On the Mechanism of the Polynucleotide Joining Reaction

Baldomero M. Olivera,† Zach W. Hall,‡ Yasuhiro Anraku, Janice R. Chien and I. R. Lehman

Department of Biochemistry, Stanford University School of Medicine, Stanford, California

Polynucleotide-joining enzymes (ligases) have been identified in uninfected and phage-infected *Escherichia coli* (Gellert, 1967; Olivera and Lehman, 1967a; Zimmerman et al., 1967; Gefter et al., 1967; Weiss and Richardson, 1967a; Cozzarelli et al., 1967). Both types of enzyme catalyze the synthesis of a phosphodiester bond between the 3′ hydroxyl and 5′ phosphoryl termini of DNA chains which have been properly aligned in a double-helical structure. Such enzymes are of interest because of their possible involvement in genetic recombination, 'dark' repair of ultraviolet-induced lesions, and in DNA synthesis. It has already been demonstrated that the polynucleotide ligase induced by phage T4 is essential for normal T4 DNA replication (Fareed and Richardson, 1967).

An interesting difference between the *E. coli* and T4-induced enzymes is in their cofactor requirement. Whereas the phage-induced ligase utilizes ATP, which is cleaved in the course of the reaction to yield 5′-AMP and inorganic pyrophosphate (Weiss and Richardson,1967a), the *E. coli* polynucleotide-joining enzyme specifically requires DPN which is split to form 5′-AMP and nicotinamide mononucleotide (Olivera and Lehman, 1967b; Zimmerman et al., 1967; Fig. 1). In both cases, the first step in the overall reaction consists of the transfer of an adenylate group from the cofactor to the enzyme to form a covalently linked enzyme-AMP intermediate (Little et al., 1967; Weiss and Richardson, 1967b; Becker et al., 1967).

† Present address: Department of Biochemistry, University of the Philippines, College of Medicine, Manila, Philippines.

‡ Present address: Department of Neurobiology, Harvard Medical School, Boston, Massachusetts.

In this paper we shall first review our studies on the formation of enzyme-AMP in the polynucleotide joining reaction and then go on to consider our more recent experimental attempts to determine the fate of the enzyme-AMP. These studies have led to the formulation of the sequence of reactions shown in Fig. 2. Once enzyme-AMP is formed by a reaction of enzyme with DPN, there is a further transfer of the AMP to the 5′ phosphoryl terminus of a DNA chain to generate a new pyrophosphate bond linking the AMP and DNA. In the final step, we presume that the DNA phosphate of the pyrophosphate is attacked by the 3′ hydroxyl group of the neighboring DNA chain displacing the activating AMP and forming the phosphodiester bond.

Stoichiometry of Polynucleotide-Joining Reaction

For each equivalent of phosphodiester bond synthesized in the joining reaction, approximately one equivalent of DPN was consumed and nearly equivalent amounts of AMP and NMN were produced (Table 1). This finding provides the basis for the equation shown in Fig. 1.

Exchange Reaction Between DPN and NMN

In the absence of polynucleotide, the *E. coli* joining enzyme catalyzes an exchange reaction between NMN and DPN. No such exchange is observed between AMP and DPN. Thus, when DPN labeled with ^{32}P in both phosphates was incubated with joining enzyme in the presence of a large excess of nonradioactive NMN and AMP, and the reaction mixture was subjected to paper electrophoresis, ^{32}P appeared in NMN but was

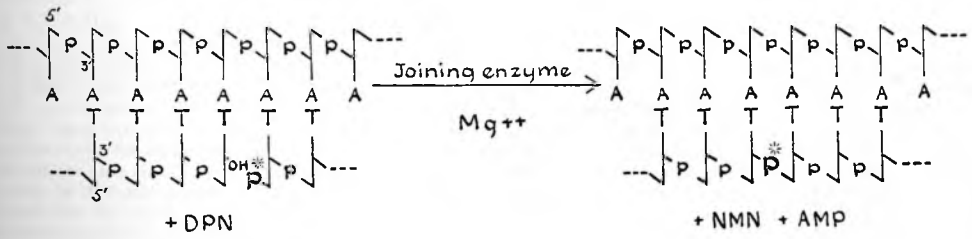

Figure 1. The reaction catalyzed by the polynucleotide-joining enzyme from *E. coli*. The reaction shown depicts the joining of short poly dT segments (about 100 residues) hydrogen-bonded to a long poly dA chain (about 3000 residues) (Olivera and Lehman, 1967a).

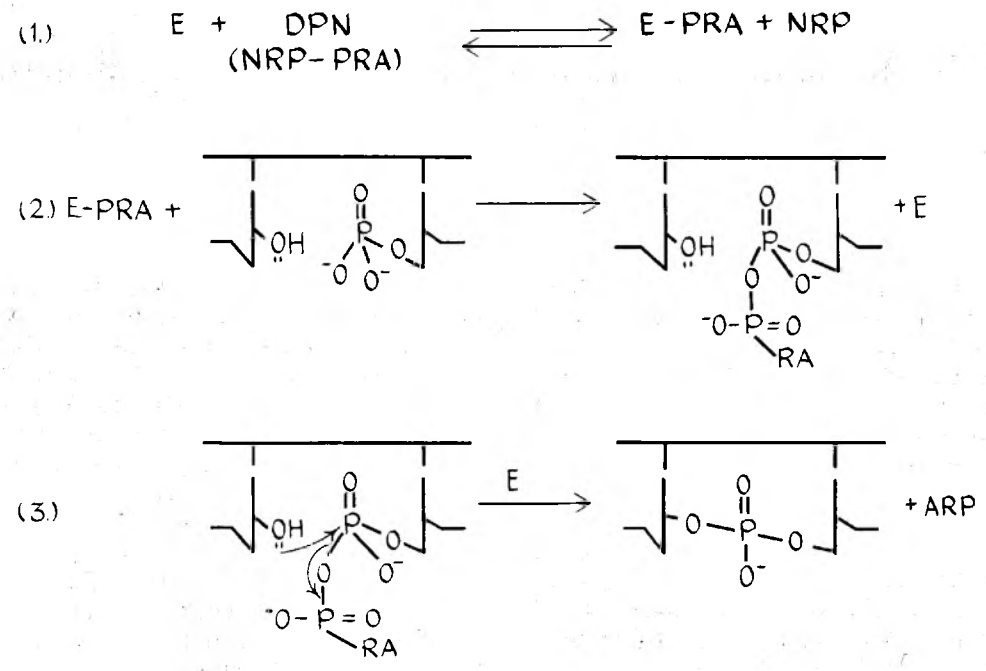

FIGURE 2. Postulated mechanism of the reaction catalyzed by the *E. coli* joining enzyme. DPN is written as NRP-PRA to emphasize the pyrophosphate bond between the nicotinamide mononucleotide (NRP) and adenylic acid (PRA) moieties of the DPN molecule. The designation of enzyme-AMP as E-PRA is not meant to imply that linkage of the AMP to the enzyme is necessarily through the phosphate group.

totally absent from the area of the electropherogram occupied by AMP. In the experiment shown in Fig. 3, approximately 30% of the ^{32}P of the DPN was recovered as NMN, indicating that 60% exchange had occurred.

These results imply that an enzyme-AMP intermediate is formed in the course of the polynucleotide-joining reaction.

Isolation of Enzyme-Adenylate

The enzyme-AMP predicted by the DPN-NMN exchange reaction could be demonstrated directly by gel electrophoresis. Joining enzyme was incubated with ^{32}P-labeled DPN in the absence of DNA under standard conditions and the reaction mixture subjected to disc gel electrophoresis (Jovin et al., 1964). When the gel was stained with amido black, two major and several minor protein bands appeared; the ^{32}P coincided with only one of the two major protein bands (Fig. 4).

Enzyme-AMP could also be isolated by density gradient sedimentation. When polynucleotide-joining enzyme was incubated with ^{32}P-labeled DPN in the absence of DNA, and the reaction

TABLE 1. STOICHIOMETRY OF THE JOINING REACTION

Reaction mixtures	DPN ($\mu\mu$moles)	AMP ($\mu\mu$moles)	NMN ($\mu\mu$moles)	Phosphodiester bonds synthesized ($\mu\mu$moles)
Control-dA:dT	30.8	<0.1	0.8	—
Complete	11.8	19.8	16.0	16.4
Δ	−19.0	+19.8	+15.2	+16.4

A reaction mixture (0.15 ml) containing 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 7.5 μg bovine plasma albumin, 0.26 μM (in termini) 5′ ^{32}P-poly dT (1.4×10^9 count/min/μmole) 0.21 μM ^{32}P-DPN (10^8 count/min/μmole), labeled in both phosphate groups (isolated from ^{32}P-labeled *E. coli* as described by Olivera and Lehman, 1967b, 0.26 mM poly dA (in nucleotide residues) and 1.2 units of joining enzyme was incubated for 120 min at 30°C, then heated at 100°C for 2 min. Two control reaction mixtures were set up, one lacking joining enzyme and the second lacking the dA:dT substrate. Phosphodiester bond synthesis was measured by treating aliquots of the reaction mixtures with phosphatase and measuring acid-precipitable ^{32}P as described previously (Olivera and Lehman, 1968). DPN and AMP were determined by paper electrophoresis as described in the legend to Fig. 3. Under these conditions, the poly dT substrate remained at the origin; the AMP and DPN were well separated and their concentrations could be determined accurately; however, NMN was not adequately resolved from the ^{32}P-poly dT. It was separated from the other components of the reaction mixture by electrophoresis at pH 3.4 in 0.02 M citrate buffer. The two control reactions (lacking dA:dT or enzyme) gave substantially the same results.

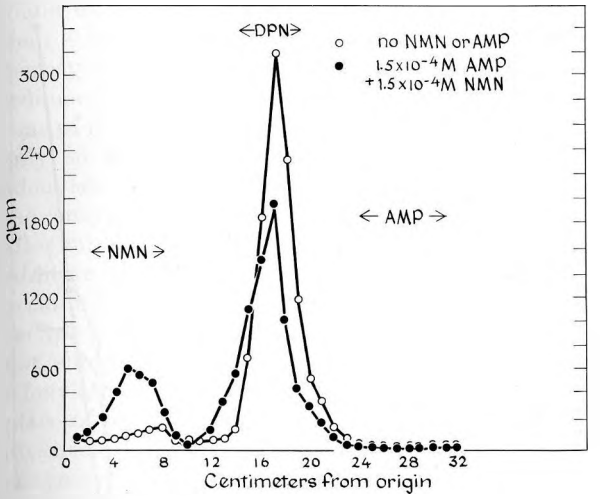

FIGURE 3. Exchange reaction between NMN and DPN. Two reaction mixtures (0.1 ml) were prepared containing 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 1.45 μM ^{32}P-DPN (2×10^8 count/min/μmole) labeled in both phosphate groups and 0.5 unit of joining enzyme; one reaction mixture was made 0.15 mM in AMP and 0.15 mM in NMN. Both reactions were incubated at 30°C for 20 min; an additional 1.8 units of enzyme were added to each and the mixtures were incubated again for 40 min at 30°. The reaction mixtures were heated at 100° for 2 min, then subjected to paper electrophoresis (5000 volts for 60 min) in 0.015 M citrate buffer, pH 5.5, with AMP, DPN and NMN as markers. The paper was cut into strips (1 × 4 cm) and their radioactivity determined. The small peak of radioactivity migrating slightly ahead of NMN in the control mixture is an impurity in the ^{32}P-DPN preparation.

mixture analyzed (after dialysis) by sucrose density gradient sedimentation, a single peak of acid-insoluble radioactivity appeared which cosedimented almost exactly with joining enzyme activity (Fig. 5).

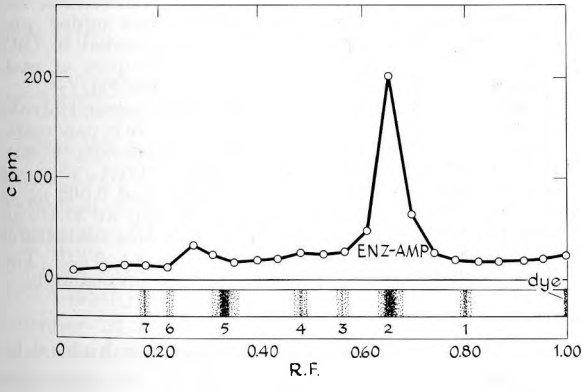

FIGURE 4. Disc gel electrophoresis of enzyme-AMP. A reaction mixture (1.0 ml) containing 0.3 μM ^{32}P-DPN (3×10^8 count/min/μmole) labeled in both phosphate groups, 10 mM Tris-HCl, pH 8.6, 3 mM $MgCl_2$ and 30 units of enzyme was incubated for 10 min at 30°C, then subjected to polyacrylamide gel electrophoresis according to Jovin et al. (1964). After staining with amido black, the gel was cut into 2 mm slices and radioactivity of the slices determined.

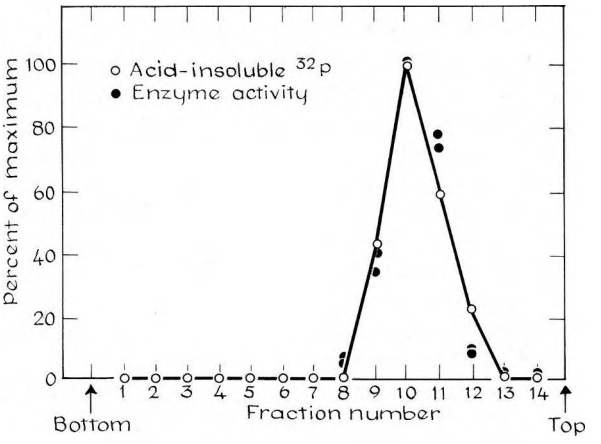

FIGURE 5. Demonstration of enzyme-AMP by sucrose density gradient sedimentation. The reaction mixture (2.0 ml) contained 10 mM Tris-HCl, pH 8.0, 7 mM $MgCl_2$, 1 mM EDTA, 50 μg/ml of bovine plasma albumin, 0.5 μM ^{3}H-DPN (1.8×10^8 count/min/μmole), and 20 units of joining enzyme. After incubation for 30 min at 30°C, 40 μmoles of EDTA, pH 8.7, and 0.2 mg of bovine plasma albumin were added, and the mixture dialyzed against 1 liter of 0.5 M $(NH_4)_2SO_4$, 10 mM Tris-HCl, pH 8.0 (3 changes). The dialyzed solution which contained 1400 count/min/ml was centrifuged in a 5–20% sucrose gradient containing 0.5 M $(NH_4)_2SO_4$, 10 mM Tris-HCl, pH 8.0 and 0.1 mg/ml albumin, at 38,000 rpm for 26 hr at 10°, using the SW39 rotor of the Spinco model L centrifuge. At the end of the run, 20-drop fractions were collected; 0.1 ml-aliquots of each fraction were heated at 100°C for 2 min and acid-precipitable ^{32}P was determined. The remainder of each fraction was used to assay joining enzyme activity (Olivera and Lehman, 1967a).

The linkage of AMP to the polynucleotide-joining enzyme was remarkably stable to treatment with both acid or alkali (Table 2). AMP was released only after exposure to 0.3 N HCl for 1 to 3 hr at 37° or after boiling in 1 N NaOH for 15 min.

TABLE 2. STABILITY OF ENZYME-AMP

Conditions	% Enzyme-AMP remaining
Control (no treatment)	100
0.3 N HCl, 60 min, 0°C	100
0.3 N HCl, 60 min, 37°C	50
0.3 N HCl, 180 min, 37°C	8
0.1 N NaOH, 60 min, 30°C	100
1.0 N NaOH, 60 min, 30°C	106
1.0 N NaOH, 15 min, 100°C	<0.1

Enzyme-AMP was prepared by incubating 16.3 $\mu\mu$moles of ^{3}H-DPN (2.0×10^9 count/min/μmole) prepared from ^{3}H-ATP and NMN using DPN-pyrophosphorylase (Kornberg, 1950), with 5.3 units of joining enzyme in a series of reaction mixtures (0.08 ml), each containing 10 mM Tris-HCl, pH 8.1, 5 mM $MgCl_2$ and 50% glycerol, for 20 min at 30°C. The reactions were terminated by the addition of 0.2 M EDTA (pH 7.5). 1 N NaOH or 0.3 N HCl were added and the reaction mixtures treated as indicated. They were then chilled to 0°C and neutralized. Acid-precipitable ^{3}H was determined as described previously (Olivera and Lehman, 1968). A value of 100% represents 0.33 $\mu\mu$mole of enzyme-AMP.

Evidence that Enzyme-Adenylate is an Intermediate in the Joining Reaction

When enzyme-AMP labeled with ^3H in the adenine moiety (synthesized with ^3H-adenine-labeled DPN) was treated with NMN, essentially all of the AMP was released into an acid-soluble form (Fig. 6). Addition of ϕX RFII (the covalently closed duplex circular form of ϕX174 DNA bearing one or more single-strand breaks [Burton and Sinsheimer, 1965]) at 20° or 30°C resulted in an equally prompt conversion of the ^3H-AMP to acid-solubility. Release of AMP from enzyme-AMP by ϕX RFII required a divalent cation (Mg^{++}) and was prevented by the addition of EDTA. A relatively slow liberation of ^3H-AMP in the presence of ϕX RFII occurred even at 0°C.

Isolation of DNA-Adenylate

The slow release of AMP from enzyme-AMP promoted by ϕX RFII at 0° suggested that, under such conditions, it might be possible to demonstrate a second intermediate compound in the joining reaction, possibly one in which a DNA terminus (3′ hydroxyl or 5′ phosphoryl) is activated by attachment of either the enzyme or the AMP group of the enzyme-AMP. In fact, a DNA-adenylate intermediate did appear under these conditions.

Joining enzyme, ^3H-adenine-labeled DPN, and phage λDNA containing multiple single-strand scissions were incubated briefly at 0°. The reaction

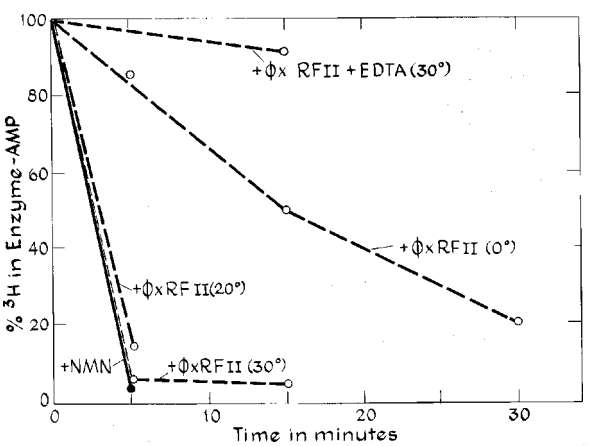

Figure 6. Release of AMP from enzyme-AMP by NMN and ϕX RFII. The reaction mixtures (0.05 ml) contained enzyme-AMP (25 count/min prepared from ^3H-DPN with a specific activity of 8.3×10^8 count/min/μmole), 10 mM Tris-HCl, pH 8.0, 7 mM $MgCl_2$, 1 mM EDTA and either 86 μM ϕX174 RFII (in nucleotide residues) or NMN (2 mM). After incubation under the conditions specified, the reactions were terminated by the addition of EDTA to a final concentration of 0.02 M; they were then heated at 100°C for 3 min and acid-precipitable ^3H was determined. A total of 500 counts over background were recorded for the enzyme-AMP sample prior to treatment.

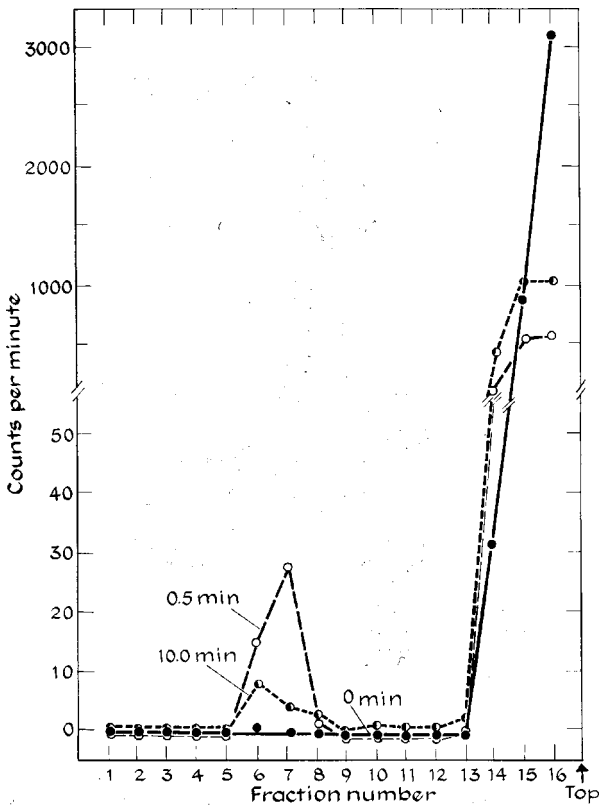

Figure 7. Demonstration of DNA-adenylate by CsCl density gradient centrifugation. Reaction mixtures (0.15 ml) containing 25 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 2.5 mM EDTA, 9 μg of bovine plasma albumin, 134 units of the polynucleotide joining enzyme and 0.8 μM ^3H-DPN (1.8×10^9 count/min/μmole) were incubated for 5 min at 30°C. Phage λ DNA (80 μg in 0.2 ml) containing multiple single-strand breaks (prepared as described below) was added, the reaction mixtures thoroughly stirred and incubated at 0° for the indicated times. The reactions were terminated by the addition of 0.03 ml of 0.66 M glycine buffer, pH 10.2 containing 0.33 M EDTA. For the '0 minute' sample, the glycine-EDTA was added just prior to the DNA. The samples were subjected to CsCl density gradient centrifugation and an aliquot of each fraction was used to determine acid-insoluble ^3H.

Phage λ DNA containing multiple single-strand breaks was prepared by treatment of the DNA with pancreatic DNase. A reaction mixture (0.6 ml) containing 17 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1.7 mM EDTA, 50 μg of bovine plasma albumin, 240 μg of λDNA and 0.002 μg of pancreatic DNase was incubated for 10 min at 37°C; an additional 0.003 μg of pancreatic DNase was added and the mixture incubated for 5 min more at 37°C. The DNase-treated DNA preparation was used immediately.

was then terminated and the mixture subjected to CsCl density gradient centrifugation. As shown in Fig. 7, a small peak of acid-precipitable ^3H appeared at the buoyant density characteristic of λ DNA. In the case of the sample incubated for 10 min, this peak amounted to approximately 0.6% of the ^3H found at the top of the gradient, where enzyme-AMP would be expected to band. In a separate run, joining enzyme activity was

found exclusively at the top of the gradient; less than 0.01% could be detected at the buoyant density of λ DNA. The level of radioactivity which sedimented with λ DNA increased with decreasing time of incubation at 0°. Thus, after incubation for only 30 seconds, the amount of ^3H in the DNA-adenylate peak increased to 4% of that present in the enzyme-AMP. Termination of the reaction after mixing ^3H-DPN and enzyme, but before the addition of DNA ('0 min' sample), prevented the accumulation of ^3H in the position of the gradient occupied by λ DNA. Similarly, in an experiment not shown here, there was no detectable DNA-adenylate when denatured λ DNA was used in place of native λ DNA. In both cases, despite the absence of radioactivity at the density of λ DNA, enzyme-AMP was formed, as judged by the appearance of high levels of acid-insoluble ^3H at the top of the density gradient. When heat-inactivated joining enzyme was used, no acid-insoluble ^3H could be observed at any position in the gradient.

Other attempts to detect an "activated" DNA in the joining reaction which have not succeeded are noteworthy because they provide additional insights into the reaction mechanism. In these experiments, single-stranded DNA or double-stranded structures containing single-stranded gaps were used in the expectation that the activated chain terminus would be insusceptible to attack by the apposing terminus (3′ hydroxyl or 5′ phosphoryl) because of its physical inaccessibility and would therefore accumulate. However, there was no evidence for the transfer of AMP or enzyme from enzyme-AMP to the DNA under any of the conditions examined, and it would appear that the enzyme is incapable of binding DNA structures of this type.

Evidence that DNA-Adenylate is an Intermediate in the Joining Reaction

The DNA-adenylate isolated after CsCl density gradient centrifugation displayed the properties expected of an intermediate in the joining reaction. Thus, the ^3H-AMP was quantitatively converted into an acid-soluble form upon treatment of the DNA-adenylate with joining enzyme in the absence of DPN (Table 3). The liberated ^3H was identified chromatographically as 5′-AMP; after treatment with *E. coli* alkaline phosphatase (Garen and Levinthal, 1960), it chromatographed with adenosine. Under conditions where there was a quantitative removal of AMP from native DNA-adenylate, no AMP was released by treatment of heat-denatured DNA-adenylate with joining enzyme. Although approximately one-third of the ^3H was made acid-soluble, chromatographic examination showed it to be associated with oligonucleotide material, suggesting that it resulted from the action of a contaminating nuclease.

Table 3. Release of AMP from DNA-Adenylate in the Absence of DPN

Units of enzyme	'Native' DNA-adenylate	Heat-denatured DNA-adenylate
	% of ^3H made acid-soluble	
6.0	100	35*
0.2	60	—
0.06	32	2

* The acid-soluble ^3H formed in this experiment was not free AMP (see text).

Reaction mixtures (0.1 ml) contained 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA and 5 μg of bovine plasma albumin, the indicated amounts of joining enzyme and either native DNA-adenylate (2 μg of DNA, 20 count/min), or DNA-adenylate which had been denatured by heating at 100°C for 2 min. After incubation at 37°C for 30 min, the reaction mixtures were heated at 100°C for 2 min and acid-soluble ^3H was determined. A minimum of 400 counts over background were recorded for the sample in which all of the AMP was retained in the DNA-adenylate.

Site of Linkage of AMP to DNA

The two plausible sites in DNA to which AMP may be bound are at the 5′ phosphoryl and 3′ hydroxyl termini. *E. coli* exonuclease I should be capable of distinguishing between these two possibilities. This enzyme attacks single-stranded DNA sequentially from the 3′ hydroxyl end, producing 5′ mononucleotides, but leaves the 5′-terminal dinucleotide intact (Lehman and Nussbaum, 1964). Thus, if the AMP were linked to the 5′ phosphoryl terminus of the chain, digestion by exonuclease I should yield a trinucleotide in which AMP is linked to the terminal dinucleotide through a pyrophosphate bond (Fig. 8a). On the other hand, if the AMP were in phosphodiester linkage at the 3′ terminus, it should be released as free AMP (Fig. 8b).

To serve as a model compound in these experiments, DNA with ^3H-labeled riboadenylate at its 3′ termini was prepared (Berg et al., 1963; Richardson and Kornberg, 1964). This DNA and the isolated DNA-adenylate intermediate were heat-denatured, then treated with exonuclease I and alkaline phosphatase, and the products chromatographed. In the case of the DNA with 3′-terminal AMP, the only product formed was adenosine (Fig. 9); AMP must therefore have been released by the action of exonuclease I. On the other hand, treatment of the isolated intermediate with exonuclease I and alkaline phosphatase yielded products which formed a broad radioactive peak on the chromatogram at the position expected of a mixture of trinucleotides (Fig. 9).

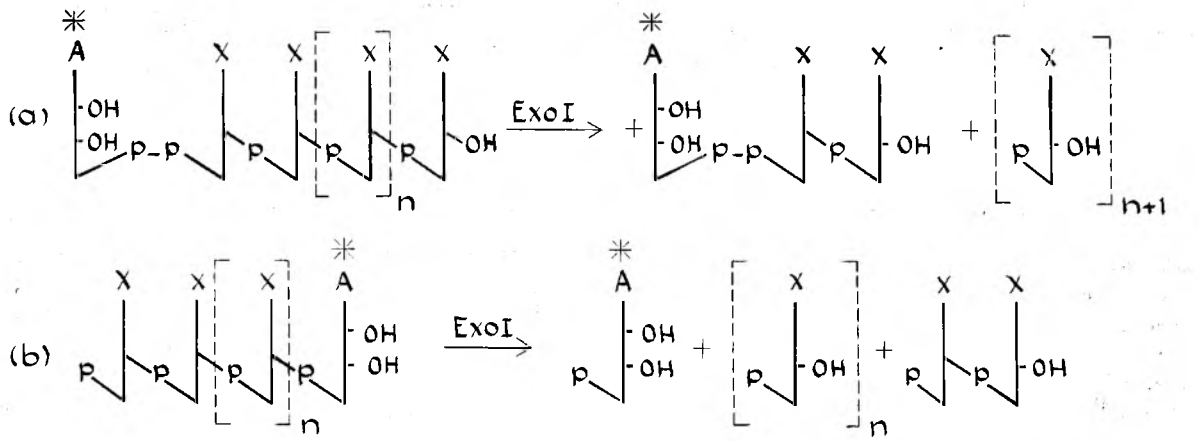

FIGURE 8. Action of exonuclease I on polynucleotide chains with (a) AMP in pyrophosphate linkage at the 5′ terminus and (b) AMP in phosphodiester linkage at the 3′ terminus.

Digestion of synthetic poly dT/adenylate[1] (see below) with these enzymes produced a similar peak of radioactivity in the trinucleotide region of the chromatogram. These data indicate that the AMP is linked at the 5′ end of the isolated DNA-adenylate. Moreover, the finding that the products of exonuclease I digestion migrated to the position on the chromatogram occupied by a trinucleoside triphosphate even after phosphatase treatment is consistent with the presence of an internal pyrophosphate group linking the AMP to the 5′ phosphoryl terminus of the DNA.

Activity of Synthetic Poly dT-Adenylate as a Substrate for the Joining Enzyme

To determine directly whether the joining reaction involves formation of a pyrophosphate linkage between AMP and the 5′ phosphoryl terminus of the polynucleotide chain, the presumptive intermediate was synthesized and tested as a substrate for the joining enzyme.

Poly dT-adenylate was prepared by condensing ^{3}H-AMP with ^{32}pTpTpT using the morpholidate method of Moffatt and Khorana (1961), and then adding additional deoxythymidylate residues to the 3′ hydroxyl end of the d(pT)$_3$ moiety by using the ^{3}H-Ap^{32}pTpTpT as an initiator in a reaction catalyzed by calf thymus deoxynucleotidyl transferase (Yoneda and Bollum,1965). The average chain length of the product was 100 deoxythymidylate residues; the ^{32}P was acid-precipitable and was insusceptible to alkaline phosphatase except after heating for 15 min at 100° in 1 N HCl. As noted above, treatment of the poly dT-adenylate with *E. coli* exonuclease I and alkaline phosphatase yielded products which migrated as a mixture of trinucleotides. Digestion with exonuclease I and venom phosphodiesterase (Sinsheimer and Koerner, 1952) resulted in the quantitative conversion of the ^{32}P to a form which cochromatographed with 5′-dTMP.

The double label in the poly dT-adenylate enabled us to measure simultaneously the release of ^{3}H-AMP from the polynucleotide and the incorporation of the ^{32}P-labeled terminal phosphate into phosphodiester linkage.

When the poly dT-adenylate was incubated with joining enzyme in the presence of poly dA (to permit its incorporation into a double-stranded structure) but in the absence of DPN, ^{3}H was released as an acid-soluble product, identified chromatographically as AMP. A nearly equivalent amount of ^{32}P was converted to a form which was insensitive to alkaline phosphatase after heating in 1 N HCl at 100° for 15 min (Table 4). Upon degradation of the product to 3′ mononucleotides by the combined action of micrococcal nuclease (Cunningham et al. 1956) and spleen phosphodiesterase (Hilmoe, 1960), all of the ^{32}P was found to be associated with 3′-dTMP, a result which is consistent with its incorporation into a phosphodiester bond. Thus, there is a stoichiometric correspondence between cleavage of the pyrophosphate bond linking poly dT and AMP on the one hand and phosphodiester bond formation on the other. Both the release of AMP and the

[1] The abbreviations are those recommended by J. Biol. Chem., *242*, 1 (1967). The following additional abbreviations are used: poly dT-adenylate, a homopolymer of deoxythymidylate residues bound in pyrophosphate linkage to AMP; pTpTpT or d(pT)$_3$, a trinucleotide composed of deoxythymidylate residues terminated by a 5′ phosphate; AppTpTpT, AMP bound in pyrophosphate linkage to the 5′ phosphate of pTpTpT; poly dA, a homopolymer of deoxyadenylate; dA:dT, poly dA hydrogen bonded to poly dT; ^{32}pTpTpT, d(pT)$_3$ in which the 5′-terminal phosphate is labeled with ^{32}P, ^{3}H-Ap^{32}pTpTpT, ^{3}H-AMP in pyrophosphate linkage to the 5′-phosphate of ^{32}pTpTpT.

incorporation of the ^{32}P into phosphodiester linkage required that poly dA be present.

The reaction mechanism for the polynucleotide joining enzyme as proposed in Fig. 2 predicts that adenylation of the enzyme (to form enzyme-AMP) would render it inactive in the reaction with poly dT-adenylate. Consistent with this prediction was the observation that preincubation of the enzyme with 0.3 mM DPN prior to the addition of poly dT-adenylate resulted in 95% inhibition of phosphodiester bond formation.

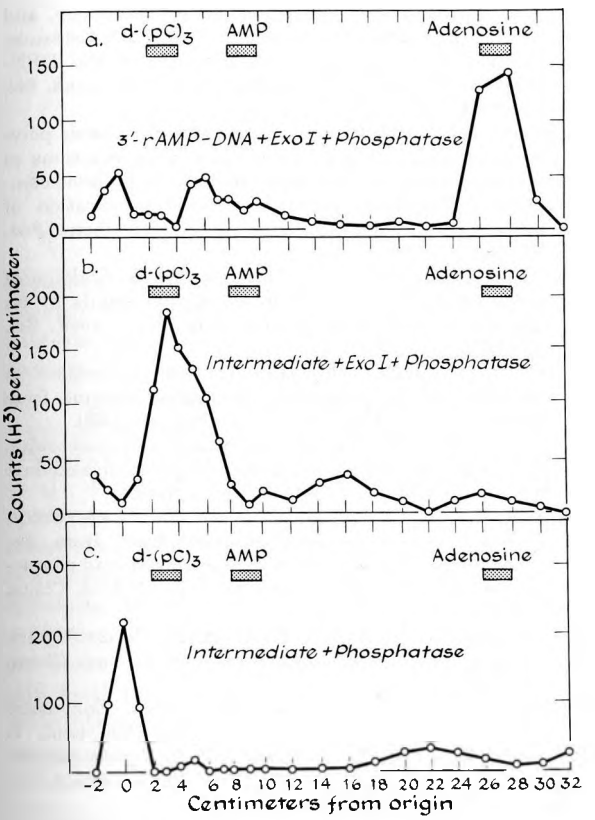

FIGURE 9. Paper electrophoresis of DNA-adenylate intermediate and DNA chains with riboadenylate at their 3′ termini (3′-rAMP-DNA) after treatment with exonuclease I (Exo I) and phosphatase. Three reaction mixtures (0.2 ml each) were prepared containing 80 mM glycine-NaOH, pH 9.5, 8 mM $MgCl_2$, and 2.5 mM β-mercaptoethanol. To one (reaction mixture *a*) were added single-stranded DNA chains with ^3H-AMP at their 3′ termini (60 μg DNA, 800 count/min), 17 units of exonuclease I (Lehman and Nussbaum, 1964) and 5 units of phosphatase. To the other two were added heat-denatured DNA-adenylate (19 μg DNA, 250 count/min) and either exonuclease I and phosphatase (*b*) or phosphatase alone (*c*). The reaction mixtures were incubated at 37°C for 30 min, then chromatographed for 18 hr in the 1-propanol ammonia-water system (Lehman et al. 1962). The paper was dried, the nucleotides identified, and the radioactivity determined. The values shown correspond to the total number of counts, corrected for background recorded for each strip.

TABLE 4. REACTIVITY OF POLY dT-ADENYLATE IN THE POLYNUCLEOTIDE-JOINING REACTION

Enzyme	poly dA	^{32}P in phosphodiester linkage ($\mu\mu$moles)	^3H-AMP released ($\mu\mu$moles)
−	+	5	7
+	+	38	46
+	−	2	3

The reaction mixtures contained, in a final volume of 0.1 ml, 10 mM Tris-HCl, pH 8.1, 3 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 10 μg bovine plasma albumin, 0.42 μM (in poly dT termini) ^{32}P-poly dT-^3H-adenylate (200 count/min of ^{32}P and 13 of ^3H per $\mu\mu$mole of termini), 48 μM (in dAMP residues) poly dA and 6 units of polynucleotide-joining enzyme, as indicated. After 1 hr at 30°C, 0.015 ml-aliquots were removed from each reaction and heated with 0.05 ml of 1 N HCl at 100°C for 15 min. 2 M Tris pH 8.1 (0.1 ml) and 0.12 unit of alkaline phosphatase (Garen and Levinthal, 1960) were then added and the reaction mixtures incubated at 37°C for 30 min. The fraction of ^{32}P adsorbable to Norit was measured as described previously (Olivera and Lehman, 1967a). To the remainder of the original incubation mixtures were added 0.01 ml 0.95 mM poly dA, 0.1 ml 0.1 M pyrophosphate, 0.1 ml 2.5 mg/ml calf thymus DNA, and 0.5 ml 3.5% perchloric acid-0.35% uranyl acetate. After 15 min at 0°C, the mixtures were centrifuged and the radioactivity of the supernatant fluid was determined.

CONCLUSIONS

The polynucleotide-joining enzyme from *E. coli* catalyzes phosphodiester bond formation between the 3′ hydroxyl and 5′ phosphoryl termini of DNA chains which have been properly aligned in a double-stranded DNA molecule, coupled to the cleavage of the pyrophosphate bond of DPN. Our attempts to understand the mechanism of this complex reaction have led to the identification of two readily distinguishable partial reactions. The first consists of a transfer of the AMP moiety of DPN to the joining enzyme to form a stable, covalently linked enzyme-AMP intermediate with the liberation of NMN. In the second reaction, the AMP is transferred from enzyme-AMP to the DNA to form a DNA-adenylate in which the AMP is attached to the 5′ phosphoryl terminus of the DNA by a pyrophosphate linkage. In the final step of the reaction sequence, we presume that the DNA phosphate in the pyrophosphate bond of the DNA-adenylate is attacked by the 3′ hydroxyl group of the neighboring DNA chain, displacing the activating AMP group and thus effecting the synthesis of the phosphodiester bond.

In the joining reaction, the energy of the pyrophosphate bond of DPN is conserved in the new pyrophosphate bond linking AMP and the 5′ phosphoryl terminus of the DNA. Interposed between DPN and DNA-adenylate is enzyme-AMP. Although the linkage of AMP to the enzyme has not yet been identified, it presumably is at the

energy level of a pyrophosphate bond. It would clearly be of interest to know the nature of the enzyme-AMP linkage.

Since the T4-induced ligase reaction also proceeds via a stable enzyme-AMP intermediate (formed by the reaction of ATP with enzyme), it seems likely that a DNA-adenylate intermediate is involved in this reaction as well.

ACKNOWLEDGMENTS

This work was made possible by a grant from the United States Public Health Service.

B. M. O. is a Postdoctoral Fellow of the Damon Runyon Memorial Fund for Cancer Research.

Z. W. H. is a Postdoctoral Fellow of the U.S. Public Health Service.

We are very grateful to Dr. John Moffatt of the Syntex Institute of Molecular Biology for his help in the synthesis of poly dT-adenylate.

REFERENCES

BECKER, A., G. LYN, M. GEFTER and J. HURWITZ. 1967. The enzymatic repair of DNA. II. Characterization of phage-induced sealase. Proc. Nat. Acad. Sci. *58:* 1996.

BERG, P., H. FANCHER and M. CHAMBERLIN. 1963. The synthesis of mixed polynucleotides containing ribo- and deoxyribo-nucleotides by purified preparations of DNA polymerase from *Escherichia coli*. pp. 467–483. *In* H. J. Vogel, V. Bryson, and J. O. Lampen [ed.]. Informational Macromolecules. Academic Press, New York.

BURTON, A., and R. L. SINSHEIMER. 1965. The process of infection with bacteriophage ϕX174. VII. Ultracentrifugal analysis of the replicative form. J. Mol. Biol. *14:* 327.

COZZARELLI, N., N. E. MELECHEN, T. M. JOVIN, and A. KORNBERG. 1967. Polynucleotide cellulose as a substrate for a polynucleotide ligase induced by phage T4. Biochem. Biophys. Res. Commun. *28:* 578.

CUNNINGHAM, L. B., B. W. CATLIN, and M. PRIVAT DE GARILHE, 1956. A deoxyribonuclease of *Micrococcus pyogenes*, J. Amer. Chem. Soc. *78:* 4642.

FAREED, G. C., and C. C. RICHARDSON. 1967. Enzymatic breakage and joining of deoxyribonucleic acid. II. The structural gene for polynucleotide ligase in bacteriophage T4. Proc. Nat. Acad. Sci. *58:* 665.

GAREN, A., and C. LEVINTHAL. 1960. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characteristization of alkaline phosphatase. Biochim. Biophys. Acta *38:* 470.

GEFTER, M. L., A. BECKER, and J. HURWITZ. 1967. The enzymatic repair of DNA. I. Formation of circular λ DNA. Proc. Nat. Acad. Sci. *58:* 240.

GELLERT, M. 1967. Formation of covalent circles of lambda DNA by *E. coli* extracts. Proc. Nat. Acad. Sci. *57:* 148.

HILMOE, R. J. 1960. Purification and properties of spleen phosphodiesterase. J. Biol. Chem. *235:* 2117.

JOVIN, T. M., A. CHRAMBACH, and M. A. NAUGHTON. 1964. An apparatus for preparative temperature-regulated polyacrylamide gel electrophoresis. Analyt. Biochem. *9:* 351.

KORNBERG, A. 1950. Reversible enzymatic synthesis of diphosphopyridine nucleotide and inorganic pyrophosphate. J. Biol. Chem. *182:* 779.

LEHMAN, I. R., and A. L. NUSSBAUM. 1964. The deoxyribonucleases of *Escherichia coli*. V. On the specificity of exonuclease I (phosphodiesterase). J. Biol. Chem. *239:* 2628.

LEHMAN, I. R., G. G. ROUSSOS and E. A. PRATT. 1962. The deoxyribonucleases of *Escherichia coli*. II. Purification and properties of a ribonucleic acid-inhibitable endonuclease. J. Biol. Chem. *237:* 819.

LITTLE, J. W., S. B. ZIMMERMAN, C. K. OSHINSKY, and M. GELLERT. 1967. Enzymatic joining of DNA strands. II. An enzyme-adenylate intermediate in the DPN-dependent DNA ligase reaction. Proc. Nat. Acad. Sci. *58:* 2004.

MOFFATT, J., and H. G. KHORANA. 1961. Nucleoside polyphosphates. X. The synthesis and some reactions of nucleoside-5′ phosphoromorpholidates and related compounds. Improved methods for the preparation of nucleoside-5′ polyphosphates. J. Amer. Chem. Soc. *83:* 649.

OLIVERA, B. M., and I. R. LEHMAN. 1967a. Linkage of polynucleotides through phosphodiester bonds by an enzyme from *Escherichia coli*. Proc. Nat. Acad. Sci. *57:* 1426.

——, ——. 1967b. Diphosphopyridine nucleotide: A cofactor for the polynucleotide-joining enzyme from *Escherichia coli*. Proc. Nat. Acad. Sci. *57:* 1700.

——, —— 1968. The enzymatic joining of polynucleotides. III. The polydeoxyadenylate:polydeoxythymidylate homopolymer pair. J. Mol. Biol., in press.

RICHARDSON, C. C., and A. KORNBERG. 1964. A deoxyribonucleic acid phosphatase-exonuclease from *Escherichia coli*. I. Purification of the enzyme and characterization of the phosphatase activity. J. Biol. Chem. *239:* 242.

SINSHEIMER, R. L., and J. F. KOERNER. 1952. A purification of venom phosphodiesterase. J. Biol. Chem. *198:* 293.

WEISS, B., and C. C. RICHARDSON. 1967a. Enzymatic breakage and joining of deoxyribonucleic acid. I. Repair of single-strand breaks in DNA by an enzyme system from *Escherichia coli* infected with T4 bacteriophage. Proc. Nat. Acad. Sci. *57:* 1021.

—— ——. 1967b. Enzymatic breakage and joining of deoxyribonucleic acid. III. An enzyme-adenylate intermediate in the polynucleotide ligase reaction. J. Biol. Chem. *242:* 4270.

YONEDA, M., and F. J. BOLLUM. 1965. Deoxynucleotide-polymerizing enzymes of calf thymus gland. I. Large scale purification of terminal and replicative deoxynucleotidyl transferases. J. Biol. Chem. *240:* 3385.

ZIMMERMAN, S. B., J. W. LITTLE, C. K. OSHINSKY, and M. GELLERT. 1967. Enzymatic joining of DNA strands: A novel reaction of diphosphopyridine nucleotide. Proc. Nat. Acad. Sci. *57:* 1841.