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Biochemical Analysis of the Naturally Repaired Sections of Bacteriophage T5 Deoxyribonucleic Acid. III. Nucleotide Analysis of Deoxyribonucleic Acid Synthesized under Nonpermissive Conditions*

Robert K. Fujimura

ABSTRACT: The DNA synthesized at a nonpermissive temperature by *Escherichia coli* infected by a phage T5 mutant that induces a temperature-sensitive DNA polymerase was examined to establish whether it is the product of residual replication or of a "repair" synthesis. *E. coli* was grown in a medium containing bromodeoxyridine (dBrUrd) and [^{32}P]-P_i, infected with ^{3}H-labeled T5 phage, and incubated at the nonpermissive temperature. The ^{32}P-labeled DNA, extracted and purified until it hybridized specifically to T5 DNA and not to *E. coli* DNA, had the same buoyant density as the parental DNA in a CsCl gradient centrifuged to equilibrium. Nucleotide analysis showed no detectable amount of dBrUrd incorporation. The amount of [^{32}P]nucleotide incorporated was about 1–5% of parental DNA, with not much dependence on the length of incubation of the infected cells. When bacteria were grown in a normal medium with [^{32}P]P_i infected with ^{3}H-labeled T5 containing dBrUrd, and incubated at the nonpermissive temperature, the isolated and purified DNA yielded no 5-[^{32}P]dBrUMP on digestion to 5′-nucleotides, but a small amount of 3′-[^{32}P]dBrUMP on digestion to 3′-nucleotides. If it is assumed that all the ^{32}P incorporation under nonpermissive conditions is due to repair, it can be calculated from the amount of 3′-[^{32}P]dBrUMP that the average chain length is very short (10–30 nucleotides). From the nucleotide analyses it is concluded also that the absence of dBrUrd incorporation from the medium (in the first experiment above) is not due to preferential reutilization of precursors coming from degraded parental T5 DNA or *E. coli* DNA. DNA synthesis was also studied in a medium containing ^{15}N and ^{2}H under nonpermissive conditions. There was a slight density shift of the isolated [^{32}P]DNA but not as much as at the permissive temperature, and the amount of DNA synthesized was less than the amount of parental DNA. It is concluded that DNA synthesis at the nonpermissive temperature is distinctly different from the bulk of synthesis under permissive conditions and is very likely a "repair" synthesis, although it may involve more than one kind of repair.

The preceding report (Fujimura, 1971) showed that in bacteria infected with T5 phage in the absence of a functional T5 DNA polymerase there is still a phage-controlled DNA synthesis that, in the presence of dBrUrd,[1] occurs without a shift in the buoyant density of the DNA synthesized. One explanation is that this is a type of repair, and that the repaired sections are too small a fraction of the total DNA molecule to affect the buoyant density. Another possibility is that there is no incorporation of the analog under nonpermissive conditions. However, Fujimura and Volkin (1968) showed that dBrUrd is incorporated covalently into DNA in positions proximal to parental DNA under nonpermissive conditions. If the dBrUrd incorporation observed is the same as the ^{32}P incorporation that occurs in the bulk of synthesis under nonpermissive conditions, there should be enough incorporation of the analog to be detected easily.

To clarify these points, nucleotide analysis of the DNA synthesized under nonpermissive conditions was carried out.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: dBrUrd, bromodeoxyuridine; dFUrd, fluorodeoxyuridine; dBrUMP, bromodeoxyuridylic acid; SSC, standard saline citrate, *i.e.*, 0.15 M NaCl–0.015 M sodium citrate (pH 7).

The main findings are that dBrUrd is not incorporated uniformly throughout the DNA, and that the ratios of the nucleotides that are incorporated—when they are uniformly labeled—are indistinguishable from those of T5 phage DNA. Furthermore, the lack of dBrUrd incorporation is not due to preferential reincorporation of degraded parental DNA. Some experiments were carried out in a medium containing [^{15}N]NH_4Cl and [^{2}H]glucose. In this medium under nonpermissive conditions there is a density shift in the isolated DNA, but the shift is slight, and the amount of DNA synthesized is less than the amount of parental DNA. Thus nucleotide incorporation under nonpermissive conditions is definitely different from normal replication; it is apparently due to repair synthesis, but probably not just of one type.

Materials and Methods

Bacteriophage T5ts53 was prepared and purified, and DNA was extracted as described by Fujimura and Volkin (1968). T5ts53 labeled with ^{3}H and dBrUrd, [^{3}H]dBrUrd-T5ts53, was prepared with [5-^{3}H]dUrd (Schwarz BioResearch) in a dBrUrd-containing medium (MGM-CA medium containing 5 μg/ml of dFUrd, 200 μg/ml of dBrUrd, and 25 μg/ml of Urd) as described in the above reference. By this method, only dCMP in DNA becomes labeled. The phage, purified in the usual manner, was further purified by equilibrium CsCl density gradient centrifugation. A phage preparation was adjusted to $A_{260} = 20$, and 5 ml of it was added per centrifuge tube, mixed with 4.27 g of CsCl, and centrifuged at 36,000 rpm for 24 hr at 0° in a no. 40 Spinco rotor. The phage fractions at the peak and denser were pooled and used with no further purification for the experiments. *Escherichia coli* F is a normal host for T5, and *E. coli* R15 is a mutant with no DNA polymerase I (Kato and Kondo, 1970).

The ^{15}N,^{2}H medium was made from MGM medium (Lanni, 1961) with 0.1% [^{15}N]NH_4Cl (Bio-Rad) and 0.2% [U-^{2}H]glucose (Mallinckrodt) instead of [^{14}N]NH_4Cl and [^{1}H]glucose.

Purification of DNA from Infected Bacteria. DNA was extracted from the infected bacteria as described by Fujimura (1971). In those cases where [^{32}P]P_i was present in the medium, the extracts were further treated after the phenol extraction step by either of the following methods.

If less than 5 mCi of [^{32}P]P_i was added per sample, the sample was dialyzed overnight against 200 volumes of SSC in a $ZnCl_2$-treated dialysis tube (McBain and Steuer, 1936).

If more than 5 mCi of [^{32}P]P_i was added per sample, the DNA extract was diluted about 10-fold with 0.02 M sodium phosphate (pH 6.8), containing 0.4 mM NaCl, 0.02 mM $MgSO_4$, and 0.2 mM Tris-HCl. The diluted sample was introduced onto a column of hydroxylapatite, 0.79 cm^2 × 10 cm (Clarkson Chemical Co.), and washed through with 1 l. of 0.05 M sodium phosphate buffer (pH 6.8). The adsorbed DNA was eluted with SSC (S. Niyogi, personal communication). Most of the adsorbed DNA was eluted in about 50 ml. Sometimes the DNA extract was washed through an agarose column before the hydroxylapatite step, in which case the sample was made 2 M in NaCl and put on a column of Sepharose 2B 4.9, cm^2 × 45 cm (Pharmacia Fine Chemicals). The fractions with appreciable amounts of parental T5 DNA were pooled, diluted 2-fold with the 0.02 M phosphate buffer containing NaCl, $MgSO_4$, and Tris-HCl, and washed through a hydroxylapatite column as described above.

The samples from either of the above steps were put in boiled dialysis tubes and dialyzed usually overnight against 200 volumes of 0.04 M potassium phosphate (pH 12). The volume of sample was adjusted to the appropriate volume for equilibrium gradient centrifugation in CsCl by concentrating with aquacide II (Calbiochem). The centrifugation was done as described by Fujimura (1971). Samples were pooled, sonicated, and hybridized to T5-nitrocellulose powder as described below. The hybridized fractions were usually pure; that is, they hybridized well to T5 DNA–membranes and very little to *E. coli* DNA–membranes.

Hybridization to T5-Nitrocellulose Powder. The T5-nitrocellulose powder was prepared as described by Riggsby (1969) at a concentration of 1 mg of DNA/1 g of the nitrocellulose (gift from Hercules). The hybridization was done in 40% formamide as follows. A sample from the CsCl centrifugation was sonicated for 3 min at the maximum setting for a Raytheon sonicator (10-kc oscillator) and diluted to about 20 ml in 40% formamide–6 × SSC. The suspension was incubated for 18 hr at 37° in a Dubnoff shaker. After the incubation, the suspension was transferred to a Schleicher & Schuell filtrator layered with two 25-mm, No. 3MM filter paper disks. It was washed until the ^{32}P activity as measured by Cerenkov radiation reached a constant value. The nitrocellulose–DNA powder was taken out, suspended in water equilibrated at 67°, incubated at 67° for 10 min, and filtered through two layers of filter paper disks. The process of elution was repeated until a negligible amount of ^{32}P was removed. The eluted fractions were assayed for specificity toward T5 and *E. coli* DNA–membranes (Fujimura, 1971). The radioactive fractions specific for T5 DNA–membranes were pooled for nucleotide analysis.

Nucleotide Analysis. The DNA was digested to 5′-nucleotides as described previously (Fujimura, 1970). For digestion to 3′-nucleotides, a DNA sample was prepared in 0.5 ml for digestion as was done for 5′-nucleotide digestion. To this were added 2 μmoles of Tris-HCl (pH 8.6), 1 μmole of $CaCl_2$, and 180 units of micrococcal nuclease (Worthington). The reaction mixture was incubated at 37° for 2 hr. The pH was reduced to 7 with HCl, and 2 μmoles of EDTA (pH 4.5), 5 μmoles of potassium phosphate (pH 6), and about 0.1 unit of spleen phosphodiesterase (Worthington lot no. 624) were added. The reaction mixture was incubated at 37° for 1 hr, and an additional 0.1 unit of spleen phosphodiesterase was added and incubated for another hour.

To a digested sample, [^{3}H]dBrUMP was added as a marker. 5′- and 3′-dBrUMP were prepared as described by Fujimura and Volkin (1968). The nucleotides were separated into five peaks by Bio-Gel DM2 chromatography and the dBrUMP region was further isolated by thin-layer electrophoresis at pH 9.3. These procedures are described in detail elsewhere (Fujimura, 1970). The composition is given as mole fractions of total nucleotides. The amount of dBrUMP was always extrapolated from analysis of an electrophoretic plate.

Results

Infection with T5ts53 Phage in Bromodeoxyuridine, ^{32}P-Containing Medium. For the first group of experiments, *E. coli* F and R15 were used. R15 is a mutant with no detectable amount of DNA polymerase I (Kornberg's polymerase) in cell extracts (Kato and Kondo, 1970). Each strain was grown in 70 ml of dBrUrd-containing medium to 3 × 10^8 cells/ml and infected with [^{3}H]T5ts53 at a multiplicity of infection of 5, as described by Fujimura (1971). After phage adsorption, each culture was split into two equal volumes

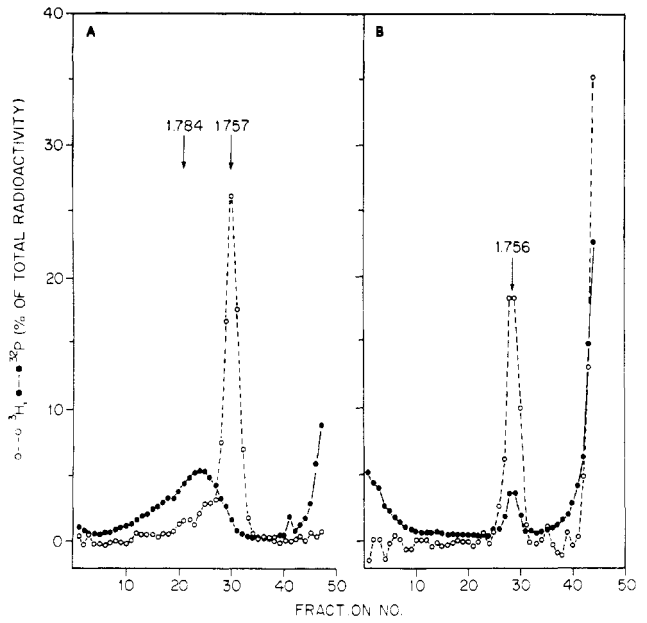

FIGURE 1: Density gradient centrifugation in alkaline CsCl of DNA extracted from *E. coli* F infected with [^{3}H]T5ts53 in a medium containing [^{32}P]P_i and dBrUrd. The conditions are listed in Table I, expt Ia and b. (A) 30° and (B) 43°. Buoyant densities around the peaks were determined from refractive index readings. ^{32}P (-----) and ^{3}H (– – – –).

and added to the fresh dBrUrd-containing medium, also containing [^{32}P]P_i, preincubated at 30 or 43° as shown in Table I, expt I. The amount of [^{32}P]P_i added was adjusted so that the final amount incorporated was not too different among the samples. These final amounts were 3, 60, 9, and 180 μCi per ml for samples a–d, respectively. The cultures were harvested at 120 min after infection, and DNA was extracted and fractionated by means of CsCl equilibrium centrifugation in alkali, as described in Methods. The results were consistent with the preceding report (Fujimura, 1971). At the permissive temperature of 30°, the buoyant density of progeny DNA shifted to the heavier side of most of the parental DNA. At the nonpermissive 43°, there was no observable shift in the density of [^{32}P]DNA. The results with infected *E. coli* F are shown Figure 1. This lack of shift in density may be due to a complete lack of incorporation of dBrUrd or to a level of synthesis too low to affect the density.

To distinguish between these two possibilities, fractions containing the [^{32}P]DNA were pooled and purified further by hybridization to T5 DNA–nitrocellulose powder as described in Methods. The eluted DNA hybridized specifically to the T5 DNA–membrane and not to the *E. coli* DNA–membrane. The DNA was digested to 5′-nucleotides and analyzed as described in Methods. The nucleotide compositions obtained are shown in expt I, a–d, in Table I. The nucleotide composition of the newly replicated DNA (expt I, a and c) is essentially the same as that of the DNA obtained from the phage grown to natural lysis in the same kind of medium with [^{32}P]P_i added from the beginning of bacterial growth (expt I, last column). The replicated DNA from R15 has a lower dBrUMP content than that from *E. coli* F (expt I, a and c), as expected from a smaller shift in density (Figure 3 in Fujimura, 1971). However, nucleotides incorporated at the nonpermissive temperature contain no detectable amount of dBrUMP (expt I, b and d). The nucleotide composition differs from that of T5 DNA, with the dCMP content markedly lower, but it resembles T5 DNA composition more than that of *E. coli*.

To test the possibility that dBrUMP may be incorporated from degraded host DNA, bacteria were grown in the medium containing [^{32}P]P_i (60 μCi/ml) and dBrUrd for one generation, and the [^{32}P]P_i was removed by washing before infection. The DNA samples were obtained and analyzed as above. The nucleotide analysis showed again that there was no detectable incorporation of dBrUMP at the nonpermissive temperature (Table I, expt II, a–c and expt IIIa). The nucleotide composition again differed from that of T5 DNA but resembled it more than that of *E. coli*, but this time with a markedly high dCMP content, regardless of when the infected cells were harvested. A sample was also prepared with [^{32}P]P_i present from one generation before infection to the time of harvest of infected cells (expt IId). The dBrUMP was again absent, and the nucleotide composition was the same as that of T5 DNA without the dBrUMP label. Experiment III in Table I confirms the above results and shows that there is no effect on nucleotide composition at the nonpermissive temperature in different host strains.

For the samples labeled from one generation before infection to the time of harvest (expts IId and IIIb), the amount of [^{32}P]P_i incorporated may be calculated, since the parental DNA was ^{3}H labeled, and it is reasonable to assume that the newly synthesized ^{32}P segments and [^{3}H]parental DNA hybridize to T5 DNA membranes with the same efficiency. For expt IId, the specific activity of [^{32}P]P_i in the medium was 3.1×10^6 cpm/μg of phosphorus. Since phosphorus is about one-tenth the molecular weight of the nucleotide, this corresponds to 3.1×10^5 cpm/μg of DNA. For [^{3}H]-DNA, the specific activity was 2.6×10^5 cpm/μg of DNA. The final purified preparation from the infected cells was hybridized to T5 DNA–membranes, and 1340 cpm of ^{32}P and 210 cpm of ^{3}H were hybridized. (The per cent of input hybridized was 43 and 48, respectively.) This corresponds to 4.3 ng of [^{32}P]DNA/81 ng of [^{3}H]DNA, so that an amount of DNA equal to 5% of the amount of parental DNA was synthesized in 60-min incubation. By a similar calculation from the data of expt IIIb, the newly synthesized segments correspond to 2% of the parental DNA after 90-min incubation. These numbers are consistent with the 2% in the preceding report (Fujimura, 1971), where the calculation was based solely on samples from the CsCl gradient profile. The time of harvest has very little effect on the amount incorporated, indicating that the incorporation rate per molecule is very low, consistent with a repair type of synthesis.

Infection with dBrUrd–T5ts53 Phage in a Medium Containing [^{32}*P*]P_i. There were two reasons for doing nucleotide analyses of ^{32}P incorporated into dBrUrd–DNA of T5ts53 under the nonpermissive temperature. One was to test the possibility that the absence of dBrUMP in the DNA in the studies in the preceding section was obtained because the parental T5 DNA had been degraded to nucleosides that were preferentially reincorporated in the residual synthesis on the remaining parental T5 DNA. If this were so, then under the present experimental conditions there would be 5′-[^{32}P]-dBrUMP incorporated from rephosphorylation of dBrUrd derived from parental DNA. The second reason was to see if the ^{32}P incorporation was due to repair. If this were so, there should be covalent linkages between the ^{32}P-labeled section and the parental DNA, and the ^{32}P-labeled section might be proximal to the dBrUrd residues in the parental DNA, so that degradation of the DNA to 3′-nucleotides would yield a small amount of 3′-[^{32}P]dBrUMP.

The experiments were done many times with slight variations in the techniques of extraction and purification of the DNA from infected cells. The following procedure was most convenient and gave the best results.

E. coli F were grown at 37° in MGM-CA medium containing one-tenth the normal amount of phosphorus (*i.e.*, 0.4 mg/100 ml) in the presence of [^{32}P]P_i at a specific activity of about 250 μCi/4 μg of phosphorus. When the bacteria were grown to 4×10^8 cells/ml, they were infected with [^{3}H]dBrUrd–T5ts53 at a multiplicity of infection of 5, as described in Methods. After adsorption, the infected bacteria were transferred back into the previous [^{32}P]P_i-containing medium, which was preequilibrated at 43°. The infected bacteria were harvested 30–60 min after infection. The DNA was extracted and purified as described in Methods, including the agarose column and hydroxylapatite column steps to wash off smaller ^{32}P-labeled substances. The eluted DNA was hybridized onto T5 DNA–nitrocellulose powder. The fractions eluted with water from the nitrocellulose powder were T5 specific; that is, when tested against T5 and *E. coli* DNA–membranes, about 50% of the material was hybridized to T5 DNA–membranes and about 1% to *E. coli* DNA–membranes. Samples were divided into two equal parts for digestions to 5′- and 3′-nucleotides. The average of the 5′-nucleotide compositions of all the experiments is shown in Table II. The composition is that of T5 DNA. There is no significant difference between samples harvested at 30 and 60 min. When the fractions from the dBrUMP region from the Bio-Gel DM2 chromatogram were analyzed by electrophoresis at pH 9.3, there was no significant amount of ^{32}P with the [^{3}H]dBrUMP in any of the preparations. A result of such an analysis at pH 9.3 is shown in Figure 2.

The detection of 3′-[^{32}P]dBrUMP was difficult because the amount present was only slightly above the limit of detection. Table II shows the results of determination of 3′-nucleotides for two of the experiments. The dBrUMP content of the parental phage DNA was determined directly by nucleotide analysis for expt I and from the buoyant density for expt II. There should have been no significant difference due to the difference in the methods (Fujimura, 1970). The specific activity of [^{3}H]-DNA was determined either by assuming $A_{260} = 20$/mg or by direct determinations of the specific activity of the dCMP obtained from the phage DNA. Since [5-^{3}H]dUrd was used as a precursor, only the dCyd residues in the DNA were labeled. Thus the specific activity of DNA is the specific activity of dCMP multiplied by the mole fraction of dCMP in the DNA. The difference in the specific activity of the DNA determined by the two methods in one of the experiments was only about 6%. The fraction of DNA labeled with ^{32}P was determined by assuming that the specific activity of phosphorous in the nucleotides was the same as that of phosphorous in the medium. It was also assumed that the change in specific activity of the parental phage DNA inside the cell is negligible at the nonpermissive temperature. This assumption was apparently justified, since the amount of ^{32}P incorporated was 4% for expt I and 1% for expt II. The 3′-nucleotide composition was the same as that of T5 DNA. The dBrUMP regions were analyzed by electrophoresis at pH 9.3 as for the 5′-nucleotide analysis. The result of one such analysis is shown in Figure 2. As can be seen, there is definitely some ^{32}P activity with the 3′-[^{3}H]dBrUMP, even though there is no detectable amount for the 5′-nucleotide. The total 3′-dBrUMP activity in a Bio-Gel DM2 chromatogram was extrapolated from such an analysis (as shown in Table II, 3′-nucleotides). The [^{32}P]dBrUMP content was about the same for two independent ex-

TABLE I: Nucleotide Composition of T5 DNA Synthesized in Bromodeoxyuridine Medium (Mole Fractions of ^{32}P-Labeled Nucleotides).

	Experiment I						Experiment II				Experiment III			
Sample:	a	b	c	d	Purified Phage[a]		a	b	c	d	a	b	c	
Temperature after infection (°C)	30	43	30	43	30		43	43	43	43	43	43	43	
Host	F	F	R15	R15	F		R15	R15	R15	R15	F	F	F	
Time of [^{32}P]P_i addition	After infection	After infection	After infection	After infection	Through-out		Before infection only	Before infection only	Before infection only	Through-out	Before infection only	Through-out	After infection	
Time of harvest[b]	120	120	120	120	240		30	60	90	60	90	90	90	
Nucleotide														
dCMP	0.179	0.107	0.199	0.096	0.199		0.259	0.246	0.250	0.195	0.292	0.194	0.123	
dAMP	0.294	0.283	0.268	0.256	0.298		0.268	0.274	0.260	0.295	0.276	0.291	0.275	
dTMP	0.233	0.360	0.237	0.377	0.214		0.277	0.273	0.297	0.294	0.269	0.303	0.321	
dBrUMP	0.091	<0.002	0.052	<0.003	0.088		<0.001	<0.002	<0.001	<0.009	<0.001	<0.003	<0.008	
dGMP	0.198	0.242	0.229	0.275	0.201		0.189	0.185	0.193	0.193	0.157	0.194	0.245	

[a] Fujimura (1970). [b] Minutes after infection.

TABLE II: Nucleotide Composition of DNA Resulting from Bacteria Infected with dBrUrd–T5ts53 in [^{32}P]P_i-Containing Medium at 43°.

	Av Composition (Fraction of Total)		
5′-Nucleotides[a]			
dCMP	0.212 ± 0.004		
dAMP	0.279 ± 0.005		
dTMP	0.306 ± 0.004		
dBrUMP	0[b]		
dGMP	0.203 ± 0.005		
	Experiment		
	I	IIa	IIb
3′-Nucleotides			
Time of harvest (min after infection)	40	30	45
dBrUMP content of parental DNA	0.14	0.19	0.19
Specific activity			
Parental [^{3}H]DNA (cpm/μg)	3.2×10^3	2.8×10^3	2.8×10^3
^{32}P in the medium (cpm/μg)	1.4×10^8	1.7×10^8	1.7×10^8
Fraction of parental DNA ^{32}P labeled	0.041	0.011	0.015
Total amount of ^{32}P activity in Bio-Gel chromatogram			
In nucleotide regions	2.27×10^5	2.25×10^5	1.92×10^5
In dBrUMP[b] (fraction of total)	$(5.2 \pm 0.7) \times 10^{-3}$	$(7.9 \pm 0.8) \times 10^{-3}$	$(9.8 \pm 0.7) \times 10^{-3}$
Average chain length	28	10	19

[a] Six independent preparations. [b] After correcting for impurity by electrophoresis, pH 9.3.

periments. The amount incorporated did not increase significantly with time.

It is assumed that these incorporations occurred as a repair synthesis, and it is assumed that such a synthesis occurs randomly with no specificity for any one nucleotide, the nucleotide composition proximal to the repaired region should reflect the nucleotide composition of the whole parental DNA. Thus the fraction of the [^{32}P]nucleotides at the end of the ^{32}P-labeled region is the fraction of 3′-[^{32}P]dBrUMP divided by the fraction of dBrUMP in the parental DNA. The average chain length of such a repaired region is the reciprocal of this or the fraction of dBrUMP divided by the fraction of [^{32}P]dBrUMP. The average chain length thus determined was about 20 and was independent of time of harvest (Table II, bottom row). A similar experiment done previously with nonradioactive parental phage DNA (Fujimura, 1969) led to the conclusion that the average chain length is about 30. Thus even with the assumption that the total ^{32}P incorporated is due to repair at the nonpermissive temperature, the average chain length is small.

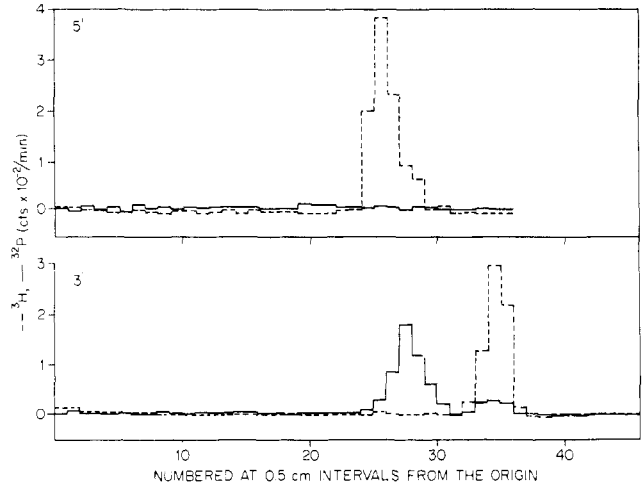

FIGURE 2: Thin-layer electrophoresis at pH 9.3. *E. coli* F in a [^{32}P]P_i-containing medium was infected with [dBrUrd]T5ts53. The conditions are listed in Table II, 3′-nucleotides, expt I. The purified DNA was divided into two equal parts and digested to 5′- and 3′-nucleotides. The nucleotides were fractionated into 5 peaks, and dBrUMP fractions were analyzed further by thin-layer electrophoresis at pH 9.3 in the presence of [^{3}H]dBrUMP as a marker. ^{32}P (——) and ^{3}H (– – – –).

Infection with T5ts53 in $^{15}N,^{2}H$ Medium. Since there is no incorporation of dBrUrd at the nonpermissive temperature, some other density marker was needed to distinguish between replication and repair. *E. coli* F was grown to 4×10^8 cells/ml in MGM medium and concentrated 10-fold and infected with [^{3}H]T5ts53 at a multiplicity of infection of 10, as described by Fujimura (1971). The infected bacteria were diluted 10-fold into $^{15}N,^{2}H$ medium preequilibrated at 43° and incubated for 15 min. According to Worcel (1970), 15 min is sufficient for equilibration of the precursors with the heavy isotopes. The culture was then divided into three fractions under the conditions shown in Table III. The specific activity of ^{32}P was adjusted so that the amount incorporated at 30 and 43° was about the same. The DNA was extracted and dialyzed against SSC in a $ZnCl_2$-treated dialysis tube as described in Methods. The samples were then fractionated by means of density gradient centrifugation in alkaline CsCl as described in Methods. About half of each CsCl fraction was analyzed for acid-insoluble radioactivity, and the other half was kept for subse-

TABLE III: DNA Synthesis in Medium Containing [^{15}N]NH_4Cl and [^{2}H]Glucose.

Conditions	Sample A	Sample B	Sample C
Temperature of incubation (°C)	30	30	43
Specific activity			
Parental DNA (cpm/μg)	1.99×10^3	1.99×10^3	1.99×10^3
^{32}P in the medium (cpm/μg)	1.7×10^6	8.5×10^4	1.7×10^6
Time of harvest (min after transfer)	5	90	90
Fraction of parental DNA ^{32}P labeled			
Whole DNA region[a]		1.4	0.097
Region d			0.56
Region e			0.018

[a] Regions are in CsCl gradient profile of Figure 3.

quent analysis. Sample A (Figure 3A) showed only a slight incorporation of ^{32}P in a 5-min incubation at 30°, even though the specific activity was 20 times that of sample B. Thus the expression of functional T5 DNA polymerase after the shift in temperature is not instantaneous. Sample B (Figure 3B) was incubated at 30° for 90 min, and normal replication occurred with a change in buoyant density of 0.025 g/ml with respect to parental DNA. The amount of newly synthesized DNA was 140% of the parental DNA. The CsCl gradient profile for sample C (Figure 3C) shows the DNA synthesis at the nonpermissive temperature after 90-min incubation. The sample was not as clean as the others. However, the specific activity of the ^{32}P was the same as for sample A, indicating that the aberrant shape of the ^{32}P curve was not the result of insufficient washing during the assay. The major peak was slightly more dense than parental DNA with a density shift of 0.008 g/ml. The total amount of ^{32}P incorporated in this peak region was about 10% of the total amount of parental DNA. The amount of ^{32}P incorporated per [^{3}H]parental DNA was calculated also at the peak regions of ^{32}P (Figure 3C, d) and ^{3}H (Figure 3C, e) from the DNA–DNA hybridization data for these regions (Table IV). At region d (Figure 3C), the amount of [^{32}P]DNA with respect to parental DNA was 56%, and at region e it was 1.8%. Thus even at heavier regions it was less than 100%. Since the density shift was also smaller than for replicated DNA, it must be covalently linked to the parental DNA. The amount of DNA synthesized is summarized in Table III.

The nature of the heavier regions observed in sample C (Figure 3) was analyzed by DNA–DNA hybridization and by digestions with DNase, even though the heavy region with major ^{32}P activity is too dense to be DNA. Sample C was pooled into fractions a–e, as marked in Figure 3C. For comparison, sample B was pooled into two fractions, marked a and b in Figure 3B. The DNA–DNA hybridization was done as described in the preceding report (Fujimura, 1971). The

TABLE IV: DNA–DNA Hybridization of Fractions from Samples in Figure 3.

	Sample B				Sample C									
	a		b		a		b		c		d		e	
	^{32}P	^{3}H	^{32}P	^{3}H	^{32}P	^{3}H	^{32}P	^{3}H	^{32}P	^{3}H	^{32}P	^{3}H	^{32}P	^{3}H
Input (cpm)	2303	47	132	890	1349	−4	472	−4	154	−6	1764	51	908	926
Nitrocellulose membrane (fraction of input)[a]														
T5 DNA	0.30	0.26	0.49	0.47	0.64		0.97		1.03		0.51	0.45	0.623	0.493
Blank	0.01	0.0	0.05	0.006	0.12		0.28		0.38		0.01	−0.04	0.058	−0.001
E. coli DNA	0.02	0.0	0.08	0.006	0.50		0.70		0.71		0.03	−0.02	0.098	0.0
Blank	0.04	0.0	0.08	0.011	0.20		0.27		0.41		0.02	−0.10	0.075	0.004
DNase-resistant fraction	0.11	0.08	0.15	0.18	0.82				0.79		0.17	0.0		

[a] Values are averages of duplicate experiments.

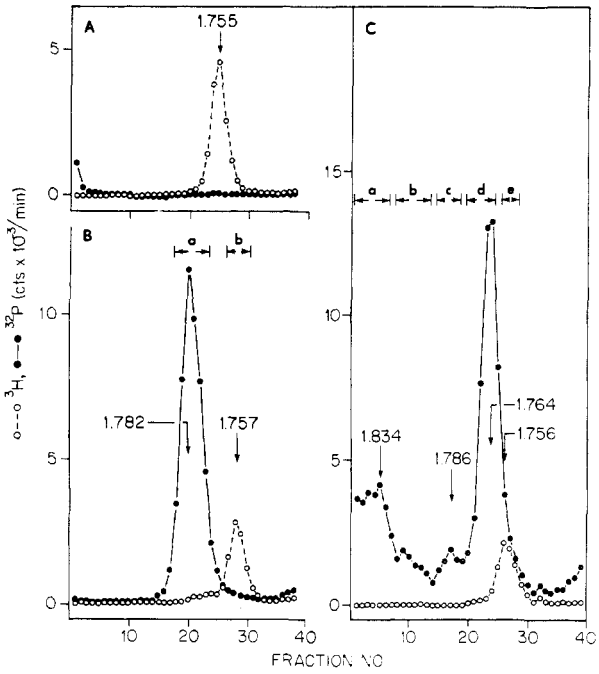

FIGURE 3: Density gradient centrifugation in alkaline CsCl of DNA synthesized in *E. coli* F infected with [^{3}H]T5ts53 in ^{15}N,^{2}H medium containing [^{32}P]P_i. The conditions are listed in Table III. The sample designation is the same. Buoyant densities around the peaks are written in the figure. ^{32}P (——) and ^{3}H (– – –).

results are shown in Table IV. Both fractions of sample B were definitely specific for T5, as were the corresponding fractions of sample C (d and e). But fractions a to c of sample C appear to hybridize almost as well to *E. coli* as to T5 DNA–membranes, and also the blanks are high. These results indicate that the ^{32}P in these fractions was bound nonspecifically. Most of these fractions were tested for sensitivity to DNase in the presence of 0.3 mg of *E. coli* DNA under the conditions described previously (Fujimura, 1970). The results are shown in Table IV. While only about 15% of fractions a and b of sample B and d of sample C remained acid insoluble after DNase treatment, fractions a and c of sample C were still 80% acid insoluble. They were further treated with venom phosphodiesterase and analyzed by electrophoresis at pH 3.5 as described by Fujimura (1970). There were no detectable nucleotides in fractions a and c of sample C. It was concluded that the ^{32}P activity in the sample C fractions a–c is not in DNA. In some experiments, bacteria were grown in ^{15}N,^{2}H medium for a few generations or for a few minutes before infection, but such bacteria become resistant to adsorption of T5.

Discussion and Conclusion

Previously (Fujimura and Volkin, 1967) it was shown that, at the nonpermissive temperature for functional T5 DNA polymerase, ^{32}P is incorporated into DNA that bands at the same position as the parental T5 DNA upon CsCl gradient centrifugation, even when dBrUrd is present in the medium. Under similar conditions, dBrUrd is incorporated proximally and is covalently linked to parental T5 DNA (Fujimura and Volkin, 1968). Thus the possibility arose that this incorporation comes from a repair synthesis of "gaps" formed in parental DNA; such gaps have been proposed as part of the recombination process of DNA molecules (Meselson, 1964; Anraku and Tomizawa, 1965a,b). The present work attempted to test this possibility.

The most surprising finding is that among the ^{32}P-labeled nucleotides incorporated into parental T5 DNA in the presence of dBrUrd there is no significant amount of [^{32}P]dBrUMP. Furthermore, no dBrUMP is incorporated either from degradation of *E. coli* DNA or parental T5 DNA containing dBrUrd. In all cases when the pools were uniformly labeled, the nucleotide composition derived from ^{32}P incorporation resembled that of normal T5 DNA. When [^{32}P]P_i was present only before the infection, the dCMP content was high, and when [^{32}P]P_i was present after the infection, the dCMP content was low. It is interesting that T5 phage induces its own deoxynucleotide kinase, which phosphorylates dCMP most slowly, even slower than dBrUMP (Bessman *et al.*, 1965).

The density shift did not distinguish between replication and repair synthesis, but the amount of nucleotide incorporated was about 1–5% of the parental DNA, with little relation to the time of incubation of the infected cells. Thus the low level of synthesis suggests that it is repair synthesis. Also, it has been reported that less bromouracil is incorporated in the repair of ultraviolet-damaged DNA than in normal replication (Hanawalt, 1967).

To distinguish between residual normal replication and repair of gaps, the nucleotide incorporation under nonpermissive conditions was also studied in ^{15}N,^{2}H medium. A density shift of newly synthesized DNA was observed, but it was not as large as it is in normal replication. The amount incorporated in heavier regions was still less than the parental DNA found in the same region of the CsCl gradient. Thus the newly incorporated material is very likely linked to the parental DNA. From the data of the ^{15}N,^{2}H experiments alone, it might be concluded that most of the DNA synthesized under nonpermissive conditions and banded in CsCl at the heavier side of parental DNA (region d, Figure 3C) is much longer than that for the repair of gaps. T5 DNA in the phage has several natural breaks (Abelson and Thomas, 1966), and it is possible that the observed synthesis occurs from the ends of some of these breaks. It is also possible that new breaks are formed *in vivo* and that these also serve as the initiation points. According to the definition by Fujimura and Volkin (1968), these syntheses are still "repair," because they do not lead to an increase in the number of integral DNA molecules. On the other hand, under permissive conditions, replication may be initiated at these breaks. Thus some of the DNA synthesis observed at the nonpermissive temperature may be an aborted replication, rather than repair. However, these regions correspond to a small fraction of the total parental DNA. Most of the parental DNA had very little synthesis (region e, Figure 3C). Most of these must be due to repair mechanism proposed previously (Fujimura and Volkin, 1968) involving the synthesis of a short stretch of oligonucleotides to fill the gaps. This mechanism is also most consistent with the experiments done with bacteria infected with dBrUrd–T5ts53 in [^{32}P]P_i medium under nonpermissive conditions. These experiments showed that the average chain length of the ^{32}P-labeled material is very short (Table II) and that it is covalently linked to parental DNA, even though the interpretation involves many assumptions.

The preceding paper and this paper deal with general nucleotide incorporation under nonpermissive conditions, while the first paper of this series (Fujimura and Volkin, 1968) dealt only with dBrUMP that happened to be incorporated proximal to a preexisting strand of DNA. Thus the failure to detect any dBrUMP in the present experiments is not neces-

sarily in conflict with those results. However, the assumption in the first paper, that dBrUMP is incorporated just as efficiently as other nucleotides, is wrong. The amount of dBrUMP incorporated must be very small. The experiment reported in that paper was repeated with the present DNA extraction method, and the result was the same as before. Therefore, further study is needed to determine the significance of the finding in our first paper.

DNA synthesis without dBrUrd incorporation is dependent on a phage gene product (Fujimura, 1971). It may be that T5 DNA polymerase incorporates dBrUrd at 30° but not at 43°. However, for *E. coli* polymerase there is less discrimination between bromouracil and thymine at higher temperature (Hanawalt, 1967). Nevertheless, the temperature-sensitive mutants of T5 DNA polymerase at the nonpermissive temperature may discriminate against dBrUrd incorporation. The isolated temperature-sensitive T5 DNA polymerase has residual polymerizing activity at the nonpermissive temperature (DeWaard *et al.*, 1965). It may not incorporate dBrUTP at 43°. Another possibility is that there is another T5-induced polymerizing enzyme that discriminates against dBrUTP. A third possibility is that thymidine kinase in bacteria infected with T5ts53 becomes refractory to the analog at high temperature. A crude preparation of thymidine kinase was prepared from the infected cells and found to phosphorylate dBrUrd at 43° better than 37° (R. K. Fujimura, unpublished experiments). The reaction occurred even in the presence of the same concentration of dFUrd and Urd as used in the *in vivo* system. Thus the third possibility is unlikely.

Couch and Hanawalt (1967) studied the effect of ultraviolet irradiation on DNA synthesis in a temperature-sensitive, DNA synthesis-deficient mutant of *E. coli*. DNA synthesis was carried out at 36 and 42° in the presence of ^{32}P and bromouracil. At both temperatures and in both irradiated and unirradiated cells they observed some DNA synthesis that did not cause a shift in density. It is possible that the cells did not incorporate any bromouracil. It is generally assumed that DNA synthesized in the presence of dBrUrd or bromouracil that does not cause a shift in buoyant density of DNA is due to incorporation of the analog to preexisting DNA in such a small amount that the density is not affected. Instead, it may be that the system was resistant to the analog. The usage of the radioactively labeled analog does not assure that the incorporated radioactive substance is the analog. The level of incorporation in such a system is so low that it could be a contaminant or the debrominated nucleoside.

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