ENZYMATIC JOINING OF DNA STRANDS, II. AN ENZYME-ADENYLATE INTERMEDIATE IN THE DPN-DEPENDENT DNA LIGASE REACTION

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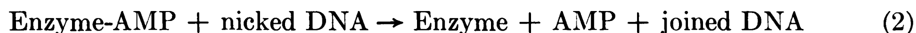
Recent work in this[1, 2] and other laboratories[3-6] has demonstrated the existence of enzymes which catalyze the covalent closure of single-strand breaks in duplex DNA. Interest in such enzymes derives in part from their probable biological importance; thus, the enzyme induced by infection with bacteriophage T4 has been shown to be necessary for multiplication of the phage.[7]

Characterization of purified enzymes from uninfected[2, 4, 5] and T4-infected[3] *Escherichia coli* has revealed a requirement in each case for a nucleotide cofactor. The enzyme from uninfected cells specifically requires DPN,[2, 5] which is broken down into AMP and NMN in the course of the reaction; the enzyme from T4-infected cells, by contrast, splits ATP into AMP and PP_i.[3] In both cases, 3'-5' phosphodiester bonds are formed by covalent joining of the two apposed DNA termini at a single-strand break.

This class of enzymes thus catalyzes a complex reaction which may be expected to proceed in several distinguishable stages. This report describes the first step of the reaction catalyzed by the DNA ligase of uninfected *E. coli*. Upon admixture with DPN in the *absence* of DNA, the enzyme forms a stable enzyme-adenylate complex, releasing NMN. The enzyme-adenylate complex, as isolated by gel filtration, can seal single-strand breaks in DNA in the absence of DPN. AMP is released in this reaction. The enzyme-adenylate complex may also be discharged with NMN to yield DPN by reversal of the reaction which formed the complex. As a consequence an exchange between DPN and NMN can be observed, as noted by Olivera and Lehman.[5]

These reactions are summarized in the following equations:

$$\text{DPN} + \text{Enzyme} \leftrightarrows \text{Enzyme-AMP} + \text{NMN} \quad (1)$$



$$\text{Enzyme-AMP} + \text{nicked DNA} \rightarrow \text{Enzyme} + \text{AMP} + \text{joined DNA} \quad (2)$$

$$\text{DPN} + \text{nicked DNA} \rightarrow \text{AMP} + \text{NMN} + \text{joined DNA} \quad (3)$$

Experimental Procedure.—Where not specified, techniques and materials are as described previously.[2]

Materials: Adenine-labeled H^3-AMP (2.7 mc/μmole) and H^3-ATP (4 mc/μmole) were products of Schwarz BioResearch, Inc. H^3-DPN labeled in the AMP moiety was synthesized from NMN and H^3-ATP with hog liver DPN-pyrophosphorylase.[8] H^3-NMN was formed from H^3-DPN (nicotinamide-labeled,[2] 2.7 mc/μmole) and PP_i by reversal of this enzymatic reaction. Labeled DPN and NMN were purified by DEAE-cellulose chromatography.[9] Nicotinamide riboside was formed by dephosphorylation of NMN with *E. coli* alkaline phosphatase and purified in the paper chromatography system described below.

Dithiothreitol and nicotinamide were products of Calbiochem. *p*-Hydroxymercuribenzoate was obtained from Sigma Chemical Co., and crystallized bovine plasma albumin from Armour Pharmaceutical Co. Sephadex G-25 (fine) was a product of Pharmacia Fine Chemicals, Inc.

DNA ligase in all cases was the fraction VI previously described[2] which had been brought to 1 mg/ml of crystallized bovine plasma albumin and dialyzed at 4° for 16–30 hr vs. 200 vol of 20% glycerol–0.02 M Tris-HCl buffer, pH 8.0. Where specified the dialysis medium included 2 mM $MgSO_4$. The specific activity of the enzyme was unchanged by these dialyses. Control samples of albumin (without fraction VI) dialyzed as above were inactive in either the exchange or the enzyme-adenylate system.

Methods: Assay of exchange reaction: The routine assay for exchange measured the incorporation of H^3 into DPN after incubation of enzyme with H^3-NMN and unlabeled DPN. The incubation mixtures (0.07 ml) contained H^3-NMN (2.9×10^{-7} M, 3.1×10^4 cpm), DPN (1.4×10^{-6} M), $MgSO_4$ (0.01 M), potassium phosphate buffer, pH 6.5 (0.05 M), and enzyme (0.01 ml of a dilution in 20% glycerol–0.02 M Tris-HCl buffer, pH 8.0). After 45 min at 37°, the tubes were heated 1 min at 100°, chilled, and marker nucleotides (0.01 ml of 8 mM DPN and 30 mM NMN) were added. The entire mixture was applied to paper (Schleicher and Schuell no. 589 orange ribbon) in four successive aliquots. The paper was developed for 16 hr by descending chromatography with solvent C of Preiss and Handler[10] modified slightly to contain 7 parts of 95% ethanol to 3 parts of 1 M ammonium acetate buffer, pH 4.8. R_f values in this system are: TPN, 0.05; DPN or desamino-DPN, 0.14; thionicotinamide-DPN, 0.19; 3-acetylpyridine-DPN, 0.22; NMN or AMP, 0.34; nicotinamide riboside, 0.69; and nicotinamide, 0.85. DPN and NMN spots were located by quenching of ultraviolet light, cut out, and counted in a toluene-base scintillator (Liquifluor, Pilot Chemicals). The recovery of added counts in the two spots was >70% in all cases. If enzyme was omitted, 7% of the H^3 was found in the DPN spot; this value has been subtracted from all exchange data reported below. The efficiency of counting H^3-NMN or H^3-DPN on paper was 14% relative to counting on a glass filter in the same scintillator. Kinetics of the exchange and proportionality to enzyme are indicated in Figure 1.

Measurement of enzyme-adenylate: Formation of enzyme-adenylate was measured by the conversion of radioactivity from the AMP moiety of DPN into an acid-insoluble form. After incubation of the DNA ligase with labeled DPN (H^3-NRPPRA* or P^{32}-NRPP*RA) in a total volume of 0.1 ml, the reaction was stopped by addition of 0.4 ml of a solution containing 2 mM EDTA and 1 mg/ml bovine plasma albumin. The sample was chilled and 0.5 ml of cold 10% trichloroacetic acid was added. After 10 min on ice, the mixture was centrifuged and the supernatant solution was discarded. The precipitate was resuspended in 1 ml of 10% trichloroacetic acid and again centrifuged. The pellet was dissolved in 0.5 ml of 0.02 M NH_4OH. Radioactivity was determined in Bray's scintillator solution.[11]

Alternatively, enzyme-adenylate was separated from unreacted DPN by the use of Sephadex G-25 columns. A typical separation is shown in Figure 2.

Release of AMP from enzyme-adenylate thus isolated was measured by the above assay; aliquots of the acid-soluble fractions were neutralized with Tris and their radioactivity was determined in Bray's solution.

Results.—An exchange reaction of NMN and DPN: Purified DNA ligase catalyzes an active exchange reaction between NMN and DPN. With high levels of enzyme, at least 70 per cent of the added H^3-NMN is recovered as H^3-DPN. Under the same conditions, the other nucleotide product of the over-all DNA-joining reaction, AMP, is not incorporated into DPN (Fig. 1). Attempts to demonstrate AMP incorporation into DPN at several other pH values and Mg^{++} concentrations were unsuccessful.

Requirements for obtaining NMN exchange into DPN are simply enzyme, NMN and DPN, and a divalent cation. The response to enzyme level is indicated in Figure 1. Responses to nucleotides and cations follow.

The apparent K_m for DPN was 7×10^{-7} M under the routine exchange assay conditions (phosphate buffer, pH 6.5, 0.01 M Mg^{++}, 3×10^{-7} M NMN; see *Methods* for details). Under conditions optimal for the over-all DNA-joining reaction, the apparent K_m for DPN in the exchange was decreased to 1×10^{-7} M; the rate of exchange was diminished fivefold under these latter conditions (Tris-

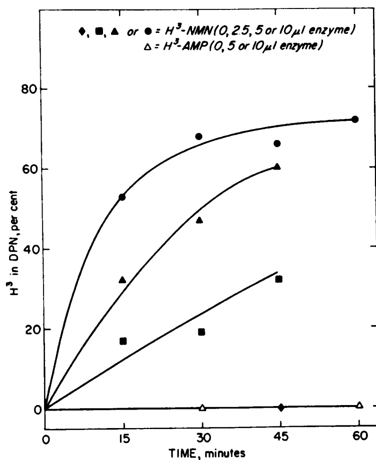


FIG. 1.—NMN-DPN exchange: Kinetics, proportionality to enzyme, and lack of H^3-AMP incorporation. H^3-NMN exchange was followed as in *Methods* for samples incubated at 37° for various times. Where indicated, H^3-AMP of the same concentration and specific radioactivity replaced the H^3-NMN. A mixture of this amount of H^3-AMP and of H^3-NMN yielded within 20% the same number of counts in DPN as did H^3-NMN alone (tested with 5 μl of enzyme at 30 or 45 min). Recovery of added H^3-AMP was >80%; radioactivity in the DPN region of the chromatogram from tubes containing H^3-AMP was <1% of the added H^3. Enzyme, 1200 units/ml.

HCl buffer, pH 8.0, and 2 mM $MgSO_4$ substituted in the routine exchange incubation mixture). A number of compounds related to DPN were tested for ability to replace DPN under exchange assay conditions (Table 1). Only the thionicotinamide analogue of DPN was clearly active. From the proposed mechanism, in a thionicotinamide-DPN-dependent exchange no label from H^3-NMN would be expected to be introduced into thionicotinamide-DPN; none was bound. If TPN or desamino-DPN supported an exchange with H^3-NMN, label would be expected to appear in TPN or in desamino-DPN, respectively; none was found. This specificity may be compared with that of the DNA-joining assay, in which only DPN and thionicotinamide-DPN (and to a much lesser extent 3-acetylpyridine-DPN) were active.[2]

Response to several divalent cations is indicated in Table 2. Mg^{++} and Mn^{++} are active, while Zn^{++} and Ca^{++} are not. This pattern is similar to that found in the over-all reaction. The optimal $MgSO_4$ concentration in the exchange under conditions more favorable to DNA-joining (Tris-HCl buffer, pH 8) was at least tenfold lower, and higher concentrations were inhibitory.

Apparent K_m values for NMN (determined over a concentration range from 3 to 35×10^{-7} M) were 6 and 9×10^{-7} M in the presence of 2×10^{-6} and 1×10^{-5} M DPN, respectively. At 5×10^{-7} M DPN, there was disproportionate inhibition of the reaction by high NMN concentrations.

Preincubation with *p*-hydroxymercuribenzoate (0.1 mM, 1 hr, 0°) completely inhibited both the exchange and the DNA-joining activity. Both activities were restored by a subsequent incubation with a sulfhydryl compound, dithiothreitol (0.01 M, 1 hr, 0°). The optimal pH for the exchange reaction in potassium phosphate buffers at 6 mM $MgSO_4$ was pH 6.5. The rate was *ca.* 50 per cent lower at pH 5.6 or pH 7.5; in Tris-HCl buffer, pH 8.0, the rate was 80 per cent lower.

Isolation of enzyme-adenylate: The exchange reaction between DPN and NMN indicates that the products of reaction between enzyme and DPN are free NMN and an enzyme-adenylate complex. Such a complex can be isolated directly by gel filtration through a Sephadex G-25 column (Fig. 2). In the experiment illustrated, two DPN preparations, one labeled with P^{32} in the AMP moiety and the other with H^3 in the NMN moiety, were mixed and incubated with the enzyme (in the absence of DNA). Upon gel filtration of the mixture, approximately half of the added P^{32}, but <0.5 per cent of the added H^3, eluted with the DNA ligase activity. Ab-

TABLE 1

EXCHANGE REACTION SPECIFICITY FOR DPN AND RELATED COMPOUNDS

Compound added	Per Cent of H^3 in DPN with Given Concentration of Compound 10^{-7} *M*	10^{-6} *M*	10^{-5} *M*	10^{-5} *M* + 10^{-5} *M* DPN
None		<5		
DPN	9	32	54	56
Thionicotinamide-DPN	<5	8	28	60
α-DPN	<5	<5	<5	58
3-Acetylpyridine-DPN	<5	<5	<5	51
TPN			<5*	42*
Desamino-DPN			<5	47
ADP-ribose			<5	67
DPNH			†	

The routine exchange assay (see *Methods*) was used with the indicated additions in place of the usual level of DPN.

* <2% of added H^3 found in TPN region of chromatogram.

† Test of DPNH was not meaningful due to significant DPNH-oxidizing activity demonstrable at the relatively high enzyme levels used in the exchange system.

TABLE 2

CATION SPECIFICITY IN THE EXCHANGE REACTION

Compound added	Per Cent of H^3 in DPN with Given Concentration of Compound 6×10^{-5} *M*	6×10^{-4} *M*	6×10^{-3} *M*	3×10^{-2} *M*
None		<5		
$MgSO_4$	12	42	65	61
$MgCl_2$	25	34	78	
$MnCl_2$	15	44	14*	
$ZnCl_2$	<5	<5	<5*	
$CaCl_2$	<5	<5	<5*	

The routine exchange assay (see *Methods*) was used with the indicated additions in place of the usual level of $MgSO_4$.

* Incipient precipitation in the incubation mixture.

TABLE 3

INCORPORATION OF THE AMP MOIETY OF DPN INTO DNA LIGASE

Conditions	Acid-precipitable radioactivity (μμmoles as AMP)
Enzyme boiled or omitted	<0.01
8 Units enzyme	0.36
20 Units enzyme	1.02
40 Units enzyme	2.31
P^{32}-DPN (0.10 μM) instead of H^3-DPN	1.04
Add 0.02 *M* EDTA	0.02
Add 10 μM NMN	0.46
Add 10 μM NMN after 10 min incubation; incubate 10 min more	0.42

The standard incubation mixture (0.1 ml) contained 0.02 *M* Tris-HCl buffer, pH 8.0, 2 mM $MgSO_4$, 10% glycerol, 0.5 mg/ml bovine plasma albumin, 0.10 μM H^3-DPN (adenine-labeled), and dialyzed DNA ligase (20 units, unless specified). P^{32}-DPN was labeled in the AMP moiety. After incubation for 10 min at 37°, mixtures were acid-precipitated as described in *Methods*.

sence of H^3 from this peak indicates that binding of DPN *per se* to the enzyme was negligible under the conditions of isolation.

The radioactivity found in the DNA ligase peak was >95 per cent acid-precipitable (none of the isotope in the peak of DPN and NMN was acid-precipitable). The acid-insolubility of the complex permitted its detection and assay by direct acid precipitation of incubation mixtures containing DNA ligase and DPN labeled in the AMP moiety with either H^3 or P^{32}. The reaction thus measured proceeded very rapidly (*ca.* two-thirds complete in 1 min at 0°); we therefore chose assay conditions under which the reaction had reached a limiting value.

The assay shows approximate proportionality with amount of enzyme added (Table 3). A divalent cation is required; Mn^{++} at low concentrations (0.2 mM)

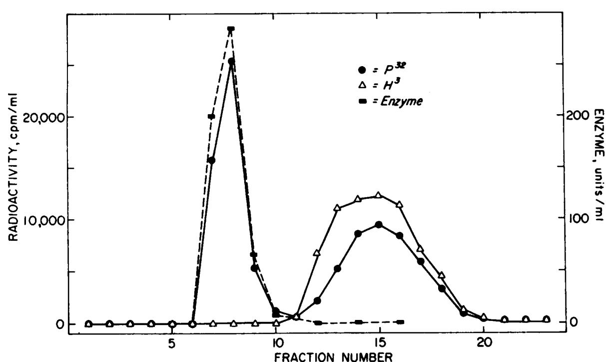


FIG. 2.—Gel filtration of AMP-enzyme specifically labeled from the AMP^{32} moiety of DPN. A mixture (30 μl, 151 $\mu\mu$moles) containing H^3-DPN (N*RPPRA) and P^{32}-DPN (NRPP*RA) at 480 and 680 cpm/$\mu\mu$mole, respectively, was added to 1.0 ml of the ligase preparation dialyzed, as described in *Methods*, to contain 2 m*M* $MgSO_4$. After 10 min at 37°, EDTA (0.08 ml of 0.1 *M*) was added, the mixture was chilled and an aliquot (0.8 ml) applied to a Sephadex G-25 column (0.9 cm^2 × 9 cm).

The column was eluted at 4° with 0.05 *M* Tris-HCl buffer, pH 8.0, at 0.5 ml/min; 0.7-ml fractions were collected. Samples were taken for counting and DNA ligase assay. The recovery from the column of H^3, P^{32}, and enzyme was 81, 103, and 63%, respectively.

The significant disparity in ratio of H^3 to P^{32} at the leading edge of the second peak is due to formation during the reaction of H^3-NMN, which is partially separated from DPN on such a column. In a control column with a mixture of H^3-NMN and P^{32}-DPN, the peaks of H^3 and of P^{32} appeared in fractions 13 and 15, respectively. In a second control column in which the reaction of the ligase with P^{32}- and H^3-DPN was prevented by prior addition of EDTA, the ratio of H^3 to P^{32} varied by <10% throughout the DPN peak.

can substitute for Mg^{++}, while Ca^{++} and Zn^{++} (0.2 or 2 mM) cannot. This pattern is the same as that shown by the DNA-joining assay and the exchange assay. The limiting value reached in the presence of 10 μM NMN was the same whether this was added before or after reaction with DPN; this implies that the limit represents an equilibrium value.

When fractions from DEAE-cellulose chromatography of a DNA ligase preparation were assayed for DNA-joining activity,[2] for exchange activity, and for the formation of enzyme-adenylate, a reasonably good correspondence between the three assays was found (Table 4), suggesting that all three activities are associated with the same protein species.

Release of the adenylate moiety from the enzyme-adenylate complex: Upon treatment of the complex with NMN or with DNA bearing single-strand breaks, the adenylate moiety becomes acid-soluble. The acid-soluble product of reaction with NMN is DPN, while AMP is released upon treatment with nicked DNA (Table 5). Thus, treatment of the complex with NMN leads to reversal of the reaction which formed the complex (see equation 1); incubation with nicked DNA, which is a substrate for the over-all DNA-joining reaction,[2] liberates the other nucleotide product of the over-all reaction, AMP (equation 2). The specificity with which

TABLE 4

CHROMATOGRAPHY OF DNA-JOINING, EXCHANGE, AND ENZYME-ADENYLATE-FORMING ACTIVITIES

DEAE-cellulose fraction	Relative Activity		
	DNA-joining	Exchange	Enzyme-AMP
47	5	2	3
50	27	35	49
53	70	83	132
56	(100)	(100)	(100)
59	80	73	52
65	31	21	12

Samples were taken through the peak of enzyme activity in the final DEAE-cellulose chromatography described previously,[2] dialyzed, and assayed as in *Methods*.

TABLE 5

NUCLEOTIDE PRODUCTS OF THE FORWARD AND REVERSE REACTIONS OF ISOLATED ENZYME-ADENYLATE

Tube	Addition	Per Cent of Radioactivity Enzyme-AMP*	AMP	DPN
1.	None	100 (100)	<1	<1
2.	Nicked DNA, 23 μg/ml	23 (21)	77	<1
3.	NMN, 0.1 mM	1 (1)	1	98

Incubation mixtures (0.15 ml) contained enzyme-adenylate (0.10 ml of enzyme-AMP^{32} from the peak ligase tube of a preparation similar to that shown in Fig. 2; 2200 cpm), 2 mM $MgSO_4$, and the additions indicated. The nicked λ DNA had *ca.* 30 breaks per strand, judged by assay with polynucleotide kinase.[12] After 1 min (tube 2) or 10 min (tubes 1 and 3) at 37°, 0.01 ml of 0.2 *M* EDTA was added and the tubes were heated 1 min at 100°. Aliquots (0.025 ml) were taken for the determination of acid-soluble radioactivity as described in *Methods*. To separate aliquots (0.075 ml) was added 0.01 ml of a marker solution of DPN and AMP (8 mM each), and the samples were analyzed by paper chromatography as in the exchange assay (see *Methods*). Radioactivity remaining at the origin is listed as enzyme-AMP. Results are expressed as per cent of radioactivity recovered from the chromatography (75, 99, and 92% of P^{32} added in tubes 1, 2, and 3, respectively). Less than 5% of the recovered radioactivity was located outside of the origin or marker nucleotide spots.

* Figures in *parentheses* are per cent acid-insoluble P^{32} at the end of the incubation period.

TABLE 6

RELEASE OF AMP MOIETY FROM ENZYME-ADENYLATE

Expt.	Additions	Concentration	Per cent radioactivity made acid-soluble
1.	None	—	2
	Intact DNA	38 μg/ml	16
	Nicked DNA	1.5 "	19
	" "	3 "	33
	" "	19 "	67
	" "	38 "	68
	Intact or nicked DNA, denatured	38 "	4
	tRNA	200 "	3
2.	None	—	<5
	NMN	10^{-5} *M*	69
	Sodium phosphate, pH 8.0	0.05 *M*	<5
	ATP, AMP, or PP_i	1 mM	<5
3.	Treatment		
	10 min at 100°, pH 8.0		<5
	" , 0.1 *N* NaOH		25
	" , 0.1 *N* HCl		>95

Expt. 1: Intact λ DNA was incubated 5 min at 60°, then quickly chilled to 0°.[15] Nicked λ DNA bore 15 breaks per strand, as determined by polynucleotide kinase assay.[12] Where indicated, DNA samples (0.36 ml, in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) were denatured by the addition of 0.04 ml 1 *N* NaOH; after 10 min at 20°, 0.04 ml of 1 *M* Tris-HCl, pH 8.0, then 0.04 ml 1 *N* HCl, were added. Incubation mixtures (0.10 ml) contained 0.03 *M* Tris-HCl, pH 8.0, 2.5 mM $MgSO_4$, 0.5 mM EDTA, 0.5 mg/ml bovine plasma albumin, enzyme-AMP^{32} (500 cpm), and further additions as indicated. Tubes were preincubated 1 min at 37°; reactions were then initiated by addition of nucleic acid (at 37°); after 1 min more at 37°, 0.15 ml of a solution containing 3 mg/ml of bovine plasma albumin and 4 mM EDTA was added and the tubes were chilled. Acid-soluble radioactivity was determined as in *Methods*. Results are expressed as per cent of added radioactivity recovered in acid-soluble form.

Expt. 2: Incubations were as in expt. 1, except that enzyme-AMP-H^3 (630 cpm) was used, and incubation was for 10 min at 37°.

Expt. 3: As expt. 2, except that incubations were for 10 min at 100°; acidic and alkaline samples were brought to pH 8.0 before acid-precipitation.

NMN and nicked DNA discharge the enzyme-adenylate complex is indicated by the relative inability of a number of other nucleotides and nucleic acids to liberate radioactivity in acid-soluble form (Table 6).

The enzyme-AMP linkage is stable to quite drastic chemical treatments, such as boiling at neutral or alkaline pH (Table 6). Boiling in 0.1 *M* HCl releases AMP, identified as such by paper chromatography.

Joining of DNA strands by enzyme-adenylate: As also depicted in equation (2), the isolated enzyme-adenylate complex can seal single-strand breaks in nicked DNA in the absence of DPN. When the complex (20 units) was allowed to react with a sample of λ DNA (4 μg) whose single-strand molecular weight[13] had been reduced about fourfold by pancreatic DNase treatment, the mean sedimentation constant

in alkali of the DNA ($s_{20,w}$) increased from 16.0S to 27.8S. This indicates a threefold increase in mean single-strand length, suggesting that a majority of the single-strand breaks were sealed by treatment with enzyme-adenylate. When a duplicate DNA sample was incubated with the same number of units of untreated DNA ligase (again in the absence of DPN), its sedimentation constant increased to 18.2S.[14]

Joining of DNA molecules by the enzyme-adenylate complex can also be demonstrated in the routine DNA-joining assay. When this assay is carried out *in the presence of saturating levels of DPN*, the isolated enzyme-adenylate complex exhibits roughly linear kinetics of DNA joining for at least 20 minutes of incubation (as does untreated ligase), and has a specific activity 30–60 per cent of that of untreated enzyme (the specific activity of untreated DNA ligase was similarly reduced upon gel filtration). When the joining assay is performed *in the absence of DPN*, the amount of DNA joined by either the isolated enzyme-adenylate complex or the untreated enzyme reaches a stable value within ten minutes of incubation; the limit reached per unit of activity is five times higher with the complex than with untreated enzyme.

Discussion.—Our results indicate that the DNA ligase reaction proceeds through a stable enzyme-adenylate intermediate.

Evidence that an enzyme-adenylate complex is formed comes first from the fact that the enzyme supports an exchange reaction between DPN and NMN, but not between DPN and AMP. This suggests that in the reaction of enzyme with DPN, the AMP moiety is bound to the enzyme while the NMN is released, and that the reaction is freely reversible:

$$\text{Enzyme} + \text{DPN} \rightleftharpoons \text{Enzyme-AMP} + \text{NMN}.$$

Moreover, enzyme-adenylate can be isolated directly by gel filtration under conditions in which free DPN does not bind to the enzyme and is completely separated from it.

The isolated enzyme-adenylate is functionally active; it can join DNA strands in the absence of added DPN, as demonstrated both by activity in the routine ligase assay and by analytical centrifugation of nicked DNA treated with the complex. The reaction which forms enzyme-adenylate may also be reversed by NMN to yield DPN. The specificity of the exchange reaction for DPN and cations is similar to that of the over-all ligase reaction. The fact that the complex is discharged efficiently only by DNA with single-strand breaks and by NMN, but by none of the other nucleotides or nucleic acids tested, is consistent with the characteristics of the over-all reaction. It is reasonable to suppose, therefore, that the enzyme-adenylate complex represents the first stable intermediate in the DNA ligase reaction.

The mechanism of the final steps of the DNA ligase reaction is presently undefined. Possibly a second intermediate compound is formed by the reaction of enzyme-adenylate with a DNA terminus, followed by attack of the other (juxtaposed) DNA terminus with concomitant phosphodiester formation and displacement of the activating group. At the present state of knowledge, there is no basis for predicting whether the activating group attached to DNA might be the enzyme or the adenylyl residue of the enzyme-adenylate complex, nor which DNA ter-

minus is the initial site of activation. Several types of DNA-dependent exchanges are predicted by these various formulations. These and other means of defining the subsequent steps of the ligase reaction are currently under investigation.

The ATP-dependent ligase from T4-infected cells[3] seals internal breaks in DNA, supports an ATP-PP_i exchange reaction, and forms an enzyme-adenylate complex.[16] Indeed, the similarities between the two enzymes suggest that the steps of the ligase reaction subsequent to enzyme-adenylate formation will be found to be identical in the reactions of the two enzymes.

Summary.—The DPN-dependent DNA ligase purified from uninfected *Escherichia coli* reacts with DPN in the absence of DNA to form a stable enzyme-adenylate complex. As a result of this reaction, the enzyme catalyzes an exchange between DPN and NMN, but not between DPN and AMP. The enzyme-adenylate complex can be isolated by gel filtration. It is discharged specifically by NMN to yield DPN, and by DNA containing single-strand breaks to give AMP. It can also seal single-strand breaks in DNA in the absence of DPN. The enzyme-adenylate complex is therefore an intermediate in the over-all DNA-joining reaction.

We are indebted to Drs. Weiss and Richardson and Drs. Olivera and Lehman for communicating results prior to publication.

The following terminology is used: DNA ligase was previously called "DNA-joining enzyme." [2] "Nicked DNA" [3] is duplex DNA containing single-strand breaks (produced by a limited exposure to pancreatic DNase). "Joined DNA" is nicked DNA in which single-strand breaks have been repaired by DNA ligase. Abbreviations are: DPN (or NRPPRA) and DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, oxidized triphosphopyridine nucleotide; NMN, nicotinamide mononucleotide; ADP-ribose, adenosine diphosphoribose; AMP, adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate; PP_i, inorganic pyrophosphate; tRNA, transfer RNA; EDTA, sodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; DEAE-cellulose, diethylaminoethyl cellulose.

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[14] This small endogenous activity is presumably due to naturally occurring enzyme-adenylate, present in untreated enzyme. Consistent with this idea, the endogenous activity per unit of enzyme was several-fold diminished by incubation with NMN followed by gel filtration (activity measured in the routine ligase assay).

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[16] Weiss, B., and C. C. Richardson, personal communication.