Enzymatic Joining of Polynucleotides

VI. ACTIVITY OF A SYNTHETIC ADENYLYLATED POLYDEOXYNUCLEOTIDE IN THE REACTION*

Zach W. Hall‡ and I. R. Lehman

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

SUMMARY

Polydeoxythymidylate in which the 5′-terminal phosphate is linked by a pyrophosphate bond to adenosine 5′-phosphate (poly dT-adenylate) serves as an intermediate in the reactions catalyzed by the *Escherichia coli* and T4-induced polynucleotide joining enzymes in the presence of polydeoxyadenylate. Adenosine 5′-phosphate release and phosphodiester bond formation occur stoichiometrically and with the same time course. The reaction is inhibited by DPN; hence it may be inferred that enzyme-adenylate is inactive in the reaction. The K_m of the *E. coli* joining enzyme for poly dT-adenylate is 1 to 3 μM; the K_m for poly dT is 0.003 μM. The specific activity of the *E. coli* enzyme measured at saturating substrate concentrations is 2 to 5 times higher with poly dT-adenylate than with poly dT.

Polynucleotide joining enzymes (ligases) have been isolated from uninfected and phage-infected *Escherichia coli* (1–6). This class of enzymes catalyzes the formation of phosphodiester bonds between the contiguous 3′-hydroxyl and 5′-phosphoryl ends of polynucleotide chains which form part of a double stranded structure. The enzymes from the two sources utilize different cofactors: the bacterial enzyme, DPN (7, 8), and the phage-induced enzyme, ATP (5). However, in both cases, the first step in the reaction is the same and consists of the transfer of an adenylate moiety from cofactor to the enzyme to form the first intermediate in the reaction, enzyme-AMP. This intermediate can be readily isolated and will promote the joining of DNA chains in the absence of cofactor (7, 9, 10).

The next step in the reaction catalyzed by the *E. coli* enzyme has recently been shown to be the transfer of the adenylate group from the enzyme to the 5′ terminus of the polynucleotide

chain to form the second intermediate in the reaction, DNA-adenylate. In the final step, the adenylate is released from the DNA and the phosphodiester bond between the polynucleotide chains is formed (11).

As part of an investigation of the mechanism of the reaction catalyzed by the *E. coli* joining enzyme, we have synthesized an adenylylated polydeoxynucleotide, poly dT-adenylate,[1] by a combination of chemical and enzymatic means, and shown it to have the properties expected of an intermediate (11).

In this paper we describe in detail the synthesis and characterization of poly dT-adenylate and provide further evidence for its participation in the joining reaction. We have also found that poly dT-adenylate serves as an intermediate in the T4 phage-induced polynucleotide ligase reaction.

EXPERIMENTAL PROCEDURE

Materials

DPN was purchased from Sigma and ³H-AMP from Schwarz Bioresearch. γ-³²P-ATP was prepared according to Glynn and Chappell (12). ³²P-poly dT and poly dA were prepared as described previously (2). d(pT)₃ was the generous gift of Dr. John Moffatt.

E. coli polynucleotide joining enzyme (Fraction V) was prepared as described previously (2) and further purified by DEAE-Sephadex chromatography.[2] T4-induced polynucleotide ligase (phosphocellulose fraction) was prepared according to Cozzarelli *et al.* (6). Polynucleotide kinase was purified as described by Richardson (13). Semen phosphatase was prepared according to Wittenberg and Kornberg (14). Exonuclease I was purified by the method of Lehman and Nussbaum (15). *E. coli* alkaline phosphatase, micrococcal nuclease, and spleen and snake venom phosphodiesterases were purchased from Worthington. Calf

[1] The abbreviations used are: d(pT)₃, a trinucleotide composed of deoxythymidylate residues terminated by a 5′-phosphate; ³²pTpTpT, d(pT)₃, in which the 5′-terminal phosphate is labeled with ³²P; ³H-Ap³²pTpTpT, ³H-AMP bound in pyrophosphate linkage to the 5′-phosphate of ³²pTpTpT; ³²P-poly dT, poly dT in which the 5′-terminal phosphate is labeled with ³²P; poly dT-adenylate, AMP bound in pyrophosphate linkage to the 5′-phosphate of poly dT.
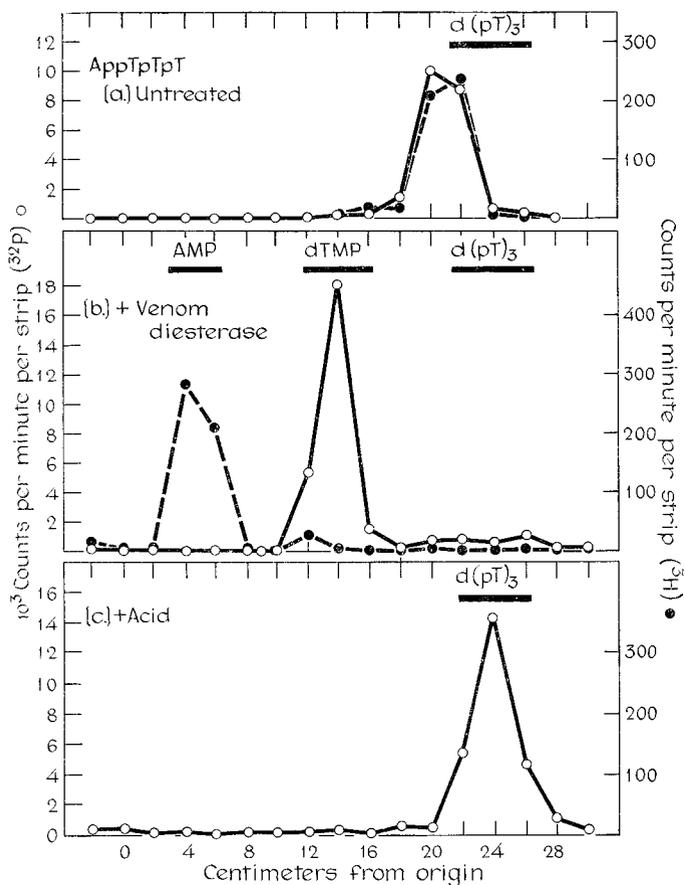
[2] Y. Anraku, N. Anraku, and I. R. Lehman, to be published.

FIG. 1. Paper electrophoresis of ^{3}H-Ap^{32}pTpTpT after treatment with snake venom phosphodiesterase or 1 N HCl. ^{3}H-Ap^{32}-pTpTpT (65 $\mu\mu$moles) was (a) left untreated; (b) treated with 2 μg of venom phosphodiesterase in 80 mM Tris-HCl (pH 8.0), 8 mM MgCl$_2$ for 45 min at 37°; or (c) heated at 100° for 20 min in 1 N HCl and then subjected to paper electrophoresis as described under "Methods." The ^{3}H-labeled products of the acid treatment were not identified.

thymus terminal deoxynucleotidyl transferase was the generous gift of Dr. F. N. Hayes.

The general scheme of preparation of ^{32}P-poly dT-^{3}H-adenylate was to synthesize ^{3}H-Ap^{32}pTpTpT chemically (16) and use it as an initiator in a nucleotide addition reaction catalyzed by terminal deoxynucleotidyl transferase (17).[3] ^{3}H-AMP-morpholidate was prepared as described by Moffatt and Khorana (16) except that the reaction was scaled down to accommodate 5 μmoles of ^{3}H-AMP as starting material. The yield of ^{3}H-AMP-morpholidate was 2.5 μmoles. The product was purified by chromatography in Solvent I and converted to the triethylammonium salt by passage over a Dowex 50 column in the triethylammonium form.

^{32}pTpTpT was prepared by treating d(pT)$_3$ with semen phosphatase to remove the terminal phosphate, and replacing it with ^{32}P, with γ-^{32}P-ATP and polynucleotide kinase as described by Richardson (13).

In the condensation reaction, 0.6 μmole of ^{32}pTpTpT and 0.75 μmole of ^{3}H-AMP-morpholidate were evaporated to dry-

ness and dissolved in pyridine, first several times separately, and then several times together. The extent of the reaction was determined by measuring alkaline phosphatase-insusceptible ^{32}P. After 48 hours at room temperature, when 20% of the ^{32}P was resistant to alkaline phosphatase, the reaction mixture was chromatographed in Solvent I and the single ^{32}P-containing band was eluted. This fraction, containing both ^{32}pTpTpT and ^{3}H-Ap^{32} pTpTpT, was treated with alkaline phosphatase to remove the ^{32}P from the unreacted ^{32}pTpTpT and applied to a column of DEAE-Sephadex (A-25, bicarbonate form, 1 × 4 cm). The column was washed with 10 ml of 0.1 M triethylammonium bicarbonate, pH 7.5, and eluted with 200 ml of a linear gradient from 0.1 to 0.5 M triethylammonium bicarbonate, pH 7.5. The product, ^{3}H-Ap^{32}pTpTpT, had the following properties. (a) The ^{32}P was not susceptible to the action of alkaline phosphatase, but was rendered greater than 95% susceptible by treatment with snake venom phosphodiesterase or by heating in 1 N HCl at 100° for 30 min. (b) Electrophoresis at pH 3.5 revealed a single radioactive peak, containing both ^{3}H and ^{32}P (Fig. 1). After incubation with venom phosphodiesterase, the ^{3}H migrated on paper electrophoresis with AMP and the ^{32}P with dTMP. After treatment with acid, the ^{32}P had the same electrophoretic mobility as authentic d(pT)$_3$. (c) The ratio of ^{3}H to ^{32}P in the product was 0.7. Since all of the ^{32}P was alkaline phosphatase-resistant, this ratio may reflect some cleavage of the AMP during the preparation, releasing the ^{3}H-adenine but leaving the terminal phosphate blocked. The presence of such termini in the final product would not affect the assay for joining since all the ^{32}P became sensitive to alkaline phosphatase by heating in acid.

The ^{3}H-Ap^{32}pTpTpT was used as an initiator in the terminal deoxynucleotidyl transferase reaction according to Hayes et al. (18). A reaction mixture containing 0.12 M potassium phosphate (pH 6.9), 1 mM β-mercaptoethanol, 8 mM MgCl$_2$, 1 mM dTTP, 10 μM ^{3}H-Ap^{32}pTpTpT, and 260 units of enzyme in a volume of 1 ml was incubated for 2 hours at 15° and then at 30° until the reaction reached a plateau value, as judged by measurement of acid-precipitable radioactivity. Yields ranged from 30 to 50% in several preparations. The product was purified by filtration through a column of Sephadex G-100 (1 × 26 cm) and fractions corresponding to the excluded column volume were pooled and concentrated by dialysis against polyethylene glycol.

The product, poly dT-adenylate, contained both ^{32}P (200 cpm per $\mu\mu$mole) and ^{3}H (15 cpm per $\mu\mu$mole) and was acid-precipitable. The average chain length was estimated from the ratio of absorbance at 260 mμ to ^{32}P and varied from 100 to 230 in different preparations. The ^{32}P in the product was insusceptible to the action of alkaline phosphatase except after heating in 1 N HCl for 30 min at 100° or after treatment with exonuclease I and venom phosphodiesterase. On chromatography in Solvent I, the product remained at the origin; after digestion with exonuclease I and alkaline phosphatase, the ^{32}P migrated to the trinucleotide region of the chromatogram. However, after digestion with exonuclease I and venom phosphodiesterase, the ^{32}P cochromatographed with 5'-dTMP (Fig. 2).

Methods

Phosphodiester Bond Formation—This assay measured ^{32}P which was insusceptible to alkaline phosphatase after treatment with acid, utilizing the fact that the pyrophosphate bond linking AMP to the polynucleotide terminus is relatively acid-labile as compared to a phosphodiester bond. Incubation mixtures (0.1
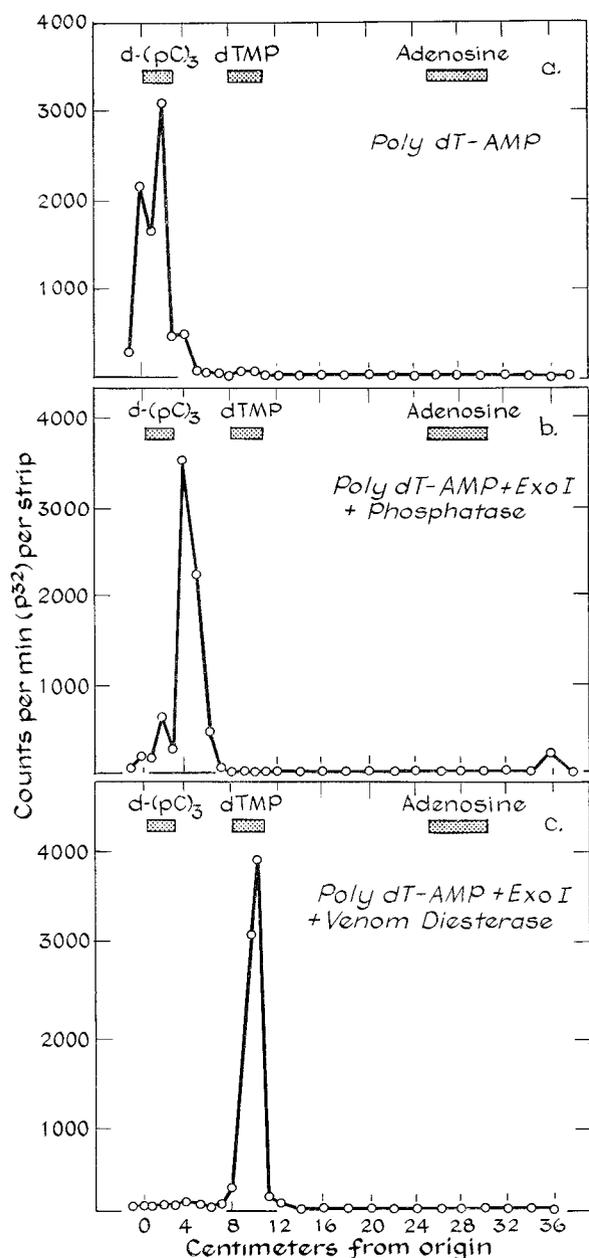
FIG. 2. Chromatography of poly dT-adenylate after enzymatic degradation. Reaction mixtures (0.02 ml) containing 80 mM glycine-NaOH (pH 9.5), 8 mM MgCl$_2$, 2.5 mM β-mercaptoethanol, 21 $\mu\mu$moles of poly dT-adenylate (in termini) and (a) no enzyme, (b) 1.2 units of exonuclease I and 0.1 unit of alkaline phosphatase, and (c) 1.2 units of exonuclease I were incubated at 37° for 30 min. The pH of Reaction c was adjusted to approximately 7.5 with 1 N HCl, 0.3 μg of snake venom phosphodiesterase was added, and the reaction mixture was incubated at 37° for 30 min. All three reactions were then chromatographed in Solvent I for 18 hours as described under "Methods."

ml) contained 20 mM Tris-HCl (pH 8.0), 2 mM MgCl$_2$, 1 mM EDTA, 100 μg per ml of bovine plasma albumin, 20% glycerol, 0.1 to 1.5 μM (in termini) poly dT-adenylate, and an amount of poly dA equivalent in nucleotide residues to the poly dT-adenylate. After incubation for 30 min at 30°, 0.03 ml of 4 N HCl was added and the mixture was heated at 100° for 30 min. Tris base, 2 M (0.1 ml), and 1 unit of alkaline phosphatase (19) were then added and the mixture was incubated at 85° for

30 min. Norit-adsorbable ^{32}P was measured as described previously (2).

AMP Release—At the end of an incubation, carried out as described above, 0.01 ml of 0.95 mM (in nucleotides) poly dA, 0.1 ml of calf thymus DNA (2.5 mg per ml), and 0.1 ml of 14% perchloric acid-1.4% uranyl acetate were added. After 15 min at 0°, the precipitate was removed by centrifugation, and 0.2 ml of the supernatant solution was added to a mixture containing 0.15 ml of 2 M Tris, 1.65 ml of water, and 20 ml of a dioxane base scintillator fluid. Radioactivity was then determined in a Nuclear-Chicago model 724 liquid scintillation counter.

Other Methods—Electrophoresis was performed at 5 kv in 0.02 M sodium citrate, pH 3.5, on S and S Orange Ribbon No. 589 paper. Descending paper chromatography was carried out on Whatman No. 3MM paper with either Solvent I, 1-propanol-concentrated ammonium hydroxide-water (6:3:1) (20); or Solvent II, 95% ethanol-1 M ammonium acetate, pH 4.8 (7:3) (21) A Zeiss PMQ II spectrophotometer was used for all optical measurements.

RESULTS

Activity of poly dT-Adenylate as Intermediate in E. coli Joining Enzyme Reaction—When poly dT-adenylate containing ^{3}H-labeled adenylate and 5'-^{32}P-labeled poly dT was incubated with poly dA and the *E. coli* joining enzyme in the absence of DPN, the ^{3}H was released as an acid-soluble product, and the ^{32}P was converted to a form which was insensitive to alkaline phosphatase after heating in 1 N HCl at 100° for 20 min. The release of ^{3}H and the conversion of ^{32}P to an acid-stable form were stoichiometric and occurred with the same time course (Fig. 3).

The identity of the acid-soluble ^{3}H-labeled product was established by chromatography of an incubation mixture in Solvent I. After incubation of the joining enzyme with poly dT-ade-
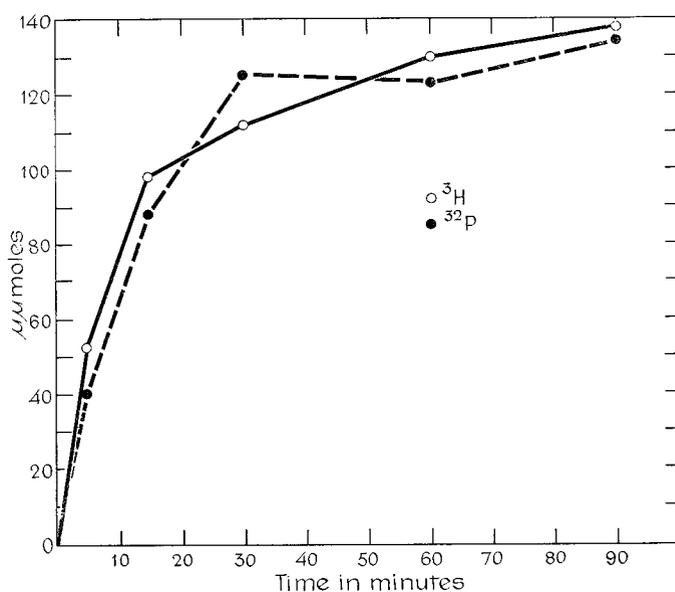

FIG. 3. Time course of phosphodiester bond formation and AMP release from poly dT-adenylate with *E. coli* joining enzyme. The standard reaction mixture (see "Methods") was scaled up to a volume of 0.3 ml. At the indicated times 0.03-ml aliquots were removed to assay AMP release and two 0.005 ml-aliquots were taken for the determination of phosphodiester bond formation (in duplicate). Both assays were performed as described under "Methods."
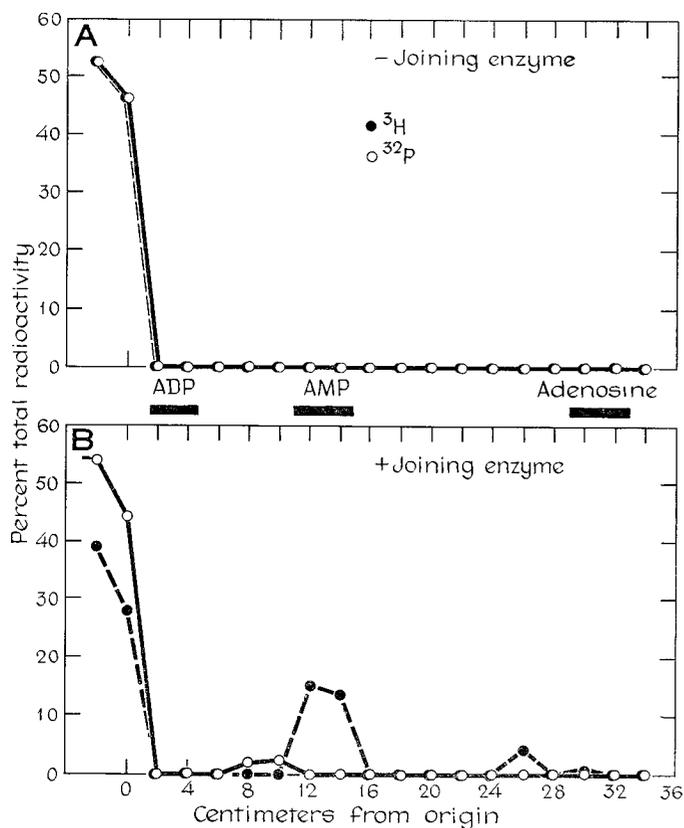
FIG. 4. Chromatography of products of the reaction of *E. coli* joining enzyme with poly dT-adenylate. Incubations were carried out in a volume of 0.1 ml as described under "Methods," with 142 $\mu\mu$moles of poly dT-adenylate (in termini) and 2 units of *E. coli* joining enzyme; the incubation mixtures were chromatographed in Solvent II as described under "Methods."

nylate under the conditions described above, 85% of the ³H released appeared at a position characteristic of AMP and 10% chromatographed just behind adenosine; the ADP spot was devoid of radioactivity. All of the ^{32}P remained at the origin, indicating that it was still associated with polynucleotide material (Fig. 4). Thus, in the joining reaction, most of the AMP is released intact from the poly dT.

The fate of the ^{32}P was examined by treating the product of the joining reaction with micrococcal nuclease (22) and spleen phosphodiesterase (23) which in combination degrade polynucleotides completely to 3'-mononucleotides. When poly dT-adenylate was incubated with these enzymes and the digestion products were chromatographed, a radioactive peak was obtained with an R_F characteristic of oligonucleotide material (Fig. 5*A*). This finding presumably reflects the inability of either enzyme to cleave the internal pyrophosphate bond. On the other hand, enzymatic digestion and then chromatography of the product of a reaction of poly dT-adenylate with the joining enzyme yielded a single radioactive peak corresponding to 3'-dTMP (Fig. 5*B*). Thus, the pyrophosphate bond of the poly dT-adenylate was cleaved in the joining reaction and the ^{32}P was incorporated into a phosphodiester bond.

Inhibition of Joining of poly dT-Adenylate by DPN—If poly dT-adenylate is an intermediate in the joining of poly dT chains by the polynucleotide joining enzyme, then adenylylation of the enzyme (to form enzyme-AMP) should render it inactive in the

reaction with poly dT-adenylate. Accordingly, it was found that preliminary incubation of the enzyme with increasing amounts of DPN resulted in progressive inhibition of the joining reaction with poly dT-adenylate (Table I). At the highest concentration of DPN used (0.3 mM), nearly quantitative inhibition was observed. These levels of DPN had essentially no effect on
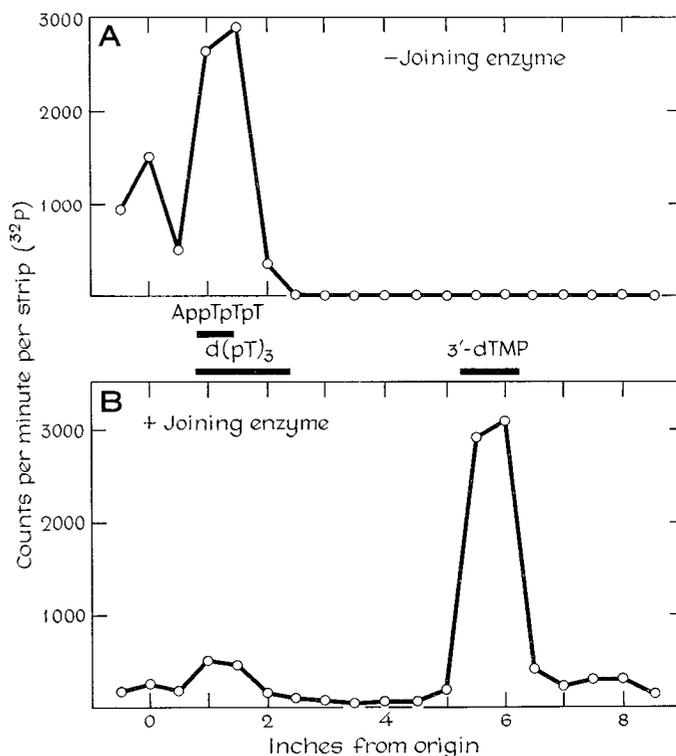

FIG. 5. Chromatography of products of the reaction of *E. coli* joining enzyme with poly dT-adenylate after digestion with micrococcal nuclease and spleen phosphodiesterase. Incubations were carried out in a volume of 0.1 ml as described under "Methods," with 26 $\mu\mu$moles of poly dT-adenylate (in termini) and 1.5 units of *E. coli* joining enzyme. CaCl₂, 0.003 ml, 0.1 M, and 8 units of micrococcal nuclease were added to each reaction mixture and the incubation was continued at 37° for an additional 60 min. The pH was adjusted to 6.5 with 0.1 N HCl and 0.005 ml of 0.5 M KF and 0.03 unit of spleen phosphodiesterase were added. After 90 min at 37°, the reaction mixtures were chromatographed in Solvent I for 32 hours, as described under "Methods."

TABLE I

Inhibition of joining of poly dT-adenylate by DPN

Incubations were carried out as described under "Methods" except that before addition of poly dA and poly dT-adenylate reaction mixtures containing all other components were incubated for 10 min at 30°. Joining enzyme, 0.2 unit, was added to each reaction mixture.

DPN concentration	^{32}P in phosphodiester linkage	Inhibition
M	$\mu\mu moles$	%
0	3.04	
1×10^{-7}	3.40	0
5×10^{-7}	1.42	53
5×10^{-6}	0.62	80
3×10^{-4}	0.15	95

TABLE II

Activity of poly dT-adenylate in T4 polynucleotide ligase reaction

The reaction mixtures contained, in a volume of 0.1 ml, 20 mM Tris-HCl (pH 7.5), 5 mM MgCl$_2$, 1 mM EDTA, 10 mM β-mercapto-ethanol, 0.97 μM (in termini) poly dT-adenylate, 0.19 mM (in nucleotides) poly dA, where indicated, and 0.6 unit (2) of the T4 ligase. Incubations were at 30° for 60 min, and the release of AMP and phosphodiester bond formation was measured as described under "Methods."

Enzyme	poly dA	^{3}H-AMP released	^{32}P incorporated into phosphodiester linkage
		μμmoles	
−	+	5	5
+	−	12	3
+	+	67	61

the rate of the joining reaction when poly dT was used as substrate.

Kinetics of Joining Reaction with poly dT and poly dT-Adenylate—The K_m for poly dT-adenylate was found in two experiments to be 0.6 and 2 μM (in termini), about two orders of magnitude higher than the K_m of 0.003 μM for poly dT in the over-all reaction. Since the K_m of poly dT-adenylate was relatively high and only limited amounts of substrate were available, measurements of activity could not be performed at saturating poly dT-adenylate concentrations, but were extrapolated with several concentrations (0.1 to 1.5 μM) and a conventional double reciprocal plot. In two determinations with a side fraction obtained after DEAE-Sephadex chromatography of the joining enzyme, specific activity values of 2900 and 7900 $\mu\mu$moles per min per mg were obtained. These compare with values of 1460 and 1420 $\mu\mu$moles per min per mg for the over-all reaction, observed with saturating amounts of poly dT. Thus, the rate of the enzyme reaction with poly dT-adenylate is the same or slightly greater than the rate with poly dT, a result which is consistent with poly dT-adenylate being an intermediate in the over-all joining reaction with poly dT.

Activity of poly dT-Adenylate in T4-induced Polynucleotide Ligase Reaction—Poly dT-adenylate served as an intermediate in the T4-induced polynucleotide ligase reaction. In an incubation carried out in the absence of ATP (the cofactor for the phage-induced enzyme), 67 $\mu\mu$moles of AMP were released and 61 $\mu\mu$moles of ^{32}P were joined in phosphodiester linkage. The identification of the ^{3}H-AMP and verification of the phosphodiester linkage were carried out as described above. As with the *E. coli* joining enzyme, poly dA was required for the reaction (Table II).

DISCUSSION

The use of a synthetic intermediate, poly dT-adenylate, has permitted an unequivocal identification of both the substrate and the products of the final step in the over-all reaction catalyzed by the *E. coli* polynucleotide joining enzyme. Phosphodiester bond formation has been shown to occur with the same time course and

to be stoichiometric with AMP release from the polynucleotide. A nonadenylated enzyme is required for the reaction, and the kinetic requirement that the rate of the joining reaction with poly dT-adenylate be as fast or faster than the rate of the over-all joining reaction has been satisfied.

Poly dT with a triphosphate group at its 5' terminus was also tested in the *E. coli* joining enzyme reaction and found to be inactive.[4] Thus, activation of the 5' terminus of a polynucleotide chain by a pyrophosphate bond is an insufficient condition for its activity in the reaction catalyzed by the *E. coli* joining enzyme, which must therefore exhibit some specificity for the adenosine moiety of the polynucleotide-adenylate intermediate.

The finding that poly dT-adenylate is utilized by the T4-induced polynucleotide ligase indicates that the joining reaction catalyzed by this enzyme also proceeds through a DNA-adenylate intermediate; it further suggests that the mechanism proposed for the *E. coli* joining enzyme (11) is a general one.

REFERENCES

1. GELLERT, M., *Proc. Nat. Acad. Sci. U. S. A.*, **57**, 148 (1967).
2. OLIVERA, B. M., AND LEHMAN, I. R., *Proc. Nat. Acad. Sci. U. S. A.*, **57**, 1426 (1967).
3. ZIMMERMAN, S. B., LITTLE, J. W., OSHINSKY, C. K., AND GELLERT, M., *Proc. Nat. Acad. Sci. U. S. A.*, **57**, 1841 (1967).
4. GEFTER, M., BECKER, A., AND HURWITZ, J., *Proc. Nat. Acad. Sci. U. S. A.*, **58**, 240 (1967).
5. WEISS, B., AND RICHARDSON, C. C., *Proc. Nat. Acad. Sci. U. S. A.*, **57**, 1021 (1967).
6. COZZARELLI, N. R., MELECHEN, N. E., JOVIN, T. M., AND KORN-BERG, A., *Biochem. Biophys. Res. Commun.*, **28**, 578 (1967).
7. LITTLE, J. W., ZIMMERMAN, S. B., OSHINSKY, C. K., AND GELLERT, M., *Proc. Nat. Acad. Sci. U. S. A.*, **58**, 2004 (1967).
8. OLIVERA, B. M., AND LEHMAN, I. R., *Proc. Nat. Acad. Sci. U. S. A.*, **57**, 1700 (1967).
9. BECKER, A., LYN, G., GEFTER, M., AND HURWITZ, J., *Proc. Nat. Acad. Sci. U. S. A.*, **58**, 1996 (1967).
10. OLIVERA, B. M., HALL, Z. W., ANRAKU, Y., CHIEN, J. R., AND LEHMAN, I. R., *Cold Spring Harbor Symp. Quant. Biol.*, **33**, in press.
11. OLIVERA, B. M., HALL, Z. W., AND LEHMAN, I. R., *Proc. Nat. Acad. Sci. U. S. A.*, **61**, 237 (1968).
12. GLYNN, I. M., AND CHAPPELL, J. B., *Biochem. J.*, **90**, 147 (1964).
13. RICHARDSON, C. C., *Proc. Nat. Acad. Sci. U. S. A.*, **54**, 158 (1965).
14. WITTENBERG, J., AND KORNBERG, A., *J. Biol. Chem.*, **202**, 431 (1953).
15. LEHMAN, I. R., AND NUSSBAUM, A. L., *J. Biol. Chem.*, **239**, 2628 (1964).
16. MOFFATT, J. G., AND KHORANA, H. G., *J. Amer. Chem. Soc.*, **83**, 649 (1961).
17. YONEDA, M., AND BOLLUM, F. J., *J. Biol. Chem.*, **240**, 3385 (1965).
18. HAYES, F. N., MITCHELL, V. E., RATLIFF, R. L., SCHWARTZ, A. W., AND WILLIAMS, D. L., *Biochemistry*, **5**, 3625 (1966).
19. MALAMY, M. H., AND HORECKER, B. L., *Biochemistry*, **3**, 1893 (1964).
20. HANES, C. S., AND ISHERWOOD, F. A., *Nature*, **164**, 1107 (1949).
21. PREISS, J., AND HANDLER, P., *J. Biol. Chem.*, **233**, 488 (1958).
22. CUNNINGHAM, L. B., CATLIN, B. W., AND PRIVAT DE GARILHE, M., *J. Amer. Chem. Soc.*, **78**, 4642 (1956).
23. HILMOE, R. J., *J. Biol. Chem.*, **235**, 2117 (1960).

---

[4] Z. Hall and N. Cozzarelli, unpublished experiments.