The Dependence of Reactions Catalyzed by Polynucleotide Phosphorylase on Oligonucleotides

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(Received for publication, July 14, 1961)

The bacterial enzyme, polynucleotide phosphorylase, catalyzes the following reactions: the synthesis of long chain polyribonucleotides from ribonucleoside diphosphates (Equation 1); the phosphorolytic cleavage of polyribonucleotides (reverse of Equation 1); and an exchange reaction between P$_{32}$ and the terminal phosphate of a nucleoside diphosphate (Equation 2). Recent review articles by Grunberg-Manago (1) and by Khorana (2) summarize our knowledge of the mechanism, specificity, and properties of enzyme preparations from several sources, and the purification procedures that have been reported.

Previous studies by Singer, Heppel, and Hilmoe (3, 4) led to the suggestion that polynucleotide phosphorylase may be unable to catalyze the initiation of new polyribonucleotide chains, rather than to catalyze the elongation of preformed polynucleotide primers. The relation of the exchange reaction (Equation 2) to the mechanism of the over-all reaction (Equation 1) has been discussed by Grunberg-Manago (1) and by Singer, Hilmoe, and Grunberg-Manago (5). Thus, although the lag in the polymerization of adenosine diphosphate, uridine diphosphate, and cytidine diphosphate noted with highly purified preparations of enzyme from Azotobacter agilis is overcome by certain oligonucleotides (3), the exchange reaction, with the same enzyme preparations, is stimulated only slightly by these compounds (5). Further, whereas relatively crude preparations from Azotobacter agilis or Escherichia coli polymerize guanosine diphosphate only when an oligonucleotide with an unesterified terminal C-3' hydroxyl group is present (4), guanosine diphosphate participates actively in the exchange reaction in the absence of such compounds (5). On the other hand, Littauer and Kornberg (6), with Escherichia coli enzyme preparations, described the activation of the adenosine diphosphate-P$_{32}$ exchange by a relatively heat-stable fraction having no effect on adenosine diphosphate polymerization. It is obvious that these experiments do not allow a clear description of the mechanism of the exchange reaction.

In certain instances the data obtained with enzyme preparations from Micrococcus lysodeikiticus differ from those described above, and Beers (7) has suggested that the observed stimulation of polymerization by oligonucleotides is actually a reversal of inhibition of the enzyme by degraded ribonucleic acid which contaminates the enzyme preparation.

A resolution of the conflicting results and interpretations and a detailed description of the mechanism of this complex reaction depend on obtaining highly purified enzyme preparations that are free of nucleic acid. In this laboratory, experiments along this line are in progress, and this paper records some of the initial data obtained. In particular, the catalysis of both the exchange reaction (Equation 2) and polymerization (Equation 1) by the enzyme preparations described below is greatly stimulated by the presence of oligonucleotides with unesterified, terminal C-3' hydroxyl groups. Under suitable conditions, as in Assay B (below), the two reactions are almost completely dependent on oligonucleotide addition. However, this dependence can be largely eliminated by apparently minor changes in the pH of the reaction medium or the concentrations of the substrates, as in Assay C (below).

Because of difficulties encountered in reproducing the Azotobacter agilis preparations (8, 9) and our unsuccessful attempts to free highly purified Azotobacter agilis preparations of nucleic acid, an alternative enzyme source was sought. Beers (10) described a procedure for obtaining extracts of Micrococcus lysodeikticus that are rich in polynucleotide phosphorylase but low in nucleic acid content, and we chose that microorganism as a starting material.

**EXPERIMENTAL PROCEDURE**

**Materials**

The Li salts of ADP and GDP were obtained from Schwarz BioResearch, Inc., and used as such. UDP and CDP were Na salts from Sigma Chemical Company. Poly A, poly U, and poly C were prepared by published procedures (11) with the use of enzyme preparations from E. coli and M. lysodeikticus. Poly A was also obtained commercially from the Takamine Division of Miles Laboratories. Polymer concentrations are expressed as moles of polymer phosphate. Spray-dried M. lysodeikticus was purchased from Miles Laboratories.

Oligonucleotides such as pApA and its homologues, which have phosphate monoesterified to the C-5' hydroxyl of one terminal adenosine residue and unesterified, terminal C-2' and C-3' hydroxyl groups, were prepared by the digestion of poly A with a nuclease from pork liver nuclei (12). The resulting mixture of oligonucleotides was separated into its components by paper chromatography.

1 The abbreviations used are: poly A, polyadenylic acid; poly U, polyuridylic acid; poly C, polycytidylic acid, for the polymers synthesized with polynucleotide phosphorylase. S.A., specific activity.
chromatography on DEAE-cellulose columns by Dr. H. A. Sober (13, 14). The chain length of each oligonucleotide was ascertained by measuring the ratio of total organic phosphate to phosphate released by purified E. coli alkaline phosphatase (15–17). The purity of each compound was also checked by paper chromatography (3, 12). Oligonucleotide concentrations are expressed as moles of compound, not organic phosphate.

Oligonucleotides such as ApAp and its homologues, which have phosphate monoesterified to the C-3′ (or C-2′ and C-3′) hydroxyl of one terminal adenosine residue, and an unesterified, terminal C-3′ hydroxyl group, were prepared by digestion of poly A with a nuclease from Staphylococcus aureus (12) or by controlled alkaline digestion of poly A (18). The compounds were isolated by paper chromatography (3) and characterized as described previously (3, 12).

Crystalline lysozyme and E. coli alkaline phosphatase were purchased from Worthington Biochemical Corporation.

Analytical Procedures

Pₐ, was determined by the method of Fiske and SubbaRow (19). Total organic phosphorus was determined by a procedure described by Ames and Dubin (20), except that 0.5 N HCl was used in place of 1 N HCl. Protein was determined by the method of Lowry et al. (21). A Beckman model DU spectrophotometer was used. The ratio of A at 280 μm to that at 260 μm (referred to as 280:260 ratio) of various enzyme solutions was determined at suitable dilutions in 0.1 N NaCl. Counting of radioactive samples was done on a thin window gas flow counter.

Enzyme Assays

Polynucleotide phosphorylase activity was measured in several ways. The various procedures are below, and each is designated by a capital letter.

Assay A—This assay measures the formation of P₃²-labeled ADP from the phosphorolytic cleavage of poly A in the presence of Pₐ, (reverse of Equation 1) (11) and was used to follow enzymatic activity during the purification procedure. It is evident from the experiments reported below that this assay is the standard of reference for enzyme activity, as there is no apparent primer requirement.

Reaction mixtures (0.1 ml) contained 0.1 M Tris buffer, pH 8.2, 5 mM MgCl₂, 0.01 M K₂HP₃₀₄O₄ (approximately 25,000 c. p. m. per μmole), 0.5 mM EDTA, 1 mM poly A, and enzyme. After 15 minutes of incubation at 37°, the reaction was stopped and nucleotides were adsorbed onto charcoal by adding 1 ml of a cold suspension of charcoal in HClO₄ (10 parts of 2.5% HClO₄ plus 1 part of a 10% (weight per weight) aqueous suspension of acid-washed Norit A). After 10 minutes at 0° with occasional stirring, the charcoal suspension was washed onto a Millipore filter (pore size, 0.45 μ; disk diameter, 25 mm), and washed three times, with suction, each time with approximately 15 ml of cold distilled water. The entire filter was placed upside down on a copper planchet, dried under an infrared lamp, and counted. The self-absorption correction for such samples was less than 10% and was ignored. A blank without enzyme was run with each set of assays and its value subtracted from each sample. One unit of activity in this phosphorolysis assay ([U]ₚ,ₚ) is equivalent to the incorporation of 1 μmole of Pₐ, into charcoal-adsorbable material per 15 minutes, under the conditions described. Specific enzyme activity is defined as [U]ₚ,ₚ per milligram of protein. Under the conditions described, the extent of phosphorolysis is proportional to enzyme concentration and to time of incubation (Fig. 1). The pH optimum is from 8.1 to 8.7 (in Tris buffer).

Assay B—This procedure determines the exchange between ADP and Pₐ, (Equation 2) under conditions found to be optimal with A. agilis polynucleotide phosphorylase (22). Although the best conditions for the M. lysodeikticus enzyme are different (see Assay C and below), this procedure was useful in studying the stimulation of the exchange reaction by oligonucleotides.

Reaction mixtures (0.1 ml) contained Tris buffer, MgCl₂, and EDTA as for Assay A, 3.5 mM K₂HP₃₀₄O₄, 2.5 mM ADP, and enzyme. The procedure for incubation and the preparation of samples for counting were the same as described for Assay A. The amount of Pₐ, incorporated into charcoal-adsorbable material was calculated from the expression (22)

\[
\frac{\text{c. p. m. incorporated}}{\text{(c. p. m. in reaction)}/(\text{μmole Pₐ} + \text{μmole ADP})}
\]

One unit of activity in this exchange assay ([U]ₚ,ₚ) is equivalent to the incorporation of one μmole of Pₐ, into ADP under the conditions described.

Assay C—This procedure also determines the exchange reaction (Equation 2) between ADP and Pₐ, but under conditions found optimal for the M. lysodeikticus preparations. Under these conditions the stimulation of exchange by oligonucleotides is much less than with Assay B (see below). The reaction mixtures were similar to those described for Assay B, with the following changes: pH 8.7; 3.8 mM ADP; 2.5 mM K₂HP₃₀₄O₄. The procedure was as described for Assay B. A unit of enzyme
\textbf{TABLE I}

Partial purification of polynucleotide phosphorylase from \textit{M. lysodeikticus}

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Total $\mu$P $/\mu$g</th>
<th>280:260 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract*</td>
<td>2.8</td>
<td>0.17</td>
<td>460</td>
</tr>
<tr>
<td>II. (NH$_4$)$_2$SO$_4$ (30-65%)</td>
<td>23.9</td>
<td>0.44</td>
<td>1020</td>
</tr>
<tr>
<td>III. (NH$_4$)$_2$SO$_4$ (43-57%)</td>
<td>11.3</td>
<td>0.24</td>
<td>619</td>
</tr>
<tr>
<td>IV. Protamine eluate</td>
<td>8.9</td>
<td>0.73</td>
<td>351</td>
</tr>
<tr>
<td>V. Protamine eluate, dialyzed</td>
<td>5.9</td>
<td>1.3†</td>
<td>450</td>
</tr>
<tr>
<td>VI. (NH$_4$)$_2$SO$_4$ (40-60%), pH 6.3</td>
<td>12.3</td>
<td>0.84</td>
<td>185</td>
</tr>
</tbody>
</table>

* A sample of the crude extract was dialyzed against H$_2$O before assay.
† This value is unusually high. The specific activity of dialyzed protamine eluate is generally the same as that of undialyzed material.

($\%$ exchange) is defined as for Assay B. Under these conditions the exchange reaction is proportional to enzyme concentration; with 8, 16, 24, 32, and 40 $\mu$g of a Fraction VI preparation (see below) per reaction mixture, 9.0, 20, 30 and 39, and 51 mmoles, respectively, of P$_{32}$ were incorporated. The pH optimum was at 8.7 in both Tris and glycine buffers.

\textit{Assay D}—This procedure measures the polymerization of nucleoside dinucleotides (Equation 1) by determining the release of P$_{32}$. Reaction mixtures are described in the text. Incubations were carried out at 37° for the indicated times. Reactions were stopped by the addition of cold HClO$_4$ (final concentration, 2.5%), and after 10 minutes at 0°, insoluble protein and polymer were removed by centrifugation. Suitable aliquots of the supernatant solutions were taken for P$_{32}$ determination.

\textbf{Purification Procedure}

The procedures for lysis of the cells and the first two (NH$_4$)$_2$SO$_4$ fractions were described by Beers and Steiner (10, 23) and Steiner and Beers (24). After lysis of a 10% suspension of 40 $g$ of spray-dried \textit{M. lysodeikticus} in 0.5% NaCl at pH 8.0, 37°, by 100 mg of lysozyme, 200 ml of saturated (NH$_4$)$_2$SO$_4$ are added, and the suspension is centrifuged for 15 minutes at 20,000 $\times$ g at 4°. A clear, yellow-brown supernatant fluid is obtained (410 to 440 ml) (Fraction I, Table I). The volume of Fraction I is a good indication of the success of the lysis step (24). Because of the critical nature of the lysis procedure (10), it is not practical to work up larger batches of cells or several batches simultaneously, although several lots of Fraction I may be combined at this point. The crude extract is brought to 65% saturation with solid (NH$_4$)$_2$SO$_4$, and the precipitate is collected by centrifugation, as above, and dissolved in 0.1 $M$ Tris buffer, pH 8.1. The resulting solution is dialyzed against cold distilled H$_2$O until free of (NH$_4$)$_2$SO$_4$ (Fraction II). It is possible to lyophilize Fraction II (23) but we have routinely stored it frozen. The 280:260 ratio of Fraction II is approximately 1.0. Lower 280:260 ratios indicate improper lysis (24).

Fraction II is diluted to 10 mg of protein per ml, and made 0.1 $M$ in Tris buffer, pH 8.1. The fraction insoluble between 43 and 57% saturation with (NH$_4$)$_2$SO$_4$ is collected as above, dissolved in 0.1 $M$ Tris buffer, pH 8.1, and dialyzed against 0.1 $M$ Tris, pH 8.1 - 0.001 $M$ EDTA, until free of (NH$_4$)$_2$SO$_4$, Fraction III. This fraction may be stored frozen.

Fraction III is diluted to 4.3 mg of protein per ml with cold glass-distilled H$_2$O. The 280:260 ratio of this fraction must be approximately 1.2 if the following steps are to be successful. With the aid of efficient mechanical stirring, 14.4 ml of a 0.5% protamine sulfate solution per 100 ml of diluted Fraction III are added, dropwise. The suspension is stirred for 1 hour and centrifuged for 10 minutes at 7000 $\times$ g, and the supernatant fluid is discarded. The precipitate is eluted with 20 ml of 0.5 $M$ Tris, pH 8.1, per 100 ml of diluted starting material. Care is taken to disperse the sticky precipitate, and the mixture is centrifuged for 1 hour with occasional stirring. The supernatant fluid (Fraction IV) is collected by centrifugation and dialyzed, in 15 ml batches, for 2 hours against 100 volumes of 0.01 $M$ Tris, pH 8.1 - 0.001 $M$ $\beta$-mercaptoethanol. The dialysis fluid is changed after 1 hour. Any precipitate that forms is removed by centrifugation. This fraction (Fraction V) may be stored frozen.

Fraction V is adjusted to pH 6.3 with 1 $M$ acetic acid. It is then brought to 40% saturation with (NH$_4$)$_2$SO$_4$ by the addition of 29.6 $g$ of solid salt per 100 ml. The suspension is stirred for 10 minutes and centrifuged for 10 minutes at 10,000 $\times$ g, and the precipitate is discarded. The supernatant fluid (pH maintained at 6.3) is made 60% saturated with (NH$_4$)$_2$SO$_4$ by the addition of 12 $g$ solid salt per 100 ml, and the precipitate is collected as above. It is dissolved in 0.2 of the volume of Fraction V of 0.5 $M$ Tris, pH 8.1, and dialyzed for 24 hours against 200 volumes of 0.01 $M$ Tris buffer, pH 8.1 - 0.001 $M$ $\beta$-mercaptoethanol-0.001 $M$ EDTA. The dialysis fluid is changed every hour. The enzyme solution (Fraction VI) may be stored frozen and is stable for at least 2 weeks at 4°.

A resume of a typical preparation is shown in Table I. We have consistently found an increase in total units in going from Fraction I to Fraction II (Table I). The reason for this finding is unknown. The over-all purification obtained thus far is 5-fold. The further purification of these preparations is being investigated, with a special effort to remove a contaminating nuclease and nuclease. The most important feature of Fraction VI is that the 280:260 ratio is consistently 1.6 or greater, suggesting relatively low nucleic acid contamination.

\textbf{RESULTS}

\textbf{Dependence of Exchange Reaction on Oligonucleotides}—The activities of the various enzyme fractions (Table I) for the ADP-P$_{32}$ exchange reaction were also determined (Assay B, Table II). The ratio of exchange activity to phosphorolysis activity (Column 4, Table II) is relatively constant in Fractions I through IV, but upon dialysis of the protamine eluate, as described above, the ratio falls markedly. An additional relative loss of exchange units accompanies (NH$_4$)$_2$SO$_4$ fractionation at pH 6.3 (Fraction VI). Thus, the ratio of exchange activity to phosphorolysis activity falls from 7.2 in Fraction I to 0.5 in Fraction VI, and values as low as 0.1 have been observed with other preparations.

The dialysis of the protamine eluate as described above is critical for this observation. Thus, if 0.001 $M$ EDTA is included in the dialysis fluid, the relative loss in exchange units (Assay B) is not found, and no additional loss occurs in Fraction VI, although the specific activities (phosphorolysis Assay A) and 280:260 ratios of the fractions are unchanged. Fractions derived from protamine eluates dialyzed against 0.001 $M$ EDTA

* The authors are indebted to Dr. R. F. Beers, Jr. for demonstrating the lysis step to us.
(in addition to the usual constituents) are described below and are designated by the letter A after the usual Roman numeral.

The exchange activity (Assay B) of Fractions V and VI is markedly stimulated by low concentrations of oligonucleotides such as pApApApA, which have unesterified, terminal C-2' and C-3' hydroxyl groups (Tables II and III). Indeed, the relative loss in exchange units in Fractions V and VI is largely recovered by conducting the exchange assay in the presence of 0.03 mM pApApApA (Table II, Columns 3 and 5). The data in Table III indicate that excellent stimulation of Fraction VI activity is obtained in the range, 0.015 to 0.060 mM pApApApA. Precise study of the effect of oligonucleotide concentration on stimulation must await more purified enzyme. Comparison of the data in Tables II and III shows that the extent of stimulation varies from one Fraction VI preparation to another. Thus, at 0.03 mM pApApApA, the enzyme used in Table II was stimulated 8.3-fold; the one used in Table III, 44-fold. The dinucleotide, pApApA, also stimulates the exchange (Table III), although not as well as pApApApA. The control for pApApA in Table III shows, in confirmation of earlier work (11), that it is not phosphorylated by these preparations.

Oligonucleotides such as ApApApApA, in which the terminal C-3' hydroxyl group is esterified by phosphate, do not stimulate the exchange reaction (Table IV) when tested over a 10-fold concentration range (0.03 mM to 0.34 mM). The data in Table IV also show that ApApApApA has little effect on the stimulation of the exchange reaction by pApApApA when the two are present in equimolar concentration, although inhibition is detected at a 2-fold excess of ApApApApA over pApApApA, and a 20-fold excess completely eliminates any stimulatory effect.

In confirmation of earlier results (11), oligonucleotides, such as ApApApApA, that are phosphorylated at the terminal C-3' hydroxyl group, are not phosphorylated by polynucleotide phosphorylase.

With the use of the same enzyme preparation described in the legend to Table III, the exchange of P$^{32}$ with UDP, CDP, and GDP was increased 136-, 58-, and 19-fold, respectively, by 0.3 mM pApApApA. The UDP and CDP experiments were carried out as described for ADP in Assay B. For the GDP experiments, GDP was 4 mM; K$_2$HPO$_4$, 25 mM; and MgCl$_2$, 5 mM. These are the conditions previously found to be optimal for GDP-P$^{32}$ exchange with A. agilis and E. coli enzyme preparations (5), and our experiments showed them to be optimal with the M. lysodeikticus enzyme as well.

Effect of Reaction Conditions on Stimulation of Exchange by Oligonucleotides Detailed investigation of the ADP-P$^{32}$ exchange reaction with M. lysodeikticus preparations revealed that the conditions of Assay B do not afford maximal reaction rates. Experiments designed to determine the best conditions for the reaction are described below and led to the set of conditions described under Assay C. Under these circumstances the stimulations described above are greatly reduced.

In general, good rates are achieved when the ADP:P ratio is greater than 1 (Fig. 2B) whereas in Assay B this ratio is 0.7. Over a wide range of ADP:P ratios, ADP concentrations of 3.8 mM or 5 mM give similar rates (Fig. 2B), although higher ADP concentrations apparently result in inhibition (Fig. 2A). Regardless of ADP concentration, or ADP:P ratio, the reaction rate is sharply dependent on the Mg$^{2+}$ concentration (Fig. 2C).

From the preliminary data obtained (Fig. 2C) it appears that maximum reaction rates occur when the concentrations of ADP and Mg$^{2+}$ are approximately equal. The conditions for Assay C (see above) are based on the data just summarized. As indicated in the description of Assay C, the reaction rate is proportional to enzyme concentration; it is also proportional to time of incubation for at least 30 minutes. The pH optimum is at 8.7 (Fig. 2D).

The differences between Assay B and Assay C are the following: ADP concentration is changed from 2.5 mM to 3.8 mM, P$^i$ concentration is changed from 3.5 mM to 2.5 mM (thus, ADP:P goes from 0.7 to 1.5), and the pH is increased from 8.2 to 8.7. These apparently minor changes in the assay conditions result in very different pictures of enzyme properties. For example, the loss of exchange units relative to phosphorylization units that was found with Assay B (Table II) is eliminated by using Assay C. In one experiment the ratios of [U]$^{32}$ per milliliter to [U]$^{32}$ per milliliter in Fractions I through VI were 6.4, 4.5, 6.2, 4.5, 3.6, and 3.5, respectively. Similarly, the ability of oligonucleotides such as pApApApA to stimulate ADP-P$^{32}$ exchange is
The dependence of polymerization on oligonucleotide varies markedly with experimental conditions, in particular with pH. The experiments in Fig. 3 were carried out at pH 8.2. Beers (25) and Olmsted and Lowe (26) reported a pH optimum for the polymerization reaction (M. lysodeikticus preparations) of 9 to 10, and our experiments confirm these findings (Fig. 4).

The data in Table VII show that when the pH of the reaction
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mixture is raised from 8.2 to 9.0, the barely detectable polymerization reaction is stimulated approximately 5-fold. Indeed, in Experiment 1, the rate at pH 9.0 is almost that at pH 8.2 in the presence of oligonucleotide. In each experiment, the large stimulation of polymerization rate by oligonucleotide at pH 8.2 is greatly diminished at pH 9.0.

It is also of interest to point out the quantitative differences in the influence of pH in the absence or presence of primer. In the absence of primer, the shift from pH 8.2 to 9.0 brings about a 5-fold increase in reaction rate; in the presence of primer the increase is less than 2-fold (Table VII). The pH curves in Fig. 4 illustrate the same point. The curves labeled A were obtained with a preparation of Fraction VI A (see above) which has no requirement for added primer; there is only a very small increase in activity when the pH is raised from 8.2 to the maximum. The curves labeled B were obtained with a preparation of Fraction VI; when the pH is raised from 8.2 to 9.0 in Tris buffer, the reaction is stimulated 5.9-fold. At the pH maximum, in glycine buffer, a further stimulation occurs.

The pH curves in Fig. 4 and the data in Table VII were obtained at an ADP:Mg++ ratio of approximately 4, as these conditions afford the best reaction rates (Fig. 5). This optimal ratio is somewhat higher than that previously reported for this enzyme (26). Consistent with the findings of previous workers (6, 10, 22, 25), the data in Fig. 5 also indicate that 0.04 M ADP is required to saturate the enzyme for polymerization.

Substrate Specificity—Olmsted and Lowe (26, 27) presented evidence suggesting that in M. lysodeikticus several polynucleotide phosphorylases, each specific for a particular nucleoside diphosphate, exist. Although the preparation described here is not of sufficient purity to warrant definite conclusions, the evidence obtained does not support the earlier conclusions. The activity of the fractions obtained in a typical fractionation (Table I) for the phosphorolysis of poly U and poly C as well as poly A was determined, and no evidence of a separation of activities toward the various polymers was obtained. In addition, Olmst and Lowe (26) reported that crude enzyme preparations were inhibited by Cu++, Zn++, Ba++, and 0.4 mM iodoacetic acid in different ways, depending on whether ADP or CDP was the substrate for polymerization. However, with a Fraction VI preparation, we found no inhibition of either ADP, UDP, or CDP polymerization by 0.4 mM iodoacetate acid. In addition, the polymerization of ADP, UDP, and CDP was inhibited 93, 97, and 100%, respectively, by 0.02 M CuSO_4, and 89, 97, and 77%, respectively, by 0.02 M ZnCl_2. Inhibition by BaCl_2 could not be tested because Ba++ precipitated the nucleoside diphosphates.

**Table V**


Reaction mixtures contained 12.3 μg (Assay B) or 6.2 μg (Assay C) of Fraction VI (S.A., 0.84; 280:260 ratio, 1.63; 12.3 mg per ml).

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Assay B</th>
<th>Assay C</th>
<th>Assey C, pH 8.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>1.4</td>
<td>27.6</td>
<td>13.5</td>
</tr>
<tr>
<td>ADP + pApApA (0.03 mm)</td>
<td>48.2</td>
<td>48.2</td>
<td>30.6</td>
</tr>
<tr>
<td>pApApA (0.03 mm)</td>
<td>0.9</td>
<td>2.2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Table VI**


Experiments were carried out as described for Assay C. Reaction mixtures contained 0 μg of a Fraction VI preparation (S.A., 0.83; 280:260 ratio, 1.61).

<table>
<thead>
<tr>
<th>Nucleotide present</th>
<th>μmole of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>8.4</td>
</tr>
<tr>
<td>ADP + 0.00 mm ApApAp</td>
<td>1.0</td>
</tr>
<tr>
<td>ADP + 0.10 mm pApApA</td>
<td>38.2</td>
</tr>
<tr>
<td>ADP + 0.10 mm pApApA + 0.09 mm ApApAp</td>
<td>19.7</td>
</tr>
<tr>
<td>ADP + 0.03 mm pApApA + 0.09 mm ApApAp</td>
<td>48.6</td>
</tr>
</tbody>
</table>

**Discussion**

Discussion of these data in relation to a detailed hypothesis for the reaction mechanism of polynucleotide phosphorylase must be postponed until purer enzyme and more detailed experi-
preparation (S.A., 0.81; 280:260 ratio, 1.63) that shows little or no stimulation by oligonucleotide. For Curve B (dashed lines) they contained 0.25 mg of a Fraction VI preparation (S.A., 0.77; 280:260 ratio, 1.59) which was stimulated approximately 27-fold by 0.3 mM pApApA, where indicated. Incubation and assay were as described for Fig. 3.

**Table VII**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No additions</th>
<th>Oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 8.2</td>
<td>pH 9.0</td>
</tr>
<tr>
<td>1</td>
<td>1.7</td>
<td>8.8</td>
</tr>
<tr>
<td>2</td>
<td>5.3</td>
<td>21.6</td>
</tr>
</tbody>
</table>

FIG. 4. The pH dependence of the polymerization of ADP. Reaction mixtures (0.1 ml) contained 0.15 M buffer (Tris, closed circles and squares, or glycine, open circles and squares) 0.04 M ADP, 0.01 M MgCl₂, 0.4 mM EDTA and enzyme. For Curve A (solid lines) the mixtures contained 0.04 mg of a Fraction VI preparation (S.A., 0.81; 280:260 ratio, 1.63) that shows little or no stimulation by oligonucleotide. For Curve B (dashed lines) they contained 0.02 mg of a Fraction VI preparation (S.A., 0.77; 280:260 ratio, 1.59) which was stimulated approximately 27-fold by 0.3 mM pApApA at pH 8.2. Incubation and assay were as described for Fig. 3.

FIG. 5. The polymerization of ADP; the relation between ADP and Mg⁺⁺ concentration. Reaction mixtures (0.1 ml) contained 0.15 M Tris buffer, pH 9.0, 0.4 mM EDTA, ADP, and MgCl₂ as indicated, and 0.12 mg of Fraction VI A (S.A., 0.81; 12.3 mg per ml). Incubation was for 30 minutes at 37°C; reactions were stopped by the addition of 0.9 ml of 2.5% HClO₄. Any precipitate was removed by centrifugation, and aliquots of the clear supernatant were used for Pi determination. Values were corrected for Pi contaminating the ADP used. ADP concentrations were 0.06 M (△), 0.04 M (○), and 0.02 M (●).
oligonucleotides of the first type in order to be polymerized. With our \textit{M. lysodeikticus} preparations, however, only compounds of the first type serve to stimulate either exchange or polymerization.

**SUMMARY**

The properties of a partially purified preparation of polynucleotide phosphorylase from \textit{Micrococcus lysodeikticus} are described. The preparations are relatively free from nucleic acid contaminants, and the ratio of $A$ at 280 nm to that at 260 nm is 1.6 or higher. Under suitable conditions, both the exchange reaction between nucleoside diphosphates and inorganic $P^{32}$ and the polymerization reaction are dependent on the presence of an oligonucleotide with an unesterified, terminal C-3' hydroxyl group. Small changes in the pH of reaction mixtures or in the concentrations of some of the reactants eliminate the oligonucleotide dependence, although some stimulation of the reaction rates is still obtained. The significance of the data with respect to the mechanism of action of polynucleotide phosphorylase is discussed.

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