Enzymatic Removal and Replacement of Nucleotides at Single Strand Breaks in Deoxyribonucleic Acid

(Received for publication, December 4, 1970)

YUKITO MASAMUNE,† ROGER A. FLEISCHMAN,§ AND CHARLES C. RICHARDSON¶
From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

SUMMARY

Pancreatic DNase has been used to introduce single phosphodiester bond interruptions (nicks) into double stranded circular DNA. Such DNA substrates have been used to study the action of several exonucleases at the site of nicks. Escherichia coli exonuclease III and the exonuclease activities of phage T4 DNA polymerase (3'→5' nuclease) and E. coli DNA polymerase (3'→5' and 3'→3' nuclease) initiate hydrolysis at these sites as well as at the termini of linear molecules. Under conditions of limited hydrolysis, these enzymes initiate hydrolysis at all nicks in the molecules. In contrast, phage λ exonuclease is unable to hydrolyze DNA containing nicks. Either the phage T4 or the E. coli DNA polymerase, in the presence of the four deoxyribonucleoside triphosphates, can completely fill in the gaps created by exonuclease action. The combined 5'→3' hydrolytic activity and the polymerizing activity of the E. coli DNA polymerase can account for the conversion of a nick displaying a 5'-hydroxyl end group to one displaying a 5'-phosphomonoester; i.e. the nick is translocated. These studies have been facilitated by the use of polynucleotide ligase to distinguish nicks containing 5'-phosphomonoesters from gaps and from nicks containing 5'-hydroxyl groups. E. coli alkaline phosphatase does not readily distinguish 5'-phosphomonoesters at gaps from those at nicks; quantitative hydrolysis of phosphomonoesters at these sites by the phosphatase requires a higher temperature than that required for the hydrolysis of phosphomonoesters at the ends of linear duplexes.

DNA molecules containing single strand breaks may serve as intermediates in the processes of replication, recombination, repair, and restriction (see References 1 to 6). In these processes exonucleases may remove nucleotides to form single stranded regions (gaps). DNA polymerase could subsequently replace the missing nucleotides and eliminate these gaps. In each reaction the initial substrate or the final product is a DNA molecule containing a single broken phosphodiester bond (nick). Although the substrate for exonuclease and DNA polymerase action in vivo may be DNA molecules containing nicks and gaps, the majority of the previous studies on these enzymes have been concerned with their action at the termini of linear molecules.

Circular duplex DNA is a convenient substrate for studying enzymatic reactions occurring at nicks and at gaps, since the possibility of reactions at external termini is eliminated. In the studies described in this paper we have used the closed circular duplexes isolated either as the replicative form of phage φX174 DNA or as the DNA of phage PM2 (Fig. 1a). Nicks are introduced into these molecules by limited treatment with pancreatic DNase (Fig. 1b). Exonucleases which can initiate hydrolysis at single strand breaks will catalyze the formation of gaps (Fig. 1c) which can be filled in by DNA polymerases (Fig. 1d). The molecules containing nicks can be distinguished from those containing gaps by incubation with polynucleotide ligase, since the latter structure is not a substrate for ligase. The covalently closed molecules can then be identified by sedimentation analysis in alkalai (7).

This report shows that Escherichia coli exonuclease III and the hydrolytic activities of the E. coli and the phage T4 polymerases can create gaps in DNA. Both DNA polymerase activities can restore these structures to yield molecules which can be covalently closed by polynucleotide ligase. Kelly et al. (8) have carried out extensive studies on the initiation of DNA synthesis by E. coli DNA polymerase at nicks in duplex DNA. They showed that synthesis entails the covalent extension of the 3'-hydroxyl terminus and a concurrent hydrolysis at the 5' terminus, resulting in translation of the nick. We show that, in this process, the 5'-hydrolytic activity of polymerase can change the 5' end from a hydroxyl to a phosphoryl group by re-

* This investigation was supported in part by Research Grant A1-08945 from the National Institutes of Health, United States Public Health Service, and Grant P-486 from the American Cancer Society, Inc.
† Present address, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan.
‡ Supported by National Science Foundation Summer Research Grant GY-6082.
¶ Recipient of Public Health Service Research Career Program Award GM-13,534.

2680
moving the 5'-terminal nucleoside. The following report (9) also describes synthesis of DNA by E. coli DNA polymerase at nicks in which displacement and conservation of the 5' strand occur.

**EXPERIMENTAL PROCEDURE**

**Materials**

Enzymes

Crystalline pancreatic DNase in standard vials (1 mg per vial) and E. coli alkaline phosphatase (chromatographically purified) were purchased from Worthington. Alkaline phosphatase was further purified as previously described (10).

Crystalline λ-exonuclease, purified and assayed by the procedure of Little, Lehman, and Kaiser (11), was a gift from Dr. A. D. Kaiser. E. coli exonuclease III was the phosphocellulose fraction purified and assayed as described previously (12).

Polynucleotide kinase was purified from E. coli infected with T4amN82 as previously described (13). The enzyme was further purified by chromatography on hydroxylapatite (14). DNA polymerase induced after the infection of E. coli B by phage T4amN82 was the hydroxylapatite fraction purified and assayed as described by Goulian, Lucas, and Kornberg (15). The preparation had a specific activity of 16,000 units per mg of protein. The exonuclease activity associated with T4 polymerase was measured as described by Oleson and Koerner (16).

E. coli DNA polymerase was the Sephadex fraction purified and assayed according to Jovin, Englund, and Bertsch (17). The preparation had a specific activity of 3600 units per mg of protein in the poly d(A-T)-primed assay. The exonuclease II activity was measured as described previously (18).

Phage T4 polynucleotide ligase was the phosphocellulose fraction purified from E. coli B infected with phage T4amN82 (19). The preparation had a specific activity of 10,000 units per mg of protein.

All enzyme preparations were examined for the presence of endonucleases in an assay with φX174 RF² DNA as substrate as previously described (20). No detectable endonuclease activity was present in the preparations of polymerase, ligase, kinase, phosphatase, exonuclease III, or λ-exonuclease.

Chemicals and Other Materials

γ-²⁵P-ATP was prepared as described previously (10). Carrier-free radioactive phosphate (²⁵P) and ³H-thymidine (specific activity 15 Ci per mmole) were purchased from New England Nuclear. Deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, and dTTP) and ATP were purchased from Schwarz BioResearch. Thymine and deoxyadenosine were purchased from Calbiochem. Bac-T-Flex membrane filters (type B-6, 25 mm) were purchased from Schleicher and Schuell. CsCl, optical grade, was purchased from Harshaw Chemical Company. Ethidium bromide was obtained from Calbiochem. Antibody to E. coli DNA polymerase (Fraction VII) was a gift from Dr. I. R. Lehman.

**DNA Preparations**

φX174 RF²—Two liters of E. coli C were infected with φX174RF² phage in the presence of chloramphenicol, and the cells were collected and lysed as described by Sinzheimer (21).

---

8 The abbreviation used is: RF, replicative form.

---

**Fig. 1. Scheme for formation of circular duplex containing nicks and gaps and their repair by polymerase and ligase.** Covalently closed circular φX174 RF or PM2 DNA (a) is treated with pancreatic DNase in order to introduce nicks displaying 3'-hydroxyl and 5'-phosphoryl end groups (b). A stepwise hydrolysis initiated at these sites leads to the formation of an internal single stranded region or gap (c). These nucleotides can be replaced by DNA polymerase in the presence of the four deoxynucleoside 5'-triphosphates to yield a molecule containing a nick (d). Molecules with a nick (b, d) can be repaired in the polynucleotide ligase reaction.

The lysate was then incubated with Pronase (Calbiochem, Grade C) for 6 hours at 37° at a concentration of 500 μg per ml. The viscous lysate was extracted twice with phenol saturated with 10 mM Tris-HCl buffer (pH 8.0). The aqueous phase was dialyzed against 10 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA-0.01 M NaCl. The dialysate was centrifuged in CsCl (p = 1.54) in the presence of ethidium bromide (500 μg per ml) with a Spinco rotor 30 at 25,000 rpm for 48 hours at 4°. The closed circular duplex RF DNA, which has a higher density than does the nicked RF DNA and linear duplex DNA (22), was collected from the side wall of the tube by using a syringe. To remove the contaminating linear DNA, the RF DNA was centrifuged again in CsCl with ethidium bromide. The RF DNA thus purified (about 10 μl) was extracted with isopropanol alcohol to remove the ethidium bromide (23). DNA was dialyzed against 10 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA-10 mM NaCl. Since the RF DNA was contaminated with RNA, it was centrifuged in a 5 to 20% linear sucrose gradient containing 10 mM Tris-HCl buffer (pH 8.0)-10 mM NaCl-1 mM EDTA in the Spinco SW 25 rotor at 25,000 rpm for 16 hours at 4°. After centrifugation, 1-mI fractions were collected from the bottom of the tube.

The fractions containing RF DNA (detected by optical density at 260 μm or radioactivity) were pooled and dialyzed against 10 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA-10 mM NaCl. The purified DNA was concentrated to approximately 7 ml by dialysis against dry Sephadex G-200. From 2 liters of culture 1 μmole of DNA phosphorus was recovered. The molar extinction coefficient at 260 μm with respect to deoxyxystonate was 6500.

³H-Labeled φX174 RF DNA was prepared in a similar manner, except that E. coli CR, a thymine-requiring mutant, was used instead of E. coli C. The cells were grown in 3XD medium (24) containing 10 μg per ml of thymine. Five minutes after infection, 1 mCi of ³H-thymidine (specific activity of 15 Ci per mmole) was added to the 500 ml of culture. Purification of the
DNA was identical with that described above for the unlabeled RF DNA. The specific activity of the purified $^{3}H$-RF was 1 to 5 $\times 10^{9}$ cpm per $\mu$ mole of DNA phosphorus.

$PM_{2}$ DNA—$PM_{2}$ phage were grown as described by Espejo and Canelo (25) and purified by the procedure described by Yamamoto et al. (26). $^{1}H$- or $^{32}P$-Labeled $PM_{2}$ phage were prepared by adding $^{3}H$-thymidine (10 $\mu$Ci per ml) and deoxyadenosine (50 $\mu$g per ml) or $^{32}P$-inorganic phosphate (2 $\mu$Ci per ml) to the AMS nutrient broth (27) 10 min after phage infection. The DNA was extracted with phenol and sodium dodecyl sulfate as described by Espejo and Canelo (27). The DNA was dialyzed against 10 mM Tris-HCl (pH 8.0)-1 mM EDTA-10 mM NaCl. The dialyzed DNA was centrifuged in a 5 to 20% neutral sucrose gradient containing 10 mM Tris-HCl buffer (pH 8.0)-10 mM NaCl-1 mM EDTA in a Spinco SW 25 rotor at 25,000 rpm for 14 hours at 4°C. After centrifugation, 1-ml fractions were collected from the bottom of the tube. The fractions containing closed circular duplex DNA were pooled, dialyzed against 10 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA-10 mM NaCl, and concentrated with Sephadex G-200. The molar extinction coefficient at 260 nm with respect to phosphorus was 6,500. The specific radioactivity of the labeled DNAs was 2 to 5 $\times 10^{9}$ cpm per $\mu$ mole of DNA phosphorus.

Now DNA—$^{3}H$-Labeled $\lambda$C857 phage were prepared by heat induction of $E$. coli W3102 ($\lambda$C857) grown in media containing $^{3}H$-thymidine. Phage growth, purification, and isolation of the DNA have been described previously (10). The specific activity of the DNA was 2.3 $\times 10^{9}$ cpm per $\mu$ mole of DNA phosphorus. Hydrogen-bonded circles of DNA were prepared by the procedure of Hershey, Burgi, and Ingraham (28) and concentrated by precipitation with ethanol as described by Celler (29). $T7$ DNA—$^{3}H$-Labeled $T7$ DNA was isolated from $T7$ phage grown and purified as previously described (30). The specific activity of the DNA was 3 $\times 10^{9}$ cpm per $\mu$ mole of DNA phosphorus.

Concentrations of DNA are expressed as equivalents of nucleotide phosphorus unless otherwise stated. In several experiments the radioactively labeled DNAs were diluted with unlabeled DNA to lower the specific radioactivity.

**Methods**

**Preparation of DNA Substrates**

**Circular DNA Containing Nicks ($3'$-Phosphomonoester)**—Circular duplexes containing nicks bearing $3'$-hydroxyl and 5'-phosphoryl end groups were prepared by incubating $H$- or $^{32}P$-labeled $\phi X174$ RF or PM2 DNA with pancreatic DNase. The reaction was carried out as previously described (10) with an amount of pancreatic DNase sufficient to produce one or two breaks per duplex molecule (approximately 2.5 $\mu$g of DNase in a 1-ml reaction mixture containing 0.1 mM DNA). After incubation at 20°C for 30 min, the reaction was stopped by the addition of 10 mM EDTA (pH 8.0), followed by heating at 65°C for 10 min. The nicked circular DNA was purified by sedimentation through a 5 to 20% sucrose gradient containing 10 mM Tris-HCl buffer (pH 8.0)-10 mM NaCl-1 mM EDTA. Sedimentation proceeded for 16 hours with $\phi X174$ RF DNA and 14 hours in the case of the PM2 DNA, in the Spinco SW 25 rotor at 25,000 rpm at 4°C. A maximum of 1 $\mu$ mole of DNA was layered on each 25-ml gradient. After centrifugation, 1-ml fractions were collected from the bottom of the tube. The radioactivity of each fraction was measured in 0.02-ml aliquots after precipitation with 1 N trichloroacetic acid and salmon sperm DNA (50 $\mu$g per fraction) as a carrier. The DNA was collected on a glass filter paper (Whatman GF/C, 24 mm), washed with 0.01 N HCl, and dried, and the radioactivity was measured as previously described (30). The nicked circular fractions were pooled and concentrated by dialysis against dry Sephadex G-200. The concentrated DNA was dialyzed against 10 mM Tris-HCl buffer (pH 8.0)-10 mM NaCl-1 mM EDTA.

**Circular DNA Containing $\delta'$-Hydroxy End Groups**—The nicked DNAs prepared by incubation of the circular duplexes with pancreatic DNase were dephosphorylated by incubation with $E$. coli alkaline phosphatase at 65°C as previously described (10). Under these conditions the internal $5'$-phosphomonoesters are hydrolyzed to yield DNA containing nicks bearing 3'- and 5'-hydroxyl end groups. The reaction was terminated by the addition of potassium phosphate buffer (pH 7.5) to a concentration of 1.4 mM (10). A portion of the dephosphorylated DNA was used without further purification; another portion was phosphorylated in the polynucleotide kinase reaction as described below.

**Circular DNA Containing $5'$-$^{32}P$-Phosphomonoester**—The circular DNA containing 5'-hydroxyl end groups was radioactively labeled in the polynucleotide kinase reaction containing $\gamma$-$^{32}P$-ATP as described previously (10). The DNA containing internal $5'$-$^{32}P$-phosphomonoester was purified by sedimentation through a neutral sucrose gradient as described above for the nicked DNA. End group analysis (10) of the purified DNA revealed that 40% of the 5'-hydroxyl end groups were phosphorylated in the kinase reaction.

**$T7$ DNA Containing External $5'$-$^{32}P$-Phosphomonoester**—Terminally labeled $H$-$T7$ DNA was prepared by incubation of the DNA with alkaline phosphatase and then with polynucleotide kinase and $\gamma$-$^{32}P$-ATP as previously described (30). The labeled DNA (0.7 $\mu$ mole) was isolated by sedimentation through a 5 to 20% neutral sucrose gradient containing 10 mM Tris-HCl buffer (pH 8.0)-10 mM NaCl-1 mM EDTA for 15 hours in the SW 25 type Spinco rotor at 22,000 rpm at 4°C. After centrifugation, 1-ml fractions were collected from the top of the gradient to minimize shearing, and the radioactivity in an aliquot of each fraction was measured as described above. The fractions containing the intact molecules of $T7$ DNA were pooled and dialyzed, first against 10 mM Tris-HCl buffer (pH 8.0)-1.0 mM NaCl-1 mM EDTA, and then against 10 mM Tris-HCl buffer (pH 8.0)-10 mM NaCl-1 mM EDTA. The dialyzed DNA was concentrated by dialysis against dry Sephadex G-200. The efficiency of labeling was more than 85%.

**$5'$-$^{32}P$-Phosphoryl $T7$ DNA Containing $5'$ Single Stranded Termini—$H$-$T7$ DNA containing external $5'$-$^{32}P$-phosphomonoester was incubated with exonuclease III until 5% of the $H$-radioactivity was released as mononucleotides. The procedure for incubation and assay of the extent of reaction has been described previously (31).

**$5'$-$^{32}P$-Phosphoryl $T7$ DNA Containing $5'$ Single Stranded Termini—$H$-$T7$ DNA was incubated with $\lambda$-exonuclease until 12% of the $H$-radioactivity was released as mononucleotides. The DNA was then dephosphorylated with alkaline phosphatase and labeled in the polynucleotide kinase reaction with $\gamma$-$^{32}P$-$^{32}P$-DNA indicates that the DNA molecules bear $5'$-$^{32}P$-labeled phosphomonoesters at their 5' termini. If the molecules are circular, this implies that the $5'$-$^{32}P$-phosphomonoester is located at a nick or gap.
ATP. The reaction mixture (0.5 ml) contained 0.6 mM $^3$H-T7 DNA, 67 mM glycine buffer (pH 9.2), 3 mM MgCl$_2$, 3 mM 2-mercaptoethanol, and 3 units of $\lambda$-exonuclease. The reaction mixture was incubated for 30 min at 37°C. The extent of hydrolysis was determined by measuring the release of acid-soluble radioactivity. The reaction mixture was then adjusted to pH 8.0 by adding Tris-HCl (pH 4.0). Bacterial alkaline phosphatase (0.2 unit) was added, and the incubation was continued at 65°C for 30 min. Additional alkaline phosphatase (0.2 unit) was added after 15 min. The 5'-hydroxyl-terminated DNA was then labeled with $^32$P in the polynucleotide kinase reaction as described previously (10). The DNA was purified by sedimentation through a sucrose gradient and then concentrated as described above.

Repair of Nicks

Initiation with Polynucleotide Ligase—We incubated DNA with polynucleotide ligase to determine whether the interruptions in the DNA were nicks containing 3'-hydroxyl and 5'-phosphoryl end groups and thus would be repairable by ligase. The incubation mixture (0.1 ml) contained 10 to 100 nM $^3$H $\phi X$174 RF DNA or PM2 DNA containing one to two breaks per molecule, 67 mM Tris-HCl buffer (pH 7.6), 67 mM MgCl$_2$, 10 mM 2-mercaptoethanol, 0.06 mM ATP, and 0.05 unit of T4 polynucleotide ligase. After incubation at 20°C for 15 min, the reaction was stopped by chilling and the addition of 20 mM EDTA (pH 8.0). The reaction mixture was then adjusted to pH 8.0 by adding Tris-HCl buffer (pH 7.6), 6.7 mM MgCl$_2$, 10 mM 2-mercaptoethanol, dATP, dTTP, dGTP, dCTP (0.03 mM each), 0.06 mM ATP, 0.05 unit of T4 polynucleotide ligase, and 1 unit of E. coli or T4 polymerase. The mixture was incubated at 20°C for 20 min. After the reaction, 20 mM EDTA was added and the reaction mixture was centrifuged in an alkaline sucrose gradient as described above. The complete replacement of nucleotides at the gaps was thus measured by the formation of phosphodiester bonds in the presence of ligase.

Treatment of Nicked DNA with Exonucleases

E. coli DNA Polymerase—E. coli DNA polymerase, in addition to its polymerizing activity, catalyzes the hydrolysis of DNA from both 3' and 5' termini. The reaction mixture (0.1 ml) contained 10 to 100 nM nicked DNA, 67 mM Tris-HCl buffer (pH 8.0), 3 mM MgCl$_2$, 10 mM 2-mercaptoethanol, and exonuclease III (0.001 to 0.1 unit). After incubation at 37°C for 30 min, the mixture was made 5 mM with EDTA and heated at 65°C for 10 min to inactivate the enzyme.

E. coli DNA Polymerase—E. coli DNA polymerase also catalyzes the hydrolysis of DNA, but only from 3' termini (34). The reaction mixture (0.1 ml) contained 10 to 100 nM nicked DNA, 67 mM Tris-HCl buffer (pH 7.6), 6.7 mM MgCl$_2$, 10 mM 2-mercaptoethanol, and 1 unit (polymerase unit) of E. coli exonuclease II. The reaction mixture was incubated for 10 min at 37°C.

T4 DNA Polymerase—T4 DNA polymerase also catalyzes the hydrolysis of DNA, but only from 3' termini (34). The reaction mixture (0.1 ml) contained 10 to 100 nM nicked DNA, 67 mM Tris-HCl buffer (pH 8.0), 6.7 mM MgCl$_2$, 10 mM 2-mercaptoethanol, and 1 unit (polymerase unit) of T4 polymerase. The reaction mixture was incubated at 37°C for 10 min.

$\lambda$-Exonuclease—The reaction mixture (0.1 ml) contained 10 to 100 nM nicked DNA, 67 mM glycine-KOH buffer (pH 9.2), 6.7 mM MgCl$_2$, 10 mM 2-mercaptoethanol, and 10 units of $\lambda$-exonuclease. After incubation for 30 min at 37°C, 100 mM NaCl and 15 mM Tris-HCl (pH 4.0) to yield pH 7.6 were added to diminish the activity of $\lambda$-exonuclease.

Since $\lambda$-exonuclease hydrolyzes DNA exclusively from 5' termini (35), DNA substrates bearing 5'-32P-phosphomonoesters were used in a specific assay. The reaction mixture is identical with that described above, except that circular DNA containing 5'-32P-phosphomonoesters was used as substrate. In some experiments the DNA was first treated with exonuclease III in order to create gaps.

Treatment of DNA with Phosphatase

The reaction mixture (0.5 ml) contained $\phi X$174 RF DNA with either nicks (9 nM) or gaps (15 nM) (exonuclease III treatment) and bearing 5'-32P-phosphomonoesters, 45 mM Tris HCl buffer (pH 8.0), 60 mM MgCl$_2$, and 0.15 unit of bacterial alkaline phosphatase. The reaction mixtures were incubated at the temperatures indicated in the text for 30 or 60 min, with an addition of 0.1 unit of enzyme after 15 min. The DNA was precipi-
open circle, with the result that the sedimentation rate in alkaline
from that of a covalently closed, twisted circle to a relaxed
acquires one or more nicks, the structure of the DNA changes
of the DNA molecules were closed circular duplexes, as distin-
was calculated from the amount of DNA remaining in the closed
at a slower rate which is characteristic of a mixture of single
stranded circular and linear molecules, respectively. The
two samples of DNA were centrifuged in separate tubes, but the
results are plotted together. In this and all subsequent sedi-
mation analyses, the direction of sedimentation is from right
to left. O- - - O, DNA incubated without pancreatic DNase;
- - - - , DNA incubated with pancreatic DNase.

Fig. 3 (right). Repair of nicked circular duplexes. The nicked
DNA described in Fig. 2 was incubated with polynucleotide ligase
(see "Methods"). A control incubation without ligase was also
carried out. The DNAs were sedimented on alkali and analyzed
as described in Fig. 2. O- - - O, incubation without ligase;
O- - - O, incubation with ligase.

Fig. 2 (left). Alkaline sedimentation analysis of nicked φX174
RF DNA. 3H-φX174 RF DNA was incubated with pancreatic
DNase in order to introduce nicks (see "Methods"). A control
incubation without DNase was also carried out. The DNAs
were then centrifuged in an alkaline sucrose gradient for 3 hours
at 40,000 rpm with the Spinco type SW 50.1 rotor. After cen-
trifugation, fractions (3 drops) were collected from the bottom of
the tube, and the radioactivity was measured. Fractions 8 and
20 correspond to covalently closed duplex circles and a mixture of
single stranded circular and linear molecules, respectively. The
results are plotted together. In this and all subsequent sedi-
mation analyses, the direction of sedimentation is from right
to left. O- - - O, DNA incubated without pancreatic DNase;
- - - - , DNA incubated with pancreatic DNase.

Calculation of Number of Nicks per Molecule

The average number of nicks introduced by pancreatic DNase
was calculated from the amount of DNA remaining in the closed
circular duplexes. For example, the relative amount of RF in

Fig. 4 (right). Repair of nicked circular duplexes. The pancreatic
DNase may have some preference for attacking a DNA strand
at any internal 5'-aaP-phosphomonoesters was purified by sedimentation
through a neutral sucrose gradient, and the open circular DNA
fraction, but was found exclusively in the nicked circular
DNA fraction. The isolated nicked DNA was then centrifuged

Identification of Single Stranded Circles and Linear Molecules in
Nicked DNA

φX174 RF DNA containing one nick per molecule (P(0) =
0.7) was dephosphorylated by incubation with bacterial alkaline
phosphatase at 65° and then rephosphorylated in the polynu-
icotide kinase reaction with γ-32P-ATP. The DNA bearing
internal 5'-32P-phosphomonoesters was purified by sedimentation
through a neutral sucrose gradient, and the open circular DNA
was isolated. 32P label was not detected in the supercoiled
DNA fraction, but was found exclusively in the nicked circular
DNA fraction. The isolated nicked DNA was then centrifuged

Fig. 5 (right). Repair of nicked circular duplexes. The pancreatic
DNase may have some preference for attacking a DNA strand
at any internal 5'-aaP-phosphomonoesters was purified by sedimentation
through a neutral sucrose gradient, and the open circular DNA
fraction, but was found exclusively in the nicked circular
DNA fraction. The isolated nicked DNA was then centrifuged

Characterization of Circular DNA Containing Nicks

Sedimentation Analysis

Limited hydrolysis of duplex DNA by pancreatic DNase
creates a number of nicks at different loci in the two DNA
strands (10, 36-39). When φX174 RF DNA or PM2 DNA
acquires one or more nicks, the structure of the DNA changes
from that of a covalently closed, twisted circle to a relaxed
open circle, with the result that the sedimentation rate in alkaline
solution is drastically changed (7). The sedimentation coeffi-
cient of φX174 RF DNA in alkali, for example, is 54 S, while the
mixture of single stranded circular and linear DNA mole-
cules which are derived from the nicked circles has a coefficient of
18 S (7). We have used zone sedimentation of DNA in an
alkaline sucrose density gradient to measure nicks in φX174 RF
DNA. As shown in Fig. 2, prior to treatment with DNase most
of the DNA molecules were closed circular duplexes, as distin-
guished by their large sedimentation velocity. However, after
treatment with DNase, a large fraction of the DNA sedimented
at a slower rate which is characteristic of a mixture of single
stranded circular and linear molecules.

RESULTS

The average number of nicks introduced by pancreatic DNase
was calculated from the amount of DNA remaining in the closed
circular duplexes. For example, the relative amount of RF in

Identification of Single Stranded Circles and Linear Molecules in
Nicked DNA

φX174 RF DNA containing one nick per molecule (P(0) =
0.7) was dephosphorylated by incubation with bacterial alkaline
phosphatase at 65° and then rephosphorylated in the polynu-
icotide kinase reaction with γ-32P-ATP. The DNA bearing
internal 5'-32P-phosphomonoesters was purified by sedimentation
through a neutral sucrose gradient, and the open circular DNA
was isolated. 32P label was not detected in the supercoiled
DNA fraction, but was found exclusively in the nicked circular
DNA fraction. The isolated nicked DNA was then centrifuged

Calculation of Number of Nicks per Molecule

The average number of nicks introduced by pancreatic DNase
was calculated from the amount of DNA remaining in the closed
circular duplexes. For example, the relative amount of RF in

Characterization of Circular DNA Containing Nicks

Sedimentation Analysis

Limited hydrolysis of duplex DNA by pancreatic DNase
creates a number of nicks at different loci in the two DNA
strands (10, 36-39). When φX174 RF DNA or PM2 DNA
acquires one or more nicks, the structure of the DNA changes
from that of a covalently closed, twisted circle to a relaxed
open circle, with the result that the sedimentation rate in alkaline
solution is drastically changed (7). The sedimentation coeffi-
cient of φX174 RF DNA in alkali, for example, is 54 S, while the
mixture of single stranded circular and linear DNA mole-
cules which are derived from the nicked circles has a coefficient of
18 S (7). We have used zone sedimentation of DNA in an
alkaline sucrose density gradient to measure nicks in φX174 RF
DNA. As shown in Fig. 2, prior to treatment with DNase most
of the DNA molecules were closed circular duplexes, as distin-
guished by their large sedimentation velocity. However, after
treatment with DNase, a large fraction of the DNA sedimented
at a slower rate which is characteristic of a mixture of single
stranded circular and linear molecules.

Calculation of Number of Nicks per Molecule

The average number of nicks introduced by pancreatic DNase
was calculated from the amount of DNA remaining in the closed
circular duplexes. For example, the relative amount of RF in

Identification of Single Stranded Circles and Linear Molecules in
Nicked DNA

φX174 RF DNA containing one nick per molecule (P(0) =
0.7) was dephosphorylated by incubation with bacterial alkaline
phosphatase at 65° and then rephosphorylated in the polynu-
icotide kinase reaction with γ-32P-ATP. The DNA bearing
internal 5'-32P-phosphomonoesters was purified by sedimentation
through a neutral sucrose gradient, and the open circular DNA
was isolated. 32P label was not detected in the supercoiled
DNA fraction, but was found exclusively in the nicked circular
DNA fraction. The isolated nicked DNA was then centrifuged
in an alkaline sucrose gradient for a length of time sufficient to resolve partially single stranded circles and single stranded linear molecules. Fig. 4 shows that the DNA was distributed between two overlapping peaks, as measured by the $^3$H radioactivity. However, the $^{32}$P located at the 5' termini of the linear strands was present only in the more slowly sedimenting peak. This result is in agreement with the earlier observation that circular single strands sediment faster than do linear single strands of the same molecular weight (7). It is also clear that the nicked duplex molecules from which these single strands were derived contain approximately one break per duplex molecule.

When the nicked DNA containing $5'$-$^{32}$P-phosphomonoesters was incubated with polydeoxyribonucleotides from both the 3' and 5' termini of the molecule (18, 32, 33). Studies similar to those described above for exonuclease III and T4 polymerase reveal that the E. coli' enzymes are capable of hydrolyzing phosphoester bonds at nicks (40). When exonuclease III was incubated with $^3$H-$\phi$X174 RF DNA containing one nick per molecule, the rate of hydrolysis as measured by the release of acid-soluble mononucleotides was identical with that observed when linear T7 DNA was used as a substrate. As shown in Fig. 6, the nicked $\phi$X174 RF DNA can no longer be repaired by incubation with ligase after it has been incubated with exonuclease III. However, as shown below, these molecules can be repaired if the polymerizing activity is permitted to act in the presence of the four deoxynucleoside triphosphates. Similar results were obtained when joining was measured in the standard ligase assay with 5'-$^{32}$P-phosphomonoesters into phosphodiester bonds was markedly inhibited by prior incubation of the DNA with T4 polymerase. This inhibition could be completely overcome by the addition of the four deoxynucleoside triphosphates, indicating that the inhibition was due to a gap and not to strand breakage.

### Hydrolytic Activity of T4 DNA Polymerase

Bacteriophage T4 induces a new DNA polymerase after infection of E. coli. The purified enzyme has been shown to possess a hydrolytic activity in addition to its polymerizing activity (15). The purified T4 polymerase catalyzes a stepwise hydrolysis of nucleotides from the 3' ends of DNA strands in single stranded or duplex configuration. Unlike exonuclease III, the T4 polymerase can initiate hydrolysis at nicks; the rate of hydrolysis of nicked $\phi$X174 RF was found to be identical with that observed with linear T7 DNA. As shown in Fig. 8, the nicked $\phi$X174 RF could not be joined by ligase after treatment with T4 polymerase. However, as discussed below, repair could be achieved if the polymerizing activity was permitted to act in the presence of the four deoxynucleoside triphosphates. Similar results were obtained when joining was measured in the standard ligase assay with 5'-$^{32}$P-$\phi$X174 RF DNA (Table I). The incorporation of the 5'-$^{32}$P-phosphomonoesters into phosphodiester bonds was markedly inhibited by prior incubation of the DNA with T4 polymerase. This inhibition could be completely overcome by the addition of the four deoxynucleoside triphosphates, indicating that the inhibition was due to a gap and not to strand breakage.

### Hydrolytic Activity of T4 DNA Polymerase

Purified E. coli DNA polymerase catalyzes the hydrolysis of polydeoxyribonucleotides from both the 3' and 5' termini of the molecule (18, 32, 33). Studies similar to those described above for exonuclease III and T4 polymerase reveal that the E. coli enzyme can initiate hydrolysis at nicks (Table II). When nicked circular PM2 DNA is incubated with DNA polymerase, the molecules can no longer be joined by incubation with ligase alone. Including the four deoxynucleoside triphosphates in the reaction mixture, however, allows the polymerizing activity to function and covalent joining to occur. Although these experiments do not distinguish between 3' $\rightarrow$ 5' and 5' $\rightarrow$ 3' hydrolytic activities of E. coli polymerase, experiments described below, as well as those carried out by others (8, 32-34), demonstrate that both activities function at nicks.
confirmed by measuring the extent of joining of 5′-32P-phosphomonoesters in nicked φX174 RF DNA before and after incubation with T4 ligase. The amount of DNA, and the removal of a single nucleotide would have prevented joining in these assays. This result was confirmed by measuring the extent of joining of 5′-32P-phosphomonoesters in nicked φX174 RF DNA before and after incubation with λ-exonuclease. Incubation of 15 μmole of the ligase substrate with the exonuclease (1.5 to 30 units) had no effect on its subsequent closure by T4 ligase. The amount of exonuclease added is sufficient to degrade 300 μmole of linear DNA, and the removal of a single nucleotide would have prevented joining in these assays.

When 3H-T7 DNA and 5′-32P-φX174 RF DNA were incubated together with λ-exonuclease, there was no significant release of the 32P under conditions in which 50% of the 3H was rendered acid-soluble (Fig. 10). This experiment shows that the exonuclease is fully active in the reaction mixture containing the nicked φX174 RF.

In order to determine whether λ-exonuclease can hydrolyze circular duplexes containing gaps, 5′-32P-φX174 RF DNA was treated with exonuclease III prior to incubation with λ-exonuclease. The introduction of a gap did permit λ-exonuclease to initiate hydrolysis at some of the 5′ termini (Fig. 11). However, when all of the nicks had been converted to gaps as judged by the lack of repair in the ligase assay, only 30% of the 5′-terminal nucleotides could be hydrolyzed with an amount of λ-exonuclease sufficient to hydrolyze completely the 5′-terminal nucleotides of linear DNA. The explanation for this observation is not known, although it may be related to the fact that λ-exonuclease has a tendency to “stick” to a given DNA molecule, particularly at low DNA concentrations (42).

**TABLE I**

**Formation and repair of gaps by T4 DNA polymerase**

<table>
<thead>
<tr>
<th>Treatment prior to ligase</th>
<th>Phosphatase-resistant 32P %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>65</td>
</tr>
<tr>
<td>T4 polymerase</td>
<td>8</td>
</tr>
<tr>
<td>T4 polymerase + dXTP</td>
<td>82</td>
</tr>
</tbody>
</table>

**TABLE II**

**Formation and repair of gaps by Escherichia coli DNA polymerase**

The reaction mixtures (0.1 ml) contained 20 μmole nicked +32P-PM2 DNA. Eight per cent of the molecules in the preparation were closed circular duplexes. After incubation with E. coli DNA polymerase (2.0 units), the DNA was then incubated with T4 ligase, either in the presence or absence of the four deoxynucleoside triphosphates (dTTP), as indicated below. Control incubations were carried out with no polymerase. The incubation and sedimentation assay for closed circular molecules is described in “Methods.”

<table>
<thead>
<tr>
<th>Treatment prior to ligase</th>
<th>Closed circular duplexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>65</td>
</tr>
<tr>
<td>E. coli polymerase</td>
<td>8</td>
</tr>
<tr>
<td>E. coli polymerase + dXTP</td>
<td>80</td>
</tr>
</tbody>
</table>

**Inability of Phage λ-Exonuclease to Initiate Hydrolysis at Nicks**

The exonuclease induced by phage λ has been shown to initiate hydrolysis from the 5′ end of a duplex DNA, producing nucleoside 5′-monophosphates (11, 35). Genetic studies have shown that the enzyme is important in the recombination system of phage λ (41). We find that, unlike the three exonucleases described above, λ-exonuclease is unable to hydrolyze nicked circular DNA duplexes. A similar observation has been made by Carter and Radding (42). As shown in Fig. 9, when an amount of exonuclease sufficient to hydrolyze 100 μmole of linear T7 DNA was added to an incubation mixture containing 10 μmole of nicked φX174 RF DNA, there was no hydrolysis at nicks, as measured in the ligase assay. This result was confirmed by measuring the extent of joining of 5′-32P-phosphomonoesters in nicked φX174 RF DNA before and after incubation with λ-exonuclease. Incubation of 15 μmole of the ligase substrate with the exonuclease (1.5 to 15 units) had no effect on its subsequent closure by T4 ligase. The amount of exonuclease added is sufficient to degrade 300 μmole of linear DNA, and the removal of a single nucleotide would have prevented joining in these assays.

When 3H-T7 DNA and 5′-32P-φX174 RF DNA were incubated together with λ-exonuclease, there was no significant release of the 32P under conditions in which 50% of the 3H was rendered acid-soluble (Fig. 10). This experiment shows that the exonuclease is fully active in the reaction mixture containing the nicked φX174 RF.

At temperatures below 37° alkaline phosphatase will quantitatively hydrolyze external phosphomonoesters (those located at either end of the duplex molecule), but will not hydrolyze phosphomonoesters located at nicks (10). However, at elevated temperatures both types of phosphomonoesters are hydrolyzed by phophatase (10). As shown in Table III, the external phosphomonoesters of T7 DNA are quantitatively hydrolyzed by alkaline phosphatase at 20° as well as at 65°, while the internal phosphomonoesters of nicked φX174 RF are susceptible to phosphatase only at 65°.

When a gap is introduced into the nicked φX174 RF, the phosphomonoesters are still resistant to hydrolysis by phosphatase, only 10% being hydrolyzed at 20° (Table III). Apparently a single stranded region adjacent to the phosphomonoester inhibits the action of phosphatase, since 5′-phosphomonoesters on linear T7 DNA molecules bearing 3′ or 5′ single stranded regions were not quantitatively hydrolyzed at 20°, whereas they were hydrolyzed at 65° (Table III). These substrates were prepared by partial digestion of T7 DNA by exonuclease III or λ-exonuclease.

**Synthesis at Gaps by DNA Polymerases**

The studies described above show that exonuclease III and the hydrolytic activities of E. coli and phage T4 polymerase can initiate hydrolysis at a nick. Their continued action leads to the formation of a gap in one of the two strands of the duplex. These DNA molecules in turn can be used to study the polymerizing activity of T4 and E. coli DNA polymerase at gaps.

**T4 DNA Polymerase**

T4 DNA polymerase can initiate synthesis at a gap created by exonuclease III, replace the missing nucleotides, and leave a nick which can then be covalently joined by ligase (Figs. 6 and 7). Similarly, after the hydrolytic activity of T4 polymerase has created a gap in nicked DNA, the addition of the four deoxyribose monophosphates stabilizes the polymerizing activity to fill in the gap (Fig. 8, Table I). Since T4 polymerase has no 5′-hydrolytic activity (34), the nicks remaining after synthesis by polymerase are located at the sites where they were originally introduced. This is clearly shown in the experiments in Fig. 7 and Table I in which the nick displays a 5′-32P-phosphomonoester.
E. coli DNA Polymerase

E. coli DNA polymerase in the presence of the four deoxynucleoside triphosphates can also fill in the gaps in DNA. When the preparation of exonuclease III-treated dX174 RF shown in Fig. 6 was incubated with E. coli polymerase (2 units), the four deoxynucleoside triphosphates, and T4 ligase (0.05 unit) as described under "Methods." The reaction mixtures were centrifuged in an alkaline sucrose gradient in the Spinco type SW 50.1 rotor at 40,000 rpm for 3 hours. One-half of the control reaction mixture lacking ligase was centrifuged.

Fig. 9 (center). The inability of phage λ-exonuclease to initiate hydrolysis of 5'-hydroxyl groups at nicks. The reaction mixture (0.1 ml) containing 100 μM nicked 3H-dX174 RF DNA was incubated first with 10 units of λ-exonuclease and then with T4 ligase, as described under "Methods." A control experiment was carried out in which the nicked DNA was incubated with 0.1 unit of exonuclease III instead of λ-exonuclease. The reaction mixtures were centrifuged in an alkaline sucrose gradient in the Spinco type SW 50.1 rotor at 40,000 rpm for 3 hours. The two samples of DNA were centrifuged in separate tubes, but the results are plotted together.

Conversion of 5'-Hydroxyl Termini to 5'-Phosphoryl Termini by E. coli DNA Polymerase

DNA bearing 5'-hydroxyl group at nicks cannot be covalently closed by polynucleotide ligase (43). However, the combined action of a 5'-exonuclease and a DNA polymerase can effectively convert a nick bearing a 5'-hydroxyl terminus to one bearing a 5'-phosphoryl terminus. Hence, E. coli DNA polymerase which has a 5'-hydrolytic activity in addition to its polymerizing activity theoretically should be able to catalyze both of these reactions. Klett, Cerami, and Reich (32) have shown that the E. coli polymerase can hydrolyze 5'-hydroxyl-terminated, as well as 5'-phosphoryl-terminated, polynucleotides. In Fig. 12 a scheme is shown for the repair of nicks bearing 5'-hydroxyl end groups by E. coli DNA polymerase and by ligase. In the first reaction the 5'-hydrolytic activity of DNA polymerase excises at least the terminal 5'-nucleoside, and perhaps several other bases. The excision of 5'-nucleotides or 5'-mononucleotides, however, exposes a 5'-phosphoryl group. In the second reaction repair synthesis by polymerase closes the gap created by the excision. The end product contains a nick displaced from its original position, but now having a 5'-phosphoryl and 3'-hydroxyl end group. Kelly et al. (8) have shown that E. coli DNA polymerase can translate a nick bearing a 5'-phosphoryl end group in such a manner. In the final step ligase catalyzes the covalent closure of the molecule.

In this section we show that E. coli DNA polymerase and the ligase can convert nicked PM2 DNA bearing 5'-hydroxyl groups and 5'-hydroxyl-terminated hydrogen-bonded circles of phage λ DNA to closed circular duplexes, and that both the polymerizing activity and the 5'-hydrolytic activity of DNA polymerase are essential in the reaction.

5'-Hydroxyl Nicked PM2 DNA

When PM2 DNA containing nicks displaying 5'- and 5'-hydroxyl groups was incubated with E. coli DNA polymerase, the four deoxynucleoside triphosphates, polynucleotide ligase, and ATP, up to 50% of the DNA molecules were converted to
closed circles (Table IV). In the absence of *E. coli* DNA polymerase or ligase, there was no significant joining. Furthermore, T4 polymerase, lacking a 5'-hydrolytic activity, could not replace *E. coli* polymerase in the reaction mixture. It is possible to isolate two fragments of *E. coli* DNA polymerase after limited hydrolysis with subtilisin (44-46). Furthermore, one of the fragments contains the polymerizing activity and 3'-hydrolytic activity and the other the 5'-hydrolytic activity. When the fragment of *E. coli* DNA polymerase lacking 5'-hydrolytic activity was incubated with 5'-hydroxyl nicked PM2 DNA in the presence of ligase, no covalent joining was observed (Table IV).

### 5'-Hydroxyl-terminated Hydrogen-bonded Circles of λ DNA

**Repair by Purified *E. coli* Polymerase and T4 Ligase**—The purified T4 ligase catalyzes the conversion of hydrogen-bonded circles of λ DNA to covalently closed molecules (19, 47), a form which can be readily identified by a 4-fold increase in sedimentation rate in alkaline (48). As shown in Table V, T4 polynucleotide ligase could catalyze the covalent closure of 17% of the molecules in a preparation of hydrogen-bonded λ circles. If the DNA was dephosphorylated prior to incubation with ligase, no covalent circles were formed. However, incubation of the 5'-hydroxyl-terminated hydrogen-bonded circles with *E. coli* DNA polymerase and the four deoxynucleoside 5'-triphosphates converted them to a form which could be covalently closed by T4 polynucleotide ligase (Table V). As in the case of the 5'-hydroxyl nicked PM2 DNA discussed above, T4 DNA polymerase could not replace *E. coli* DNA polymerase.

**Role of DNA Polymerase in Repair by Extracts**—The results shown in Table VI show that extracts of wild type *E. coli* can catalyze the covalent closure of 5' hydroxyl terminated circles of λ DNA and that DNA polymerase is an essential component of this reaction. When an extract of *E. coli* K12 endonuclease I-negative cells, supplemented with DPN (the cofactor of the *E. coli* DNA ligase), was incubated with 5'-phosphoryl hydrogen-bonded circles, 11% of the DNA was converted to covalently closed circles. On the other hand, only 2% of the 5'-hydroxyl-terminated circles could be covalently closed (Table VI). The addition of the four deoxynucleoside 5'-triphosphates not only increased the joining of 5' phosphoryl terminated circles 3 fold, but also made possible the joining of a comparable amount of the 5'-hydroxyl-terminated circles. Adding antiserum to *E. coli* DNA polymerase to the extract caused a marked decrease in the joining of both forms of λ DNA (Table VI).

DeLucia and Cairns (49) have isolated a mutant of *E. coli*, *E. coli* PolAl, which has less than 1% of wild type DNA polymerase activity in cell-free extracts. When the λ circles described above were incubated with extracts of a strain of *E. coli* PolAl, also lacking endonuclease I (50), the results were similar to those obtained with wild type extracts containing antiserum to DNA polymerase; there was no detectable joining of 5'-hydroxyl-terminated λ DNA circles and only 3% of 5'-phosphoryl-terminated circles (Table VI). The addition of purified *E. coli* DNA polymerase initiates hydrolysis at the 5' terminus of the nick releasing the terminal base as a nucleoside or oligonucleotide. In the process, a 5'-phosphoryl end group is exposed. Repair synthesis by polymerase closes the gap, and the nick is repaired by ligase.

**Table III**

Action of *Escherichia coli* alkaline phosphatase at nicks and gaps

The reaction mixtures (0.1 ml) containing the DNAs listed below at a concentration of 10 μM were incubated with 0.3 unit of *E. coli* alkaline phosphatase at either 20° or 65°. The percentage of 32P made acid-soluble was measured as described under “Methods.” The preparation and characterization of the various DNA substrates are described under “Methods.”

<table>
<thead>
<tr>
<th>DNA substrate labeled at 5' end with 32P</th>
<th>Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20°</td>
</tr>
<tr>
<td>%</td>
<td></td>
</tr>
<tr>
<td>T7</td>
<td>92</td>
</tr>
<tr>
<td>φX174 RF (nick)</td>
<td>4</td>
</tr>
<tr>
<td>φX174 RF (gap)</td>
<td>10</td>
</tr>
<tr>
<td>T7 DNA with 5' single stranded termini</td>
<td>37</td>
</tr>
<tr>
<td>T7 DNA with 3' single stranded termini</td>
<td>64</td>
</tr>
</tbody>
</table>

**Fig. 11.** Action of λ-exonuclease on DNA containing gaps. The reaction mixture (0.1 ml) contained 10 μM 5'-32P-φX174 RF DNA, 67 mM Tris-HCl buffer (pH 8.0), 3 mM MgCl2, 10 mM 2-mercaptoethanol, and varying amounts of exonuclease III as indicated. After incubation at 37° for 30 min, 3 units of λ-exonuclease were added to each tube, and incubation was continued for 30 min. Then 0.65 unit of T4 ligase and 0.06 μM ATP were added and, after incubation for 15 min, the extent of repair and the acid-soluble radioactivity were determined as described under “Methods.”

**Fig. 12.** Scheme for the repair of DNA containing nicks bearing 5'-hydroxyl end groups. The 5'-hydrolytic activity of *E. coli* DNA polymerase initiates hydrolysis at the 5' terminus of the nick releasing the terminal base as a nucleoside or oligonucleotide. In the process, a 5'-phosphoryl end group is exposed. Repair synthesis by polymerase closes the gap, and the nick is repaired by ligase.
TABLE IV
Covalent closure of circular DNA containing nicks with 5'-hydroxyl end groups

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>Closed circular duplexes %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-P-nicked PM2 DNA</td>
<td></td>
</tr>
<tr>
<td>Minus T4 ligase</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Plus T4 ligase</td>
<td>55</td>
</tr>
<tr>
<td>5'-OH-nicked PM2 DNA</td>
<td></td>
</tr>
<tr>
<td>Minus T4 ligase</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Plus T4 ligase</td>
<td>3</td>
</tr>
<tr>
<td>Plus E. coli polymerase</td>
<td>50</td>
</tr>
<tr>
<td>Plus E. coli polymerase lacking 5'-hydrolytic activity and T4 ligase</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Plus T4 polymerase and T4 ligase</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

TABLE V
Covalent closure of 5'-hydroxyl-terminated circles of λ DNA by Escherichia coli DNA polymerase and T4 ligase

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>Closed circular duplexes % 5'-P</th>
<th>5'-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 ligase</td>
<td>17</td>
<td>&lt;0.8</td>
</tr>
<tr>
<td>T4 DNA polymerase, 4dXTP, T4 ligase</td>
<td>17</td>
<td>1.5</td>
</tr>
<tr>
<td>E. coli DNA polymerase, 4dXTP, T4 ligase</td>
<td>17</td>
<td>12</td>
</tr>
</tbody>
</table>

The stimulation in joining seen with 5'-phosphoryl-terminated circles of λ DNA when deoxynucleoside triphosphates are present and the decrease in joining when polymerase is removed suggest that repair synthesis is necessary to counteract the action of exonucleases in the extract. The complete absence of joining of 5'-hydroxyl-terminated circles of λ DNA by extracts in which polymerase is inactive can be explained by the strict requirement for a 5'-hydroxyl activity to remove the 5'-hydroxyl terminus in addition to a polymerizing activity to fill in the resulting gap.

DISCUSSION

The exonuclease and DNA polymerases used in these studies have already been thoroughly characterized (6) although mainly in regard to their action on the termini of linear molecules. In this report we have systematically examined their action on circular duplex DNA molecules containing nicks and gaps. The use of such DNA substrates has several advantages for this type of study. (a) The relatively low molecular weight and the homogeneity of some of the circular duplexes, such as φX174 RF and PM2 DNA, facilitate their manipulation. (b) The marked effect of a single interruption in their strands on their physical properties makes possible an accurate analysis of the number of interruptions. (c) The absence of external termini eliminates concern over reactions occurring at these sites. In our studies DNA ligase has been useful in distinguishing between nicks and gaps, since ligase can repair only the former.

Earlier studies (10) on E. coli alkaline phosphatase revealed that results obtained for the action of enzymes at external termini of linear molecules cannot always be extended to their action at internal sites. While the phosphatase can hydrolyze external phosphomonoesters at 25°, significantly higher tempera-
tures are required to hydrolyze internal phosphomonoesters. In the present report we show that elevated temperatures are required to hydrolyze phosphomonoesters at gaps as well as at nicks. It is evident from our studies that similar considerations apply to the action of exonucleases at nicks and gaps. Thus, while E. coli exonuclease III and the hydrolytic activities of T4 polymerase can initiate hydrolysis at nicks, the phage λ exonuclease cannot do so.

E. coli exonuclease III was found to hydrolyze nicked DNA at a rate comparable to that observed with linear duplex DNA. Furthermore, the fact that an average of only five nucleotides need be removed from a nick to inhibit joining by ligase suggests that the enzyme initiates hydrolysis at all of these sites. Similar results were found with the hydrolytic activities of the T4 and E. coli polymerases. Sadowski and Hurwitz (51) have shown that T4 exonuclease IV can create gaps in nicked DNA, and it seems likely that this activity is identical with that associated with the T4-induced DNA polymerase. From our results it is clear that the E. coli polymerase can create gaps, and that hydrolysis can occur in a 5' → 3' direction. Although these studies do not establish whether or not simultaneous 3' → 5' hydrolysis is occurring, the work of Cozzarelli, Kelly, and Kornberg (34) indicates that both hydrolytic activities function at nicks. Furthermore, Englund, Kelly, and Kornberg (52) have shown that E. coli DNA polymerase can bind to nicks and that there is only a single DNA-binding site.

The inability of λ-exonuclease to initiate hydrolysis at nicks was shown in assays involving either the closing of circles in the presence of ligase or the hydrolysis of 5'-32P-nicked circular DNA. Both assays are capable of detecting the removal of a single nucleotide from the 5' terminus, the known site of initiation for the exonuclease (35), and the former assay could detect the removal of a single nucleotide from the 3' terminus as well. The lack of activity on such substrates was not due specifically to the structure of a nick, since circular DNA containing gaps was also resistant to its action.

The gaps produced by exonuclease action at nicks are readily filled in by either the T4 or the E. coli DNA polymerase. There are several experiments which show that the single stranded ends produced by exonuclease III can be filled in by DNA polymerase (31, 53). A more extensive type of repair synthesis involves the synthesis of the complementary strand of M13 or φX174 DNA. Both assays are capable of detecting the removal of a single nucleotide from the 5' terminus, the known site of initiation for the exonuclease (35), and the former assay could detect the removal of a single nucleotide from the 3' terminus as well. The lack of activity on such substrates was not due specifically to the structure of a nick, since circular DNA containing gaps was also resistant to its action.

The formation and repair of gaps are phenomena which probably occur in vivo in a number of biological processes including the repair of ultraviolet-induced lesions in DNA and genetic recombination. Since the 5' → 3'-hydrolytic activity of the E. coli DNA polymerase can excise thymine dimers (58), this activity in conjunction with the polymerizing activity of the enzyme could account for the removal of such lesions and their replacement by normal nucleotides. The sensitivity of E. coli PolA1, a mutant lacking this DNA polymerase in extracts, to ultraviolet light (59) could be due to the absence of one or both of these activities.

We also report a special, but related, type of repair synthesis which occurs through a mechanism of nick translation of the type shown by Kelly et al. (8). Although nicked DNA bearing 5'-hydroxyl end groups cannot be covalently joined by polyadenylate ligase alone, it can be joined in a reaction mixture containing E. coli DNA polymerase, the four deoxynucleoside 5'-triphosphates, and ligase. Our results suggest that the joining process involves the removal of nucleotides from the 5' terminus by the 5' → 3'-hydrolytic activity, and their replacement by DNA polymerase. Since the polymerase can hydrolyze 5'-hydroxyl-terminated polymers (32), it will excise the 5' terminus, simultaneously exposing a 5'-phosphoryl group. Wu and Kaiser (60) found that 5'-phosphoryl- and 5'-hydroxyl-terminated λ DNA were equally infectious in a transfection assay with helper phage. If covalently closed circles are a prerequisite for replication or lysogeny (or both), then a mechanism for the conversion of 5'-hydroxyl-terminated λ DNA to closed circular duplexes must exist. The combined actions of the 5'-hydrolytic activity and the polymerizing activity of E. coli polymerase provide such a mechanism. Our results show that extracts of wild type E. coli can catalyze the joining of 5'-hydroxyl-terminated circles of λ DNA, and that DNA polymerase is an essential component of this reaction.

The role of exonuclease and DNA polymerases in recombination is more difficult to evaluate. In the case of phage T4, it has been shown that hydrogen-bonded recombinant molecules produced in cells infected with phage defective in the synthesis of ligase and polymerase can be subsequently covalently joined by polymerase and ligase in vivo (61, 62). This result suggests that the recombination process in phage T4 may involve the repair of gaps. Recombination in phage λ also appears to involve limited synthesis of DNA (63). λ-Exonuclease is an essential component in the recombination system of λ phage (41) as is the β-protein (64). Carter and Radding (42) have reported that the λ-exonuclease cannot initiate hydrolysis at single strand breaks even in the presence of the β-protein whose function has not yet been established. The finding that the exonuclease is unable to hydrolyze DNA containing nicks or gaps suggests that an early step in recombination in vivo might be the introduction of double strand breaks or the exposure of single strand regions by strand separation rather than by hydrolysis.

REFERENCES

1. thomas, c. a., progr. nucleic acid res., 5, 315 (1966).
2. melson, m., in r. a. dalen (editor), heredity from mendel, university of wisconsin press, madison, wisconsin, 1967, p. 81.
5. abbe, w., and linn, s., annu. rev. biochem., 38, 467 (1969).