Purification and Properties of Polynucleotide Phosphorylase from Escherichia coli*

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SUMMARY
A method for the purification of polynucleotide phosphorylase from Escherichia coli has been developed. The purified enzyme has a specific activity 700-fold higher than the crude extract. When enzyme fractions obtained at different stages of the purification were assayed by phosphorylation of polyadenylic acid (poly A) or 32P-orthophosphate exchange with the 5' phosphates of adenosine, uridine, cytidine, guanosine, and inosine, the degree of purification at each step was approximately the same.

Various factors affecting the exchange reactions between labeled orthophosphate and different ribonucleoside diphosphates were examined. An activating factor which enhances the rate of the ADP-Pi exchange reaction 3- to 6-fold was isolated during the purification of the enzyme. It affects only the exchange reaction and not the phosphorylation of poly A. It was found to be susceptible to proteolytic enzyme action, but not to RNase degradation.

The effect of the dinucleoside monophosphate ApA on the polymerization and exchange reactions was studied. At low Mg++ concentration, it could overcome the lag period in the polymerization of ADP and also stimulate the rate of the ADP-Pi exchange reaction.

The results presented in this report support the hypothesis that the polymerization and exchange reactions are catalyzed by the same enzyme. Their relation to the mechanism of the exchange reaction is discussed.

The enzyme polynucleotide phosphorylase (EC 2.7.7.8) catalyzes the polymerization of various ribonucleoside diphosphates and the reverse reaction, the phosphorolytic cleavage of polyribonucleotides, as well as an exchange reaction between 32P-orthophosphate and the β-phosphate of several ribonucleoside diphosphates. These reactions are summarized in the following two equations.

\[ n \text{ ribonucleoside-P-P} \rightleftharpoons (\text{ribonucleoside-P})_n + n \text{ P}_i \] (1)

Ribonucleoside-P-3P + 32P \rightleftharpoons ribonucleoside-P-32P + P_i \] (2)

The enzyme is widely distributed among different bacteria (1) and has been purified quite extensively from cells of Azotobacter agilis (2, 3), Micrococcus lysodeikticus (4), Clostridium perfringens (5), and Escherichia coli (6, 7). Littauer and Kornberg (6) described a purification procedure for an E. coli enzyme, by which 200-fold purification was achieved. A more thorough purification and some studies on the enzyme from E. coli and its properties are described in this report.

In a previous procedure for the purification of polynucleotide phosphorylase from E. coli cells (6), an “activating” factor for the ADP-32P exchange reaction was described. Such a factor was not reported for the same enzyme purified from A. agilis (3) or M. lysodeikticus (4), nor was it reported by Williams and Grunberg-Manago for the purification of E. coli enzyme (7). Examination of the ADP-32P exchange reaction carried out by the enzyme in the various steps of a new purification procedure led to the redemonstration of such a factor. Studies on some of the properties of this activating factor and its effect on the exchange reaction between 32P and various ribonucleoside diphosphates are described. The data presented also support the hypothesis that the reactions described by Equation 1 as well as by Equation 2 are catalyzed by the same single enzyme.

METHODS

Materials

ADP, UDP, GDP, CDP, and IDP were obtained from Sigma or Schwarz; 3H-UDP came from Schwarz. Poly A \(^1\) was purchased from Takamine Division of Miles Laboratories. 32P-Orthophosphate was purchased from the Radiochemical Centre, treated by boiling in 1 N HCl for 1 hour at 100°, and neutralized before use. Streptomycin sulfate, spermine, spermidine, putrescine, and Pronase, Grade B, were obtained from Calbiochem. Poly I. lyeine, poly L ornithine, poly L tyrosine, poly L glutamic

\(^1\) The abbreviations used are: poly A, polyadenylic acid; poly U, polyuridylic acid.
acid, and poly-L-hydroxyproline were obtained from Yeda Fine Chemicals, Rehovoth, Israel. Trypsin (once crystallized) and RNase (three times crystallized) were purchased from Worthington. ApA was purchased from Zellstofffabrik, Waldhof, Germany.

\[ {\text{14C}-}\text{Labeled RNA was isolated from } E. \text{ coli (pyrimidine-requiring mutant 6386) cells grown in the presence of } {\text{14C-uracil}} \]

(8). The ribosomal RNA was separated from the soluble RNA by chromatography on Sephadex G-200 columns.

**Columns**

DEAE-Sephadex and Sephadex G-200 were purchased from Pharmacia and processed as follows before use. DEAE-Sephadex A-25, medium size, was suspended in water, and fine particles were removed by suction. It was then washed successively with 0.5 \( \times \) NaOH, water, 0.5 \( \times \) HCl, water, 0.5 \( \times \) NaOH, and water until the pH was lower than 10. A column (8.5 \( \times \) 50 cm) was then packed with the suspension and washed with about 60 liters of Tris-HCl buffer (pH 7.4, 0.02 M) until the pH of the effluent reached a constant value of 7.4. The same procedure was applied for preparing the material for reuse. Sephadex G-200 was suspended twice in boiling Tris-HCl buffer (pH 8.0, 0.01 M) for 10 min, and the fine particles were then removed by suction. The material was equilibrated with 0.1 \( \times \) Tris-HCl buffer, pH 8.0. An open funnel was placed over the column, and a low hydrostatic pressure was kept during the operation of the column by raising the outlet tube to 10 cm below the liquid level in the funnel.

Hydroxyapatite was prepared according to Anacker and Stog (9). Care was taken to add the CaCl\(_2\) and KH\(_2\)PO\(_4\) solution at constant and equal rates of flow.

**Enzyme Assays**

**Assay I: Phosphorolysis of Poly A**—The reaction mixture (0.1 ml) contained the following (in micromoles): Tris-HCl buffer (pH 8.0), 10; MgCl\(_2\), 0.5; EDTA (pH 8.0), 0.05; \(^{32}\)P-potassium phosphate buffer (pH 8.0), 1 \( \times \) 10\(^4\) cpm per \( \mu \)mole of phosphate); polyadenylic acid, 0.1; and enzyme, 0.005 to 0.006 unit. In some experiments 25 \( \mu \)g of crystalline serum albumin were also added to the reaction mixture. Incubation at 37\(^\circ\)C was carried out for 15 min, after which the tubes were cooled and 0.9 ml of perchloric acid (1\%) was added. Extraction of inorganic phosphate was carried out as follows (10). Water saturated with isobutyl alcohol-benzene (1:1, v/v) was added to the reaction mixture to give a final volume of 2.5 ml, and 0.8 ml of ammonium molybdate reagent (5\% in 4 \( \times \) HCl) was then added. The phosphomolybdate complex was mixed on a Vortex mixer with 7 ml of isobutyl alcohol-benzene (1:1, v/v). The upper organic layer was removed by suction, and the extraction was repeated with 7 ml of isobutyl alcohol-benzene. Special care was taken to remove traces of the organic solvent. One-milliliter aliquots (out of a 3.3-ml total volume) were dried in copper planchets and counted in a gas flow counter. One unit of enzyme is defined as the amount of enzyme which exchanges 1 \( \mu \)mole of ADP in 1 hour.

When ADP exchange reaction was measured in the presence of the activator (6), the phosphate concentration was reduced to 0.125 or 0.250 \( \mu \)mole as indicated. In some experiments, the concentrated enzyme solution was diluted before assay in 0.02 \( \times \) Tris-HCl buffer (pH 8.3) containing 0.5 mg of crystalline bovine albumin per ml, and aliquots of this solution were removed for assay of enzyme activity. In such cases the reaction mixture contained 50 \( \mu \)g of serum albumin. When serum albumin was present in the reaction mixture (0.5 ml), the phosphate concentration was increased to 1.0 \( \mu \)mole in the absence of activator or 0.375 \( \mu \)mole in its presence. All the other concentrations and conditions were the same as described above.

When the exchange reaction was measured with UDP, CDP, IDP, or GDP, the phosphate concentration was changed to give optimal rates of reaction, as indicated.

**Assay II: Liberation of Inorganic Orthophosphate from ADP**—The reaction mixture (1.0 ml) contained the following (in micromoles): Tris-HCl buffer (pH 8.3), 10; MgCl\(_2\), 0.5; ADP, 1.0; and less than 0.1 unit of enzyme. After a 20-min incubation at 37\(^\circ\)C, the tubes were cooled in an ice bucket, and 0.5 ml of perchloric acid (3\%) was added. The suspension was centrifuged, and 0.3 ml of the supernatant was removed for inorganic orthophosphate determination according to the method of Chen, Toribara, and Warner (11). One unit of enzyme is defined as the amount of enzyme which liberates 1 \( \mu \)mole of Pi in 20 min.

**Assay IV**—This procedure was used to demonstrate the stimulation of the exchange reaction by ApA and is described under Fig. 5.

**Preparation of Activator Solution**

Ten milliliters of the ammonium sulfate fraction (see "Results") were heated in a water bath for 5 min at 100\(^\circ\)C. The precipitate formed was removed by centrifugation, and the clear supernatant was dialyzed against Tris-HCl buffer (pH 8.0, 0.01 M). In some cases this solution was mixed with 7 ml of 70\% phenol for 60 min at 20\(^\circ\)C. The suspension was cooled to 4\(^\circ\)C, then centrifuged at 12,000 \( \times \) g for 10 min at 4\(^\circ\)C, and the phenol phase was saved. To 5 ml of the phenol phase, 5 ml of ethanol and 5 ml of ethyl ether were added. After 30 min at 4\(^\circ\)C, the precipitate was collected by centrifugation, washed three times with ethanol, dissolved in 0.01 \( \times \) Tris-HCl buffer (pH 8.0), and dialyzed against 0.002 \( \times \) Tris-HCl buffer (pH 8.0) at 4\(^\circ\)C.

**Protein Determination**

Through the acetone step of the enzyme purification (Table 1), protein was determined by the method of Lowry et al. (12), with crystalline bovine serum albumin as a standard. Thereafter, it was measured either by the above phenol method or spectrophotometrically according to Warburg and Christian (13). These two methods gave almost identical results with purified enzyme fractions.

**Growth of Cells**

E. coli strain B cells were grown with forced aeration at 37\(^\circ\)C in a medium containing 2.18\% K\(_2\)HPO\(_4\), 1.7\% KH\(_2\)PO\(_4\), 1\%
yeast extract (Difco), and 1% glucose (O). The glucose was autoclaved separately and added to the cooled medium. Ten liters of the medium, in 20-liter bottles, were inoculated with 500 ml of an overnight culture and incubated at 37° with vigorous forced aeration. Growth was continued to the end of the exponential phase; the culture was then cooled to 10-15°, and the cells were harvested in a Sharples supercentrifuge. The yield was about 5 to 7 g of cells, wet weight, per liter of medium. Cells were kept frozen at -20° until they were used.

RESULTS

Purification of Enzyme

All the work described was performed at 0-4° unless otherwise stated.

Step 1: Crude Extract—Frozen cells (540 g) were thawed and suspended in 400 ml of Tris-HCl buffer (pH 7.4, 0.01 M) containing magnesium acetate (0.01 M) and DNase (0.5 μg per ml) at 4°. The suspension was homogenized in a Mantan-Gaulin homogenizer (Everett, Massachusetts) by recycling it four times at 4,000 p.s.i. The temperature was kept below 15° during the entire operation. After the homogenization, Tris-HCl buffer (pH 7.4, 0.01 M) containing magnesium acetate (0.01 M) was added to a final volume of 1.7 liters, and the solution was stirred for an additional 20 min at 4°. The suspension was centrifuged for 20 min at 16,000 × g, and the supernatant was decanted and saved (1,400 ml). The viscous precipitate was re-extracted by suspension in 300 ml of Tris-MgCl₂ buffer and then centrifuged once more for 10 min at 25,000 × g. The precipitate was discarded, and the two supernatant solutions were combined and used for the next step.

Step 2: Precipitation of Ribosomes—The combined supernatant solutions (1,700 ml) were centrifuged for 18 hours at 78,000 × g (Spinco No. 30 rotor) Under these conditions, the ribosomes and the relatively heavy enzyme precipitated toward the bottom of the tube. The upper, clear two-thirds of the supernatant solution was therefore removed by suction and discarded; the remaining brown supernatant, which contained the enzyme, was then decanted and saved. The pellet, which contained ribosomes and some enzyme, was re-extracted by suspending it in Tris-MgCl₂ buffer to a final volume of 850 ml with the use of a magnetic stirrer. The resuspension was complete in 6 hours.

(To facilitate the removal of the pellet, the centrifuge tubes containing the sticky pellet were cooled in Dry Ice and the frozen precipitate was removed with a spatula.) This suspension was centrifuged for 6 hours at 78,000 × g. The supernatant was decanted and combined with the supernatant kept from the first precipitation. The combined solution will be referred to as 78,000 × g supernatant.

Step 3: Streptomycin Precipitation and Ammonium Sulfate Fractionation—To 1,150 ml of the 78,000 × g supernatant, 57.5 ml of 10% streptomycin sulfate solution (adjusted to pH 7.0) were added dropwise with mechanical stirring. The stirring was continued for an additional 10 min, and the precipitate that formed was removed by centrifugation for 10 min at 10,000 × g.

To the supernatant (1,100 ml), a saturated solution of ammonium sulfate (760 ml) was added dropwise. The stirring was continued for an additional 30 min, and the precipitate that formed was removed by centrifugation for 10 min at 16,000 × g. To the supernatant, 530 ml of saturated ammonium sulfate solution were added dropwise. After 30 min of additional stirring, the precipitate was collected by centrifugation for 10 min at 16,000 × g. The pellet was then suspended in 100 ml of Tris-HCl buffer (pH 7.4, 0.02 M) and dialyzed for 15 to 20 hours against two successive 6-liter portions of Tris-HCl buffer (pH 7.4, 0.02 M).

Step 4: Autolysis—To the diylized ammonium sulfate fraction (146 ml), 16.2 ml of 1 M potassium phosphate buffer (pH 8.0) were added, and the solution was incubated at 37°. The autolysis was followed by removing 1-ml aliquots and determining the increase in A₅₆₅ of the acid-soluble (5% perchloric acid) fraction. The incubation was continued until the A₅₆₅ reached a constant value (2 to 4 hours). The solution was then cooled and centrifuged. The precipitate was discarded, and the supernatant was dialyzed against 6 liters of Tris-HCl buffer (pH 7.4, 0.02 M) at 4°.

Step 5: Acetone Fractionation—Sodium acetate buffer (8.4 ml, pH 5.7, 1.0 M) was added to the dialyzed solution (168 ml), followed by 111 ml of acetone (precooled to -20°), and the temperature was maintained at -5°. After additional stirring for 10 min, the precipitate was removed by centrifugation for 5 min at 20,000 × g at 4°. To the supernatant, 56 ml of acetone were added, and, after additional stirring for 10 min, the precipitate was collected by centrifugation for 10 min at 20,000 × g.

The pellet was then dissolved in Tris-HCl buffer (pH 7.4, 0.02 M), and the turbid solution was dialyzed for 12 hours against 6 liters of the same buffer (final volume, 255 ml).

Step 6: DEAE-Sephadex Chromatography—The enzyme solution (255 ml) was diluted to 400 ml with Tris-HCl buffer (pH 7.4, 0.02 M) and applied to a DEAE-Sephadex column (8.5 × 50 cm) previously equilibrated with Tris-HCl buffer (pH 7.4, 0.02 M). The column was washed first with 1 liter of Tris-HCl buffer (pH 7.4, 0.02 M), followed by NaCl solution (0.15 M) containing Tris-HCl buffer (pH 7.4, 0.02 M), until the A₂₆₀ of the effluent dropped to a value of 0.15 (after approximately 3 liters). The enzyme was then eluted with a linear gradient consisting of 3 liters of 0.15 M NaCl solution containing Tris-HCl buffer (pH 7.4, 0.02 M) and 3 liters of 0.4 M NaCl solution containing Tris buffer as above. Twenty-milliliter fractions were collected at a flow rate of 13 ml per min, and the elution of protein was followed by the A₂₆₀ of the effluent. The enzyme was eluted between 0.30 and 0.35 M NaCl. The fractions which had relatively high specific activity and contained about 40% of the total enzyme activity were combined (700 to 900 ml). The pooled solution was then dialyzed against 6 liters of an ammonium sulfate suspension (90 g per liter). Precipitation was generally completed within 4 to 6 hours. The precipitate formed was collected by centrifugation, dissolved in a minimal volume of Tris-HCl buffer (pH 8.0, 0.02 M), and dialyzed overnight against the same buffer (final volume, 4.8 ml).

Step 7: Sephadex G-200 Column—The enzyme solution was applied to a column (2.5 × 110 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.0. Five-milliliter fractions were collected, at a flow rate of 20 ml per hour. The enzyme appeared as a sharp peak, usually followed by another shoulder of polynucleotide phosphorylase activity. The peak tubes were pooled and concentrated by dialysis for 2 to 3 hours against ammonium sulfate suspension. The precipitate was collected by centrifugation, dissolved in a minimal volume of Tris-HCl buffer (pH 8.0, 0.02 M), and dialyzed overnight against the same buffer.

The purification procedure is summarized in Table I. The enzyme was purified about 700-fold (Sephadex G-200 peak
Purification of polynucleotide phosphorylase of E. coli

Phosphorolysis assay was used. The reaction mixture was as described for Assay I, and contained in addition 25 µg of serum albumin.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Yield</th>
<th>Specific activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>88,400 mg</td>
<td>13,200 units</td>
<td>100%</td>
<td>0.15 (1.0)</td>
</tr>
<tr>
<td>78,000 × g supernatant</td>
<td>44,800 mg</td>
<td>13,100 units</td>
<td>99%</td>
<td>0.29 (1.9)</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>16,100 mg</td>
<td>11,000 units</td>
<td>83%</td>
<td>0.68 (4.5)</td>
</tr>
<tr>
<td>Autolysate</td>
<td>13,300 mg</td>
<td>9,900 units</td>
<td>75%</td>
<td>0.74 (5.0)</td>
</tr>
<tr>
<td>Acetone</td>
<td>3,550 mg</td>
<td>8,100 units</td>
<td>61%</td>
<td>2.3 (15)</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>135 units</td>
<td>2,890 units</td>
<td>22%</td>
<td>21.3 (142)</td>
</tr>
</tbody>
</table>

### Comments on Purification Procedure

**Streptomycin Step**—Protamine was avoided during the development of this purification procedure, although it has been used in the previously published methods (6, 7). This was done for the following reasons: (a) it was found that several preparations of protamine contain nuclease activity; (b) the presence of protamine causes an activation of the exchange reaction; and (c) the enzyme yield with protamine was variable and not reproducible. Streptomycin, which can be readily removed by dialysis, was therefore preferred for precipitating nucleic acids.

**Ammonium Sulfate**—The enzyme is stable in non-neutralized solutions of ammonium sulfate, although it is unstable below pH 5.5 in the absence of ammonium sulfate.

**Sephadex G-200**—The main peak of polynucleotide phosphorylase activity is always followed by a shoulder of activity. One of the purification procedures tried during the course of this work involved autolysis of the crude extract for 5 hours at 37°C in 0.1 M phosphate buffer, pH 7.0, with further purification according to the method described above. This particular preparation, when chromatographed on Sephadex G-200, gave two distinct peaks of activity. This phenomenon has not been further examined.

**Hydroxylapatite Chromatography**—In some experiments the enzyme was also chromatographed on a hydroxylapatite column. The DEAE-Sephadex fraction was applied to the column (4 × 8 cm) at a flow rate of 2 ml per min. The column was then washed with 50 ml of Tris-HCl buffer (pH 7.4, 0.01 M), and the enzyme was eluted with a linear gradient consisting of 1 liter of Tris-HCl buffer (pH 7.4, 0.01 M) and 1 liter of neutralized ammonium sulfate solution (10%, w/v) containing the same buffer. a, enzymatic activity was assayed with 5-µl aliquots of each fraction. For the phosphorolysis of poly A (□ — □), the reaction mixture (see “Methods”) contained 1 µmole of ATP (0.01 M) and 1 liter of neutralized ammonium sulfate solution (10%, w/v) containing the same buffer. b, ribonucleoside diphosphate-SIPi exchange assay for UDP (□ — □), GDP (□ — □), and CDP (□ — □) was assayed with 3-µl aliquots of each fraction. For the UDP exchange reaction, the incubation mixture contained 1.25 and 0.50 µmole of **Pi, respectively (1.35 × 10^6 cpm per µmole of phosphate).** The liberation of Pi from ADP (□ — □) was assayed as described under “Methods.” a, optical density at 280 nm.
glutathione, 0.1 enzyme. After 5 days at 4°C in the presence of 1 mM reduced Higher enzyme activity was also obtained when the enzyme was solution (in 0.02 crystalline bovine albumin per ml stabilized the diluted enzyme of the dilute enzyme activity was lost. Addition of 0.5 mg of on freezing and thawing, over 95% week at 4°C and 25°C, respectively; after 2 months, 54% and 18% HCl, pH 8.3, Assay II) were less stable. The diluted enzyme ever, diluted enzyme solutions (0.25 unit per ml in 0.02 ATPase; 0.6 unit (Assay I) of Sephadex G-200 fraction liberated of Sephadex G-200, peak fraction catalyzed the formation of 2.4 mpmoles of ADP the enzyme was kept in 0.02 NaCl, 6 mM β-mercaptoethanol, or 1 mM M

Molecular Weight—By the schlieren method, sedimentation analysis of the G-200 peak fraction in the ultracentrifuge revealed a major component with an s20 value of about 8.2, comprising about 70% of the total protein. In addition, there was one minor peak with an s20 value of about 4.4. The main 8.2 S component contained all the enzymatic activity as judged from centrifugation in sucrose gradients or chromatography on Sephadex G-200 columns. From these studies a molecular weight of around 200,000 was estimated for this enzyme, which is in agreement with the observation of Williams and Grunberg-Manago (7).

Presence of Other Enzymes—RNase activity was detected in trace amounts only. Incubation of 14C-poly U (3.21 mmoles of nucleotides, 8,580 cpm) or 14C ribosomal RNA (7.65 mmoles of nucleotides, 10,300 cpm) in the presence of 3.7 units (Assay I) of Sephadex G-200 peak fraction resulted in the release of 230 mmoles of mononucleotides from poly U or 92 mmoles of mononucleotides from ribosomal RNA after a 40-min incubation in the presence of 0.05 M Tris-HCl buffer (pH 8.0) and 0.2 M NaCl at 37°C. When the reaction was carried out under conditions optimal for RNase II activity (16), 3.7 units of Sephadex G-200 peak fraction released 160 mmoles of mononucleotide from 13C-poly U or 180 mmoles from 13C-ribosomal RNA after a 40-min incubation in the presence of 0.01 M Tris-HCl buffer (pH 7.5), 0.5 mM MgCl2, and 0.1 M KC1 at 37°C. It should be noted that the amount of enzyme used in these assays was very high and was equivalent to the amount that liberates 9.57 mmoles of ADP from poly A in 40 min. Therefore, the amount of nuclease detected was negligible.

The enzyme preparation did not contain phosphatase or ATPase; 0.6 unit (Assay I) of Sephadex G-200 fraction liberated less than 10 mmoles of 32P1 from ATP-1-32P (2.25 x 106 cpm per μmole) (10, 15) after a 30-min incubation at 37°C. Adenylate kinase activity was very low; 1.0 unit (Assay I) of Sephadex G-200 fraction catalyzed the formation of 2.4 mmoles of ADP per hour from ATP and AMP when measured with a coupled pyruvate phosphokinase-lactate dehydrogenase system (17).

Stability of Enzyme—The concentrated Sephadex G-200 peak fraction retained more than 90% of its ADP-32P1 exchange activity after storage for 1 year at -20°C or for 5 days at 4°C. However, diluted enzyme solutions (0.25 unit per ml in 0.02 M Tris-HCl, pH 8.3, Assay II) were less stable. The diluted enzyme fraction retained 71% and 57% of its activity after storage for 1 week at 4°C and 25°C, respectively; after 2 months, 54% and 18% of the activity remained. On freezing and thawing, over 95% of the dilute enzyme activity was lost. Addition of 0.5 mg of crystalline bovine albumin per ml stabilized the diluted enzyme solution (in 0.02 M Tris-HCl, pH 8.3). Under these conditions full enzyme activity was retained after freezing and thawing. Higher enzyme activity was also obtained when the enzyme was assayed in the presence of serum albumin (see "Methods").

Several substances enhanced the inactivation of the undiluted enzyme. After 5 days at 4°C in the presence of 1 mM reduced glutathione, 0.1 mM NaCl, 6 mM β-mercaptoethanol, or 1 mM MgCl2, 18%, 22%, 36%, and 46% of the activity was lost, respectively. However, full enzyme activity was retained when the enzyme was kept in 0.02 M Tris-HCl buffer (pH 8.0) or 0.01 mM EDTA (adjusted to pH 8.0). Heating the enzyme for 5 min at 50°C in the presence of 0.1 mM MgCl2 caused complete inactivation. On the other hand, the presence of poly A (18) or transfer RNA protected the enzyme from inactivation even at 60°C.

### Table II

<table>
<thead>
<tr>
<th>Heated extract added</th>
<th>Amount of heated extract per reaction mixture</th>
<th>32P1 liberated</th>
<th>Stimulation of polymerization by heated extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>320</td>
<td>150</td>
<td>2.1</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>500</td>
<td>320</td>
<td>2.4</td>
</tr>
<tr>
<td>Sephadex G-200, peak fraction</td>
<td>150</td>
<td>70</td>
<td>3.4</td>
</tr>
<tr>
<td>34</td>
<td>510</td>
<td>120</td>
<td>1.9</td>
</tr>
<tr>
<td>85</td>
<td>230</td>
<td>90</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Determined prior to heating
* Determined prior to heating; Assay I

Heated extract added

MgCl2, 18%, 22%, 36%, and 46% of the activity was lost, respectively. However, full enzyme activity was retained when the enzyme was kept in 0.02 M Tris-HCl buffer (pH 8.0) or 0.01 mM EDTA (adjusted to pH 8.0). Heating the enzyme for 5 min at 50°C in the presence of 0.1 mM MgCl2 caused complete inactivation.

Contamination of Purified Enzyme with Oligonucleotides—The presence of contaminating oligonucleotide "primers" in the ammonium sulfate and Sephadex G-200 fractions was assayed by measuring the ability of heated extracts of the above to stimulate ADP polymerization under suboptimal conditions (see "Effect of ApA on Polymerization and Exchange of ADP"). As shown in Table II, there was a 7-fold stimulation of poly A synthesis on addition of a heated extract of the ammonium sulfate fractions (equivalent to 0.019 phosphorolysis unit prior to heating). On the other hand, the addition of an equivalent amount of a heated Sephadex G-200 extract caused very little stimulation. However, when the concentration of the heated Sephadex G-200 extract was increased 500-fold (equivalent to 9.5 phosphorolysis units prior to heating), a low stimulation was observed. These results indicate that the purest enzyme fraction still contained traces of contaminating oligonucleotide primers.

Specificity toward Different Ribonucleotide Diphosphates—Polynucleotide phosphorylase catalyzes the exchange of 32P1 with various ribonucleoside diphosphates (Equation 2). It was of interest to examine whether the various enzyme fractions obtained during the different steps of purification would show the same relative degree of purification when assayed by the exchange reaction with five different ribonucleoside diphosphates. Com-
The reaction mixture (0.5 ml) contained 100 μmoles of Tris-HCl buffer (pH 8.3), 0.4 μmole of ribonucleoside diphosphate, 0.2 μmole of MgCl₂, 0.001 to 0.05 unit of enzyme, 50 μg of serum albumin, and 0.01 ml of activator solution (36 μg of heated ammonium sulfate fraction). For the ADP, CDP, GDP, IDP, and UDP exchange reactions, the following amounts of ³²P₁ were added: 0.375, 0.35, 1.0, 0.25, and 0.20 μmoles, respectively (5 × 10⁶ cpm per μmole of phosphate).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ADP</th>
<th>CDP</th>
<th>GDP</th>
<th>IDP</th>
<th>UDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>0.28 (1.0)*</td>
<td>0.39 (1.0)</td>
<td>0.31 (1.0)</td>
<td>0.47 (1.0)</td>
<td>0.72 (1.0)</td>
</tr>
<tr>
<td>78,000 × g supernatant</td>
<td>0.57 (2.0)</td>
<td>0.60 (1.5)</td>
<td>0.56 (1.8)</td>
<td>0.98 (2.0)</td>
<td>1.5 (2.1)</td>
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<tr>
<td>Ammonium sulfate</td>
<td>1.5 (5.3)</td>
<td>1.5 (3.8)</td>
<td>1.3 (4.2)</td>
<td>2.3 (4.9)</td>
<td>3.6 (5.9)</td>
</tr>
<tr>
<td>Autolysate</td>
<td>1.6 (5.7)</td>
<td>1.8 (4.6)</td>
<td>1.5 (4.8)</td>
<td>2.6 (5.5)</td>
<td>4.1 (5.7)</td>
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<tr>
<td>Acetone</td>
<td>4.5 (10)</td>
<td>5.8 (14)</td>
<td>4.7 (15)</td>
<td>7.0 (16)</td>
<td>12 (17)</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>42 (150)</td>
<td>51 (131)</td>
<td>48 (155)</td>
<td>69 (147)</td>
<td>109 (151)</td>
</tr>
<tr>
<td>Sephadex G-200, peak fraction</td>
<td>146 (221)</td>
<td>200 (312)</td>
<td>157 (306)</td>
<td>265 (364)</td>
<td>413 (574)</td>
</tr>
</tbody>
</table>

* The relative purification values are given in parentheses.

The relative purification values are given in parentheses.

---

Fig. 2. Effect of cations on the rate of the ADP-³²P₁ exchange reaction. Experiments were carried out as described for Assay II. Each tube contained the standard reaction mixture, 3.8 × 10⁻³ unit of enzyme (Sephadex G-200 peak fraction; 100 units per mg of protein; Assay I), 50 μg of serum albumin, 1.0 μmole of ³²P₁, and the indicated cation concentrations: ○, MgCl₂; □, MnCl₂; ■, CdSO₄; △, CoCl₂; ▲, ZnSO₄; ●, 4 mM MgCl₂ and 0.2 mM MnCl₂; ◊, 2 mM MgCl₂ and 0.1 mM MnCl₂.

The reaction mixture, the optimal Mg ++ concentration was somewhat changed to 3 mM.

When present in low concentrations (0.09 mM), Mn ++ stimulated the exchange reaction to about 40% of the rate found with Mg ++. However, on addition of Mn ++ to a reaction mixture containing Mg ++, a slight inhibition of the ADP-³²P₁ exchange reaction was observed. The rate of the ADP-³²P₁ exchange was stimulated to only a small extent in the presence of Cd ++, Co ++, and Zn ++. No activity was detected when the enzyme was assayed in the presence of Ca ++ or Ni ++ in the concentration range of 0.01 to 0.4 mM.

Effect of Orthophosphate Concentrations on Ribonucleoside Diphosphate-³²P₁ Exchange Reaction—A study of the rate of ribonucleoside diphosphate-³²P₁ exchange as a function of P₁ concentration (Fig. 3) shows that the optimal concentration of orthophosphate varied for the different ribonucleoside diphosphates used (0.4 mM, 0.6 mM, 0.8 mM, 2.0 mM, and 2.5 mM for UDP, IDP, GDP, CDP, and ADP, respectively). The optimal molar ratio of ribonucleoside diphosphate over orthophosphate shifted from a value of 2.0 for UDP to 1.33 for IDP, 1.0 for GDP, 0.4 for CDP, and 0.32 for ADP. Slight variations in the optimal P₁ concentrations were noticed with different lots of commercially purchased ribonucleoside diphosphates or when serum albumin was omitted from the reaction mixture, although the over-all pattern remained the same. The optimal ribonucleoside diphosphate to P₁ ratios for UDP, IDP, CDP, and ADP are in close agreement with the values reported for the A. agilis enzyme (1, 2) while the GDP : P₁ value is similar to the value reported previously for the E. coli enzyme (19). The effect of P₁ concentrations on the rate of the exchange with UDP is similar to that found for UDP, a pyrimidine, rather than that found with the purine ribonucleotides, ADP and GDP. The rates of exchange at optimal P₁ concentrations were quite similar for ADP, CDP, IDP, and GDP, but were about twice as high when UDP was used.

Influence of Temperature on Ribonucleoside Diphosphate-³²P₁ Exchange Reaction—The effect of temperature on the rate of ³²P₁ exchange with ADP and UDP was examined at different orthophosphate concentrations. For the ADP-³²P₁ exchange reaction, the optimal orthophosphate concentration decreased markedly with temperature (Fig. 4), from 2.25 mM at 30° to 0.5 mM at 55°. When the UDP-³²P₁ exchange reaction was ex-
examined, the optimal orthophosphate concentration decreased too, but to a lesser extent, from 0.5 mM at 30° to 0.2 mM at 55°. In addition, the curve for the dependence of the rate of reaction on orthophosphate concentration with ADP and UDP was much broader at low temperatures than at higher ones. It was also noticed that at 50° the rate of exchange was almost identical for ADP and UDP.

Stimulation of Exchange Reaction

Activating Factor—In a previous study (6), an activating factor for the ADP-32P1 exchange reaction was described. This heat-stable factor was found to stimulate the rate of the ADP-32P1 exchange reaction, while the rate of polymer formation was unaffected. These observations were further examined by using a more extensively purified enzyme according to the procedure described above.

The effect of the activator on the rate of the ADP-32P1 exchange reaction was first examined at different Pi concentrations (Fig. 3). In the presence of the activator the optimal Pi concentration shifted from 2 mM to a lower value of 0.65 to 0.75 mM. The optimal molar ratio of ADP:Pi shifted accordingly, from 0.4 to about 1.1, and this higher optimal value was maintained at various ADP concentrations (0.8 to 3.2 mM).

At Pi concentrations lower than 1.3 mM, the rate of the exchange reaction was stimulated by the addition of the activator to the reaction mixture, and an apparent activation of the reaction was observed. These requirements were met by conditions of Assay C, which were used previously (6). On the other hand, at Pi concentrations greater than 1.3 mM, addition of the activator caused inhibition of the reaction. When serum albumin was absent from the reaction mixture, the same over-all picture was maintained, but the optimal Pi concentrations were lower (1.75 mM in the absence of the activator and 0.25 to 0.5 mM in its presence).

The effect of the activator on the 32Pi exchange with UDP, CDP, GDP, and IDP was also examined (Fig. 3). In contrast to its effect on the ADP-32P1 exchange reaction, addition of the activator caused almost no change in the optimal ribonucleoside diphosphate to orthophosphate ratio for UDP, CDP, and IDP. At optimal Pi concentrations, addition of the activator to the reaction mixture caused only a small stimulation of UDP-32Pi and IDP-32Pi exchange, but caused a slight inhibition of CDP-32Pi exchange. In the case of GDP, addition of the activator lowered the optimal Pi concentration from 2.5 mM to 1.8 mM. At this lower Pi concentration, the GDP-32Pi exchange was stimulated only 1.3-fold. When the Pi concentrations were increased above the optimal value, addition of the activator caused an apparent stimulation of the exchange reaction with UDP, CDP, IDP, and GDP.

As noted above, the ADP-32P1 exchange reaction was stimulated by the activator when assayed at low Pi concentrations. Under these conditions, the stimulation of the ADP-32P1 exchange reaction by the activator was assayed with the different enzyme fractions obtained during the different stages of purification. As shown in Table IV, the degree of activation was found to rise during the purification of the enzyme, indicating that the activator was removed during the purification procedure. On the other hand, the activator had no effect on the rate of poly A phosphorolysis.
Table IV
Effect of activator on ADP-32P exchange

Experiments were carried out as described for Assay II. Each tube contained the standard reaction mixture, 50 µg of serum albumin, 0.375 µmole of 32P-ADP (4.0 × 10^4 cpm per µmole), and 0.01 µmole of activator solution (3.6 mg of protein per ml of heated ammonium sulfate fraction) where indicated.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Activation of ADP exchange*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With activator</td>
<td>Without activator</td>
</tr>
<tr>
<td></td>
<td>units/mg protein</td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>0.35</td>
<td>0.47</td>
</tr>
<tr>
<td>78,000 x g supernatant</td>
<td>0.67</td>
<td>0.72</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>1.4</td>
<td>0.75</td>
</tr>
<tr>
<td>Autolysate</td>
<td>1.5</td>
<td>0.72</td>
</tr>
<tr>
<td>Acetone</td>
<td>4.2</td>
<td>1.7</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>38</td>
<td>13</td>
</tr>
<tr>
<td>Sephadex G-200, peak fraction</td>
<td>195</td>
<td>58</td>
</tr>
</tbody>
</table>

* Ratio of activity of ADP-32P exchange in the presence of activator to the exchange in its absence.

Properties of Activator—Some properties of the activator solution were examined. The activating factor is nondenatured and is not destroyed after heating for 5 min at 100°C. The same degree of activation was obtained when the ammonium sulfate fraction was kept at 5 min at 60°C, 70°C, or 100°C. For different activator preparations, the maximal stimulation of the ADP-32P exchange reaction varied between 3- and 6-fold. The activator could be separated from the enzyme by passing the ammonium sulfate fraction through a Sephadex G-200 column. The enzyme appeared in the first protein shoulder eluted from the column, while the activator was retarded and eluted later. The position of the activator on the Sephadex G-200 chromatogram indicated that it has a relatively low molecular weight. On electrophoresis in Geon (22 hours at 7 volts per cm in 0.02 M Tris-HCl, pH 8.0), the activator appeared as a heterogeneous mixture of substances, most of which migrated toward the cathode.

Treatment of the heated ammonium sulfate fraction with phenol revealed that this factor was recovered in the phenol phase (see “Methods”). After this treatment, the activator was somewhat heat-labile. When this fraction was kept at 60°C, 80°C, or 100°C for 5 min, 100%, 72%, and 54% of the stimulating activity was recovered, respectively. The phenol treatment probably removed some bound nucleic acids from the activator, thereby rendering it more heat-labile.

The fact that the activator was found in the phenol phase, like a protein, coupled with the finding that incubation with RNase did not affect the activating capacity of the ADP-32P exchange reaction, led to treatment of the activator solution with Pronase or trypsin. It was found that this treatment destroyed its ability to stimulate the ADP-32P exchange reaction (Table V).

These experiments indicated that the activation of the enzyme preparation with the activator was due to a polypeptide nature of the activator. The ability of various synthetic polypeptide and polynucleotides to stimulate the rate of the ADP-32P exchange reaction was studied.

Of those tested, spermine and spermidine (10^-4 to 10^-3 M) activated the reaction about 2 fold, whereas poly-L-lysine and poly-L-ornithine (10^-5 M) hardly affected it, and even caused inhibition at lower or higher concentrations. Neutral polyamino acids had no effect. The highest stimulation was observed when the “natural activator” was added to the reaction mixture.

Effect of ApA on Polymerization and Exchange of ADP

It was shown that at relatively low Mg++ concentrations, the formation of polymers from ribonucleotide diphosphates occurred only after an initial lag period. In the presence of polyribonucleotides or short oligonucleotides, this lag period was almost abolished (3, 7, 20, 21). A similar observation was made with the present purified enzyme preparation.

ApA, a dinucleoside monophosphate, does not undergo phosphorylation by polynucleotide phosphorylase (22). This simple primer was therefore chosen to study the effect of oligonucleotides in abolishing the lag in the polymerization reaction. As shown in Table VI, there was no stimulation of ADP polymerization by ApA.
upon addition of ApA to the ammonium sulfate fraction as compared with polymerization in its absence. ApA did stimulate the polymerization reaction after the enzyme had passed through a DEAE-Sephadex column. This stimulation reached a value of 10- to 14-fold in the peak Sephadex G-200 fraction. The acetone

fraction showed the lowest rate of polymerization in the presence of ApA, although an equal amount of enzyme was added with all enzyme fractions, as measured with the phosphorolysis assay.

Under the standard conditions (Assay II), ApA virtually did not stimulate the rate of the ADP-32P1 exchange when tested at a wide range of phosphate concentrations. However, when the conditions of the reaction were changed to those used to demonstrate the stimulation exerted by ApA on the polymerization of ADP (Assay IV, except that 32P1 was added to the reaction mixture), a 2.5- to 3.5-fold stimulation of the exchange reaction could be observed (Fig. 5). The differences between Assays II and IV are the following: P1 concentration is increased from 1.75 mM to 75 mM; ADP concentration is increased from 0.6 mM to 25 mM; MgCl2 concentration is decreased from 4 mM to 1 mM; and enzyme concentration is increased 250-fold from 3.8 × 10⁻⁴ unit to 0.95 unit (Assay I). Under the conditions of Assay II, the exchange reaction was proportional to enzyme concentration and to time of incubation for at least 40 min. By contrast, under the conditions of Assay IV, an initial lag of the exchange reaction was observed, this lag was greatly reduced (but not abolished) when ApA was present in the reaction mixture.

The conditions of Assay IV did not afford maximal reaction rates; in addition, owing to the initial lag of the ADP-32P1 exchange, the activity determined was not a linear measure of the enzyme concentration. It should also be noted that, under the conditions of Assay IV, addition of the activator did not stimulate the exchange reaction.

### DISCUSSION

The present studies describe a simple, fast, and reproducible method for the preparation of *E. coli* polynucleotide phosphorylase. The specific activity of the purified fraction is 700-fold higher than that of the crude extract, with a 10 to 15% yield. The purified enzyme fraction may still contain a low amount of nucleic acid-like material, since the $A_{260}/A_{280}$ ratio is about 1.5. The lag period in the polymerization of ADP, occurring at low concentrations of Mg²⁺ (3, 7, 20, 21), was largely overcome in our experiments by adding trace amounts of a dinucleoside monophosphate, ApA. The data in Table VI indicate that addition of ApA did not affect the rate of ADP polymerization when the reaction was carried out with a relatively crude enzyme, whereas a 10-fold stimulation was seen with the Sephadex G-200 peak fraction. However, addition of relatively large amounts of heated G-200 fractions to the reaction mixture did stimulate the ADP polymerization, thus indicating that the purified enzyme (after Sephadex G-200 column chromatography) still contained trace amounts of oligonucleotides (Table II).

Chromatography of the DEAE-Sephadex fraction on a hydroxylapatite column revealed two distinct peaks showing polynucleotide phosphorylase activity. This phenomenon was carefully studied in order to examine a possible separation of exchange and phosphorolysis activities or a possible separation of the exchange activities toward various ribonucleoside diphosphates. The results shown in Fig. 1 indicate that such differences were not found in any of the assays used. The possibility of a monomer-dimer separation was also examined. This was done by comparing the sedimentation velocities of the enzyme in the two protein peaks by centrifugation in a linear density sucrose gradient (with alcohol dehydrogenase as a marker) or chromatography on identical Sephadex G-200 columns. The

### Table VI

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Amount of enzyme per reaction mixture</th>
<th>P1 liberated</th>
<th>Stimilation of polymerization by ApA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ug</td>
<td>nmoles</td>
<td>-fold</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>2750</td>
<td>1.9</td>
<td>750 740</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.99</td>
</tr>
<tr>
<td>Acetone</td>
<td>834</td>
<td>1.9</td>
<td>450 360</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>DEAE-Sephadex G-200 peak</td>
<td>65</td>
<td>1.6</td>
<td>860 100</td>
</tr>
<tr>
<td>fraction</td>
<td></td>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>1.9</td>
<td>800 79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Assay I.
results showed that the enzyme activity found in both peaks was associated with molecules of the same size. One explanation for this phenomenon is that the DEAE-Sephadex fraction still contained small amounts of oligonucleotides of heterogeneous composition, mediating the binding of the enzyme to the column. The decrease of the $A_{260}$/$A_{280}$ ratio (Fig. 1) in the enzyme region may substantiate this explanation.

These findings and the data presented in Tables I and II support the hypothesis that a single enzyme carries out the phosphorolysis of poly A as well as the exchange of orthophosphate with the $\beta$-phosphate of different ribonucleoside diphosphates.

The study of the rate of ribonucleoside diphosphate-$\Pi$ exchange as a function of orthophosphate concentration (Fig. 3) shows that the optimal concentration of orthophosphate varies for different ribonucleoside diphosphates. Apparently the interaction of the various ribonucleoside diphosphates with the active site (or sites) of the enzyme differs according to the ribonucleoside diphosphate used. This is also illustrated when the effect of temperature on the rate and optimal orthophosphate concentration for ADP-$\Pi$P; and UDP-$\Pi$P; exchange reactions are compared (Fig. 4).

Examination of the ADP-$\Pi$P; exchange reaction carried out by the enzyme in the various stages of the enzyme purification led to the redemonstration of an activation factor (6). In the presence of this activator, the optimal orthophosphate concentration for ADP-$\Pi$P; exchange shifted from 2 mm to a lower value of 0.65 to 0.75 mm. At low phosphate concentrations, the activator caused a 3- to 6-fold stimulation of the exchange reaction when assayed with the more purified enzyme fractions. The preliminary results suggest that the activator is of peptide nature, since it is susceptible to digestion with Pronase or trypsin and is resistant to degradation by RNase. The difference in the effect of the activator on the rate of the ADP-$\Pi$P; exchange compared to that found with other ribonucleoside diphosphates should be noted. No explanation can be suggested at present for this phenomenon.

Two mechanisms were considered for the ribonucleoside diphosphate-$\Pi$P; exchange reaction: (I) that the observed exchange reflects a reversible formation of a ribonucleoside monophosphate-enzyme complex and is independent of polymer formation; or (II) that the exchange is the result of polymerization-phosphorylase reactions occurring under approximately equilibrium conditions (1, 19, 23).

In the case of the $M$. lysodeikticus enzyme, the data (4, 23) support the hypothesis that the reaction exchange reflects a reversible polymerization-phosphorolysis reaction (Mechanism II).

The situation is more complex with $E$. coli polynucleotide phosphorylase. No single activator was found to activate all the reactions catalyzed by the enzyme when the reactions were assayed under identical conditions. Furthermore, it is obvious from the data presented that several factors affected the phosphorylation-polymerization reaction and the exchange reaction to different extents. ApA and short oligonucleotides overcame a lag period in polymerization of ADP, under conditions wherein the so-called activator had no effect. On the other hand, only the ADP-$\Pi$P; exchange reaction was stimulated by this protease-sensitive material, which did not affect the rate of polymer formation (6) or the phosphorolysis of poly A. These results therefore suggest Mechanism I for the exchange reaction. However, before these results can be accepted as indicating the formation of an AMP-enzyme complex, the enzyme should be completely free of any contaminating oligonucleotides. As shown above, trace amounts of oligonucleotides still contaminate the $E$. coli enzyme. The observations that under suboptimal conditions (Assay IV) ApA stimulated the ADP-$\Pi$P; exchange as well as the polymerization reaction, and that under these conditions the presence of the activator did not stimulate the exchange reaction, show a similar pattern for these two reactions, which would support the postulation of a common step for the exchange and polymerization reaction. This, in turn, would favor Mechanism II. However, the stimulation of the rate of ADP polymerization when ApA was used to overcome the lag period was relatively greater than the increase observed in the rate of ADP-$\Pi$P; exchange.

In view of the results presented in this report, it seems that conclusions concerning the relation between the exchange mechanism and the polymerization-phosphorylase reaction depend very much on the assay conditions used, and further work is necessary to understand this relation fully.

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Purification and Properties of Polynucleotide Phosphorylase from *Escherichia coli*

Yosef Kimhi and Uriel Z. Littauer


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