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## Physical Characterization and Simultaneous Purification of Bacteriophage T<sub>4</sub> Induced Polynucleotide Kinase, Polynucleotide Ligase, and Deoxyribonucleic Acid Polymerase†

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**ABSTRACT:** The initial steps in the existing procedures for the purification of the bacteriophage T<sub>4</sub> induced polynucleotide kinase, ligase, and the DNA polymerase have been modified so as to enable their simultaneous preparation from one batch of the infected *Escherichia coli* cells. Procedures were devised to remove an exonuclease which often contaminated the preparations of the kinase and ligase. The kinase was shown to be homogeneous by gel electrophoresis. Its molecular weight

under native conditions using gel filtration was found to be 140,000 ± 10% daltons. Under denaturing conditions employing sodium dodecyl sulfate electrophoresis the mol wt was estimated to be 33,000 ± 5% daltons. The ligase also gave a single band upon gel electrophoresis and the mol wt under denaturing conditions was 63,000 ± 5% daltons. Gel filtration of the latter enzyme in the absence of denaturing agents gave a mol wt of 68,000 ± 10% daltons.

The bacteriophage T<sub>4</sub> induced enzymes, polynucleotide kinase, polynucleotide ligase, and DNA polymerase, have proved to be important and useful in chemical and biological studies of DNA (Richardson, 1965; Weiss and Richardson, 1967a,b; Goulian *et al.*, 1968). The kinase and ligase form parts of the methodology for the laboratory synthesis of bi-helical DNA of specific nucleotide sequence (Khorana *et al.*, 1972).

While satisfactory methods are available for the separate purification of each one of the three enzymes (Goulian *et al.*, 1968; Weiss *et al.*, 1968; Richardson, 1972), considerable saving in time and material could be achieved if a procedure could be developed which enables simultaneous preparation of the three enzymes from one batch of the T<sub>4</sub>-infected *Escherichia coli* cells. Such a procedure was especially desirable for the work on the total synthesis of the transfer RNA genes where

relatively large amounts of the enzymes are continually required (Khorana *et al.*, 1972).

In this paper we first describe a procedure which modifies the existing procedures to a single procedure for preparation of the enzymes. Procedures have also been developed for removing or completely inhibiting an exonuclease which sometimes is present after the final step in the purification of the kinase. Further physical characterizations of the kinase and the ligase are reported. The kinase is concluded to have a mol wt of ~140,000 daltons with subunits of ~33,000 daltons. The ligase appears to be a single polypeptide chain of mol wt ~68,000 daltons.

### Experimental Section

#### Materials

**T<sub>4</sub>-Infected *E. coli* Cells.** *E. coli* strain B62 was grown in 3XD medium (Fraser and Jerrel, 1953) to a cell density of 5 × 10<sup>8</sup>/ml and then infected with T<sub>4</sub> amN82 phage at a multiplicity of 5:8. Thirty minutes after infection, the culture was quickly chilled on ice and the cells were harvested by centrifugation in a continuous flow centrifuge (DeLaval).

Ion exchangers, DEAE-cellulose (DE-23) and phosphocellulose (P11), were purchased from Whatman and DEAE-Sephadex A-50 was obtained from Sigma Chemical Co. Hydroxylapatite, Bio-Gel HTP, was the product of Bio-Rad Laboratories and Alumina 350 was obtained from Sigma Chemical Co.

[γ-<sup>32</sup>P]ATP was prepared according to the published procedure (Glynn and Chappell, 1964). [α-<sup>32</sup>P]ATP and [<sup>14</sup>C]dATP were purchased from New England Nuclear and [<sup>3</sup>H]ATP

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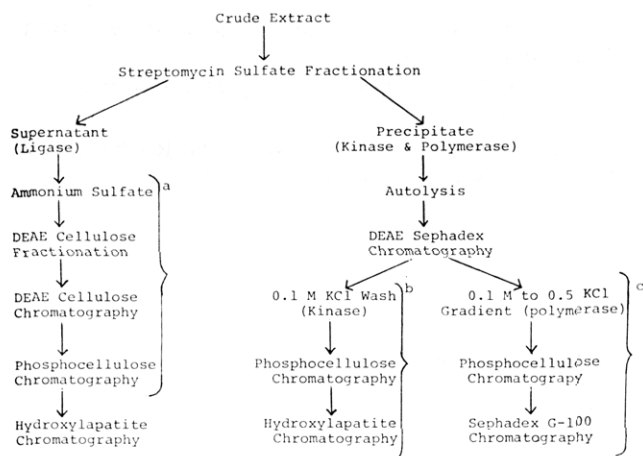


FIGURE 1: Flow diagram for the simultaneous purification of polynucleotide ligase, polynucleotide kinase, and DNA polymerase from *T*<sub>4</sub> amN82 phage-infected *E. coli* cells: (a) as described by Weiss *et al.* (1968); (b) as described by Richardson (1972); (c) as described by Goulian *et al.* (1968).

was obtained from Amersham/Searle. [<sup>3</sup>H]Poly[d(A-T)·d(A-T)] was a gift from Dr. I. F. Nes.

#### Methods

**Gel Electrophoresis.** The procedure described by Weber and Osborn (1969) was followed using 10% gels prepared from acrylamide and 2.7% methylene bisacrylamide (Eastman). The gels were polymerized in 75 mm × 5 mm tubes and electrophoresis was carried out at a constant current of 8 mA/gel for 4 hr.

**Enzyme Assays.** *T*<sub>4</sub> polynucleotide kinase was assayed essentially as described by Richardson (1965). One unit of enzyme is defined as the amount of enzyme which catalyzes the formation of 1 nmol of acid-soluble <sup>32</sup>P in 30 min (Richardson, 1965).

*T*<sub>4</sub> polynucleotide ligase was assayed as described previously (Gupta *et al.*, 1968). One unit of ligase is defined as the amount of enzyme which catalyzes the conversion of 1 pmol of the <sup>32</sup>P-labeled 5'-phosphoryl termini of dT<sub>10</sub> to a form insensitive to bacterial alkaline phosphatase in 1 min at 20° (Kleppe *et al.*, 1970). During the later steps of the ligase purification, the enzyme-catalyzed ATP-PP<sub>i</sub> exchange assay described by Weiss *et al.* (1968) was also used.

The *T*<sub>4</sub> DNA polymerase was assayed similar to the published procedure (Goulian *et al.*, 1968). One unit of enzyme is defined as the amount of polymerase catalyzing the incorporation of 10 nmol of total nucleotide into an acid-insoluble product in 30 min.

**Preparation of [<sup>32</sup>P]AMP- or [<sup>3</sup>H]AMP-Ligase.** This was prepared similarly to the procedure of Weiss and Richardson (1967a,b). The reaction mixture contained 66 mM Tris-HCl (pH 7.6), 5 mM dithiothreitol, 6 mM MgCl<sub>2</sub>, 0.25 nmol of [<sup>3</sup>H]ATP (sp act. 11 Ci/mmol) or 0.50 nmol of [α-<sup>32</sup>P]ATP (sp act. 5 Ci/mmol), and 700 units of ligase (fraction VI). The reaction mixture was incubated at 37° for 10 min and then used directly for gel filtration or sodium dodecyl sulfate gel electrophoresis.

**Deoxyribonuclease Assays.** The enzymes were assayed for endonuclease contamination by a modification of the procedure of Weiss *et al.* (1968). The incubation mixture (100 μl) contained 0.01 M MgCl<sub>2</sub>, 0.01 M dithiothreitol, 0.02 M Tris-HCl (pH 7.6), tritiated *T*<sub>7</sub> DNA (5000 cpm), and 20–40 units of polynucleotide kinase or ligase. After incubation at 37° for

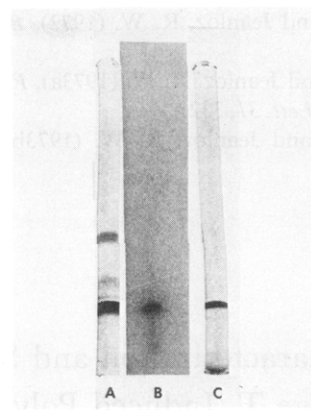


FIGURE 2: Sodium dodecyl sulfate gel electrophoresis of polynucleotide ligase and [<sup>32</sup>P]AMP-ligase. Gel electrophoresis was performed as described under Methods: (A) polynucleotide ligase fraction VI, 18 enzyme units, 5 μg of protein; (B) [<sup>32</sup>P]AMP-polynucleotide ligase (prepared as described under Methods), 18 enzyme units; (C) polynucleotide ligase fraction VII (from hydroxylapatite column), 16 enzyme units, 2 μg of protein.

30 min, the reaction mixture was chilled and made up to 0.05 M EDTA and 0.3 M sodium hydroxide. After addition of 2000–4000 cpm of <sup>32</sup>P-labeled *T*<sub>7</sub> DNA, the solution was layered on top of a 5–20% sucrose gradient (in 0.2 M sodium hydroxide–1 M NaCl–0.001 M EDTA) and centrifuged for 2.5 hr at 45,000 rpm in an SW 50.1 rotor. Fractions of the gradients were collected on GF/C filters, dried, and counted.

Polynucleotide kinase and ligase were also assayed for possible exonuclease contamination by two different methods.

(1) A 5'-<sup>32</sup>P-labeled decanucleotide was incubated with either enzyme under the usual assay conditions. Degradation of the oligonucleotide was followed by one of two methods: (a) aliquots were removed and analyzed by anion exchange paper chromatography (DE-81) in 0.35 M ammonium formate for the appearance of smaller oligonucleotides or (b) aliquots were subjected to electrophoresis on 19% polyacrylamide slab gels using the gel buffer 90 mM Tris-borate (pH 8.3)–7 M urea–4 mM EDTA.

(2) The standard assay mixture was made 20 μM in [<sup>3</sup>H]poly-[d(A-T)·d(A-T)] (sp act. ~20 mCi/mmol). Aliquots were removed at various times and mixed with 100 μl of calf thymus DNA (2 mg/ml) and then 100 μl of 15% ice-cold trichloroacetic acid was added. This solution was next centrifuged for 20 min at 10,000g. The supernatant was removed and counted in a liquid scintillation counter using the aqueous solvent system Unisolve 1 (Koch-Light).

**Protein Determinations.** The protein concentration was determined by one of two methods, that of Lowry *et al.* (1951) or that of Layne (1957).

#### Results

**Preparation of the Enzymes.** A flow diagram for the preparation of the three enzymes is shown in Figure 1. All procedures were carried out at 4°; centrifugations were at 10,000g for 15 min.

**Crude Extract.** *T*<sub>4</sub> amN82 phage infected *E. coli* cells were ground with alumina in a ratio of 1 g of cells to 2 g of alumina until a smooth paste resulted. The paste from 200 g of cells was suspended in 800 ml of TG buffer (50 mM Tris-HCl (pH 7.6) and 1 mM glutathione) and the extract collected by centrifugation. The paste was extracted twice more with 400 ml of TG buffer.

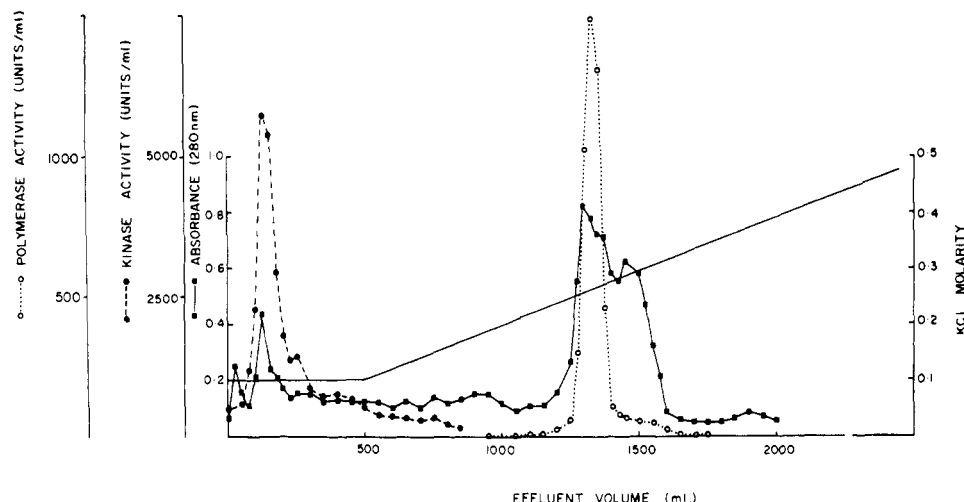


FIGURE 3: DEAE-Sephadex column chromatography of polynucleotide kinase and DNA polymerase. The ammonium sulfate fraction containing polynucleotide kinase and DNA polymerase activities was charged onto a DEAE-Sephadex A-50 column ( $3.5 \times 30$  cm) which had been preequilibrated with 0.01 M potassium phosphate (pH 7.5) containing 0.01 M 2-mercaptoethanol. The column was first washed with 500 ml of 0.1 M KCl in equilibrating buffer, followed by a gradient of 21 from 0.1 to 0.5 M KCl in equilibrating buffer. The fractions were assayed for polynucleotide kinase and DNA polymerase activity: (●) polynucleotide kinase activity; (■) absorbance (280 nm); (○) DNA polymerase activity.

**Streptomycin Fractionation.** This step separated the T<sub>4</sub> polynucleotide ligase from the other two enzymes. A small scale streptomycin sulfate titration using 5% streptomycin sulfate solution showed that no polynucleotide kinase or DNA polymerase activity was left in the supernatant at a concentration of 0.6–0.7% streptomycin sulfate. On the other hand, the precipitate contained both the polynucleotide kinase and DNA polymerase activity, but no polynucleotide ligase activity.

The bulk of the crude extract was brought to 0.7% streptomycin sulfate as described above. The supernatant containing the polynucleotide ligase was taken through the remainder of the steps for the ligase purification, while the pellet served for the polynucleotide kinase and DNA polymerase purification.

**Polynucleotide Ligase Purification.** The subsequent steps in the ligase purification were as described by Weiss *et al.* (1968) and included an ammonium sulfate fractionation (35–55% saturation), a DEAE-cellulose fractionation, DEAE-cellulose chromatography, and phosphocellulose chromatography. The specific activity and yields were also approximately the same as previously described (Weiss *et al.*, 1968).

In several preparations of polynucleotide ligase, the enzyme obtained from the phosphocellulose step (fraction VI) contained some nuclease as assayed by alkaline zone sedimentation of radioactive T<sub>7</sub> DNA (see Methods). When fraction VI of polynucleotide ligase was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the pattern shown in Figure 2A as obtained. The gel contained one major and three minor protein bands, the major band being the protein with the largest molecular weight. The <sup>32</sup>P-labeled ligase–AMP intermediate was prepared as described under Methods and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. As seen in Figure 2B, the intermediate had the same mobility on gel as the major protein band of Figure 2A. Therefore, the major protein band of fraction VI consisted of the polynucleotide ligase.

To further purify the polynucleotide ligase, chromatography on hydroxylapatite was carried out. A column ( $1.3 \times 10$  cm) of hydroxylapatite was washed with 0.5 M potassium phosphate buffer (pH 7.6) and then equilibrated with 0.01 M potassium phosphate containing 0.01 M 2-mercaptoethanol and 0.1 M KCl. Fraction VI was dialyzed against 0.01 M potassium phos-

phate (pH 7.6), 0.01 M 2-mercaptoethanol, 0.05 M KCl, and 5% glycerol, and subsequently charged onto the column at a flow rate of 50 ml/hr. The column was washed with equilibrating buffer and a gradient was run from 0 to 12.5% ammonium sulfate saturation in equilibrating buffer (total volume 180 ml). The enzyme eluted at 4.7% ammonium sulfate saturation as a single peak and was pooled and dialyzed against 0.01 M potassium phosphate (pH 7.6), in 50% glycerol containing 0.001 M dithiothreitol and 0.05 M KCl.

After this step, the enzyme was free from any deoxyribonuclease. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of this fraction showed a single protein band (Figure 2C). A 2.6-fold purification of the ligase was obtained during this step.

**Separation of the Kinase and the DNA Polymerase.** The pellet from the streptomycin sulfate precipitation was resuspended in 0.1 M potassium phosphate (pH 7.4)–0.01 M 2-mercaptoethanol with the help of a Teflon homogenizer, and autolysis was carried out until 95% of the ultraviolet-absorbing material at 260 nm had become acid soluble (Richardson *et al.*, 1964). After centrifugation, ammonium sulfate fractionation was carried out on the supernatant. Both the polynucleotide kinase and the DNA polymerase precipitated between 30 and 50% saturation of ammonium sulfate.

The two enzymes were separated by DEAE-Sephadex column chromatography, as shown in Figure 3. The resuspended ammonium sulfate precipitate was dialyzed against 0.01 M potassium phosphate (pH 7.5)–0.01 M 2-mercaptoethanol and charged on a DEAE-Sephadex column ( $3.5 \times 30$  cm) equilibrated with the dialysis buffer. The column was washed with equilibrating buffer until the optical density of the effluent dropped below 0.03, and the polynucleotide kinase was eluted with equilibrating buffer containing 0.1 M KCl. The DNA polymerase activity was then eluted by applying a gradient from 0.1 to 0.5 M KCl in equilibrating buffer. The T<sub>4</sub> DNA polymerase eluted from the column at 0.26 M KCl.

**Polynucleotide Kinase Purification.** The remaining steps in the polynucleotide kinase purification were as described by Richardson (1972). The specific activity and yield were also approximately as reported by this author. ATP (0.1 mM) was added to all solutions during the last two columns to stabilize

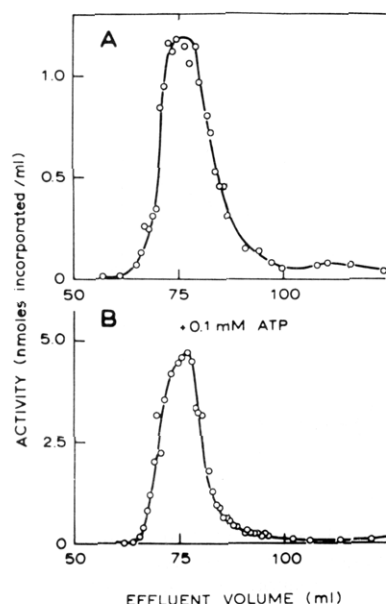


FIGURE 4: Gel filtration of  $T_4$  polynucleotide kinase. (A) Approximately 74 units of polynucleotide kinase were applied to a Sephadex G-200 column ( $1.4 \times 106$  cm) which had been equilibrated at  $4^\circ$  with 0.05 M Tris-HCl (pH 7.6), 0.005 M dithiothreitol, 0.1 M KCl, and 5% sucrose (w/v). The column was eluted with the equilibrating buffer and aliquots of each fraction were assayed for polynucleotide kinase activity as described under Methods. Approximately 20% of the applied activity was recovered. (B) Similar to the filtration above, 75 units of polynucleotide kinase were passed through the Sephadex G-200 column, but the equilibrating buffer contained also 0.1 mM ATP. Approximately 75% of the applied kinase activity was recovered from the column.

the enzyme (Wu and Kaiser, 1967). Some of the polynucleotide kinase preparations were contaminated by a nuclease which appeared to be a 3'-exonuclease. Thus, using  $^{32}\text{P}$ -labeled 5'-decanucleotides as substrates, electrophoresis on 19% gels (Methods) showed the formation of lower homologs.

The nuclease contamination could be removed by loading the enzyme (fraction VII), 10,000 enzyme units, on a phosphocellulose column (9 cm  $\times$  1 cm). The column was washed first with 50 ml of equilibration buffer (20 mM potassium phosphate (pH 7.0)–0.1 M KCl–2 mM dithiothreitol–50  $\mu\text{M}$  ATP–5% glycerol) and then eluted with a linear gradient of salt and pH as follows: 180 ml of equilibration buffer in the mixing vessel and 180 ml of equilibration buffer (pH 8) containing 0.5 M KCl in the reservoir.

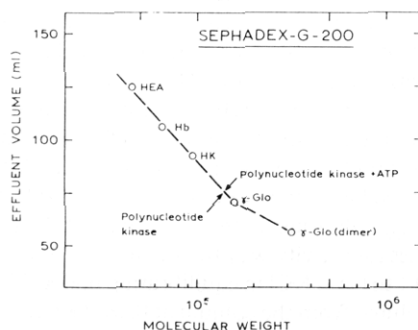


FIGURE 5: Elution volumes of protein standards used for molecular weight determination on a Sephadex G-200 column ( $1.4 \times 106$  cm): hen egg albumin (HEA), 43,000 daltons; hemoglobin (Hb), 64,500 daltons; yeast hexokinase (HK), 96,600 daltons;  $\gamma$ -globulin ( $\gamma$ -GLO), 156,000 daltons.



FIGURE 6: Sodium dodecyl sulfate gel electrophoresis of polynucleotide kinase. Sodium dodecyl sulfate gel electrophoresis was conducted as described under Methods with polynucleotide kinase, 270 enzyme units, 4  $\mu\text{g}$  of protein.

The trace of nuclease could also be separated from the kinase by gel filtration on a column of Sephadex G-100 ( $1.5 \times 96$  cm) equilibrated with 50 mM Tris (pH 7.6), 5 mM dithiothreitol, 0.1 mM ATP, and 5% sucrose. The kinase was excluded on this column whereas the nuclease was well included and the latter enzyme had a mol wt of  $\sim 40,000$  daltons. The nuclease did not degrade native or denatured  $T_7$  DNA. Poly[d(A-T)·d(A-T)] was, however, hydrolyzed, the products being di- and trioligonucleotides. Addition of spermine to a final concentration of 2 mM completely inhibited the nuclease activity, whereas the polynucleotide kinase activity increased. Further studies on the properties of the nuclease are in progress. These will be reported *in extenso* elsewhere.

**DNA Polymerase Purification.** The remaining steps in the DNA polymerase purification were those described by Goulian *et al.* (1968) and involved a phosphocellulose column chromatography and a Sephadex G-150 gel filtration.

**Molecular Weight Determination of Polynucleotide Ligase and Polynucleotide Kinase.** Two methods were used to determine the molecular weights of the two enzymes. The first method determined the size of the enzyme in native state by gel filtration through Sephadex G-100 or G-200 and assaying the column effluents for the particular enzyme activity. The second determination, under denaturing conditions, was carried out by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Shapiro and Maizel, 1969). The maximum errors by the two methods have been reported to be  $\pm 10$  and  $\pm 5\%$ , respectively (Andrews, 1964; Dunker and Rueckert, 1969).

The polynucleotide kinase activity eluted from a Sephadex G-200 column as a single peak of activity as shown in Figure 4. The molecular weight of the polynucleotide kinase was obtained after calibration of the column with several proteins of known molecular weight. As shown in Figure 5, a straight line was obtained by plotting the effluent volumes of the various marker proteins against the logarithm of their molecular weights. The mol wt of polynucleotide kinase obtained from the plot was  $140,000$  daltons  $\pm 10\%$ . Addition of ATP, a substrate, to the elution buffer did not make any difference in the value for the molecular weight of the polynucleotide kinase (Figure 5). The ATP did, however, protect the kinase from inactivation at low concentrations, which resulted in an increase in the yield of recovered polynucleotide kinase activity from 20 to 75%.

The polynucleotide kinase was also analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and only

one protein band was observed (Figure 6). The mobility of polynucleotide kinase on gels was compared to that of several proteins of known molecular weight. The relative mobilities of polynucleotide kinase and the marker proteins on sodium dodecyl sulfate gels were: polynucleotide kinase, 0.45; bovine serum albumin, 0.21; hen egg albumin, 0.35; pepsin 0.43; and pancreatic ribonuclease, 0.81. From the linear relationship between the mobilities of the marker proteins and their molecular weights, the mol wt of T<sub>4</sub> polynucleotide kinase under denaturing conditions was determined to be 33,000 daltons  $\pm$  5%. The large difference between the molecular weight found under native conditions (140,000 daltons) and denaturing conditions (33,000 daltons) suggests that polynucleotide kinase is a subunit enzyme consisting of four subunits of similar molecular weight. It is not yet known whether these subunits are identical or just different subunits with similar molecular weights.

The molecular weight of polynucleotide ligase was obtained from its elution volume on a Sephadex G-100 column. The elution pattern shown in Figure 7A indicates one major peak of ligase activity and at least one minor peak of activity. Gel filtration of the [<sup>3</sup>H]AMP-ligase intermediate on the same Sephadex G-100 column resulted in one major peak and one minor peak of the labeled intermediate (Figure 7B). The molecular weight of polynucleotide ligase was calculated by comparing its effluent volume, 72.9 ml (major peak) and 112.9 ml (minor peak), to that of several marker proteins: yeast hexokinase, 64.7 ml; hemoglobin, 76.5 ml; hen egg albumin, 83.5 ml; chymotrypsinogen, 97.6 ml; and horse heart cytochrome c, 107.0 ml. The mol wt of the major peak of ligase activity was 68,000 daltons  $\pm$  10% and of the minor peak about 10,000 daltons  $\pm$  10%. The same results were obtained from the elution pattern of the [<sup>3</sup>H]AMP-ligase intermediate.

A comparison of the mobility of polynucleotide ligase (Figure 2C) or the [<sup>32</sup>P]AMP-ligase intermediate (Figure 2B) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the mobilities of several other proteins gave a mol wt value of 63,000  $\pm$  5% daltons for polynucleotide ligase. Since the molecular weight determination of the enzyme gave similar values under either native or denatured conditions, 68,000 and 63,000 daltons, respectively, the polynucleotide ligase appears to consist of a single polypeptide chain. It is similar in this respect to the *E. coli* polynucleotide ligase which has been shown to consist of a single polypeptide chain with a mol wt of 75,000  $\pm$  4000 daltons (Modrich and Lehman, 1972).

## Discussion

Polynucleotide ligase is often used in conjunction with polynucleotide kinase. The simultaneous preparation of these enzymes and of the DNA polymerase from T<sub>4</sub>-infected *E. coli* cells should facilitate the work with these enzymes.

The polynucleotide ligase and kinase were physically characterized by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The polynucleotide kinase was found to have a mol wt of 140,000 daltons under native conditions (gel filtration through Sephadex G-200) and a mol wt of 33,000 daltons under denaturing conditions (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). This indicates that the enzyme acts as a subunit enzyme although no information is available to say whether the subunits are identical or simply have similar molecular weights. The polynucleotide ligase was found to have a similar molecular weight under either native or denatured conditions, 68,000 and 63,000 daltons, respec-

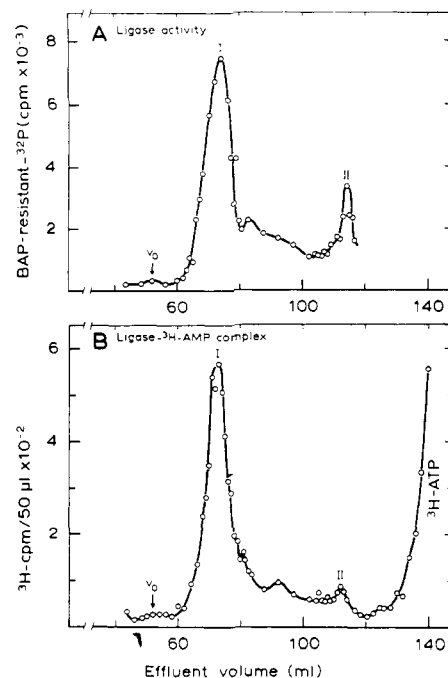


FIGURE 7: Gel filtration of T<sub>4</sub> polynucleotide ligase. (A) Approximately 700 units of T<sub>4</sub> polynucleotide ligase were applied to a Sephadex G-100 column (1.5  $\times$  90 cm) which had been equilibrated at 4° with 0.02 M Tris-KCl (pH 7.6), 0.01 M 2-mercaptoethanol, and 0.1 M KCl. The column was eluted with equilibrating buffer and aliquots of each fraction were assayed for polynucleotide ligase activity as described under Methods. Approximately 25% of the activity applied to the column was recovered. (B) The [<sup>3</sup>H]AMP-ligase intermediate was prepared as described under Methods. The AMP-ligase intermediate was passed through the Sephadex G-100 column under the same conditions as described above.

tively, indicating that the enzyme consists of a single polypeptide chain.

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## Reversal of Bacteriophage T<sub>4</sub> Induced Polynucleotide Kinase Action†

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**ABSTRACT:** The mechanism of action of T<sub>4</sub>-induced polynucleotide kinase on short single-stranded deoxyoligonucleotides has been investigated. It was found that the phosphorylation reaction catalyzed by T<sub>4</sub> polynucleotide kinase could be reversed. Thus, a 5'-<sup>32</sup>P-labeled deoxyoligonucleotide and ADP on incubation with the enzyme formed [ $\gamma$ -<sup>32</sup>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'-[ $\delta$ -<sup>32</sup>P]tetra-

phosphate. 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<sub>4</sub> polynucleotide kinase action, it was shown that deoxyoligonucleotides containing unlabeled phosphate at the 5' end could be quantitatively labeled with <sup>32</sup>P at the 5' end without prior removal of the unlabeled phosphate by phosphatase.

The bacteriophage T<sub>4</sub> induced polynucleotide kinase catalyzes the transfer of the  $\gamma$ -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  $\lambda$  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; Geftter *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. [<sup>3</sup>H]ADP was obtained from New England Nuclear Corporation. [ $\gamma$ -<sup>32</sup>P]ATP was prepared according to a published procedure (Glynn and Chappell, 1964).

**Enzymes.** Polynucleotide kinase was isolated from T<sub>4</sub> mN82 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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