was to determine the size of the antigenic mass required to elicit a state of immunologic preparedness in contrast to the recognized vaccination regimen which elicits demonstrable antibodies in the majority of persons. Field aspects of these studies, undertaken in collaboration with the Navy, Army, and University of Maryland, are essentially completed but not the testing of specimens. Nevertheless, observations on groups of 75 Marines inoculated with progressively diminishing doses of commercial vaccine have revealed that a single injection of a 1:64 dilution resulted in the appearance of small but detectable amounts of antibodies in about 40 percent of persons while 10 percent responded to the 1:256 dilution. The antibody response to these groups, particularly those with a large proportion of negatives, to the booster injection of 0.5 ml. of undiluted vaccine should provide crucial data on the minimum antigenic mass of typhus antigen required to prepare persons for a booster response. The ultimate objective of this work is to find a means of inducing the prepared state of recruits by inoculating a multiple vaccine with a small typhus component. Such prepared persons could then be rendered solidly resistant by a booster dose of monovalent typhus vaccine given when subjected to risk of exposure.

New Antimicrobial Agents

Dr. C. P. Li, the members of his immediate group and his collaborators in Bethesda, have continued to study the antibacterial and antiviral substances found in mollusks. During the year, they obtained in a highly purified state that fraction from abalone which has antibacterial activity. The purified material, which was homogeneous by ultracentrifugation, yielded 18 amino-acids on hydrolysis and contained some carbohydrate. The substance was considered to be a mucoprotein. They extended the study to oysters and found antiviral materials to be present. The heat stable oyster material, like the abalone, inhibited paralytic disease in mice caused by poliomyelitis virus and interfered with growth of influenza virus in tissue cultures. The physicochemical properties of the antiviral material are under investigation.

Intracellular Infections

Studies designed to gain a better understanding of Brill's disease and the typhoid carrier, employing tissue culture models infected with rickettsiae

or salmonella were essentially abandoned during the year because of the press of work on SV–40. However, before this happened, experiments dealing with the cure of such tissue culture models by antibiotic therapy were completed; references are included in the bibliography of one of the projects of the laboratory. In the future, attention will be devoted to a study of the means by which the infected cell, without the aid of antibiotics, begins to overpower the invading rickettsial organisms. Earlier observations had indicated that under certain conditions of deficient nutrition of the tissue culture cells, the rickettsiae are killed off at almost the same rate as in the well-nourished cell maintained in the presence of specific antirickettsial antibiotics.

LABORATORY OF BIOPHYSICS AND BIOCHEMISTRY

The Laboratory of Biophysics and Biochemistry was established in 1960 to provide within DBS a centralized facility for biophysical instrumentation and to conduct a program of research in the physical sciences related to control of biological products.

The Laboratory is divided into two units: one engaged in physical measurements, the other in experimental research organized around physical concepts and disciplines but not necessarily utilizing physical measurements or complex instruments. Activities of either unit can freely cross boundaries of the several fields of biology without any restriction resulting from prior specialization in a limited biological subject area.

The physical instrumentation unit consists of two trained research assistants specializing in the operation of the ultracentrifuge and electron microscope. More than 60 percent of the man hours devoted to these two instruments have been applied to service activities or cooperative research in conjunction with other laboratories. As an independent intramural research project, the members of this unit have been engaged in developing and evaluating methods for the physical and chemical characterization of animal viruses. This function, which will increase in productivity as the investigators gain in experience and proficiency, has an important bearing upon the regulatory responsibilities of DBS since identification and description of adventitious viruses in viral vaccines constitutes a major problem.

The research conducted by the other three professional members of this laboratory, although quite different in approach, is also related to the problem of adventitious viruses in viral vaccines. A long-range study of methods of inactivating viruses has led to development of the basic concept of "differential inactivation" as a means of eliminating contaminating viruses in live virus vaccines, and practical as well as theoretical advances have been made in this area.

LABORATORY OF BLOOD AND BLOOD PRODUCTS

Research Activities

The research program of the Laboratory of Blood and Blood Products has as its goal the improvement of testing procedures used for the control of biological products derived from blood. The various projects cover all phases of blood collection, processing, shipment, and storage. Investigations encompass not only improvements for existing control procedures but also are directed toward the perfection of new tests for existing products as well as new products.

Many of these new products which are under development have immunological or biological activities for which there are no established test criteria. As a result, it is often required that research of a fundamental nature be carried out before definitive and useful control tests can be devised. An example of this is the continuing investigation of the components of the coagulation system which is being pursued with vigor in the Laboratory. Before protocols for meaningful control tests can be set up, it has been necessary to do considerable work on the purification of these components. Thus the successful preparation of a plasminogen free fibrinogen has brought one step closer the defining of control tests that will be independent of the reagents used.

Other examples of research interests designed to strengthen the control program include:

Studies of the effects of freezing, thawing, and drying on various elements of blood.

Some of the newer techniques offer intriguing possibilities for the preservation of ordinarily labile components.

Studies of methods of collection of platelets and their use in specific disease states. Particularly interesting are the effects on donors of repeated large platelet donations. This information leads to criteria for effective standards for platelets as a licensed biological.

A long-term interest is the effect of storage, that is, temperature, time, light, container material, etc. on the properties of whole plasma and the separate plasma proteins. One biological, Normal Serum Albumin (Human) now has a dating period of as long as 10 years under certain specified conditions. It is therefore of interest to study by all means available the nature of any changes in plasma protein molecules brought about as a result of aging.

Co-operative Research and Other Activities

Because of the nature of the control functions of the Laboratory, many contacts are made with outside organizations for various reasons.

During the year, the Laboratory completed its part of the experimental aspects of study on interchangeability of disposable transfusion equipment being carried out in cooperation with the American Standards Association and the International Standards Organization.

The Laboratory participates in a program, in cooperation with several national groups, of storing blood of rare and unusual types at subzero temperatures. This blood is available to meet actual transfusion needs. In addition, this program offers an excellent opportunity to evaluate under actual conditions the best procedures for storing, shipping, and administering blood that has been stored utilizing these relatively new techniques.

The rapid expansion in the last few years of the number of licensed establishments, coupled with the increasing complexity of techniques used in blood banking has created a need for an effective means of disseminating up-to-date information on details of the techniques. The Laboratory has devoted as much time as possible in working with other groups on this problem.

The Laboratory of Blood and Blood Products has carried out its primary function of controlling the safety, purity, and potency of blood and blood products. Chief among these control functions are the following:

1. As reqnired by regulations, each establishment producing blood products was inspected at least once during the fiscal year. During the year, there have been 10 qualified inspectors available to perform this work in addition to their other duties. In fiscal 1961, the qualified inspectors performed a total of 304 inspections requiring 374 man days on travel status. In the last quarter of this calendar year, two major investigations of possible violations of the Public Health Service Act have taken a significant amount of effort on the part of the Laboratory staff. As of December 1, 1961 these two investigations have required 220 man days on travel status with the task still not completed. As a result, some research projects and other work not of immediate importance have been delayed.

2. Control testing has been carried out on the various blood and blood products produced by 157 licensees operating 229 principle locations and 219 donor centers and other subsidiary facilities. These licensees hold 593 product licenses for blood and blood products. This control testing includes all of the physical, chemical, immunological and related testing found necessary for action on the various blood products submitted for licensure or release or as the result of inspections. In 1961 control testing involved over 2,200 lots of blood products including 410 inspection samples. In excess of 14,000 tests were performed on these products.

3. License applications for 25 blood products were reviewed and the licenses were granted. Four licenses were granted for establishments to produce blood and blood products. There are 12 licenses pending.

4. Labels and circulars were reviewed for all biological products from licensed manufacturers. Over 500 groups of labels and circulars were received and reviewed for compliance with the regulations. This work involved the labels and circulars from the currently 193 licensed establishments which are licensed for 284 different

biological products and who hold 1,213 active product licenses each having its own label.

5. Reference standards are maintained for seven blood products. Work was continued on 10 new preparations for ultimate adoption.

6. The preparation of regulations and standards for the control of blood products has continued. Additional Standards for Packed Red Blood Cells (Human) and for Heparinized Whole Blood (Human) have had final publication in the *Federal Register*. A draft of Additional Standards for Plasma (Human) has been circulated for comment before initial publication in the *Federal Register*.

7. The blood bank serving the needs of patients in the Clinical Center is operated as a Section of this Laboratory. This experience helps to maintain a realistic attitude toward control functions as the Laboratory continues to be aware of the requirements of meeting the needs of a research hospital. The equivalent of over 11,000 units of blood were processed in the blood bank in 1961 for patient care either in the form of whole blood or as special blood derivatives. Emphasis is being placed on the importance of fresh blood to support surgery utilizing extracorporeal circulation and on the use of platelet therapy in the treatment of thrombocytopenic states. The increased need for platelets has resulted in increased use of plasmapheresis.

8. An additional 453 NIH employees were grouped and typed for inclusion in the panel of typed donors. These employees are available as a valuable source of extensively characterized red cells for use in the control and research program of the Laboratory. At present there are 1,050 employees listed on the panel which is utilized at least weekly.

9. When travel schedules permit members of the Laboratory staff are participating in blood bank workshops and training programs. This activity, while performed on an informal basis, nevertheless allows more efficient dissemination and illustration of information on blood banking techniques. Ways and means are being explored that will allow greater participation by the Laboratory's trained staff in these training programs as a contribution to the raising of the standards of achievement of blood banks.

10. During the year, the Laboratory completed the experimental aspects of a study on interchangeability of disposable transfusion equipment being carried out in cooperation with the American Standards Association and the International Standards Organization. A report of this work is in preparation.

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