

Bacteriophage: An Essay on Virus Reproduction¹

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IN A DISCUSSION of the mechanisms involved in virus reproduction, it is well to start with a critical revision of concepts and definitions, because some of the ideas conceived in other fields have carried into virology implications not justified by the methodology of virus research.

The term *virus* itself can be operationally defined as "an exogenous submicroscopic unit capable of multiplication only inside specific living cells." This definition gives a methodological unity to the field of virology and, by leaving ambiguous two borderline fields—that of obligate parasitic microbes, on the one hand, and that of protoplasmic components transmissible by graft only, on the other hand—suggests some of the possible natural relationships of viruses.

The concept of reproduction requires closer scrutiny. What we observe is the appearance of increased virus activity, associated with an increased number of specific material particles, in a population of virus-infected cells. Virus is produced by the only observable entity, the virus-infected cell, and the mechanism intervening between infection and appearance of the new virus activity cannot be postulated by analogy. In many minds the terms *reproduction* and *self-reproduction* are connected with the idea of increase in size followed by division. Closer scrutiny reveals that increase in size followed by division is bound to be an epiphenomenon of some critical event of reproduction, which must involve point-to-point replication of some elementary structures responsible for the conservation of specificity from generation to generation. Thus, in dealing with cell growth and division we trace the critical event to gene and chromosome duplication. Even a bag of enzymes could grow and multiply only by duplication of discrete enzyme molecules, which can hardly be supposed to grow individually in size and then split. In a repeat, crystal-like structure, such as has been suggested for rod-shaped particles of plant viruses (2), the elementary repeated unit must be replicated. In other words, all growth and reproduction should ultimately be traceable to *replication of specific chemical configurations by an essentially discontinuous appearance of discrete replicas*.

One of the first tasks in virus research is to uncover

the relation of the virus particle, as we know it in the extracellular state, to what is replicated inside the infected host. Misunderstandings may arise, however, if we fail to distinguish between replication and the more general category of chemical synthesis. There is something peculiar to homologous replication that sets it aside from other types of synthetic reactions. The replication of specific biological units must involve the building of complex specific molecules or molecular aggregates, the only permissible limitation to identity of model and replica being the production of "mutated" structures—the production, that is, of modified elements replicated in the modified form. The fact that the presence of the initial model (gene, virus) is indispensable indicates that this model plays a role in replication; but this role is by no means an obvious one. The model might carry within itself all the enzymes needed for its own synthesis from nonspecific building blocks, or it might act as a directive pattern for synthesis—a pattern in which building blocks are assembled by synthetic enzymes not pertaining to the model itself (this may require a two-dimensional unfolding of the model, to allow point-to-point replication followed by separation of the newly formed unit [33])—or it might function as a directive pattern for folding a pluripotential macromolecule into a specific tridimensional replica, possibly with the intervention of a negative template, by analogy with Pauling's theory of antibody formation (31).

The study of virus reproduction constitutes one of the best approaches to bridging the gap between growth and replication. I shall deal primarily with the study of bacterial viruses as exemplified by the system of the "T" phages (T1-T7) active on *Escherichia coli* strain B (5). Reproduction takes place in a short "latent period" (13 to 45 minutes for different viruses under standard conditions) between the infection of a bacterial cell and its dissolution or lysis, with a rise in phage activity traceable to liberation upon lysis of large numbers of specific phage particles. The number of infected cells, the number of phage particles infecting each cell ("multiplicity of infection"), the time between infection and liberation, and the amount of virus liberated by each cell can be determined accurately (7). Moreover, the infecting virus may be "labeled" with easily recognizable properties arising by mutations (15, 23). Our

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problem is: How are the newly produced phage particles related to the infecting particles?

On the one hand, the continuity between the two is evidenced not only by the general specificity of reproduction, but also by the observation that mixed infection of a bacterial cell with two closely related phages, such as T2 and its mutant T2r, causes a mixed yield of both infecting types in proportions similar to the ones in the infecting mixture (15). The continuity of virus material after infection is also proved by irradiation experiments, which show that the radiation sensitivity of phage inside a bacterium remains the same as that of free phage for a few minutes after infection, indicating that the total amount of radiation-sensitive material remains unchanged (21, 26).

On the other hand, there is ample evidence of a deep-reaching alteration of the virus after infection. Doermann, breaking open phage-infected bacteria at different times after infection, found that for several minutes no phage activity could be recovered, and only around the middle of the "latent period" did the first active particles make their appearance (8). A similar conclusion had been reached in my laboratory from the study of the effect on phage-infected bacteria of the dye proflavine, which apparently stops phage production, but allows bacteria to lyse and liberate whatever particles were already present when the dye was added (12). Thus, there is an "eclipse" of recoverable phage activity between the disappearance of the infecting particles and the appearance of new ones. To the relation between the two I shall return later.

Another line of evidence indicating that the final particles are not a direct product of reproduction of the infecting particles *as such* is the occurrence of complex interactions revealed by experiments with mixed infection. Mixed infection of bacteria with pairs of unrelated phages, such as T1 and T2, or T1 and T7, gives "mutual exclusion": only one type of particle is liberated by each bacterium and the infecting particle of the other type is lost, again suggesting an alteration of the infecting particles (4, 7). Phages T2, T4, and T6, however, form a related group and mixed infection of a bacterium with two of them gives rise to a mixed yield. If the two infecting types differ in a character whose alternative forms can manifest themselves in both types—for example, T2r⁺ and T4r— they give progeny containing, besides the parental types, some new types also, which result from a recombination of the alternative characters r and r⁺ present in the parental types: T2r⁺, T2r, T4r⁺, T4r. This fundamental result, obtained by Delbrück and Bailey (6), was greatly extended by Hershey and Rotman (16, 17) who, study-

ing recombination among different mutants of phage T2 infecting the same host cells, showed that recombinant types occur with definite specific frequencies for different characters, suggesting a localization of the hereditary properties of bacteriophage in discrete material determinants. We can then, at least formally, interpret recombination experiments according to the model of a phage particle composed of a number of discrete recombinable genetic units, whose number is probably quite large, of the order of 100 or more.

Another kind of interaction is "multiplicity reactivation," which consists in the production of active bacteriophage inside bacteria infected with *two or more* particles of some bacteriophages previously exposed to ultraviolet light and "inactivated"—in the sense that infection of a bacterium with *one* such irradiated particle, while killing the bacterium, would not cause any phage production (24, 25). For reactivation to take place, the inactive infecting particles must be of the same type or of genetically related types (T2, T4, T6). This can be interpreted on the basis that reactivation is due to replacement of damaged portions or units of the genetic material of one infecting particle by homologous undamaged portions supplied by the other particles, by the same (unknown) mechanism responsible for the genetic recombinations discussed above. This interpretation of multiplicity reactivation leads to specific expectations concerning the frequency with which bacteria infected with inactive phage particles should produce active phage. On the one hand, the greater the dose of radiation, the smaller should be the frequency of the bacteria that receive two or more particles which can successfully supplement one another, because of more frequent damage in homologous genetic units. On the other hand, for a given dose of radiation, the frequency of bacteria producing active phage should increase with increasing "multiplicity of infection," since this increases the fraction of bacteria that contain mutually supplementing groups of inactive particles. Both expectations are borne out by experiment, and the results agree reasonably well with quantitative expectations derived from a mathematical rationalization of the genetic hypothesis of reactivation. This hypothesis, however, should be considered simply as a working hypothesis until it is substantiated by independent evidence; for the time being it rests mainly on analogy and on a mathematical analysis involving several unproved assumptions. Dulbecco (10, 11) has recently discovered in my laboratory that ultraviolet-inactivated phage attached to its host bacterium can be reactivated by exposure to visible light ("photoreactivation," [20]). The results of this work may affect, in a way that

is not yet clear, the interpretation of multiplicity reactivation as well.

Be this as it may, the interactions among phage types in mixed infection indicate that in phage reproduction specificity is perpetuated not as a whole, but subdivided into discrete units; we must then look upon these units as the elements whose specific structure is replicated. This does not mean, however, that the units are replicated *separately*: one could imagine that, after the initial eclipse, each new phage particle is produced as a whole and that all recombinations result from late interactions among the newly formed particles. To gain information on this point let us return to the experiments on the kinetics of intracellular phage production.

We have seen that active phage particles appear only around the middle of the latent period; afterwards, their number increases at an approximately linear rate, as shown by Doermann's breakage experiments (8). Using mixed infection with different mutants, as in Hershey and Rotman's experiments (17), Doermann has recently proved that the very first crop of active particles to appear inside infected bacteria already comprehends the same variety of parental and recombinant types, and in the same proportions (9). This indicates that the interactions leading to recombination must take place before or concurrently with the formation of active particles. It seems indeed a reasonable working hypothesis to assume that the active phage particles, which appear at a linear rate in the late phases of intracellular growth, are the end products of reproduction and play no role in further phage production.

This point of view is supported by the following line of evidence. The writer has recently analyzed the production of spontaneous phage mutations during reproduction, and discovered that the new mutants are present in individual bacteria in clones, each clone containing all mutant particles derived from one mutation (24a). The distribution of the number of mutants per clone is similar to the one expected from the assumption that the genetic determinants of phage reproduce at a logarithmic rate by successive reduplications, with a constant probability of mutations per reduplication. This suggests the existence of a phase of reproduction of phage in which each new element acts in turn as a source for new replicas.

Does genetic recombination occur either before or during this reproductive phase? Apparently not. In mixed infection experiments (9, 17) the particles of any one recombinant type are *not* produced in clones, but are found distributed very nearly at random in

individual bacteria. This shows that recombination must follow the logarithmic phase of reproduction, since if recombination occurred earlier each recombinant would give rise to a clone, just as a mutant does. The same experiments also give evidence against recombination's taking place after the formation of the active particles, since it could then occur only by exchange of genetic materials between particles, and the recombinants of reciprocal types should be in equal numbers. Instead, there is no correlation between the numbers of reciprocal recombinants in individual bacteria (9, 17).

We conclude, then, that genetic recombination follows the reproduction of the genetic material and does not occur later than the formation of the active particles. It seems probable that recombination and active particle formation occur together, as though the same event that created an active particle also settled its genetic constitution. A hypothesis that fulfills these requirements and that was proposed earlier in a different connection (24) is independent replication—and, we should now say, logarithmic replication (24a)—of the genetic units composing the phage, followed by their final reorganization into complete, mature phage particles. It should be clearly remembered that no direct evidence for this mechanism of independent reproduction of genetic units has as yet been obtained.

This seems to be as far as we can go at present in analyzing phage production from evidence supplied by the end products. The biochemist has recently thrown some interesting light on phage reproduction, approaching it from the direction of the non-specific building blocks. The main results (see reference 3), obtained by determination of total protein and nucleic acids in infected bacteria and by isotope techniques, indicate that the material of the phage particles—which consist entirely or almost entirely of protein and desoxyribosenucleic acid (DNA)—derives in the greatest part from compounds assimilated from the medium after infection. The rate of assimilation of these new materials is similar to the rate of synthesis of bacterial protoplasm in noninfected cells immediately before infection. This suggests that the pre-existing synthetic enzymes of the bacterium are responsible and rate-limiting for the formation of the building blocks for phage synthesis. DNA synthesis immediately precedes and parallels the appearance of active phage particles and fails to take place in bacteria infected with inactive, nonreactivated phage T2, which suggests that DNA may be involved mainly in the final steps of the "baking" of active particles.

Failure of phage-infected bacteria to produce specific bacterial components, as distinct from phage substance, is shown by the elegant experiment of Monod and Wollman (30) on the absence of adaptive enzyme formation in phage-infected bacteria. A similar failure of enzymatic adaptation has been observed in bacteria infected by ultraviolet-inactivated phage under conditions in which no reactivation occurs (27).

A rationale for the suppression by phage infection of specific bacterial syntheses is suggested, in the light of current theories of gene action, by cytological observations (28). The first result of infection of a bacterium, with either active or irradiated phage T2, is a rapid disruption of its nuclear apparatus represented by the Feulgen-positive "chromatin" bodies. In the case of infection with active phage, nuclear disruption is followed by the appearance of a granular type of chromatin, which probably represents the new phage itself, as indicated by the failure of this new chromatin to accumulate either after infection with inactive, nonreproducing phage, or after infection with active phage in certain abnormal bacterial strains which upon lysis fail to produce any active phage. These observations suggest that the suppression of synthesis of specific bacterial components in a phage-infected bacterium results from a disruption of the genetic apparatus of the bacterium and its replacement with the genetic apparatus of the virus, resulting in viral rather than bacterial specificity of the protoplasm newly synthesized by the available bacterial enzymatic machinery. The disruption of the genetic apparatus of bacteria infected with inactive phage explains the failure of these bacteria to undergo any further multiplication.

According to this hypothesis, the virus introduces into the host bacterium not only an *additional* organizer of specificity, but a completely *predominant* one, in what could be called parasitism at the genetic level. In the so-called "lysogenic" strains of bacteria, which carry and occasionally liberate phage (29), the genetic patterns of host and virus may coexist and function side by side in genetic symbiosis. The difference between phage infection followed by death of the host and phage infection followed by lysogenicity may thus be interpreted as a difference in compatibility relations between the genetic materials of virus and host. The compatibility in lysogenic systems may be more or less stable and its changes may be connected with the sporadic character of phage liberation by lysogenic bacteria (29).

Stretching the available evidence, one may construct the following picture of the reproduction of a bacteriophage such as T2: Infection produces a dis-

ruption of the genetic organization of the host and a change in the organization of the infecting virus, leading to the formation of a new unit system, the *virus-infected cell*, containing the existing enzymatic machinery of the host and, superimposed upon it, a genetic pattern derived from the virus and directing the synthesis of virus material from nonspecific building blocks. This genetic pattern is resolved into a number of discrete, more or less independent units, the genetic determinants of the virus. The process of formation of the new virus is such that it allows for complex reorganizations to take place and results in the appearance of a population of virus particles that represent the end products of the process as a whole.

This picture, which admittedly has a heuristic rather than descriptive function, presents several major gaps. First, there is a time gap between the disappearance of the initial virus and the appearance of the mature virus. Second, there is a chemical gap between the nonspecific building blocks and the final specific nucleoprotein particles. Third, we have a genetic gap between the genetic determinants of the phage and the phage particle itself, the former being responsible for the inherited specificities, the latter being the carrier of infectivity and, therefore, the only operationally definable unit in the extracellular state. Finally we have a technological gap, in our ignorance of the enzymatic machinery involved in the synthesis of phage from the newly assimilated building blocks. I do not emphasize these gaps in a spirit of pessimism, since it is clear that they involve phases of biological replication about which no biologist possesses any information. The very fact that these gaps can be clearly visualized and delimited in phage analysis suggests that they may be filled more easily by work on bacteriophage than by work on other biological systems.

How far results of phage research can throw light specifically on the events of other virus infections, we do not know. Virus-host relationship may include systems so different that the only similarities to be postulated a priori are those implied in our definition of *virus*. Nevertheless, the picture of reproduction emerging from phage research is likely to bear instructive similarities to other virus infections. Disappearance of recoverable virus activity following infection of a host cell is of general occurrence. Disruption of the genetic apparatus of the host is certainly not general, since cells infected by any one of several plant or animal viruses can still grow and divide. Changes in the synthetic pattern of virus-infected animal cells similar to those of phage-infected bacteria, however, have been recognized (19).

Influenza virus in the allantoic membrane of the chick embryo behaves very much like bacteriophage in a culture of a susceptible bacterium, with discrete cycles of intracellular growth and liberation, mutual exclusion in cells infected by two virus strains, and other similarities (13); a genetic analysis of this situation would be very desirable. As virus reproduction is apparently more on a level with the reproduction of the genetic material of other cells than with the reproduction of the whole cell itself, it does not seem rash to assume that in all virus infections the material carrying virus activity will be found to be differently organized in its intracellular, replicating, "dynamic" state than in the extracellular, "static" condition. This makes it unlikely that even the most careful and painstaking work on the physical properties of extracellular virus particles (22, 32), although very interesting from other points of view, can throw much light on the fundamental problem of virology—virus reproduction. The limitation appears to be an operational one—the alteration, upon infection, of the very properties that the physicochemist analyzes.

In contrast, the limitation of chemical studies on virus-infected cells is merely a technological one—the inadequacy of present-day organic chemistry to deal with the level of organization at which biological specificities are encountered. A sharp refinement of

the chemical tool is available, however—immunochemistry. Viruses are good antigens and are in general completely distinct serologically from the uninfected host cells. Can virus specificity be traced serologically during virus reproduction, even in the absence of demonstrable virus activity, to reveal to us the "intermediates" of virus synthesis? The beautiful work of Hoyle (18) and of Henle (14) on the complement-fixing antigens in the various phases of influenza virus infection shows that these antigens, which carry virus specificity without virus activity, increase in amount *before* virus activity appears. Similar methods now being applied to the study of the early phases of phage production should yield very valuable information.

What will the "intermediates" of phage reproduction, if any, be like? Will they disclose the structure of the hereditary material represented by the postulated genetic units of replication? The recent discovery of an osmotic membrane around the phage particle (1) suggests that the latter may consist of both genetic and nongenetic specific materials; caution will be necessary in distinguishing between the two. Nevertheless, it is not unreasonable to hope that this line of work will bring us one step closer to our ultimate goal, the identification of the elementary "replicating units" of biological material and the clarification of their mode of reproduction.

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