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Reversal of Bacteriophage T_4 Induced Polynucleotide Kinase Action†

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ABSTRACT: The mechanism of action of T_4-induced polynucleotide kinase on short single-stranded deoxyoligonucleotides has been investigated. It was found that the phosphorylation reaction catalyzed by T_4 polynucleotide kinase could be reversed. Thus, a 5′-^{32}P-labeled deoxyoligonucleotide and ADP on incubation with the enzyme formed [γ-^{32}P]ATP. In addition some radioactive inorganic phosphate was produced. In the presence of ATP the major radioactive product of the reverse reaction was identified as adenosine 5′-[δ-^{32}P]tetraphosphate. The reverse reaction was found to be optimal at pH 6.5, whereas maximum rate for the forward reaction was observed at approximately pH 9.0. At pH 7.6 the rate of the reverse reaction was 2% of that of the forward reaction. Utilizing this reversibility of the T_4 polynucleotide kinase action, it was shown that deoxyoligonucleotides containing unlabeled phosphate at the 5′ end could be quantitatively labeled with ^{32}P at the 5′ end without prior removal of the unlabeled phosphate by phosphatase.

The bacteriophage T_4 induced polynucleotide kinase catalyzes the transfer of the γ-phosphate from ATP to the 5′-hydroxyl terminus of polynucleotides, oligonucleotides, 3′-mononucleotides (Richardson, 1965), and N-protected deoxyoligonucleotides (van de Sande and Bilsker, 1973). The enzyme has proved to be an extremely useful tool in structural work on nucleic acids. Thus, labeling of the 5′ end groups has been used extensively for determining the end groups and terminal sequences of macromolecular DNAs (Weiss and Richardson, 1967; Jacquemin-Sablon and Richardson, 1970; Wu and Kaiser, 1967) and for structural study of the cohesive ends of the bacteriophage λ DNA (Wu and Kaiser, 1968; Richardson *et al.*, 1968). Similarly, the fingerprinting of short oligonucleotides (pyrimidine tracts from DNAs, nuclease digests of tRNAs) is facilitated by the labeling of the 5′-end groups (Székely and Sanger, 1969; Southern, 1970; Murray, 1973; Simsek *et al.*, 1973).

Further, the techniques of introducing label at the termini of polynucleotide chains facilitate studies of the polynucleotide ligase and of DNA enzymology (Weiss *et al.*, 1968b; Gefter *et al.*, 1967; Olivera and Lehman, 1967) and 5′-phosphomonoesterases (Becker and Hurwitz, 1967; Weiss *et al.*, 1968a). In this laboratory, it has served as an indispensable tool in the characterization of short synthetic deoxyribopolynucleotides and for monitoring their subsequent joining to form defined bihelical DNAs (Khorana *et al.*, 1972; Panet *et al.*, 1973; van de Sande and Bilsker 1973).

In view of the usefulness and our continued interest in the polynucleotide kinase, we have carried out a closer study of the reaction catalyzed by this enzyme. A number of interesting observations have been made and these form the content of this paper. A particularly interesting finding is the reversibility of the phosphorylation of the 5′-OH groups in polynucleotides. A practical application of this finding is that if a 5′-phosphate group is already present at the terminus of a polynucleotide chain, it is unnecessary to remove it by a phosphatase treatment in order to introduce the radioactively labeled phosphate group.

Materials and Methods

Chemicals. ATP and ADP were purchased from P-L Biochemicals Inc. Adenosine 5′-tetraphosphate was the product of Sigma Chemical Co. [^{3}H]ADP was obtained from New England Nuclear Corporation. [γ-^{32}P]ATP was prepared according to a published procedure (Glynn and Chappell, 1964).

Enzymes. Polynucleotide kinase was isolated from T_4 am N82 phage infected *Escherichia coli* B62 by a modification (Panet *et al.*, 1973) of the method of Richardson (1965). The enzyme had a specific activity of 65,000 units/mg of protein and showed a single band on sodium dodecyl sulfate gel electrophoresis. The enzyme preparation contained no endonuclease activity as assayed by the method of Weiss *et al.* (1969a,b), or exonuclease activity as assayed by method 1a of Panet *et al.* (1973). Prolonged incubation (12 hr at 37°) of the

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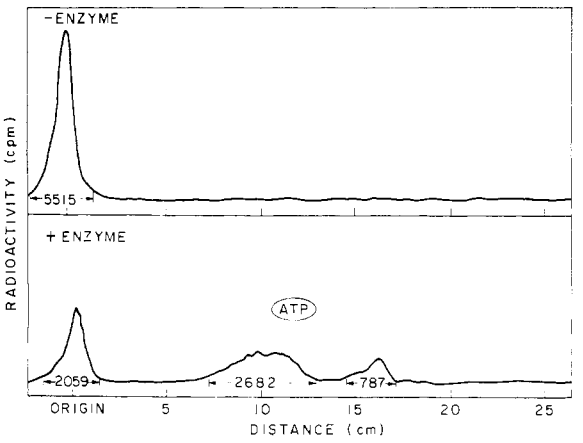

FIGURE 1: Radiochromatographic scan of the separated reaction products formed by incubating d-^{32}pT(pTpTpCpT)$_2$pTpT and [^{3}H]ATP with T_4 polynucleotide kinase. The reaction mixture (100 μl) contained 40 pmol of d-^{32}pT(pTpTCpT)$_2$pTpT and 10 nmol of [^{3}H]ATP in 10 mM $MgCl_2$–10 mM 2-mercaptoethanol–66 mM Tris-HCl (pH 7.6). After the addition of T_4 polynucleotide kinase (9 units), aliquots were taken out at timed intervals and analyzed by DE-81 paper chromatography in solvent C. The scans of the 30-min incubation aliquots from reactions with (bottom) or without (top) enzyme are shown.

enzyme with *p*-nitrophenyl phosphate according to Garen and Levinthal (1960) released no *p*-nitrophenol. Incubation of polynucleotide kinase (9 units) with [γ-^{32}P]ATP (0.5 nmol in 100 μl) at pH 6.2 for up to 2 hr at 37° did not result in any [^{32}P]orthophosphate formation.

Chromatography. Paper chromatography was carried out on Whatman No. 1 paper. Solvents used were: (A) 0.1 M sodium phosphate (pH 6.8)–ammonium sulfate–*n*-propyl alcohol (100 ml:60 g:2 ml); (B) isobutyric acid–concentrated ammonia–water (66:1:33, v/v). Assays using anion exchange paper were carried out on DEAE-cellulose anion exchange paper (DE-81). The solvent used was 0.35 M ammonium formate (pH 5.5) (solvent C).

Oligonucleotides. The undecanucleotide d-T(pTpTpCpT)$_2$-pTpT has been described previously (Jacob *et al.*, 1967). The decanucleotide dA-C-C-G-A-C-T-A-G-C was a gift of Dr. M. H. Caruthers (Caruthers and Khorana, 1972). The nonacosanucleotide dC-T-A-A-A-T-C-T-G-C-C-G-T-C-A-T-C-G-A-C-T-T-C-G-A-A-G-G-T was prepared as described by Besmer *et al.* (1972).

The oligonucleotides carrying 5′-OH end groups were phosphorylated by incubating with polynucleotide kinase and [γ-^{32}P]ATP as described previously (Sgaramella and Khorana, 1972; van de Sande *et al.*, 1972). The phosphorylated oligonucleotides were separated from excess ATP by gel filtration through Sephadex G-50.

Assay for the Reversal of Polynucleotide Kinase Action. A reversibility in the polynucleotide kinase action can be represented by the following equation.

$$5'\text{-OH-oligonucleotide} + \text{ATP} \rightleftharpoons 5'\text{-P-oligonucleotide} + \text{ADP}$$

The reverse reaction was assayed by using a ^{32}P-labeled oligonucleotide and ADP. After incubation with the enzyme, radioactivity in ATP was measured. Details of the assay conditions are given in the legends to the figures. The kinetics of this reverse reaction are followed by taking aliquots from the reaction and applying these to DE-81 paper strips previously spotted with 50 μl of a solution of 1 mM ATP containing 0.05 M EDTA. The strips were subjected to descending chromatography in solvent C. The dried strips were scanned in a Packard 7200 radiochromatogram scanner, cut into strips, and counted in a liquid scintillation counter.

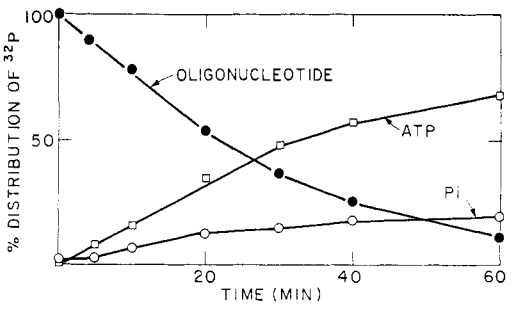

FIGURE 2: Kinetics of exchange between d-^{32}pT(pTpTpCpT)$_2$pTpT and [^{3}H]ATP. Time aliquots from the reaction mixture, described in Figure 1, were analyzed by DE-81 paper chromatography and scanned and the strips cut appropriately and counted.

Results

ATP-Catalyzed Exchange. Incubation of d-^{32}pT(pTpTpCpT)$_2$pTpT with a large excess (250-fold) of [^{3}H]ATP and polynucleotide kinase resulted in the formation of several ^{32}P-labeled products (Figure 1). Thus, after a 30-min incubation, the distribution of radioactivity was as follows: 35% in starting material, 15% in inorganic phosphate, and 50% in a product(s) which partially chromatographs as ATP. The kinetics for the exchange reaction are shown in Figure 2. In 1 hr, only 10% of the ^{32}P radioactivity remained in the starting material and over 70% of the radioactivity is incorporated into a product with a similar mobility as ATP. Incubation of d-^{32}pT(pTpTpCpT)$_2$pTpT with polynucleotide kinase in the absence of ATP resulted only in the slow formation of [^{32}P]P_i (7% after 1-hr incubation under identical conditions). The release of inorganic phosphate was not due to a phosphatase contamination of the polynucleotide kinase, since no hydrolysis of *p*-nitrophenyl phosphate or [γ-^{32}P]ATP could be

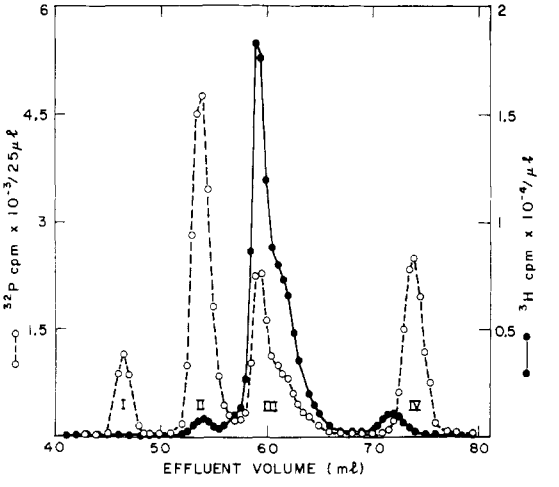

FIGURE 3: Gel filtration of the reaction products from the exchange between d-^{32}pT(pTpTpCpT)$_2$pTpT and [^{3}H]ATP. The reaction mixture from Figure 1 was made 0.04 M in EDTA after 1-hr incubation with polynucleotide kinase, and then passed through a column (1.1 × 85 cm) of Sephadex G-25. The column was eluted at 4° with 0.05 M triethylammonium bicarbonate and aliquots of the fractions were counted for both ^{32}P and ^{3}H. Characterization of the peaks is given under Results.

TABLE I:[a] Identification of Adenosine 5′-Tetraphosphate.

Treatment	Radioactivity (cpm)	
	Total	Norit Adsorbable
None	7720	7633
Phosphatase	6319	133

[a] One aliquot from peak II of Figure 3 was added to 0.2 ml of 20% Norit suspension in 0.25 M HCl–0.25 mM P_i–0.25 mM PP_i. The suspension was thoroughly mixed and the amount of Norit-adsorbable ^{32}P radioactivity was determined. Another aliquot was first treated with *E. coli* alkaline phosphatase (5 μg) for 30 min at 65° before the Norit-adsorbable ^{32}P radioactivity was determined.

observed at even higher enzyme concentrations (Materials and Methods).

The exchange reaction mixture after 1-hr incubation (Figure 2) was analyzed by gel filtration using Sephadex G-25. The elution pattern is shown in Figure 3. Peak I contained only ^{32}P radioactivity and was characterized as starting material, d-^{32}pT(pTpTpCpT)$_2$pTpT. Peak III contained the large excess of [^{3}H]ATP, but it also showed the presence of some ^{32}P-labeled material, which was identified as [γ-^{32}P]ATP. A trace of ADP which contaminated the [^{3}H]ATP preparation served as an acceptor for the [^{32}P]phosphate group from the undecanucleotide to form [γ-^{32}P]ATP. Peak IV contained ^{32}P-labeled inorganic phosphate. All these products were characterized by chromatography in solvent A in the presence of authentic markers.

The major ^{32}P-labeled product, peak II, also contained a trace of ^3H radioactivity, which indicated that this product was formed from [^{3}H]ATP. The ^{32}P radioactivity in peak II was Norit adsorbable before but not after phosphatase treatment (Table I), indicating that the product was nucleotidic. The radioactivity in both ^{32}P and ^3H cochromatographed with a sample of adenosine 5′-tetraphosphate (Figure 4) and based on these data the product from peak II was concluded to be adenosine 5′-tetraphosphate. This compound was formed by [^{3}H]ATP acting as the acceptor for the [^{32}P]orthophosphate group from the oligonucleotide.

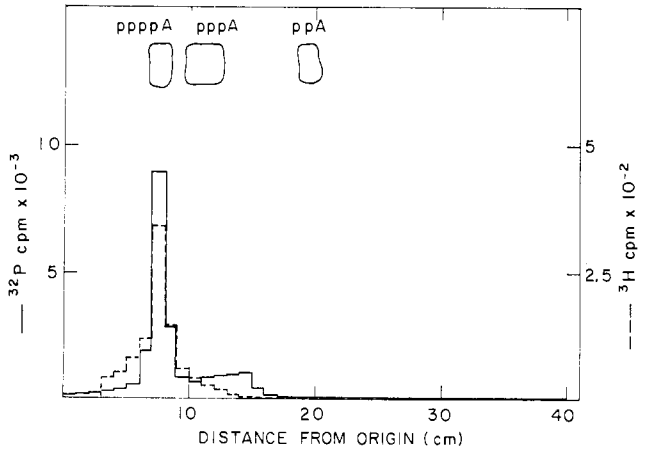

FIGURE 4: Characterization of peak II (Figure 3) as adenosine 5′-tetraphosphate. An aliquot from peak II (Figure 3) was analyzed by paper chromatography in solvent A in the presence of markers of ppppA, pppA, and ppA. The chromatogram was cut in 1-cm strips and counted for both ^{32}P and ^3H.

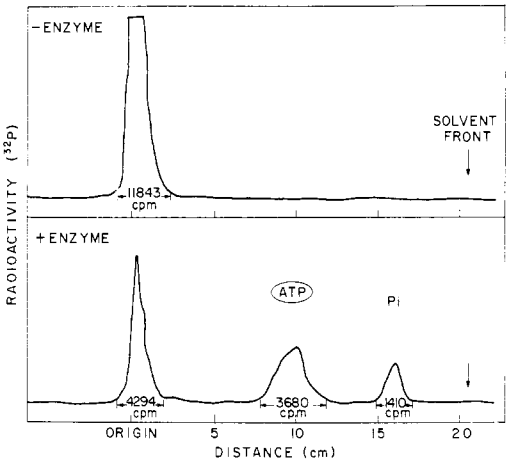

FIGURE 5: Radiochromatographic scan of a DE-81 paper chromatographic separation of the reaction products formed on incubating d-^{32}pT(pTpTpCpT)$_2$pTpT and ADP with T_4 polynucleotide kinase. The reaction mixture (50 μl) contained 25 pmol of d-^{32}pT-(pTpTpCpT)$_2$pTpT and 1 nmol of ADP in 10 mM $MgCl_2$, 10 mM dithiothreitol, and 50 mM imidazole-HCl (pH 6.2). The reaction was started by adding 0.45 unit of polynucleotide kinase and aliquots were withdrawn at various times and analyzed by DE-81 paper chromatography in solvent C. Scans of the 20-min aliquots from reactions with (bottom) or without (top) enzyme are shown.

ADP-Catalyzed Exchange. Since ADP is a product of the forward reaction, it would appear possible that it could act as an acceptor in the reverse reaction to give [γ-^{32}P]ATP. Indeed, the formation of a small amount of [γ-^{32}P]ATP in the above-described ATP-catalyzed exchange reaction suggested that this was the case. The products from the incubation of d^{32}pT(pTpTpCpT)$_2$pTpT and ADP only were [γ-^{32}P]ATP and [^{32}P]P_i and the starting undecanucleotide (Figure 5). In a 20-min incubation of d^{32}pT(pTpTpCpT)$_2$pTpT and ADP, 40% of the ^{32}P radioactivity was transferred to [γ-^{32}P]ATP and 15% to [^{32}P]P_i. When the exchange was carried out with [^{3}H]ADP, double-labeled ATP (γ-^{32}P and ^3H) was obtained. The kinetics of ADP-catalyzed exchange are shown in Figure 6. This reaction was carried out at pH 6.2, which probably resulted in the high yield of inorganic phosphate formation.

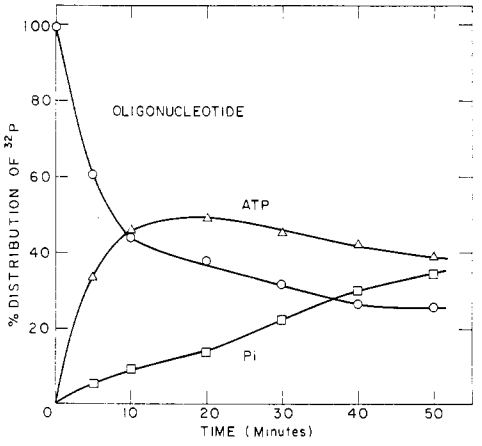

FIGURE 6: Kinetics of exchange between d-^{32}pT(pTpTpCpT)$_2$pTpT and ADP. The reaction mixture was as described in the legend of Figure 5, except that the concentration of ADP was 0.04 mM. Aliquots were withdrawn at intervals, analyzed by DE-81 paper chromatography, and scanned, and the spots corresponding to oligonucleotide, ATP, and inorganic phosphate were cut out and counted.

TABLE II:[a] Requirements for Kinase Exchange.

	^{32}P Released (pmol)
Complete	322
Omit enzyme	<2
Omit ATP	159
Omit ADP	119
Omit ATP + ADP	18
Omit $MgCl_2$	6
Omit 2-mercaptoethanol[b]	120
Complete + KP_i (0.01 M)	338
Complete + KP_i (0.05 M)	193

[a] The complete reaction mixture contained 600 pmol of d-^{32}pACCGACTAGC and 12 units of polynucleotide kinase in 1 ml of 0.01 M $MgCl_2$, 0.01 M 2-mercaptoethanol, 0.05 M imidazole-HCl (pH 6.8), 0.05 mM ATP, and 0.05 mM ADP. The reactions were stopped after 30 min and analyzed by DE-81 paper chromatography in solvent C. The ^{32}P radioactivity released from the oligonucleotide was measured as the total radioactivity in ^{32}ppppA, ^{32}pppA, and ^{32}P-labeled orthophosphate. [b] This reaction contained 0.0005 M 2-mercaptoethanol introduced from the enzyme diluent.

Requirements of Exchange Reaction. The requirements of the kinase-catalyzed exchange reaction were studied by measuring the release of ^{32}P radioactivity from the substrate oligonucleotide d-^{32}pApCpCpGpApCpTpApGpC. The reaction required magnesium ions for maximal activity. In the absence of $MgCl_2$, less than 2% of the activity was observed (Table II). This table also shows that either ADP or ATP is required to effect the reverse reaction but that ADP is a better phosphate acceptor than ATP, since a higher activity was observed in the presence of the former (49% of the total activity) than in the presence of the latter (37% of the total activity). A reducing agent seems necessary for the enzyme to effect the reverse reaction. Phosphate ions inhibit the exchange reaction; thus, in the presence of 50 mM potassium phosphate the enzyme showed only 60% of the maximal activity. All these requirements are similar to the ones reported by Richardson (1965) for the forward (phosphorylation) reaction catalyzed by T_4 polynucleotide kinase.

The affinity of the enzyme for ADP in the reverse reaction was determined with the oligonucleotide d-^{32}pT(pTpTpCpT)$_2$pTpT as the phosphate donor (Figure 7). The K_m for ADP was found to be 2.2×10^{-5} M. No binding constant of T_4 polynucleotide kinase for ATP has been reported, but the K_m for ATP in the case of T_2 polynucleotide kinase has been reported as 1.43×10^{-5} M (Novogrodsky and Hurwitz, 1966). Figure 7 also showed that a higher rate of phosphate exchange was observed in the presence of 0.1 mM ATP. Since ATP can act as both a phosphate acceptor and phosphate donor, the concentration of phosphorylated oligonucleotide substrate will remain essentially constant, with the result of a high rate of exchange.

pH Optimum. A pH profile for the reverse reaction of polynucleotide kinase was determined, as shown in Figure 8. The optimal range was found to be pH 6.0–7.0 in 50 mM imidazole-HCl. At pH 5.8 in 50 mM sodium acetate and pH 7.6 in 50 mM Tris-HCl the enzyme showed 70 and 20%, respectively, of its activity at pH 6.2 in 50 mM imidazole-HCl. The optimal pH range for the T_4 polynucleotide kinase catalyzed phosphorylation of DNA was reported to be pH 7.6–8.0 in 66 mM Tris-HCl (Richardson, 1965). A pH profile for the phosphorylation of the oligonucleotide d-T(pTpTpCpT)$_2$pTpT by polynucleotide kinase was determined (Figure 9). The optimal pH for the phosphorylation was found to be at approximately pH 9.5 in 50 mM glycine–NaOH, in contrast to the reported optimal pH range for the phosphorylation of DNA. The equilibrium constant for phosphorylation–dephosphorylation of a deoxyoligonucleotide at pH 7.6 was calculated to be ~50, indicating that the rate of the reverse reaction was 2% of that of the forward reaction.

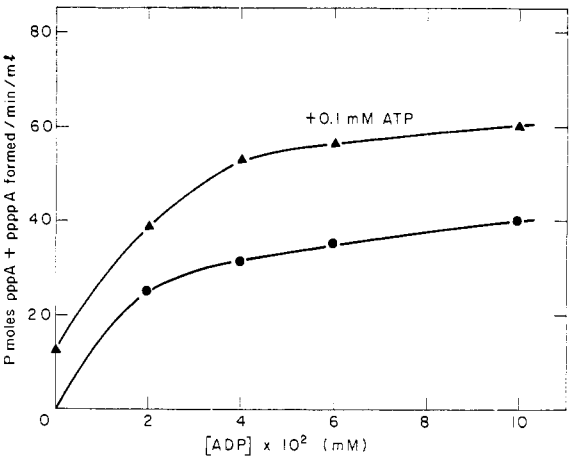

FIGURE 7: Effect of ADP concentration on initial rate of exchange between d-^{32}pT(pTpTpCpT)$_2$pTpT and ADP (ATP). The reaction conditions were the same as described in the legend to Figure 5, except that the concentration of ADP varied as shown. One set of experiments was carried out in the presence of 0.1 mM ATP.

Labeling of Deoxy-5'-phosphooligonucleotides by Exchange with [γ-^{32}P]ATP. In the experiments described above, the reversal of T_4 polynucleotide kinase action has been followed by measuring the decrease of ^{32}P radioactivity in [^{32}P]deoxyoligonucleotide with the concomitant formation of ^{32}P-

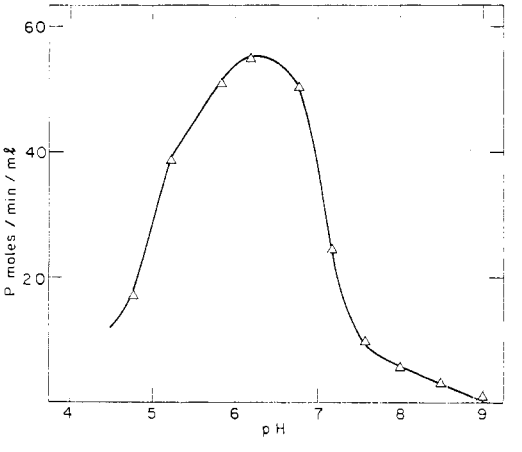

FIGURE 8: Effect of pH on initial rate of exchange between d-^{32}pT-(pTpTpCpT)$_2$pTpT and ATP–ADP. The reaction mixtures contained 0.5 nmol of d-^{32}pT(pTpTpCpT)$_2$pTpT/ml, in 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM ATP, 0.01 mM ADP, 18 units/ml of T_4 polynucleotide kinase, and 50 mM of the appropriate buffer. The buffers used were sodium acetate (pH 4.7–5.8), imidazole-HCl (pH 6.2–7.2), Tris-HCl (pH 7.6–9.0), and glycine–NaOH (pH 9.5–10.5). The reactions were carried out at 37° and for each one the time course was followed as shown in Figure 6. The initial rate at each pH measurement was calculated and expressed as the synthesis of total radioactive (pppA + ppppA)/min per ml.

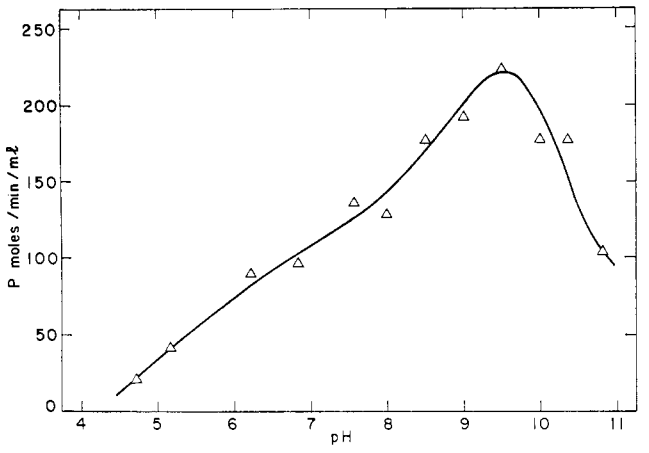

FIGURE 9: Effect of pH on initial rate of phosphorylation of d-T(pTpTpCpT)$_2$pTpT. The reaction mixtures contained 0.1 mM [γ-^{32}P]ATP (sp act. 1600 cpm/pmol), 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 mM of the appropriate buffer (described in the legend of Figure 8), 2 nmol of d-T(pTpTpCpT)$_2$pTpT/ml, and 0.9 unit of T_4 polynucleotide kinase/ml. The reactions were carried out at 37° and for each pH value, the time course of the reaction was followed as shown in Figure 6. The initial rate of phosphorylation was calculated for each reaction and expressed as the synthesis of phosphorylated undecanucleotide/min per ml.

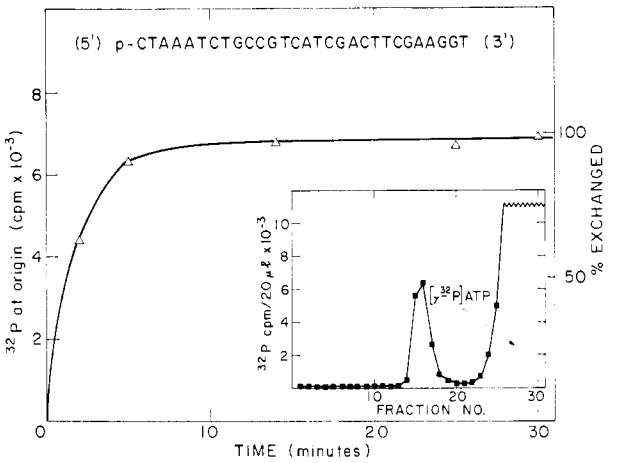

FIGURE 10: Kinetics of exchange between [γ-^{32}P]ATP and d-pCTAAATCTGCCGTCATCGACTTCGAAGGT. The reaction mixture (100 μl) contained 20 pmol of nonacosanucleotide, 500 pmol of [γ-^{32}P]ATP (sp act. ~3500 cpm/pmol), 500 pmol of ADP, 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 mM Tris-HCl (pH 7.6), and 9 units of T_4 polynucleotide kinase. The reaction was carried out at 37° and, after 15 min, the reaction mixture was heated to 100° for 2 min (to destroy any secondary structure of the oligonucleotide) and 0.1 μmol of dithiothreitol and 9 units of kinase were added and the reaction resumed at 37°. The reaction was followed by the usual DE-81 paper chromatography assay. After 30 min of reaction the solution was made 0.03 M in EDTA, boiled for 2 min, and applied to a column (1 × 15 cm) of Sephadex G-50, equilibrated at 4° with 0.05 M triethylammonium bicarbonate. Fractions of approximately 0.3 ml were collected, and 20 μl of each fraction was counted. The elution pattern is shown in the inset.

labeled adenosine tetra- or triphosphate and inorganic phosphate. In order to use this reaction as a preparative procedure for labeling 5′-phosphate terminated oligonucleotides, the exchange must be carried out in the presence of a large excess of [γ-^{32}P]ATP and ADP. Preliminary experiments using the oligonucleotide d-pT(pT)$_9$ indicated that at pH 7.6 a nearly quantitative (95%) labeling of the 5′-terminal phosphate could be achieved. The kinetics of the labeling of a 5′-phosphorylated nonacosanucleotide, *via* exchange with [γ-^{32}P]ATP in the presence of ADP, are shown in Figure 10. A fast exchange takes place which resulted in a 96% exchange of the 5′-terminal phosphate of the nonacosanucleotide. The isolation of the labeled oligonucleotide by gel filtration is also shown in this figure. The isolated nonacosanucleotide was characterized by polyacrylamide gel electrophoresis (T. Maniatis and J. H. van de Sande, manuscript in preparation) and upon digestion to 5′-mononucleotides essentially all the radioactivity (1480 cpm out of 1568 cpm) was found in d-pC.

Discussion

The results from the present study show that the reaction catalyzed by T_4-induced polynucleotide kinase is reversible. The experimental data can be best rationalized by the mediation of a phosphorylated kinase as shown by the series of reactions of Scheme I.

SCHEME I

$$\text{kinase} + {}^*\text{pppA} \rightleftharpoons \text{kinase} - {}^*\text{p} + \text{ppA} \quad (1)$$

$$\text{kinase} - {}^*\text{p} + \text{5′-HO-oligonucleotide} \rightleftharpoons \text{kinase} + \text{5′-}{}^*\text{p-oligonucleotide} \quad (2)$$

$$\text{kinase} - {}^*\text{p} + \text{pppA} \rightleftharpoons \text{kinase} + {}^*\text{ppppA} \quad (3)$$

$$\text{kinase} - {}^*\text{p} + H_2O \longrightarrow \text{kinase} + {}^*P_i \quad (4)$$

The first step is the formation of a phosphorylated kinase intermediate (eq 1), which will subsequently transfer its phosphate to the 5′-hydroxyl group of an oligonucleotide (eq 2). Similar phosphoenzyme intermediates have been postulated for the action of several enzymes which catalyze phosphate cleavage or transfer reactions (Bridger, 1973). In the present work no attempt has been made to isolate the postulated phosphorylated kinase intermediate.

The formation of radioactive labeled inorganic phosphate by T_4 polynucleotide kinase (eq 4) may also proceed *via* a phosphorylated kinase intermediate. In this case H_2O is the acceptor rather than ADP, ATP, or oligonucleotide. Since no inorganic phosphate is liberated by incubating [γ-^{32}P]ATP with polynucleotide kinase, the possibility of a phosphatase contamination can be ruled out. Production of inorganic phosphate occurs only in the presence of phosphorylated oligonucleotides *via* the phosphorylated kinase intermediate which is hydrolyzed by water. An alternate explanation for the formation of inorganic phosphate is that the polynucleotide kinase preparation contains a highly specific 5′-nucleotidase contamination. This explanation is very unlikely, but cannot be ruled out at this time.

Attempts to detect an ATP $\rightleftarrows$ ADP exchange, which would reflect the step involving the formation of a phosphorylated kinase, have been unsuccessful. The ATP $\rightleftarrows$ ADP exchange can be detected, however, in the presence of the phosphorylated oligonucleotide, which could play a synergistic role in this partial reaction. The effect of the oligonucleotide substrate on the ATP $\rightleftarrows$ ADP exchange could be analogous to the substrate stimulation of the succinyl coenzyme A synthetase catalyzed ATP $\rightleftarrows$ ADP exchange (Bridger *et al.*, 1968).

Little information is currently available regarding the active-site and subunit composition of T_4 polynucleotide kinase (Panet *et al.*, 1973). It seems reasonable to assume that there is one binding site for the oligonucleotide and one for ATP and ADP. The finding that adenosine 5′-tetraphosphate is produced in the presence of ATP suggests that the spe-

cificity of the binding site for ATP–ADP is low. The different pH optima for the forward and reverse reactions are probably a reflection of different ionizable groups at the two binding sites.

The finding that the T_4 polynucleotide kinase catalyzed reaction is reversible is analogous to the observed reversal of reaction of several enzymes of DNA metabolism. *E. coli* DNA polymerase I will depolymerize DNA in the presence of inorganic pyrophosphate (Deutscher and Kornberg, 1969), *E. coli* RNA polymerase catalyzes a pyrophosphate exchange (Furth *et al.*, 1962), and recently it was found that *E. coli* polynucleotide ligase will act as an AMP-dependent endonuclease (Modrich *et al.*, 1972).

T_4 polynucleotide kinase is currently extensively used to label DNA, deoxy-, and ribooligonucleotides at their 5′-hydroxyl end. The majority of naturally occurring polynucleotides contain 5′-monophosphate groups which have to be removed prior to labeling with radioactive phosphate using polynucleotide kinase. If not all the 5′-phosphate groups have been removed before phosphorylation, some exchange is likely to occur particularly if high enzyme concentrations are used (Sgaramella *et al.*, 1970). This exchange property must always be considered when the enzyme is used in sequence analysis (Székely and Sanger, 1969). The exchange property of T_4 polynucleotide kinase should be useful in labeling DNA or oligonucleotides without prior removal of the cold 5′-phosphate group.

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