Biochemical Analysis of the Naturally Repaired Sections of Bacteriophage T5 Deoxyribonucleic Acid. I. Bromodeoxyuridine Incorporation into Parental Deoxyribonucleic Acid in the Absence of Deoxyribonucleic Acid Replication*

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ABSTRACT: The evidence accumulated on the repair synthesis of deoxyribonucleic acid, including the final step in genetic recombination, indicates that the rejoining process requires the synthesis of a short stretch of oligonucleotide to fill the gap between the deoxyribonucleic acid fragments. This process can occur in the absence of semiconservative replication of deoxyribonucleic acid. The current report describes a biochemical analysis of the nucleotides incorporated during the repair process. The deoxyribonucleic acid analyzed was nonreplicated T5 deoxyribonucleic acid extracted from *Escherichia coli* infected at 43° with a temperature-sensitive deoxyribonucleic acid polymerase mutant of the phage.

The T5 was labeled with ^{32}P, and the bacteria were grown throughout in a medium containing bromodeoxyuridine. Analysis of the bromodeoxyuridylic acid incorporated into T5 deoxyribonucleic acid showed that only the 3′-bromodeoxyuridylic acid and not the 5′-bromodeoxyuridylic acid was labeled with ^{32}P. Several possible explanations can account for the data obtained, but it will be pointed out that in particular these data are consistent with a model which states that deoxyribonucleic acid strands, when broken by an endonuclease, yield fragments phosphorylated at the 5′ end and not at the 3′ end. During the repair process, nucleotides are incorporated and joined by phosphodiester linkage to the 5′-phosphate ends of the preexisting strands. We find about 29 ^{32}P-labeled 3′-bromodeoxyuridylic acid molecules per T5 molecule.

As reviewed by one of us (Volkin, 1965), genetic recombination in bacteriophage occurs very likely by a breakage and rejoining of DNA strands, and this process may occur in the absence of DNA replication. The data, particularly of Meselson (1964), Anraku and Tomizawa (1965a,b), Tomizawa (1967), and Kozinski and Felgenhauer (1967), indicate that the rejoining process requires the synthesis of a short stretch of oligonucleotide to fill the gap between DNA fragments. This final process may involve the same mechanism as the repair process for DNA damaged by ultraviolet light (Pettijohn and Hanawalt, 1964) and by thymine starvation (Pauling and Hanawalt, 1965). The evidence in all these cases for a covalent linkage of the rejoined or repaired section to the rest of the DNA is that such a DNA strand remains intact after denaturation. The current study is an attempt to analyze by a more direct biochemical approach the structure of the repaired section of DNA. In order to minimize any misunderstanding we shall define two words used frequently in this report. By "replication" we mean the semiconservative, sequential polymerization of a complementary strand of a replicating unit which leads to an increase in the number of DNA molecules. "Repair" means all other modes of synthesis that take part in the maintenance of preexisting DNA molecules. Thus, repaired DNA would include any recombinant DNA formed by a breakage and rejoining process.

T5 bacteriophage was selected in this work because it has a relative high frequency of recombination and its DNA is nonglucosylated. To ensure the complete separation of the repair process from the replication of DNA, a temperature-sensitive DNA polymerase mutant of the phage, T5ts53, was used at 43°. The host bacteria were grown in a medium containing an excess of the dBrUrd[1] to ensure that parental phage DNA of normal density and newly replicated DNA, if any, could be separated on the basis of density difference. However, the limited extent of incorporation of dBrUrd into repaired sections of parental DNA would not be enough to change the buoyant density of the DNA. Bacteriophage T5ts53 whose DNA was uniformly labeled with ^{32}P was used for the infection. After incubation at 43°, DNA was extracted from infected

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: dBrUrd, bromodeoxyuridine; dBrUMP, bromodeoxyuridylic acid; dFUrd, fluorodeoxyuridine; pfu, plaque-forming unit; SDS, sodium dodecyl sulfate; P-diesterase, phosphodiesterase; SSC, standard saline citrate; TES, Tris–EDTA–saline.

bacteria and the DNA of parental density was purified by CsCl centrifugation. One fraction was hydrolyzed to 5′-nucleotides, another fraction to 3′-nucleotides, and the nucleotides were analyzed for the possible presence of ^{32}P-labeled dBrUMP. The results showed that only 3′-dBrUMP was labeled with ^{32}P. The significance of the finding will be discussed in terms of known enzymic pathways involving DNA.

Materials and Methods

Escherichia coli F, a host that rapidly adsorbs T5, T5st (a wild-type bacteriophage), and T5ts53 (a mutant with a temperature-sensitive DNA polymerase) were obtained from the late F. Lanni. The following media were used: 3XDM (Fraser and Jerrel medium as modified by Guthrie and Sinsheimer, 1960), MGM (Lanni, 1961), and MGM–CA (MGM with 0.2% Casamino Acids). A dBrUrd medium consisted of one of the above media plus dFUrd (5 μg/ml), dBrUrd (200 μg/ml), and Urd (25 μg/ml).

Preparation of Phage Lysate. An overnight culture of *E. coli* was diluted 50-fold into a fresh medium (3XDM or MGM–CA) and grown at 30° with aeration for about four generations until the bacterial titer was 4×10^8/ml. $CaCl_2$ solution was added to the final concentration of 1×10^{-3} M. Phage T5ts53 was added to a multiplicity of infection of 1. Incubation was continued for 8 hr. The culture was treated with chloroform and immediately centrifuged to remove bacterial debris. For phage T5st, a culture was prepared at 37° and incubated for only about 5 hr after infection. ^{32}P-Labeled T5 was generally prepared by adding 2 mCi of ^{32}P/100 ml of MGM–CA at the time of dilution of an overnight culture. ^3H-Labeled T5ts53 was prepared by adding 200 μCi of [^3H]dUrd/100 ml of MGM–CA at the time of dilution of an overnight culture, and an additional 100 μCi/100 ml was added after the infection with the phage. A dBrUrd-labeled T5ts53 was obtained from *E. coli* F grown in dBrUrd–3XDM or dBrUrd–MGM–CA. Phage was added when the bacterial titer was 2×10^8/ml. An aeration bottle was kept in semidarkness throughout. [^3H]dBrUrd–T5ts53 was prepared by adding 400 μCi of [^3H]dBrUrd/100 ml of dBrUrd medium at the time of dilution of an overnight culture.

Purification of Phage. The procedure described below is a modification of the method of Miyazawa and Thomas (1965). The phage lysate from above was treated with 10 μg/ml each of DNase and RNase at room temperature for about 30 min, then left in a refrigerator overnight. A 4-ml suspension of commercially prepared hydroxylapatite (Hypatite C, Clarkson Chemical Co.) was added per 100 ml of a lysate. The lysate was stirred for 15 min, and the supernatant fraction was decanted into a beaker. Unlike the results reported by Miyazawa and Thomas (1965), the phage remained in solution. To this, an additional 10 ml of hydroxylapatite suspension was added per 100 ml. The suspension was stirred for 15 min, then left standing until the hydroxylapatite had settled. The supernatant fraction was decanted, and then the hydroxylapatite was packed into a column and washed with 0.02 M phosphate buffer (0.02 M sodium phosphate (pH 6.8), 4×10^{-4} M NaCl, 2×10^{-5} M $MgSO_4$, and 2×10^{-4} M Tris-HCl; Miyazawa and Thomas, 1965). Washing was continued until the A_{260} stopped decreasing; then the phage was eluted with 0.1 M phosphate buffer. The eluent fractions that were opalescent were pooled together and centrifuged in a Spinco LII centrifuge with a no. 30 rotor at 15,000 rpm for 1 hr. Each pellet was resuspended in 1–2 ml of 0.02 M phosphate buffer. The yield of phage was about 20–40% of the original plaque-forming units. The first treatment with hydroxylapatite adsorbs some material that has absorption at 260 mμ and sediments faster than phage in sucrose gradient centrifugation. The phage purified by this method usually exhibits these properties: pfu/$A_{260} = 1.2 \times 10^{11}$ and 2.6×10^{10}, and $A_{260}/A_{280} = 1.51$ and 1.54 for T5st and T5ts53, respectively.

Extraction of DNA from Phage. The concentration of phage was adjusted to $A_{260} = 20$. SDS was added to 0.67% (w/v), and the phage suspension was incubated at 50° for 10 min. The suspension was made to 1 M in NaCl, and an equal volume of phenol was added dropwise. The mixture was shaken gently for a few seconds and left at 50° for 5 min. The suspension was cooled to room temperature and centrifuged to separate the phases. The aqueous layer was dialyzed against at least two changes of about 200 times the sample volume of 0.1 SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate (pH 7); Marmur, 1961). The yield of DNA was more than 80%.

Extraction of DNA from Infected Bacteria. The infected bacteria were centrifuged and resuspended in $^1/_{10}$–$^1/_{50}$ of the original volume of TES buffer (0.1 M Tris-HCl, 0.05 M EDTA, and 0.2 M NaCl, pH 8; Smith and Burton, 1966). The following method represents a combination of procedures used by Smith and Burton (1966) and by Massie and Zimm (1965a,b). The bacterial suspension was heated at 70° for 20 min and cooled. Lysozyme was added to 330 μg/ml and the suspension was incubated at 37° for 30 min. The sample was then digested with 1 mg/ml of Pronase (CalBiochem, preincubated at 37° for 30 min) at 50° for 5 hr. This sample was treated with SDS and phenol as described for phage DNA above, except that the interphase and phenol layer were mixed with a small volume of TES buffer once more. The aqueous layers were combined. The yield of parental phage DNA from the infected cells was about 80%.

Density Gradient Centrifugation. For CsCl equilibrium and density gradient centrifugation (Meselson *et al.*, 1957), a sample previously adjusted to the desired volume with 0.1 SSC was made 55.5% w/w in CsCl by addition of solid CsCl. Analytical experiments were usually performed with a SW-39 rotor, and preparatory procedures were carried out with a no. 40 rotor. The runs were made at 36,000 rpm for about 48–72 hr. After centrifugation in a SW-39 rotor, the fractions were collected from the bottom of the centrifuge tube directly through a hole or through a metal cannula pierced through the bottom. After centrifugation in a no. 40 rotor, the fractions were collected by a Techni-

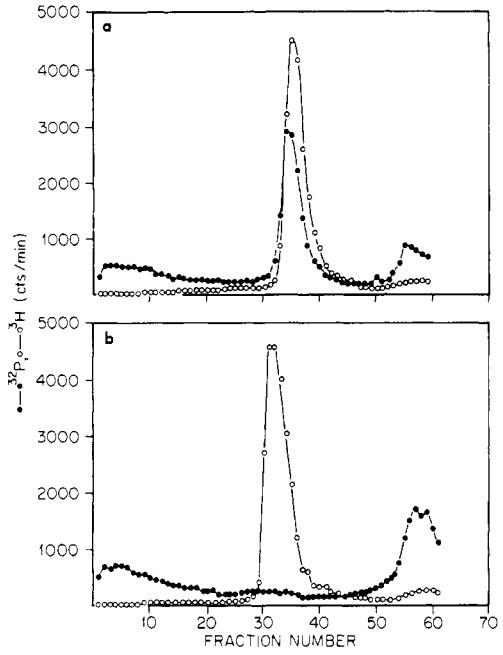

FIGURE 1: Temperature effect on DNA synthesis in T5ts53-infected cells. A 30-ml culture of bacteria was infected with T5 at a multiplicity of infection of 5, and 1.5 mCi of ^{32}P was added 5 min after the infection and incubated for 45 min (a) at 30° and (b) at 43°. The DNA extracts (0.1 and 0.5 ml) were each diluted to 4 ml, and ^{3}H-labeled T5 DNA as marker and 5.09 g of CsCl were added; then the samples were centrifuged in a no. 40 rotor at 36,000 rpm for 60 hr. (●) ^{32}P and (○) ^{3}H activities were alkaline resistant and trichloroacetic acid insoluble.

con pump through a metal tube that reached to the bottom of the centrifuge tube.

For CsCl density gradient centrifugations at an alkaline pH (Vinograd *et al.*, 1963), a sample in 0.1 SSC was dialyzed against 0.04 M potassium phosphate buffer (pH 12). The volume was adjusted with the same buffer, and CsCl was added to 55.5% w/w and centrifuged in a SW-39 rotor.

The refractive index of every tenth fraction of the CsCl solution was measured with a Zeiss refractometer and converted into density by the equation of Ifft *et al.* (1961).

For sucrose density gradient centrifugations, a linear gradient was made with 2.4 ml each of 5 and 20% w/w sucrose in the buffer in which the sample was prepared (Britten and Roberts, 1960). A sample of 0.1–0.3 ml was layered on top of the gradient. The centrifugation was performed at 36,000 rpm for 3 hr at 4° with a SW-39 rotor.

Preparation of Samples for Counting in a Liquid Scintillation Counter. For assay of total radioactivity, the sample was placed directly in a scintillation vial diluted to 1.5 ml with water, and 15 ml of dioxane scintillator (Butler, 1961) was added. For determination of acid-insoluble material in a preparation containing a small amount of acid-soluble radioactivity, an aliquot was placed on a Whatman No. 3MM filter paper disk, treated with 5% trichloroacetic acid, washed, and counted as described by Bollum (1959). For assay of DNA in the presence of RNA and acid-soluble nucleotides, a sample was hydrolyzed with 1 N KOH (total volume 1 ml) at 37° for 120 min, acidified with 0.7 ml of 50% trichloroacetic acid at 0°, mixed, and collected on a Millipore filter (Oppenheim and Riley, 1966). The filter was washed with 5 ml of cold 5% trichloroacetic acid and 5 ml of cold 70% ethanol, dried at room temperature for at least 1 hr, and counted in a toluene scintillator. In some instances, alkaline digestion was done in 0.1 N NaOH at 37° overnight. There was no significant difference in the results. When DNA is to be assayed in the presence of a large amount of acid-soluble radioactivity, the Millipore method is preferred over the filter paper method.

Digestion of DNA to Nucleotides. Digestion of the DNA to 5′-nucleotides was accomplished by the sequential action of pancreatic DNase and snake venom phosphodiesterase. To a DNA sample in 9 ml of water, $MgCl_2$ was added to 0.01 M, $CaCl_2$ to 0.005 M, Tris-HCl (pH 7.4) to 0.025 M, and 5 mg of DNase. The sample was incubated overnight at 37° in the presence of 0.1 ml of *n*-heptane, then 0.6 ml of 1 M NH_3–NH_4Cl buffer (pH 9.0) and 0.08 ml of snake venom phosphodiesterase (5 mg/ml) were added, and the incubation was continued for another 3.5 hr.

Digestion of the DNA to 3′-nucleotides was performed by the sequential action of micrococcal nuclease and spleen phosphodiesterase. A DNA sample in 9 ml of water was made to 0.005 M $CaCl_2$ and 0.1 M Tris-HCl (pH 8.8), then 4500 units of micrococcal nuclease was added. Digestion proceeded overnight at 37° in the presence of 0.1 ml of *n*-heptane. Then 0.7 ml of 0.1 M EDTA (pH 4.5), 0.5 ml of 1 M HCl, 0.5 ml of 1 M KH_2PO_4 (pH 6), and 0.4 ml of spleen phosphodiesterase (15–20 units/ml) were added, and incubation was continued for 3.5 hr. In both cases, about 98% of the samples was recovered as mononucleotides.

All of the enzymes employed were obtained from Worthington Biochemical Corp.

Isolation of dBrUMP. The enzymic digests of DNA were made to 0.15 N in NH_4OH, and 5′- and 3′-[^{3}H]-dBrUMP were added as markers to the respective digests. Each of the samples was adsorbed on a Dowex 1 (Cl^-) column (1 × 15 cm). Most of the four major 5′-nucleotides were eluted with 750 ml of 0.006 N HCl and most of the four major 3′-nucleotides were eluted with 750 ml of 0.009 N HCl. The 5′-dBrUMP was then eluted with 750 ml of 0.009 N HCl. The 5′-dBrUMP was then eluted with a linear gradient formed by 500 ml each of 0.006 and 0.016 N HCl, and the 3′-dBrUMP was eluted with a linear gradient of 0.009 and 0.03 N HCl. The fractions with appreciable amounts of ^{3}H activity of the corresponding nucleotides were pooled, neutralized with chloroform–Alamine 336 (General Mills) solution as described by Lester Smith and Page (1948), and evaporated to dryness by flash evaporation. The residues were resuspended in water for paper electrophoresis analysis.

Preparation of Marker [^{3}H]dBrUMP. To 500 ml of 3XDM, 1 ml of overnight culture of *E. coli* F was added. At the same time 2 mCi/100 mg of [^{3}H]dBrUrd, 12.5 mg of Urd, and 2.5 μg of dFUrd were added and the

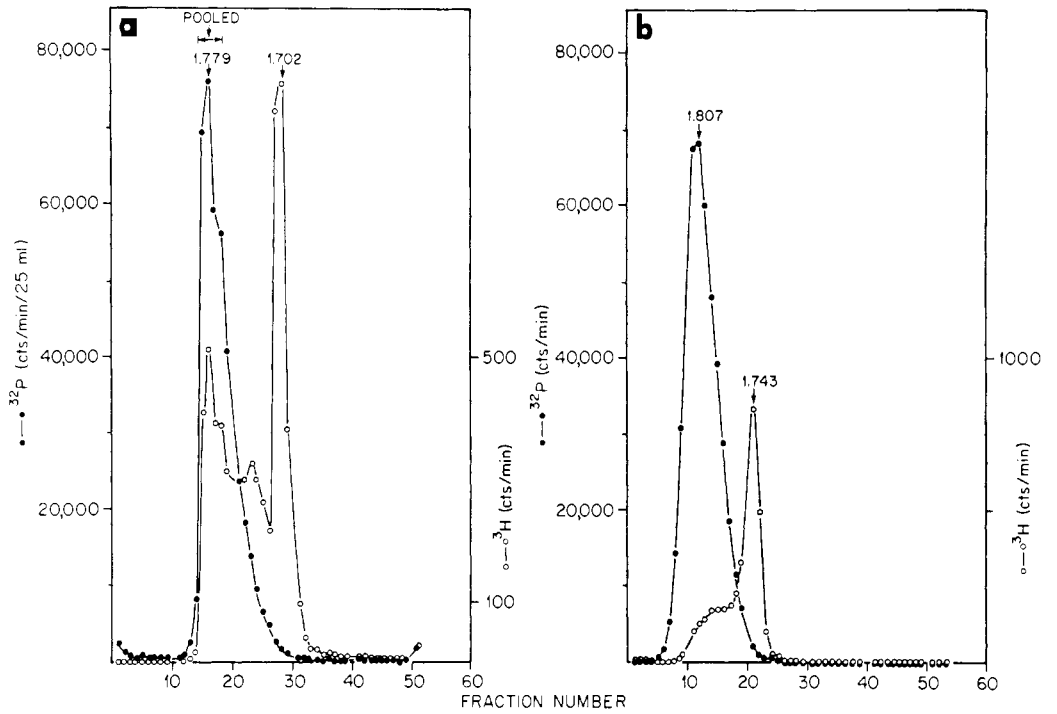

FIGURE 2: T5st DNA replication in a dBrUrd medium. A 100-ml culture of bacteria in MGM–CA–dBrUrd was infected with [^{3}H]T5st at a multiplicity of infection of 5, and 2 min later 0.3 mCi of ^{32}P was added; bacteria were harvested at 40 min after the infection. (a) The extracted DNA in 3.5 ml was made into a 55.5% (w/w) CsCl solution and centrifuged in a SW 50 rotor for 48 hr at 36,000 rpm. Radioactivity was measured on the alkaline-resistant, trichloroacetic acid insoluble fraction. (b) Fractions 15–18 from part a were pooled and diluted to 5 ml with a 55.5% (w/w) CsCl solution in 0.04 M K_2HPO_4 (pH 12) and then centrifuged in a SW 50 rotor for 60 hr at 36,000 rpm. Radioactivity was measured on trichloroacetic acid insoluble fractions. (●) ^{32}P and (○) ^{3}H.

bacteria were grown for about 12 hr. The total nucleic acid was extracted by a hot salt method (Tyner *et al.*, 1953; Volkin and Astrachan, 1956), and RNA was separated by alkaline digestion followed by precipitation of DNA by three volumes of ethanol. The DNA sample was divided into two equal fractions, and 5′- and 3′-nucleotides were obtained as described. The paper electrophoresis of both 5′ and 3′ samples in 0.05 M borate (pH 9.3) showed only one ^{3}H-active spot. The recovery of ^{3}H activity as dBrUMP from [^{3}H]DNA was about 90% for both 5′- and 3′-nucleotides.

Paper Electrophoresis. Electrophoresis was used to identify the dBrUMP and was carried out in two steps on Whatman No. 3MM paper in a Markham and Smith (1952) apparatus at 1000 V. The sample was applied on a 1-cm line, and the first separation was made in 0.02 N HCOOH for 3 hr. After the run, the radioactive spot was located as follows. Two lines were drawn parallel to the direction of the sample movement at the second and third centimeter from the center of the original sample line but off to one side. The space between the two lines was cut at 1-cm intervals. Each square was put in a counting vial, 1.5 ml of water was added followed by 15 ml of dioxane scintillator, and the sample was counted. The space corresponding to the ^{3}H activity was located, cut, and eluted with water. It was then concentrated to less than 30 μl, and spotted on new Whatman No. 3MM paper for electrophoresis in 0.05 M glycine or 0.05 M borate (pH 9.3). After a 2-hr run, the paper was dried and cut into strips 5-cm wide at 1-cm intervals in the direction of the movement, and the strips were counted as described above.

Digestion of Nucleotides. 5′-NUCLEOTIDES. Lyophilized venom (10 mg) from *Crotalus adamanteus* (Ross Allen, Silver Springs, Fla.) was dissolved in 10 ml of water, and the insoluble materials were separated by filtration. This solution was used as the 5′-nucleotidase, since it is completely free of 3′-nucleotidase activity.

ALKALINE PHOSPHATASE. The enzyme (Worthington Biochemical Corp.) was made to 0.5 mg/ml in 0.05 M Tris-HCl (pH 8).

To 25 μl of a sample whose pH was adjusted to approximately eight, 10 μl of an enzyme and 20 μl of a buffer were added. The reaction mixture was incubated at 37° for 1 hr. The buffer for the 5′-nucleotidase reaction was 0.2 M glycine-NaOH–0.02 M MgCl (pH 8) (Heppel and Hilmoe, 1955), and the buffer for the alkaline phosphatase reaction was 0.1 M Tris-HCl (pH 8). After the incubations, the samples were concentrated twofold and the products assayed by paper electrophoresis in pH 9.3 glycine buffer as described.

Results

Preliminary Experiments. A. SEPARATION OF NEWLY REPLICATED DNA FROM PARENTAL PHAGE DNA AND *E. coli* DNA. For analysis of repaired sections of

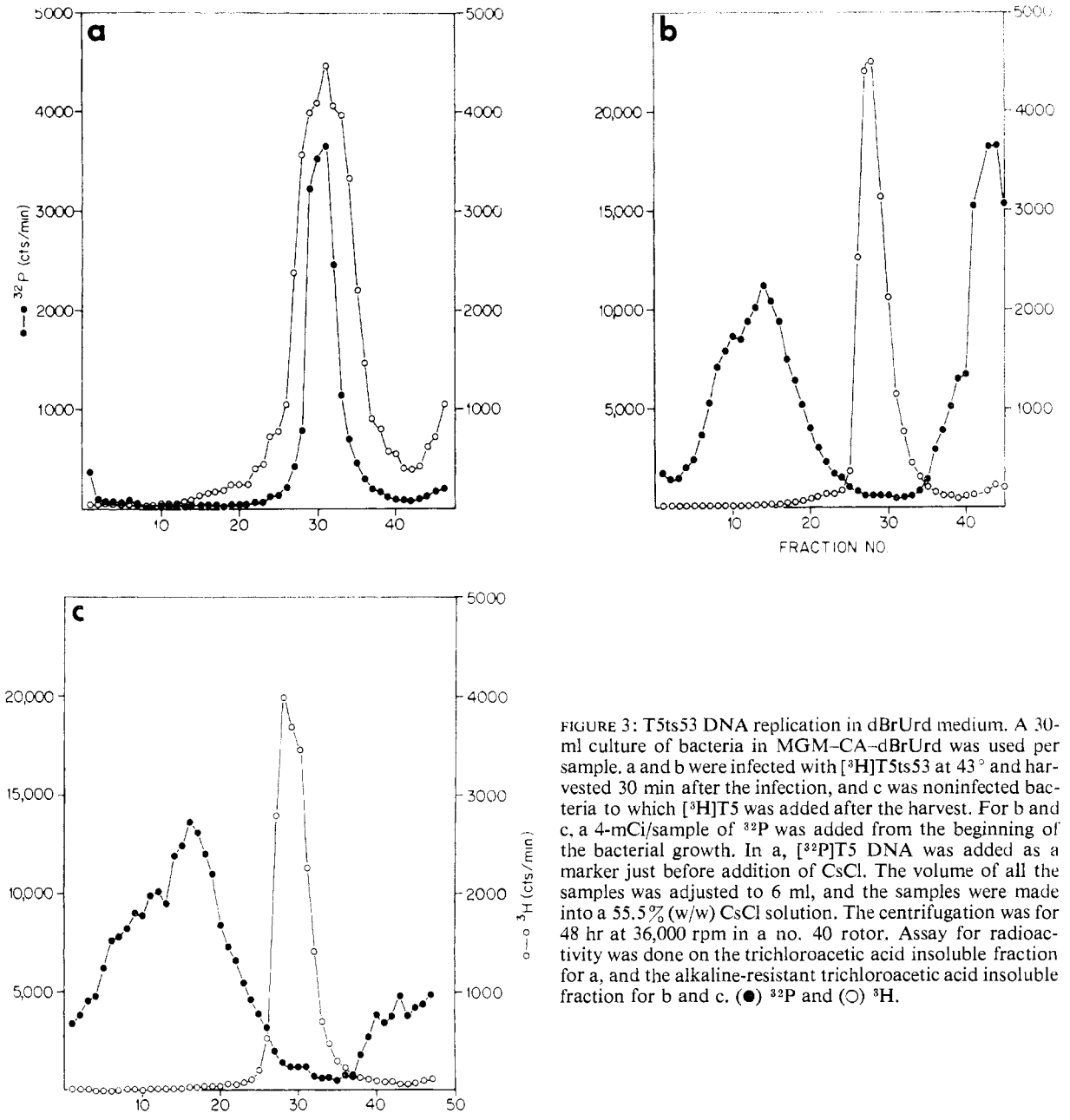

FIGURE 3: T5ts53 DNA replication in dBrUrd medium. A 30-ml culture of bacteria in MGM–CA–dBrUrd was used per sample. a and b were infected with [^{3}H]T5ts53 at 43° and harvested 30 min after the infection, and c was noninfected bacteria to which [^{3}H]T5 was added after the harvest. For b and c, a 4-mCi/sample of ^{32}P was added from the beginning of the bacterial growth. In a, [^{32}P]T5 DNA was added as a marker just before addition of CsCl. The volume of all the samples was adjusted to 6 ml, and the samples were made into a 55.5% (w/w) CsCl solution. The centrifugation was for 48 hr at 36,000 rpm in a no. 40 rotor. Assay for radioactivity was done on the trichloroacetic acid insoluble fraction for a, and the alkaline-resistant trichloroacetic acid insoluble fraction for b and c. (●) ^{32}P and (○) ^{3}H.

DNA, it is essential that such DNA be distinguished from newly replicated DNA. DNA replication is markedly inhibited by the use of the temperature-sensitive mutant, T5ts53, at 43° (DeWaard *et al.*, 1965). As shown in Figure 1, the high temperature does inhibit the DNA replication.

The growth of bacteria in a dBrUrd medium ensures the separation of any residual replicated DNA from the parental DNA. In this medium, it can be shown that for T5st, a wild-type phage, the density of the newly replicated DNA is different from that of the parental DNA. Bacteria growing in dBrUrd medium were infected at a multiplicity of infection of 5 with [^{3}H]T5st, and 0.3 mCi/100 ml of ^{32}P was added. Bacteria were harvested at 40 min after the infection, and the DNA was extracted and centrifuged in CsCl to equilibrium as described in Methods. As shown in Figure 2a, over one-half of the parental DNA has shifted to a heavier density. When the fractions from heavier DNA were pooled and recentrifuged in the alkaline CsCl solution, most of the parental DNA and newly replicated DNA were separated (Figure 2b). The parental DNA has a buoyant density of 1.743, which is the same as that of the normal phage DNA centrifuged in the alkali. There is still some parental DNA in the denser strand, presumably because of recombination with the newly replicated heavy strands. These studies indicate that the replication process can be detected by a marked shift in the density of parental DNA to a heavier density, and that the great majority

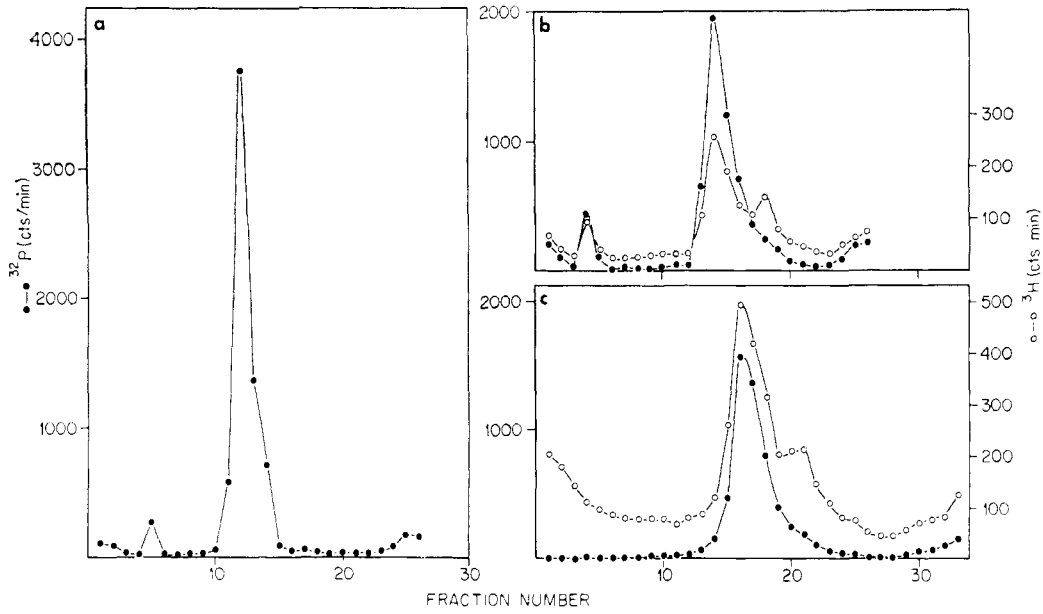

FIGURE 4: Integrity of T5 DNA. For a, [^{32}P]DNA was extracted from the phage and 9 μg was used for centrifugation. For b and c, the sample in Figure 3a was used. For b, the DNA was extracted as described in Methods. For c, the infected bacteria were lysed by SDS only. In b and c [^{32}P]T5 DNA was added as a marker. Centrifugation was done in a 5–20% linear gradient at 36,000 rpm for 3 hr. (●) ^{32}P and (○) ^{3}H.

of the newly replicated DNA is clearly separable from the parental nonreplicated DNA.

When a similar experiment was done with T5ts53 phage at 43°, there was no detectable shift of ^{3}H-labeled parental DNA to a heavier density (Figure 3a). ^{32}P-Labeled DNA from T5 phage of normal density was added as a marker. Concomitantly, experiments were performed in which ^{32}P was added from the beginning of bacterial growth; one experiment employed infection with [^{3}H]T5 at 43° (Figure 3b), and the other, infection with [^{3}H]T5 at 30°. Under both conditions, there was essentially no ^{32}P-labeled material under the [^{3}H]T5 DNA. The alkaline-resistant, trichloroacetic acid insoluble ^{32}P-labeled material at the heavier region was probably *E. coli* DNA, since it was present and in a greater quantity in noninfected bacteria to which [^{3}H]T5 was added after the harvest (Figure 3c). These experiments indicate that there is no appreciable amount of DNA replication by 30 min after the infection at 43°, the conditions under which infected bacteria were harvested for the principal experiments described below. These experiments also demonstrate that *E. coli* DNA is well separated from T5 parental DNA. The identity of ^{32}P-labeled material lighter than [^{3}H]T5 DNA was not investigated.

B. INTEGRITY OF VIRAL DNA. The recovery of T5 DNA by our method was the same whether the extraction was from free bacteriophage or from infected bacteria. The extracted DNA was analyzed by sucrose density centrifugation. DNA from free phage was found to be intact (Figure 4a), but the T5 DNA from infected bacteria was partially fragmented (Figure 4b). However, the absence of low molecular weight material in the latter preparation could be demonstrated by the lack of labeled material at the meniscus. Furthermore, the DNA label could be quantitatively recovered in an acid-insoluble form. About the same amount of DNA was fragmented when a part of the same bacterial suspension was treated only with SDS and centrifuged through a sucrose gradient (Figure 4c). This indicates that T5 phage DNA existing under these conditions inside the cells is fragile, possibly owing to the formation of

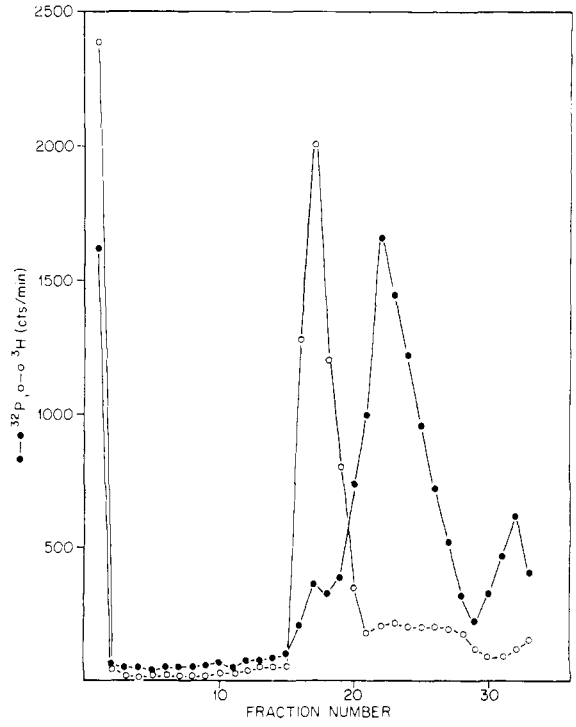

FIGURE 5: Integrity of T5 DNA used for expt 2. [^{3}H]T5 DNA was added as a marker. DNA was extracted and centrifuged in a sucrose gradient as described in Methods. (●) ^{32}P and (○) ^{3}H.

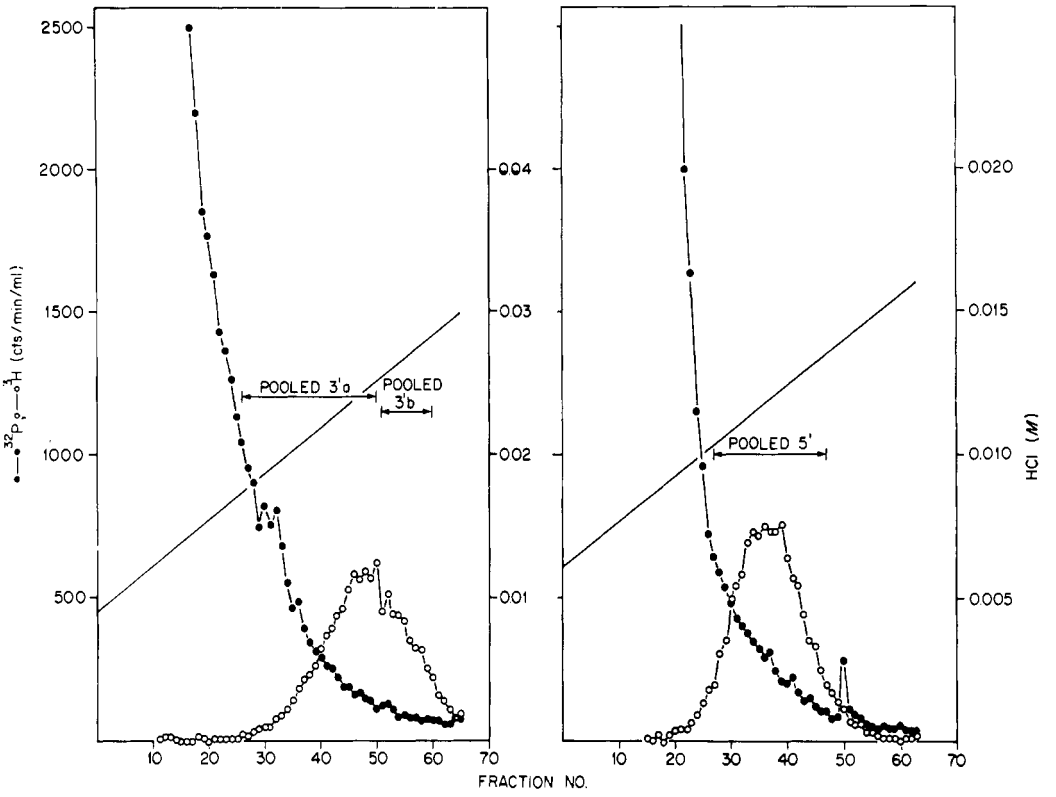

FIGURE 6: Elution patterns at the dBrUMP region from Dowex 1 chromatography of digests of the DNA of expt 2. The conditions are described in Methods. The 3'-nucleotides were pooled into two fractions indicated on the figure as 3'a and 3'b. (●) [^{32}P]Nucleotides and (○) [^{3}H]dBrUMP.

TABLE I: Recovery of Parental [^{32}P]DNA during Extraction.

Extraction Step	Experiment 1		Experiment 2	
	Total TCA[a]-Insol Act. Cor for Decay (cpm × 10^{-8})	% Yield[a]	Total TCA-Insol Act. Cor for Decay (cpm × 10^{-8})	% Yield[a]
Phage inoculum	7.05		11.2	
After centrifugation				
In supernatant fraction	2.04		1.38	
In bacterial suspension	5.05	100	4.28	100
Sum	7.09		5.66	
Lysozyme, Pronase, and SDS	4.96	98	4.94	115
Phenol extraction and dialysis	3.84	76	3.67	86
CsCl density gradient centrifugation	1.8	36	2.61	71
Alkali-resistant 5% TCA and ethanol precipitate	1.55	31		

[a] Yield refers to the bacterial suspension after centrifugation; TCA, trichloroacetic acid.

nicks. T5 DNA has preferred breakage points when subjected to shear (Burgi *et al.*, 1966).

Deoxybromouridine Incorporation in the Absence of DNA Replication. On the basis of the above data, we concluded that it is possible to look for the incorporation of dBrUrd into DNA that could have resulted from a repair process independent of the replication process. The two experiments described below were done independently of each other with different preparations of phage.

Bacteria were grown in 3XDM–dBrUrd medium to 2 × 10^{8}/ml, the temperature was shifted to 43°, and

[^{32}P]T5ts53 was added at a multiplicity of infection of 5. The incubation was continued for 30 min; then the infected bacteria were harvested. DNA was extracted and purified by CsCl density gradient centrifugation in a Spinco no. 40 rotor as described in Methods. Table I shows the recovery of parental DNA at the various purification steps. In expt 1, all the ^{32}P-active material added was accounted for as trichloroacetic acid insoluble material in the bacterial suspension or the supernatant medium. In expt 2, only 51% was accounted for. In an analysis by sucrose density-gradient centrifugation of the preparation in expt 2, practically all the material sediments slower than the marker [^{3}H]T5 DNA (Figure 5). This is probably because there is a greater degree of fragmentation when the bacterial concentration is made relatively high before lysis. (The concentration of bacteria before lysis was fivefold higher than in Figure 4.) But a relatively small amount was at the meniscus, indicating that most of the ^{32}P unaccounted for was in the growth medium and not in the bacteria, and was thus lost during the centrifugation of the culture. In a preparatory CsCl gradient centrifugation, all the ^{32}P activity was banded at one peak at the expected position, for both expt 1 and 2. In expt 1, the sample pooled after the CsCl gradient centrifugation was incubated in alkali overnight to digest any RNA present, but little ^{32}P material was lost by this process. This step was thus omitted from expt 2.

Before enzymatic hydrolysis of DNA, the samples in both experiments were precipitated with three volumes of ethanol and resuspended in water. Each sample was split into two equal fractions; one fraction was hydrolyzed to 5′-nucleotides and the other was hydrolyzed to 3′-nucleotides. After hydrolysis, the corresponding [^{3}H]dBrUMPs were added as markers. The fractions containing dBrUMP were partially isolated from the rest of the nucleotides by Dowex 1 chromatography as described in Methods. The recovery of ^{32}P activity in the nucleotide elution region was 98% for both 5′- and 3′-nucleotides for expt 1, and 89% for 5′-nucleotides and 100% for 3′-nucleotides in expt 2. The chromatographic elution patterns for the dBrUMP regions of the samples from expt 2 are shown in Figure 6. It is clear that this technique allows only a gross separation of the four major mononucleotides from the dBrUMPs, and that a more refined method is necessary to demonstrate the presence of authentic ^{32}P-labeled dBrUMP. A two-step paper electrophoresis procedure was found that could remove the contaminating nucleotides. For this purpose the samples pooled under the marker regions were concentrated as described in Methods.

Electrophoresis was carried out first in 0.02 N formic acid. This separates the four normal nucleotides. dBrUMP as detected by ^{3}H activity was near dTMP. This area was cut out, eluted, and concentrated. Electrophoresis was then carried out at pH 9.3 with borate or glycine buffer. At this pH, dBrUMP acquires another charge and moves faster than other normal deoxyribonucleotides (Dunn and Smith, 1957). The results, as shown in Figure 7a for expt 1 and in Figure 7b for expt 2, clearly demonstrate that only 3′-dBrUMP was ^{32}P

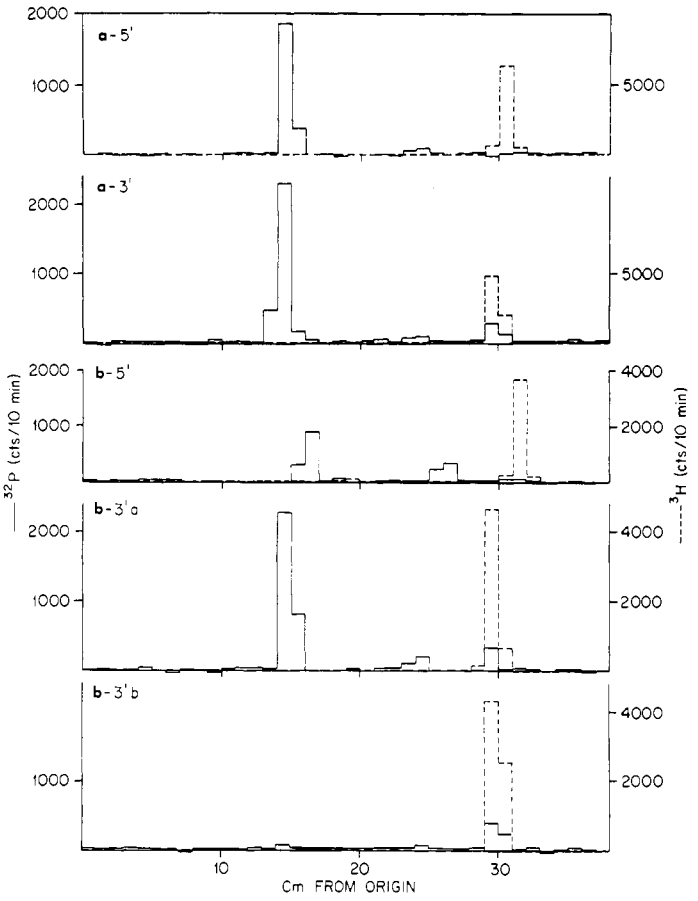

FIGURE 7: The two-step electrophoresis results. The conditions are described in Methods. Papers were cut at 1-cm intervals for the assay. (——) [^{32}P]Nucleotides and (– – – –) [^{3}H]dBrUMP. (a) Results for expt 1 after electrophoresis in 0.05 M borate (pH 9.3). (b) For expt 2, the samples were from the regions indicated in Figure 6. The patterns shown were obtained after electrophoresis in 0.05 M glycine buffer (pH 9.3).

labeled and that there was no detectable amount of ^{32}P with 5′-dBrUMP. The results were the same in glycine or borate buffer, indicating no contamination with UMP. The identity was further confirmed by treatment with venom 5′-nucleotidase and alkaline phosphatase. The 5′-nucleotidase degraded [^{3}H]5′-dBrUMP to the nucleoside but did not attack [^{3}H]3′-dBrUMP or the ^{32}P under it (Figure 8a,b). Alkaline phosphatase removed both ^{3}H and ^{32}P activity from that position for 3′-nucleotides (Figure 8c). Thus it is quite certain that this compound was 3′-dBrUMP. The identity of a slower moving component (Figure 7) that had most of the ^{32}P activity (except in expt 2, 3′b) is unknown, but it is not dUMP, and since it was resistant to alkaline phosphatase, it is not a mononucleotide.

The fraction of ^{32}P due to 3′-dBrUMP in the sample used for paper electrophoresis can be calculated. The recovery of 3′-dBrUMP after Dowex 1 chromatography and the concentration process can be determined from the recovery of [^{3}H]dBrUMP used as a marker. From these two calculations the ^{32}P in the original preparation that was due to 3′-dBrUMP was calculated to be 9.7

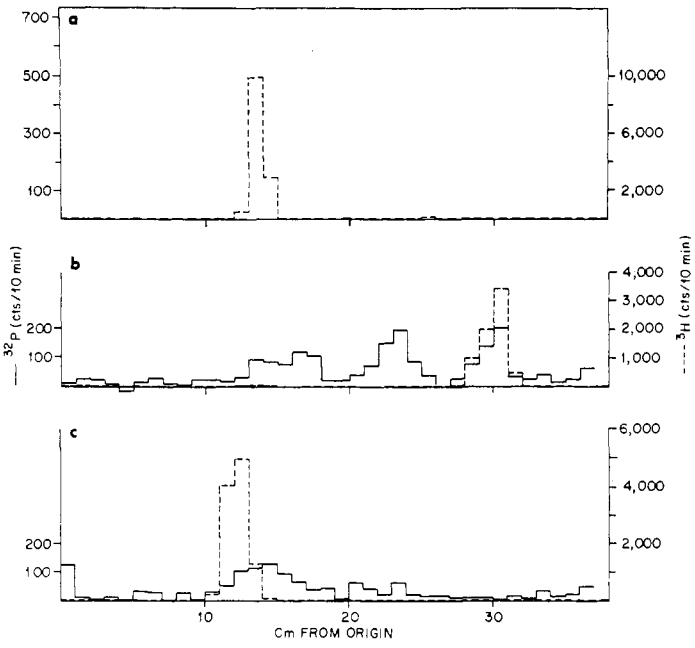

FIGURE 8: Electrophoresis of nucleotide digests. The nucleotides of expt 2 were digested with the indicated enzymes and assayed by paper electrophoresis in 0.05 M glycine buffer (pH 9.3), as described in Methods. (a) 5′-Nucleotide sample treated with 5′-nucleotidase. For clarity, the ^{32}P activity in the sample was not shown. (b) 3′b-Nucleotide sample treated with 5′-nucleotidase. (c) 3′b-Nucleotide sample treated with alkaline phosphatase. (——) ^{32}P and (– – – –) ^{3}H.

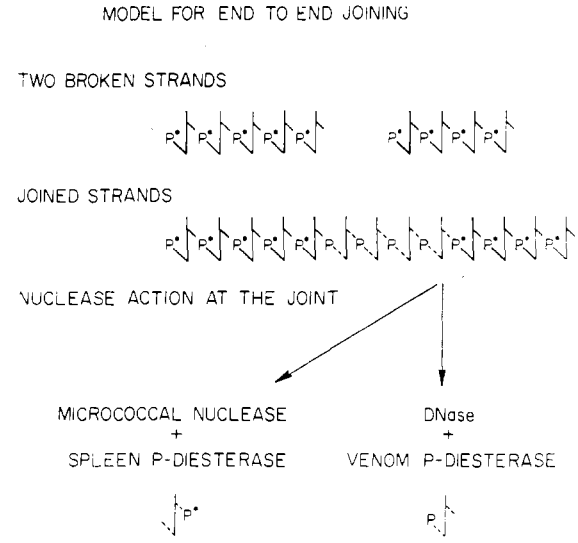

FIGURE 9: Rejoining model consistent with the experimental results and effect of nuclease action on such a rejoined DNA.

$\times 10^{-3}$% for expt 1 and 1.3×10^{-2}% for experiment 2. Since there are 2.6×10^{5} nucleotides/T5 molecule (calculated from a molecular weight of 8.0×10^{7}; Hershey *et al.*, 1962; Burgi and Hershey, 1963), there are 25 and 34 dBrUMPs/T5 DNA molecule at the points of joining of a repaired section to the preexisting strand according to expt 1 and 2, respectively.

Discussion and Conclusion

There are several possible interpretations of these experimental results, some of which are discussed below. (1) The most attractive possibility from our viewpoint is that these data describe a repair of DNA involving the synthesis of a short stretch of oligonucleotides. It has been suggested by many that the repair of DNA by such a mechanism can be done by a combination of enzymes similar to those already identified and characterized (Fareed and Richardson, 1967; Hurwitz *et al.*, 1967; Takagi *et al.*, 1967; Grossman *et al.*, 1967). This model, as applied to our system, is shown on Figure 9 and is as follows. (a) Single-strand nicks are formed by endonuclease action, then further degraded by an exonuclease in such a way that gaps are formed at the point of breakage. The specificities of the enzymes are such that one DNA fragment is left with a phosphorylated 5′ end, whereas the 3′ end of the other fragment is nonphosphorylated. (b) New 5′-nucleotides are incorporated into the gaps by a polymerizing enzyme, then a ligase (Weiss and Richardson, 1967; Olivera and Lehman, 1967; Hurwitz *et al.*, 1967) rejoins the DNA strands. The polymerizing enzyme is very likely not the T5 DNA polymerase. There is a report that the enzyme system for repair is different from that of replication (Couch and Hanawalt, 1967). (c) If such a rejoined DNA is digested to 5′- and 3′-nucleotides, as was done in the present work, ^{32}P label from a polynucleotide is transferred only to the 3′ position of newly incorporated nucleotides at the joint. The total number of joints per molecule can be estimated from the number of dBrUMP molecules incorporated at the joints, if the nucleotides incorporated adjacent to the polynucleotide fragment containing the 5′-phosphoryl are random and similar to the total base composition of the T5 DNA. The fraction of the bases that is dBrUrd in [^{32}P]DNA extracted from dBrUrd-phage prepared under similar conditions was found to be 0.16 by two-dimensional thin-layer electrophoresis under conditions essentially the same as those described for two-step electrophoresis. Thus, if all the 3′-[^{32}P]dBrUMP were derived by this mechanism, one can estimate that there are 29/0.16 = 180 rejoined sections per molecule.

(2) The formation of [^{32}P]3′-dBrUMP could be merely a result of the degradation of parental [^{32}P]DNA to [^{32}P]mononucleotides, then the reincorporation of these labeled mononucleotides into newly synthesized DNA. The degraded mononucleotides would be diluted by the pool of nonradioactive nucleotides, which includes deoxybromouridine nucleotide, but the extent of dilution is unknown. We felt that the most feasible approach to this problem was to prevent DNA replication, and this was effected by use of the mutant T5ts53. With this mutant in a dBrUrd medium, there was no detectable DNA replication even at 30° until about 90 min after the infection. However, the possibility that replication at much slower rate occurs under our experimental conditions is being investigated with more rigorous methods. Preliminary results indicate

that the average chain length of the newly synthesized section is only about 40 nucleotides long even 40 min after infection at 43°. This value is consistent with the interpretation that these nucleotides represent repaired sections.

We have attempted to assay for any degradation of parental labeled DNA by measuring the recovery of the total radioactive material by our extraction procedure and by sucrose density gradient centrifugation. The fractions from centrifugation were analyzed for total radioactivity (acid soluble plus insoluble). The sample obtained from the centrifugation showed (Figure 4b) that the only significant difference between DNA from the purified phage and the phage DNA from infected cells is that there is a slower moving fraction in the latter DNA. The peak height of this fraction changes from preparation to preparation. Its position in the gradient is about that of half-molecules; thus these fragments could arise from molecules broken at the weak point which is present at about 0.6 of the way from one end of a molecule (Burgi *et al.*, 1966). It is difficult to determine whether this fragmentation occurs inside the cell or during the lysis, but it is present even when the cells are lysed with SDS only. The data for radioactivity recoveries for the two major experiments (Table I) reveal that all of the ^{32}P was recovered as acid-insoluble material in expt 1, whereas the recovery of acid-insoluble ^{32}P was only about 50% in expt 2. Yet in both expt 1 and 2, the amount of 3′-[^{32}P]dBrUMP found was about the same. If the label in this compound originated *via* the breakdown of parental DNA and reutilization of the resultant mononucleotide, there should be far more of it in expt 2. These observations make it appear unlikely that the label in 3′-dBrUMP was due to this kind of degradation and reincorporation into replicated DNA. The same argument would tend to rule out this mechanism as a significant source of label incorporated into repaired sections of DNA.

(3) [^{32}P]3′-dBrUMP could originate by the replication of DNA near the initiation site(s). In some *in vitro* systems, it has been shown that newly replicated DNA can be covalently linked to template DNA (Bollum, 1963; Goulian *et al.*, 1968). It also has been proposed as a possible mode of replication of *B. subtilis* (Yoshikawa, 1967). In the case of T5, there is also the possibility that replicated DNA is linked to template DNA, since it was found that the replicative form is larger than one phage DNA unit (Smith and Skalka, 1966). However, since we find about 29 3′-dBrUMP molecules at the joint between a repaired section and the preexisting strand and since about half of dTMP is displaced by dBrUMP in our conditions, this mean that about 60 initiation sites/molecule would start with thymidylic acid. This seems to be highly unlikely.

(4) There are four natural nicks existing in T5 DNA (Abelson and Thomas, 1966), but such nicks are repaired *in vitro* without incorporation of nucleotides (Jacquemin-Sablon and Richardson, 1968). In our *in vivo* system, even if incorporation of nucleotides were involved in the repair of these nicks, it would only account for a small fraction of the [^{32}P]dBrUMP found.

Thus we are encouraged to believe that most of the 3′-[^{32}P]dBrUMP found resulted from mechanism 1 above. We feel that these data demonstrate that a repair synthesis can occur in phage-infected cells in the absence of DNA replication and can be analyzed biochemically. We intend to utilize various biochemical and physical techniques in order to determine whether such a process is involved in the final rejoining process of the recombination of T5 DNA molecules as proposed and rather indirectly observed by Meselson (1964), Anraku and Tomizawa (1965a,b), Tomizawa (1967), and Kozinski and Felgenhauer (1967) for other phage systems.

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