THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 239, No. 1, January 1964
Printed in U.S.A.

A Deoxyribonucleic Acid Phosphatase-Exonuclease from *Escherichia coli*

II. CHARACTERIZATION OF THE EXONUCLEASE ACTIVITY*

CHARLES C. RICHARDSON,† I. R. LEHMAN, AND ARTHUR KORNBERG

From the Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California

(Received for publication, July 18, 1963)

An enzyme detected initially by its capacity to increase the priming capacity of deoxyribonucleic acid was identified upon its purification from *Escherichia coli* extracts as a phosphatase acting specifically on 3′-phosphoryl-terminated deoxyribonucleic acid chains (1, 2). Concurrent with the purification of this DNA phosphatase, there was an enrichment for an exonucleolytic activity which could be distinguished from the two known exonucleases of *E. coli*. Evidence for the identity of the phosphatase and exonuclease activities with a single enzyme is the subject of this report and the basis for naming this enzyme a DNA phosphatase-exonuclease. The specificity of the enzyme in attacking native double stranded DNA and its inability to act on small polynucleotides make it a unique and useful reagent in studies of DNA structure and metabolism.

EXPERIMENTAL PROCEDURE

Materials

The ^{32}P- and ^{3}H-labeled DNAs and RNA, the mixed polymer of ribo- and deoxyribonucleotides, the synthetic oligonucleotides, and the 3′- and 5′-phosphoryl-terminated DNA substrates were prepared as described in the previous paper (1). Heat-denatured DNA was prepared by heating ^{32}P-labeled *E. coli* DNA (0.3 μmole per ml) in a boiling water bath for 10 minutes in 0.05 M KCl and then quickly cooling it in an ice bath. Unlabeled deoxyribonucleoside triphosphates were purchased from the California Corporation for Biochemical Research. Deoxythymidine-2-^{14}C was purchased from the New England Nuclear Corporation; it was phosphorylated to 5′-deoxythymidylate by *E. coli* deoxythymidine kinase, and subsequently to 5′-deoxythymidine triphosphate as previously described (3). The synthetic dAT copolymer[1] was prepared by synthesis *de novo* (4) with ^{14}C-labeled dTTP, dATP, and purified *E. coli* DNA polymerase. The synthetic rAU copolymer was prepared with *E. coli* RNA polymerase in a dAT-primed synthesis utilizing ^{14}C-ATP and UTP as substrates (5). *Bacillus subtilis* DNA labeled with ^{14}C-deoxythymidylate at its 3′-hydroxyl end was prepared by treating *B. subtilis* DNA with ^{14}C-deoxythymidylate in the presence of purified *E. coli* DNA polymerase (6). Hybrid DNA with one strand ^{15}N-^{2}H-labeled and the other strand normal, isolated from *B. subtilis*, was a gift of Dr. A. T. Ganesan. The latter DNA was labeled in both strands with ^{3}H (specific activity, 2×10^5 c.p.m. per μmole). The deoxythymidine 3′- and 5′-*p*-nitrophenyl esters were prepared by Dr. A. Nussbaum.

The fractions of *E. coli* DNA phosphatase-exonuclease were prepared and assayed as described in the previous paper (1). Fraction VI, the phosphocellulose pervaporate, was used as the purified enzyme source in all the experiments to be described. *E. coli* phosphodiesterase, specific for single stranded DNA, and specific 5′-nucleotidase from *Crotalus adamanteus* venom were purified and assayed as previously described (7, 8). *E. coli* alkaline phosphatase was obtained from the Worthington Biochemical Corporation and characterized as before (1).

Methods

Assay of Exonuclease Activity of E. coli DNA Phosphatase-exonuclease—This assay measures the conversion of a ^{32}P-labeled native DNA to acid-soluble fragments. The incubation mixture (0.3 ml) contained 20 μmoles of Tris-HCl buffer, pH 8.0, 0.2 μmole of $MgCl_2$, 0.3 μmole of 2-mercaptoethanol, 50 mμmoles of ^{32}P-labeled native *E. coli* DNA, and 0.1 to 2.0 units of enzyme.[2] The reaction mixture was incubated for 30 minutes at 37°; 0.2 ml of a cold solution of calf thymus DNA (2.5 mg per ml) and 0.5 ml of cold 7% perchloric acid were added. After 5 minutes at 0°, the resulting precipitate was removed by centrifugation at 10,000 × *g* for 5 minutes; 0.2 ml of the supernatant fluid was pipetted into a planchet, and 1 drop of 1 N KOH was added to the aliquot. The solution was taken to dryness, and the radioactivity was measured. The supernatant fluids obtained from control incubations with enzyme omitted contained 0.3 to 0.5% of the added radioactivity. A unit of exonuclease activity is defined as the amount causing the production of 1.0 mμmole of acid-soluble ^{32}P in 30 minutes. The radioactivity produced was proportional to the enzyme concentration at levels of 0.1 to 2.0 units of enzyme. Thus, with the addition of 0.005, 0.01, 0.02, and 0.03 ml of a 1:100 dilution of Fraction VI, specific activities of 6,300, 6,250, 6,400, and 6,200, respectively, were obtained.

Unless otherwise noted, all enzyme units in this paper refer to those obtained with the exonuclease assay.

* This work was supported in part by Grants GM 07581 and 5T1 Gm 196 from the National Institutes of Health, United States Public Health Service.

† Postdoctoral Fellow, United States Public Health Service (Grant GF 13,634). Present address, Department of Biological Chemistry, Harvard Medical School, Boston 15, Massachusetts.

[1] The abbreviations used are: dAT copolymer, copolymer of deoxyadenylate and deoxythymidylate; rAU copolymer, copolymer of adenylate and uridylate; the prefix "r" denotes "ribo."

[2] Enzyme dilutions were made as previously described for the DNA phosphatase assay (1).

TABLE I

Identification of acid-soluble product of DNA phosphatase-exonuclease action on native DNA as 5′-mononucleotides

The reaction mixtures (0.3 ml) contained 20 μmoles of Tris-HCl buffer, pH 8.0, 0.2 μmole of $MgCl_2$, 0.3 μmole of 2-mercaptoethanol, 25 mμmoles of ^{32}P-labeled *E. coli* DNA, and 30 units of DNA phosphatase-exonuclease. After incubation for 30 minutes, a 0.05-ml aliquot was assayed in the routine manner for the production of acid-soluble ^{32}P. Radioactivity susceptible to *E. coli* alkaline phosphatase (20 μg) in a 0.05-ml aliquot was determined in a Norit assay as described for the DNA phosphatase activity (1); for susceptibility to 5′-nucleotidase, a 0.05-ml aliquot was assayed in a reaction mixture (0.3 ml) containing 30 μmoles of glycine buffer, pH 9.6, μmoles of $MgCl_2$, and 5 units of venom 5′-nucleotidase.

Experiment	Acid-soluble ^{32}P	Acid-soluble, Norit-nonadsorbable ^{32}P		
		Control	*E. coli* alkaline phosphatase	Venom 5′-nucleotidase
	mμmoles	*mμmoles*		
1	7.8	<0.1	7.5	
2	5.7	<0.1	5.3	5.3

The hydrolysis of the 3′- and 5′-*p*-nitrophenyl thymidylate esters was measured by the formation of *p*-nitrophenol according to Razzell and Khorana (9). The release of mononucleotides from the synthetic substrate pTpTpTpTpT was measured by the formation of phosphate esters susceptible to *E. coli* alkaline phosphatase or 5′-nucleotidase.

Density Gradient Centrifugation—The technique described by Meselson, Stahl, and Vinograd (10) was followed. Solid CsCl (Harshaw Chemical Company, optical grade) was added to the DNA solution to raise the density to values between 1.725 and 1.750 g per cm^3, depending on the sample being examined. The exact density of the solution was determined by means of the linear relation between refractive index and density (11). Approximately 0.75 ml of the final CsCl solution, containing 0.05 M Tris at pH 8.0, was placed in a cell (plastic Kel-F centerpiece) and centrifuged in a Spinco model E analytical ultracentrifuge at 44,770 r.p.m. at 25°. After 20 hours of centrifugation, ultraviolet absorption photographs were taken on Kodak commercial film. Tracings were then made with a Joyce-Loebl double beam recording microdensitometer with an effective slit width of 30 μ in the film dimension. Densities were calculated by using the position of *Tetrahymena pyriformis* (buoyant density of 1.684 g per cm^3) as a reference (12).

Other Methods—Protein, inorganic orthophosphate, and deoxyribose were determined as described in the previous paper (1). ^{32}P was counted in a windowless gas flow counter; ^{14}C and ^{3}H were measured in the Packard Tri-Carb liquid scintillation counter.

RESULTS

Exonucleolytic Action of Enzyme

Identification of Acid-soluble Product of Reaction—When native ^{32}P-labeled *E. coli* DNA was incubated with the enzyme, 31% of the radioactivity was rendered acid-soluble. Greater than 95% of the acid-soluble label was susceptible to *E. coli* alkaline phosphatase as measured by the formation of Norit-nonadsorbable ^{32}P. Treatment of the product with the specific venom 5′-nucleotidase also rendered 91% of the radioactivity Norit-nonadsorbable, thus identifying the acid-soluble product as 5′-mononucleotides (Table I).

Exonuclease Action on ^{14}C-Terminally Labeled DNA—When DNA terminally labeled at its 3′-hydroxyl end with ^{14}C-deoxythymidylate was treated with the *E. coli* DNA phosphatase-exonuclease, 90% of the radioactive deoxynucleotides of the molecules were made acid-soluble in 15 minutes. At this time, less than 1% of the unlabeled nucleotides had been released as judged by the appearance of acid-soluble ultraviolet-absorbing material (Fig. 1). This result is similar to that observed with venom diesterase (6, 7), an enzyme which attacks DNA or polynucleotides stepwise from the end bearing a free 3′-hydroxyl group (13). Digestion of a terminally labeled DNA with pancreatic DNase, an endonuclease which attacks DNA in a random manner (14), has been previously shown to release unlabeled nucleotides at a rate similar to the release of radioactivity (7). It therefore appears that the DNA phosphatase-exonuclease carries out a stepwise attack on DNA, starting from the 3′-hydroxyl end and producing 5′-mononucleotides in a manner analogous to venom diesterase.

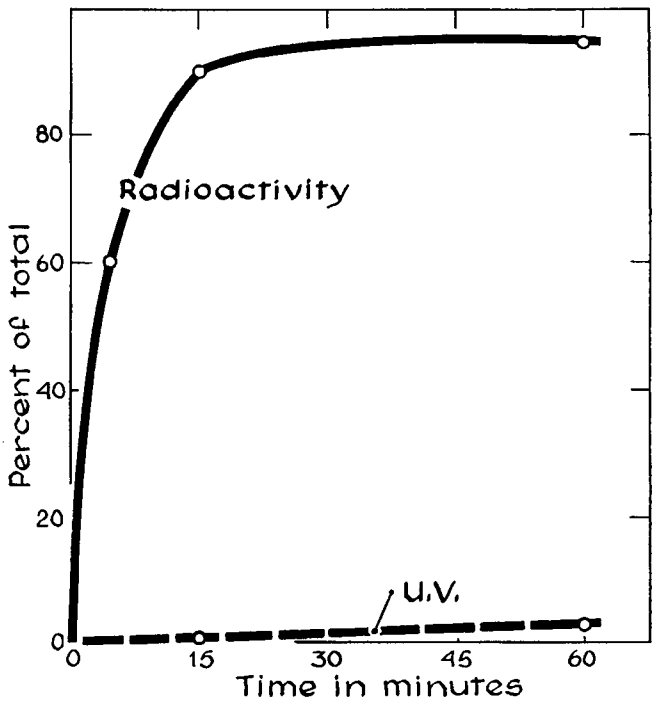

FIG. 1. DNA phosphatase-exonuclease action on DNA terminally labeled with ^{14}C-deoxythymidylate. The incubation mixture (2.4 ml) contained 0.64 μmole of DNA phosphate terminally labeled with ^{14}C-deoxythymidylate (2250 c.p.m. per μmole of DNA phosphate), 160 μmoles of Tris-HCl, pH 8.0, 1.6 μmoles of $MgCl_2$, 2.4 μmoles of 2-mercaptoethanol, and 16 units of the purified enzyme (Fraction VI). Incubation was at 37°. At the times indicated, 0.3-ml aliquots were removed; 0.2 ml of calf thymus "carrier" DNA (2.5 mg per ml) and 0.5 ml of cold 7% perchloric acid were added. After 5 minutes at 0°, the suspensions were centrifuged and the optical density of the supernatant fluids was determined at 260 mμ. The radioactivity in the supernatant fluid was determined by pipetting 0.2 ml into an aqueous scintillator solution and is expressed as a percentage of the total radioactivity in the DNA added at zero time. Measurement of the radioactivity in the precipitate as previously described (7) gave values for hydrolysis that agreed within 8%.

Sequential Release of P_i and Mononucleotides from 3'-Phosphoryl-terminated DNA—Unlike venom diesterase (13) or *E. coli* phosphodiesterase,[3] the DNA phosphatase-exonuclease attacks a 3'-phosphoryl-terminated DNA. The enzyme first removes the 3'-phosphoryl group, as described in the previous paper (1), and then carries out a stepwise attack on the molecule, starting from the newly formed 3'-hydroxyl end. When ^{32}P-labeled 3'-phosphoryl-terminated DNA was incubated with the enzyme, there was an immediate release of P_i, which reached a limit in approximately 30 minutes (Fig. 2). The release of Norit-adsorbable radioactivity (5'-mononucleotides) occurred only after a lag of approximately 5 minutes. However, the rate of release of mononucleotides rapidly increased, and reached a linear rate which corresponded to that initially seen with the P_i release. It appears that the enzyme attacks the 3'-hydroxyl end of the molecule only after having first removed the 3'-phosphoryl end group. The enzyme apparently has the same specificity for this monoesterified group and cleaves it at about the same rate as the phosphodiester linkage.

Effect of DNA Structure on Rate of Reaction—The DNA phosphatase-exonuclease hydrolyzed *B. subtilis* DNA, *E. coli* DNA, and the synthetic dAT copolymer at similar rates (Table I). ^{32}P-Labeled *E. coli* DNA, partially digested with either micrococcal nuclease or *E. coli* endonuclease (see "Methods"), was acted on by the enzyme at the same rate as the untreated DNA. Heat-denatured *E. coli* DNA is hydrolyzed at approximately one-fourth the rate seen with the native DNA. The addition of heat-denatured DNA (30 mμmoles) to the standard reaction mixture produced no inhibition.

When ^{32}P-labeled *E. coli* ribosomal RNA was added to the reaction mixture, acid-soluble ^{32}P was released at a rate 2% of that seen with native DNA. However, the hydrolysis of the RNA was inhibited by Mg^{++}, and the products of the hydrolysis were found to be oligonucleotides as judged by the limited susceptibility to *E. coli* alkaline phosphatase. No 5'-mononucleotides were produced as tested by the formation of venom 5'-nucleotidase-sensitive phosphate. These results suggest that the DNA phosphatase-exonuclease does not attack ribosomal RNA and that the slow degradation of RNA to oligonucleotides was due to a contaminant of *E. coli* ribonuclease (15). Since the secondary structure of the DNA substrate clearly influenced the rate and extent of hydrolysis, the hydrogen-bonded, double stranded rAU copolymer (5) was incubated with the purified enzyme. However, this synthetic RNA was not hydrolyzed (less than 0.1% the rate on native DNA) as measured by the formation of acid-soluble radioactivity. The synthetic oligonucleotide pTpTpTpTpT is not a substrate for the enzyme, nor are the synthetic thymidine 3'- and 5'-*p*-nitrophenyl esters (Table II). The addition of pTpTpTpTpT to the standard reaction mixture produced no detectable inhibition.

Effect of DNA Structure on Extent of Reaction—The exonuclease hydrolyzes native *E. coli* or *B. subtilis* DNA to only 35 to 45% acid solubility (Table II). The addition of more enzyme or prolonged incubation did not result in further hydrolysis of the native DNA. However, if an additional 50 mμmoles of ^{32}P-labeled *E. coli* DNA were added, there was a further release of radioactivity at a rate similar to that initially seen with the native DNA, indicating that the enzyme had not been inactivated and that the products were not inhibitory.

The dAT copolymer, which, because of its alternating, repeating sequence, remains double stranded even after extensive hydrolysis (16), was hydrolyzed almost to completion.

[3] I. R. Lehman, personal communication.

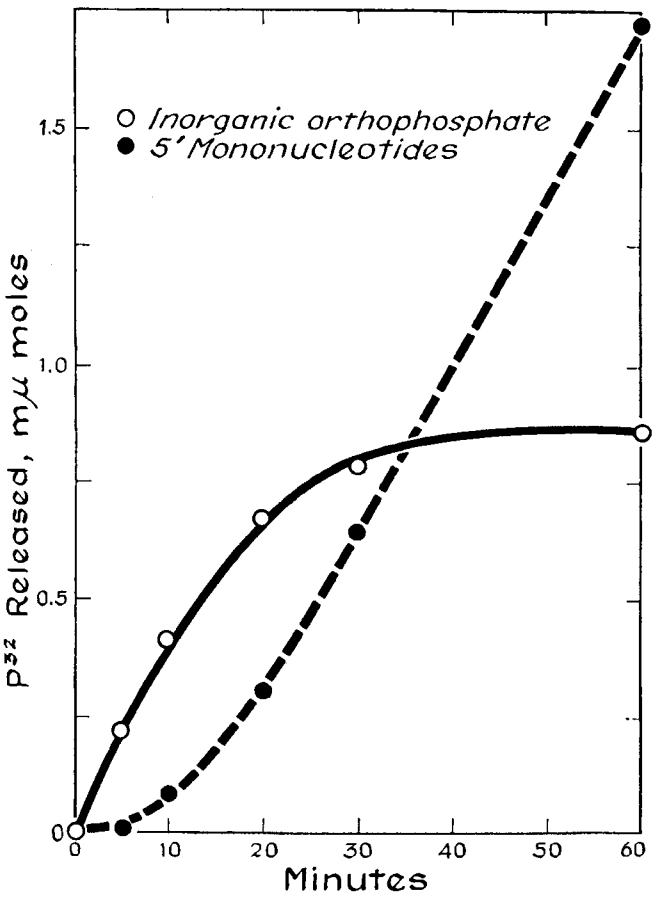

FIG. 2. Sequential release of P_i and mononucleotides from 3'-phosphoryl-terminated DNA. The reaction mixture (2.4 ml) contained 400 mμmoles of ^{32}P-labeled 3'-phosphoryl-terminated DNA, 160 μmoles of potassium phosphate buffer, pH 7.0, 24 μmoles of $MgCl_2$, 2.4 μmoles of 2-mercaptoethanol, and 1.2 units of DNA phosphatase-exonuclease (DNA phsophatase units). Incubation was at 37°. At the times indicated, 0.3-ml aliquots were removed; 0.2 ml of calf thymus "carrier" DNA (2.5 mg per ml) and 0.5 ml of cold 10% trichloroacetic acid were added. After 5 minutes at 0°, the suspensions were centrifuged and 0.2 ml of the supernatant fluid was pipetted into a planchet for determination of the acid-soluble ^{32}P. A similar aliquot of 0.5 ml of the supernatant fluid was treated in a manner identical with the routine DNA phosphatase assay (1) to obtain the acid-soluble, Norit-nonadsorbable ^{32}P (P_i). The difference between the $^{32}P_i$ released and the acid-soluble ^{32}P represents the release of mononucleotides.

With heat-denatured *E. coli* DNA, the initial rate of hydrolysis was slow and decreased progressively, but could be carried to 18% by the addition of large amounts of enzyme (150 units) and prolonged incubation (2 hours).

Effect of Incubation at 45° on Limit Reached with Native and Heat-denatured DNA—Additional evidence that the secondary structure of the DNA plays a significant role in the catalytic property of the enzyme is shown by experiments in which incubation was carried out at 45°, a temperature which decreases the nonspecific hydrogen bonding in heat-denatured DNA (17). A preparation of the heat-denatured DNA is degraded to a limit of only 3% at 45°, as compared to 18% at 37° (Table III). When the native DNA is incubated at 45°, a limit of 32% is obtained as compared to one of 38% at 37°.

TABLE II

Effect of DNA structure on exonuclease activity

The rate of reaction was measured in the standard assay with replacement of the usual native *E. coli* DNA by the compounds listed. The preparation and characterization of these substrates were described in the previous paper (1). Native *E. coli* DNA, native *B. subtilis* DNA, dAT copolymer, heat-denatured *E. coli* DNA, ribosomal RNA, and the endonuclease-treated DNAs (40 mμmoles of each) were incubated in the standard reaction mixture (0.3 ml) containing 20 μmoles of Tris-HCl buffer, pH 8.0, 0.2 μmole of $MgCl_2$, 0.3 μmole of 2-mercaptoethanol, and 0.5 to 2.0 units of enzyme. Because of a small contamination of the enzyme preparation wiyh *E. coli* RNase as described in the text, the release of 5′-mononucleotides from the RNA substrates was assayed with venom 5′-nucleotidase (see Table I). The synthetic substrates were tested at several concentrations ranging from 40 to 200 mμmoles per reaction mixture and at several pH values and $MgCl_2$ concentrations with 10 to 100 units of enzyme. The natural DNAs, treated and untreated, as well as the dAT copolymer, were assayed by determination of acid-soluble radioactivity. Hydrolysis of pTpTpTpTpT was assayed by the formation of phosphate esters susceptible to 5′-nucleotidase as described in Table I. Hydrolysis of the thymidine 3′- and 5′-*p*-nitrophenyl esters was determined by spectrophotometric assay (see "Methods"). For determination of *the extent of reaction*, the reaction mixtures contained these compounds and an excess of enzyme (50 to 100 units); the total release of acid-soluble radioactivity was determined after 60 minutes. In each case, additional enzyme (50 units) and incubation for 30 minutes resulted in no further release of radioactivity. The amount of radioactivity made acid-soluble is recorded as the percentage of the total radioactivity present in the reaction mixture.

Compound	Rate	Extent
	mμmoles/min/mg	*% total*
dAT copolymer	1370	92
Native *E. coli* DNA	1130	38
Native *B. subtilis* DNA	2100	39
3′-Hydroxyl-terminated DNA*	1460	44
3′-Phosphoryl-terminated DNA*	1391	50
Heat-denatured *E. coli* DNA	350†	18
Ribosomal RNA	<1	
rAU copolymer	<1	
pTpTpTpTpT	<6‡	
Thymidine 3′-*p*-nitrophenyl ester	<13	
Thymidine 5′-*p*-nitrophenyl ester	<13	

* The acid-soluble radioactivity obtained with the partially digested substrates reflects not only mononucleotides released but also those oligonucleotides whose size decreased to the "acid-soluble" range.

† Proportionality to enzyme concentration with the heat-denatured DNA as substrate was obtained only up to the release of 0.40 mμmole of ^{32}P. Addition of 20 mμmoles of heat-denatured DNA to the standard assay produced no inhibition.

‡ Addition of 100 mμmoles of pTpTpTpTpT to the standard assay produced no inhibition.

The DNA phosphatase-exonuclease appears to require double stranded regions in the molecule in order to hydrolyze the DNA substrate. Heat-denatured DNA apparently contains enough such regions at 37° to enable the enzyme to act on it at a reduced rate and to a limited extent. At 45°, where these regions are eliminated, the heat-denatured DNA is hardly degraded at all.

Action of E. coli Exonuclease I (Phosphodiesterase) on Acid-precipitable Product of Reaction—The acid-insoluble product which remains as a result of the extensive action of the DNA phosphatase-exonuclease is largely single stranded, as judged by its susceptibility to *E. coli* exonuclease I, an enzyme specific for single stranded DNA (7). The susceptibility of the acid-insoluble ^{32}P to this enzyme increases with the progressive action of the DNA phosphatase-exonuclease on native DNA (Fig. 3). When 10% of the DNA has been converted to acid-soluble mononucleotides, only 2% of the residual DNA is susceptible to exonuclease I. As the DNA phosphatase-exonuclease approaches its limit of degradation (approximately 35% of the total radioactivity), there is a rapid increase in the susceptibility of the acid-precipitable DNA to exonuclease I. Since exonuclease I requires a single stranded region terminated by a free 3′-hydroxyl group, these findings indicate that the 3′-hydroxyl termini are protected in double stranded regions until the limit of action of the DNA phosphatase-exonuclease is approached.

Buoyant Density Distribution in CsCl of Acid-precipitable Product of Extensive DNA Phosphatase-Exonuclease Action—Further evidence for the formation of single stranded DNA by the extensive action of the DNA phosphatase-exonuclease was obtained by following the buoyant density distribution in CsCl of a hybrid *B. subtilis* DNA, one strand labeled with ^{15}N-^{2}H and one with ^{14}N-^{1}H. As shown in Fig. 4, such a density-labeled hybrid bands in CsCl at a buoyant density of 1.728 g per cm^3, and on heating and fast cooling the strands separate and band at 1.770 and 1.721 g per cm^3, the expected densities of single stranded heavy (^{15}N-^{2}H-labeled) and single stranded light *B. subtilis* DNA (18). After extensive digestion with the DNA phosphatase-exonuclease (42% acid-soluble nucleotides), two new bands appeared, one at a density (1.770 g per cm^3) corresponding to that of single stranded heavy DNA, and the other at a density (1.721 g per cm^3) corresponding to that of single stranded light DNA. Furthermore, the original hybrid band at a density of 1.728 g per cm^3 had increased in density, with a major portion banding in the region (1.735 g per cm^3) expected of hybrid molecules which were predominately single stranded but unable to completely separate and band separately.

Action on Mixed Ribo-Deoxyribonucleotide Polymer—DNA chains which contain interspersed ^{32}P-rCMP residues served as substrates for the enzyme. The initial rates were from 3 to 6% of those observed with native *E. coli* DNA, but the extent of hydrolysis ranged from 77 to 82% (Fig.5); of the labeled nucleo-

TABLE III

Extent of hydrolysis of native and heat-denatured DNA at 37° and 45°

The extent of hydrolysis of native and heat-denatured ^{32}P-labeled *E. coli* DNA was determined under the standard assay conditions at 37° and 45°. In each reaction mixture were present 40 mμmoles of DNA phosphate. At both temperatures and with each substrate, 50 units of DNA phosphatase-exonuclease were added at 15-minute intervals until no further release of acid-soluble radioactivity was obtained. The limit is expressed as the percentage of total ^{32}P present in the reaction mixture.

DNA	Extent	
	37°	45°
	%	%
Native	38	32
Heat-denatured	18	3

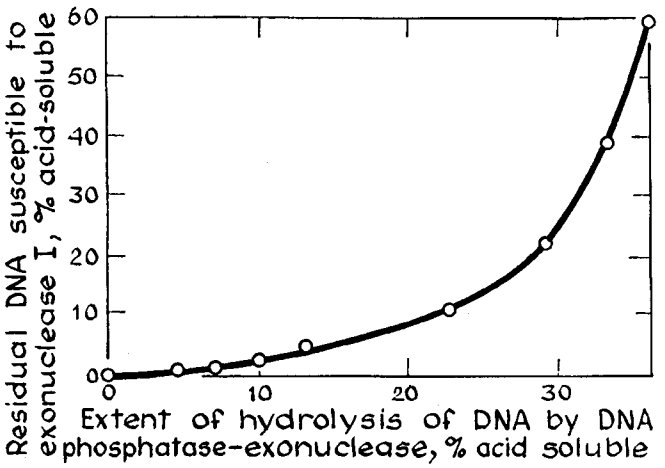

FIG. 3. Action of *E. coli* exonuclease I ("phosphodiesterase") on the acid-precipitable product of DNA phosphatase-exonuclease reaction. The reaction mixture (2.2 ml) contained 0.27 μmole of ^{32}P-labeled *E. coli* native DNA, 140 μmoles of Tris-HCl buffer, pH 8.0, 1.4 μmoles of $MgCl_2$, 2.1 μmoles of 2-mercaptoethanol, and 80 units of *E. coli* DNA phosphatase-exonuclease. Incubation was at 37°. At intervals, 0.1-ml aliquots were removed and added to 0.2 ml of water. The acid-soluble ^{32}P was determined as in the standard exonuclease assay. Similar aliquots (0.1 ml) were also removed and added to reaction mixtures (0.3 ml) containing 20 μmoles of glycine buffer, pH 9.2, 2 μmoles of $MgCl_2$, 0.02 μmole of $ZnCl_2$ (to inhibit DNA phosphatase-exonuclease), and 150 units of *E. coli* exonuclease I. After incubation at 37° for 30 minutes, the reaction mixture was assayed for acid-soluble ^{32}P as above. The percentage of acid-insoluble ^{32}P made acid-soluble by the *E. coli* exonuclease I is plotted against the percentage of total DNA made acid-soluble by the DNA phosphatase-exonuclease.

tides rendered acid-soluble, 95% were attacked by 5′-nucleotidase to P_i (nonadsorbable to Norit), indicating that 5′-rCMP was released by the enzyme. The rather extensive digestion of the mixed polymers may be explained by the assumption that these mixed polymers are synthesized by addition to chains of native DNA primer as a repair process (6, 19) rather than as new strands. Therefore, even extensive removal of the mixed polymers would not be expected to alter the essentially native character of the DNA to which they were initially attached; the DNA exonuclease is thus binding a native DNA rather than single stranded substrate.

Evidence that DNA Phosphatase and Exonuclease are Part of a Single Enzyme

Constant Ratio of Activities during Purification—During the course of a 1300-fold purification, the ratio of DNA phosphatase to exonuclease activity remained essentially constant (Table IV);[4] the ratio was also constant throughout the DEAE-cellulose and phosphocellulose peaks.

Similar Reaction Rates—When the enzyme acts on ^{32}P-labeled 3′-phosphoryl-terminated DNA under the conditions found optimal for the DNA phosphatase activity, it is possible to distinguish the rate of release of orthophosphate and of mononucleotides (Fig. 2). The linear rate of release of P_i from the 3′-phosphoryl end groups (1.26 mμmoles in 30 minutes) was followed by an identical rate of release of mononucleotides (1.30 mμmoles in 30 minutes).

Dependence on Divalent Cations and pH—Optimal conditions for each activity depend on complicated relationships between pH, buffer, divalent cation concentration, and the nature and amount of the DNA substrate. For example, the optimal pH for exonuclease activity at 7×10^{-4} M $MgCl_2$ is 7.7 to 8.4, but with a 10-fold increase in $MgCl_2$ the pH optimum is 7.0 to 7.4. The latter conditions are just those which were found to be optimal for DNA phosphatase activity (1). In the absence of added Mg^{++}, exonuclease activity (standard assay) is only 6% of the optimal value, and with 0.01 M EDTA, it is undetectable.

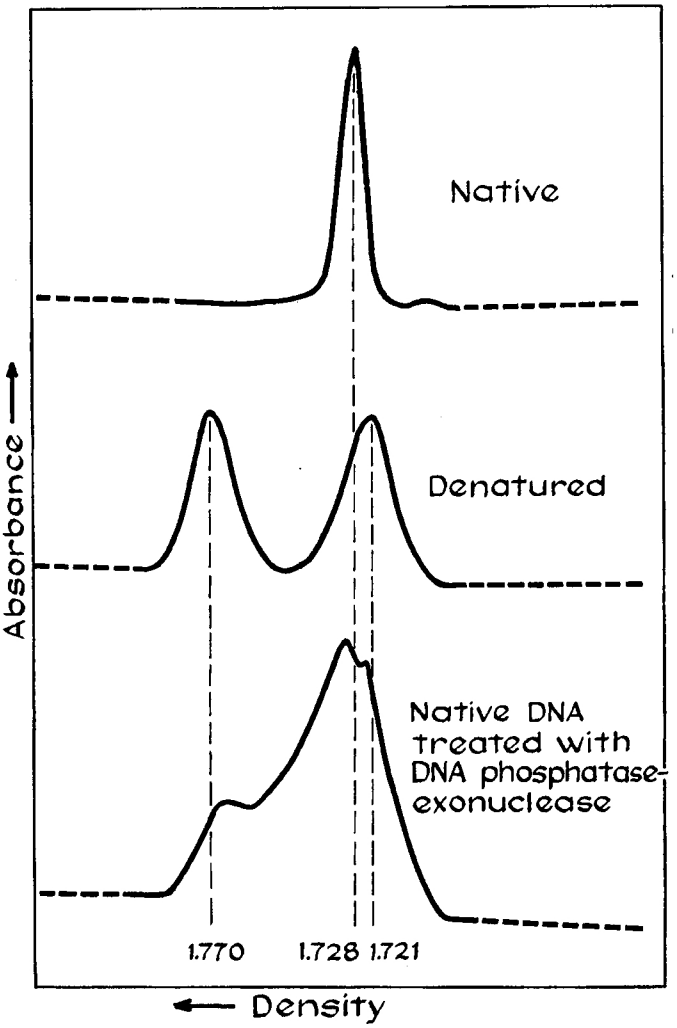

FIG. 4. Buoyant density distribution in CsCl of the product of extensive digestion of ^{15}N-^{2}H-hybrid *B. subtilis* DNA by DNA phosphatase-exonuclease. When a solution of native ^{15}N-^{2}H-hybrid DNA labeled in both strands with ^{3}H (band profile shown in *top tracing*) was heated for 5 minutes at a concentration of 30 mμmoles per ml at 100° in 0.05 M KCl and quickly cooled, the strands separated and banded at the expected density for single stranded heavy and single stranded light *B. subtilis* DNA (*second tracing*). Treatment of 60 mμmoles of the native hybrid DNA with 16 μg of the DNA phosphatase-exonuclease (Fraction VI) under standard assay conditions resulted in a limit of 42% of the radioactivity becoming acid-soluble. After dialysis first against 1.0 M KCl and then 0.05 M KCl, the product banded as shown in the *third tracing*.

[4] The exonuclease assay could be used to follow the purification of the DNA phosphatase-exonuclease provided that soluble RNA and the antiserum to *E. coli* endonuclease were present in the assay mixture (see Table IV).

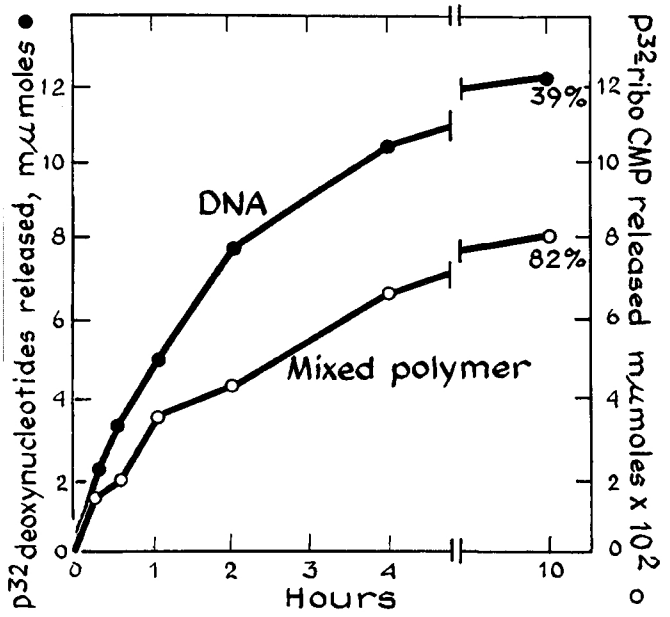

FIG. 5. Hydrolysis of a mixed ribo-deoxyribonucleotide polymer compared to DNA. The mixed polymer contained ^{32}P-rCMP interspersed among the four deoxyribonucleotides with a frequency of about 1 per 10 nucleotides (1). The incubation mixtures (0.3 ml) contained 0.07 M potassium phosphate buffer, pH 7.0, 0.01 M $MgCl_2$, 0.001 M 2-mercaptoethanol, mixed polymer containing 0.98 mμmole of rCMP (1), and 8 μg of Fraction VI. For DNA hydrolysis, the mixture contained 31 mμmoles of ^{32}P-native *E. coli* DNA (1.3×10^6 c.p.m. per μmole) and 4 μg of Fraction VI. Acid-soluble ^{32}P was determined as in the standard exonuclease assay.

TABLE IV

Ratio of DNA phosphatase and exonuclease activities during purification

The standard DNA phosphatase assay, utilizing the 3'-phosphoryl-terminated DNA as substrate, was used to determine the DNA phosphatase activity. The standard exonuclease assay, with native DNA as substrate, was used to determine the exonuclease activity. In assaying the exonuclease activity in the crude extract and acetone fractions, 10 mμ moles of soluble RNA and 0.005 ml of a 1:5 dilution of rabbit antiserum prepared against *E. coli* endonuclease were added.

Fraction and step	DNA phosphatase	Exonuclease	Ratio of phosphatase to exonuclease
	units/ml		
I. Extract	780	270	2.9
III. Acetone	1,040	400	2.6
IV. DEAE-cellulose	393	123	3.2
VI. Phosphocellulose pervaporate	19,500	6,400	3.0

$MnCl_2$ was about equally effective as $MgCl_2$ when tested in the range of 2 to 7×10^{-4} M.

Inhibition by $ZnCl_2$ and p-Chloromercuribenzoate—$ZnCl_2$ at 3.3×10^{-5} M inhibited DNA phosphatase by 90% (1) and the exonuclease activity by 88%. $CaCl_2$ (3×10^{-3} M) inhibited neither activity. The DNA phosphatase activity requires protective sulfhydryl compounds for prolonged incubation (see footnote 5 in (1)); both the DNA phosphatase and exonuclease activities are sensitive to *p*-chloromercuribenzoate. At 1×10^{-4} M in the standard assays (2-mercaptoethanol omitted), *p*-chloromercuribenzoate inhibited the DNA phosphatase activity by 50% and the exonuclease activity by 90%.

Heat Inactivation—The two activities follow an identical heat inactivation curve (Fig. 6). After 30 minutes at 37° in the absence of Mg^{++} or substrate, only 7% of each activity remained.

DISCUSSION

The enzyme described here is an exonuclease attacking stepwise from the 3'-hydroxyl end of a DNA molecule. This is established by (*a*) the formation of 5'-mononucleotides as the only acid-soluble products, (*b*) the preferential removal of labeled nucleotides from a DNA molecule labeled at the 3'-hydroxyl end, and (*c*) the sequence of reaction on a DNA terminated by a 3'-phosphoryl end; liberation of a 5'-nucleotide occurs only after the 3'-phosphoryl group is removed.

The exonuclease attacks denatured DNA at a slow rate and to a very limited extent. The requirement for a double stranded helical structure is suggested by the almost total lack of hydrolysis (less than 5%) of heat-denatured DNA at an elevated temperature. The inability of the enzyme to hydrolyze short oligonucleotides or model phosphodiester substrates and its capacity

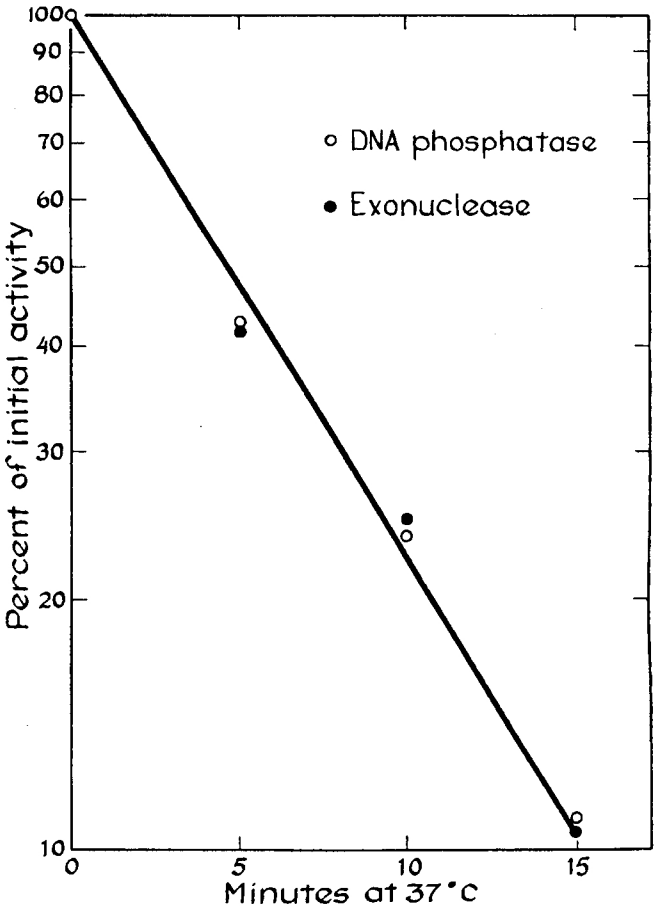

FIG. 6. Heat inactivation of DNA phosphatase-exonuclease. Fraction VI (1.6 μg) was diluted to 1.5 ml with the standard diluent and incubated at 37°. At the times indicated, 0.005-ml aliquots were added to the standard DNA phosphatase assay and 0.05-ml aliquots to the standard exonuclease assay. The percentage decrease in activity is relative to the zero time aliquots set at 100.

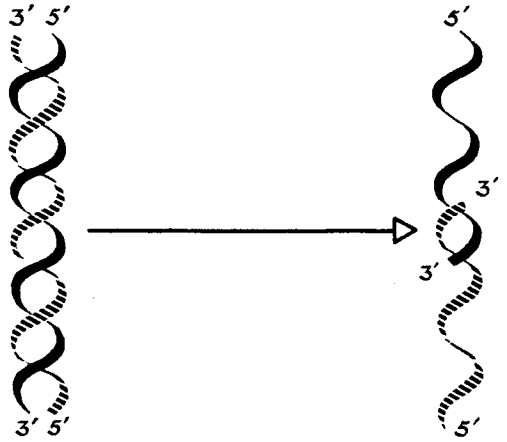

FIG. 7. Postulated mechanism of action of the exonucleolytic activity of the DNA phosphatase-exonuclease.

to digest the dAT copolymer virtually to completion also demonstrate the importance of secondary structure of the substrate in reactions catalyzed by this enzyme. The inability to attack RNA, even when double stranded, must therefore be due to a specificity for the DNA structure.

Consistent with the requirement for a double stranded DNA is the fact that its degradation stops when 35 to 45% has been digested. Assuming that the enzyme initiates its stepwise attack from both 3′-hydroxyl ends of the double stranded molecule, then at or near 50% degradation, all of the residual acid-insoluble DNA should be single stranded (Fig. 7) and resistant to further attack. Supporting this is the observation that the residual DNA is largely susceptible to the *E. coli* phosphodiesterase, an enzyme specific for single stranded DNA.

Further evidence for this model is provided by the examination of the buoyant density in CsCl of the acid-insoluble product formed as a result of extensive digestion of a density-labeled hybrid DNA (one strand containing ^{15}N and ^{2}H atoms). Banding of the product in CsCl clearly reveals that single strands of both light and heavy buoyant density are formed. The finding that a certain proportion of the hybrid DNA increases in density but does not give rise to strand separation suggests that hydrogen bonding still exists to some extent in these molecules. It is also significant that the dAT copolymer, which, because of its alternating structure, remains double stranded even after extensive degradation, is digested to 94% of completion.

A most interesting and unusual property of the enzyme is its ability to cleave 3′-phosphoryl groups from the terminus of a DNA chain, a feature which distinguishes it from known exonucleases, which are either blocked or inhibited by such end groups. These two activities appear to reside on the same protein molecule and reflect the ability of this enzyme to cleave a 3′-phosphoryl linkage whether it is involved in a mono- or diester linkage.

TABLE V

Comparison of E. coli exonucleases

* Exonuclease I is the *E. coli* phosphodiesterase, specific for single stranded DNA; exonuclease II is the *E. coli* nuclease purified with DNA polymerase; and exonuclease III is the *E. coli* DNA phosphatase-exonuclease described in this paper. For a comprehensive review of the nucleases of *E. coli*, see (20).

	Exonuclease I	Exonuclease II	Exonuclease III
Required end group on DNA:			
---p p p OH	Active	Active	Active
---p p p P	Inactive	Inactive	Active; because initial attack removes P_i terminus
Required DNA structure	Single stranded	Single or double stranded	Double stranded
Extent of action	Up to terminal dinucleotide	Complete	Up to 40% degradation; residual single stranded chains are resistant
Type of attack	Stepwise beginning at 3′-hydroxyl end of chain	Stepwise beginning at 3′-hydroxyl end of chain	Stepwise beginning at 3′-hydroxyl or phosphoryl end of chain
Products from DNA	Mono- and dinucleotides with 5′-phosphodiester group	5′-Mononucleotides	P_i, 5′-mononucleotides, and large molecular weight single stranded oligonucleotides
Action on synthetic oligonucleotides:			
pTpTpTpTpT	Produces 5′-mononucleotides and pTpT	Produces 5′-mononucleotides	Inactive
TpTpTp	Inactive	Inactive	Inactive

In addition to the endonuclease in *E. coli*, there are now three characterized exonucleases. Their common and distinguishing properties are summarized in Table V.

The DNA phosphatase-exonuclease has properties which may be exploited in several ways. Unlike venom diesterase, spleen diesterase, and *E. coli* exonuclease I, the DNA phosphatase-exonuclease prefers and may therefore be used for stepwise degradation of native, double stranded DNA. Moreover, regardless of the presence of 3′-hydroxyl or 3′-phosphoryl end groups, the enzyme is able to initiate its exonucleolytic action and therefore attack all the DNA molecules.

These two properties make it possible to dissect away a biologically active DNA by observing the retention as well as loss of selected markers. A preliminary study with *B. subtilis* DNA showed that when 5% of the DNA had been converted to mononucleotides, 50% of the control transforming activity remained, as measured by transformation of the Try_2^+ (indole) marker.[5] Since kinetics of this inactivation was consistent with an exonucleolytic attack on the DNA, the absence of endonuclease activity in the purified enzyme was confirmed.

Combined with the use of another phosphatase such as the *E. coli* alkaline phosphatase, which acts on both 5′- and 3′-phosphoryl termini, it should be possible to determine the occurrence of (and factors producing) such termini in DNA.

SUMMARY

1. A deoxyribonucleic acid phosphatase purified extensively from extracts of *Escherichia coli* (described in the preceding paper) is also an exonuclease carrying out a stepwise attack from the 3′-hydroxyl end of the deoxyribonucleic acid chain, releasing 5′-mononucleotides.

2. The enzyme preferentially attacks native, double stranded DNA, degrading it to an extent of 35 to 45% mononucleotides; the residual, predominately single stranded DNA is resistant to further attack.

3. A mixed polymer of ribo- and deoxyribonucleotides is cleaved by the enzyme, although at a reduced rate. The enzyme can therefore liberate a 5′-ribonucleotide when it terminates a DNA chain.

4. The enzyme is unable to hydrolyze single stranded short oligonucleotides, ribosomal ribonucleic acid, or the adenylate-uridylate copolymer.

5. Identity of the phosphatase and exonuclease activities is supported by the constant ratio of the activities throughout purification, similar reaction rates, inhibition by $ZnCl_2$ and *p*-chloromercuribenzoate, and the rates of heat inactivation.

[5] We are indebted to Dr. Walter Bodmer of the Department of Genetics, Stanford University, for carrying out the *B. subtilis*-transformation assays.

REFERENCES

1. Richardson, C. C., and Kornberg, A., *J. Biol. Chem.*, **239**, 242 (1964).
2. Richardson, C. C., Schildkraut, C. L., Aposhian, H. V., Kornberg, A., Bodmer, W., and Lederberg, J., in H. Vogel (Editor), *Symposium on informational macromolecules*, Academic Press, Inc., New York, 1963, p. 13.
3. Okazaki, R., and Kornberg, A., *J. Biol. Chem.*, **239**, 269 (1964).
4. Schachman, H. K., Adler, J., Radding, C. M., Lehman, I. R., and Kornberg, A., *J. Biol. Chem.*, **235**, 3242 (1960).
5. Chamberlin, M., Baldwin, R. L., and Berg, P., *J. Molecular Biol.*, in press.
6. Adler, J., Lehman, I. R., Bessman, M. J., Simms, E. S., and Kornberg, A., *Proc. Natl. Acad. Sci. U. S.*, **44**, 641 (1958).
7. Lehman, I. R., *J. Biol. Chem.*, **235**, 1479 (1960).
8. Lehman, I. R., Roussos, G. G., and Pratt, E. A., *J. Biol. Chem.*, **237**, 819 (1962).
9. Razzell, W. E., and Khorana, H. G., *J. Biol. Chem.*, **236**, 1144 (1961).
10. Meselson, M., Stahl, F. W., and Vinograd, J., *Proc. Natl. Acad. Sci. U. S.*, **43**, 581 (1957).
11. Ifft, J., Voet, P. H., and Vinograd, J., *J. Phys. Chem.*, **65**, 1138 (1961).
12. Sueoka, N., *J. Molecular Biol.*, **3**, 31 (1961).
13. Razzell, W. E., and Khorana, H. G., *J. Biol. Chem.*, **234**, 2114 (1959).
14. Dekker, C. A., and Schachman, H. K., *Proc. Natl. Acad. Sci. U. S.*, **40**, 894 (1954).
15. Spahr, P. F., and Hollingworth, B. R., *J. Biol. Chem.*, **236**, 823 (1961).
16. Inman, R. B., and Baldwin, R. L., *J. Molecular Biol.*, **5**, 185 (1962).
17. Stollar, D., and Grossman, L., *J. Molecular Biol.*, **4**, 31 (1962).
18. Schildkraut, C. L., Marmur, J., and Doty, P., *J. Molecular Biol.*, **3**, 595 (1961).
19. Richardson, C. C., Schildkraut, C. L., and Kornberg, A. *Cold Spring Harbor symposia on quantitative biology, Vol. 28*, Long Island Biological Association, Cold Spring Harbor, New York, 1963, in press.
20. Lehman, I. R., in W. Cohn and J. N. Davidson (Editors), *Progress in nucleic acid research, Vol. II*, Academic Press, Inc., New York, in press.