A deoxyadenylate kinase activity associated with polynucleotide phosphorylase from Micrococcus luteus.

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ABSTRACT

We report here the presence of two enzymatic activities associated with highly purified preparations of polynucleotide phosphorylase from Micrococcus luteus. The first, a nuclease activity, which is not separated from the phosphorylase on hydroxylapatite, may be due to substitution of rLO for phosphate in the phosphorolysis reaction. The second activity, a deoxyadenylate kinase, the bulk of which is not resolved from the phosphorylase using gel filtration, sucrose density gradient centrifugation, DEAE-Sephadex, or hydroxylapatite chromatography, may represent a new activity of polynucleotide phosphorylase or be due to an enzyme which is tightly bound to the phosphorylase. Several properties of the kinase are described and its possible significance with respect to the overall enzyme mechanism is discussed.

Previous work with polynucleotide phosphorylase from E. coli showed that this enzyme synthesizes polynucleotides containing a 5'-monophosphate terminus (1). Under the conditions of the experiments carried out in that study, long polynucleotide chains were synthesized. More recently, Chou and Singer (2) showed that deoxyadenosine diphosphate can serve as a substrate in the de novo synthesis of oligo- and polynucleotides using the enzyme from M. luteus. Under the conditions employed by the latter investigators, a large percentage of the product was found to be composed of oligonucleotides containing seven or less dAMP residues.

We therefore decided to investigate the nature of the initiation reaction (i.e., the formation of the first internucleotide bond) by studying the polymerization of dADP catalyzed by M. luteus polynucleotide phosphorylase. Our initial experiments demonstrated, however, that a deoxyadenylate kinase reaction was occurring at a rate at least 100 times greater than the incorporation of dAMP residues into polymer. We therefore undertook to characterize this other reaction and the results are presented here.

MATERIALS AND METHODS

Unlabeled nucleotides were obtained from Schwarz/Mann and Sigma

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Chemical Co. \( [\beta^{-14}C]dADP, [\beta^{-3}H]dADP, [\beta^{-14}C]dATP, [\gamma^{-14}C]dCDP, [\gamma^{-14}C]dCDP, [\gamma^{-14}C]dAMP, [\gamma^{-14}C]Poly(A), \) and unlabeled Poly(A) were purchased from Schwarz/Mann. \( [\gamma^{32}P] \) phosphoric acid was obtained from New England Nuclear Corp. \( Ap^\gammaA \) was purchased from Boehringer. \( [beta^{32}P]dADP \) was prepared and purified as previously described (2). Highly purified, primer-independent (3) polynucleotide phosphorylase, prepared by a modification\(^2\) of the published procedure (4), was used in all experiments. The enzyme, which had a specific activity of 470 polymerization units/mg (in the presence of \( Ap^\gammaA \)) and a stimulation by primer of 1.1-1.3 fold, contained no visible contaminant protein bands when subjected to polyacrylamide gel electrophoresis as described (4).

Polynucleotide phosphorylase was assayed for the polymerization of \( AUP \) in the presence or absence of primer (\( Ap^\gammaA \)) at pH 9.0 and for the phosphorolysis of Poly(A) at pH 8.2 as previously described (4).

The deoxyadenylate kinase reaction was carried out at pH 6.5 and contained the following components: Na cacodylate, pH 6.5, 0.06 M; dADP (containing \( 5 \times 10^4 \) cpm per nmole), 0.18 mM; \( MgCl_2 \), 0.9 mM; and enzyme in a final volume of 0.016 ml. The \( [\gamma^{14}C]dADP \) used was contaminated to the extent of 0.2% with dATP and 1% with dAMP. Ten \( \mu \)l of enzyme, usually in \( 0.01 \) M Tris-HCl, pH 8.2 - 0.1 M NaCl - 1 mM \( MgCl_2 \) - 1 mM mercaptoethanol, was added to a 6 \( \mu \)l reaction mixture.

Two separate methods were used to analyze the products of the reaction. In the first method, the reaction was terminated by the addition of 1.0 ml of 1 mM EDTA. AMP, \( K_2HPO_4 \), and ADP (2.5 \( \mu \)moles each in a total volume of 30 \( \mu \)l) were added to the mixture which was then applied to a 1.5 ml column of Bio-Rad AG1X2, 200-400 mesh. The column was eluted sequentially with 2 x 5 ml of 25 mM \( NH_4Cl \), 10 x 1 ml of 0.01 N HCl (AMP or dAMP eluted), 10 x 1 ml of 0.01 N HCl - 0.02 M NaCl (phosphate eluted), 10 x 1 ml of 0.01 N HCl - 0.05 M NaCl (ADF or dADF eluted), and 10 x 1 ml of 0.01 N HCl - 0.2 M NaCl (ATP or dATP eluted). One-milliliter fractions were collected in vials and counted with 7 ml of counting fluid (liquifluor-Triton X-100-toluene, 1:6:12). In the second procedure, the reaction was terminated by the abbreviation used is: \( Ap_3A \), \( (Adenylyl)_3 \) adenosine (ApApApA).

\(^2\) The major modification is the incorporation of a Poly U Sepharose affinity chromatography step in the purification procedure. Craine, J. E., Barbehenn, E. K. and Klee, C. B., manuscript in preparation.
the addition of 1 μl of 0.1 M EDTA followed by the addition of 1 μl of a mixture of AMP and ATP (0.1 M each). Ten microliters of the mixture were spotted on PEI-cellulose plates (Brinkmann) which had previously been washed in water. Chromatography was carried out in 0.75 M KH₂PO₄, pH 3.6, until the solvent front was 15 cm from the origin. The ATP spots (and in some experiments, the AMP spots as well) were localized under ultraviolet light, cut out, and counted in 1 ml H₂O and 7 ml of counting fluid. This system separates nucleoside mono-, di-, and triphosphates, but does not separate ribo- and deoxyribo- compounds.

The kinase assays were carried out by the second procedure (chromatography on PEI-cellulose plates) unless otherwise specified.

One unit of kinase activity is defined as that amount of enzyme required to convert 2 nmol of dADP to 1 nmole of dATP and 1 nmole of dAMP per hour.

Ribonuclease activity was assayed by a method similar to that of the kinase. The reaction mixture contained the following components: Na cacodylate, pH 6.5, 0.06 M; [¹⁴C]Poly(A) (containing 2500 cpm per nmole), 0.25 mM (with respect to AMP residues); MgCl₂, 7 mM; and enzyme in a final volume of 0.016 ml. The enzyme, in the buffer described above, was added to 6 μl of the other components in a volume of 1 μl. Incubation was carried out at 37° for 20 min. The reaction was terminated by the addition of 1 μl of 0.1 M EDTA followed by the addition of 1 μl of 0.2 M AMP. Ten microliter aliquots were spotted on PEI-cellulose plates. Chromatography was carried out in 1.0 M LiCl until the solvent front was 15 cm from the origin. The AMP spots were localized, cut out, and counted as described above. One unit of nuclease is defined as that amount of enzyme required to liberate 1 nmole of AMP from Poly(A) in 1 hour. Units of polynucleotide phosphorylase are as previously described (4).

RESULTS

Identification of the Reaction Products of dADP Polymerization: To identify the nature of the 5'-terminus of polynucleotide phosphorylase synthesized polynucleotides, experiments were carried out with the use of double-labeled ([³H] and [β-³²P])dADP. When the reaction mixture was applied to a Sephadex G-50 column, a small amount (0.02% of the starting material) of ³²P- and ³H-containing material was eluted with the void
volume. However, we also observed a partial separation of the $^{32}$P- and $^3$H-containing compounds in the included material which had been presumed to be unreacted dADP (Fig. 1).

Fig. 1. Separation of reaction products of dADP polymerization on Sephadex G-50. The reaction mixture contained the components described under "Materials and Methods." The specific activities of dADP were $1.3 \times 10^5$ cpm of $[^3H]dADP$ and $4.7 \times 10^6$ cpm ($[^{32}P]dADP$) per nmole). The final reaction volume was 0.16 ml and contained 0.22 mg of enzyme. Following incubation at 37°C for 150 min., 0.08 ml of the reaction was applied to a Sephadex G-50 column (0.9 x 13.5 cm) equilibrated with 0.01 M Tris-HCl, pH 8.2 - 0.1 M NaCl - 1 mM EDTA. The column was eluted with the above buffer and fractions (0.27 ml) were collected and 0.15 ml aliquots were monitored for radioactivity. $\circ\circ\circ$, $^{32}$P and $\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\bullet\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The experiments were repeated, but this time the products were analyzed using the ion-exchange chromatography method described above. It was found that an equal amount of both dAMP and dATP had been produced, and that the amounts produced could be accounted for by the disappearance of dADP (Table I). The dAMP produced contains no $^{32}$P, whereas the dATP produced contains 2 moles of $^{32}$P per mole of tritium since both $\beta$ and $\gamma$-phosphate of dATP comes from the $\beta$-phosphate of the reactant dADP. The data from this column also indicated the absence of detectable phosphatase activity in that no $^{32}$P-phosphate was formed under these conditions.

The identity of the dATP produced was confirmed by chromatography of duplicate samples of the reaction mixture on PEI-cellulose in 0.75 M KH$_2$PO$_4$, pH 3.6, and in 1.0 M LiCl. In these experiments (data not shown) ultraviolet light quenching material (ATP marker) corresponded exactly with the spots on an autoradiogram of the plates.

Figure 2 shows a time course for the deoxyadenylate kinase reaction. As can be seen, the rates of formation of dAMP and dATP are identical and linear for one hour under the experimental conditions employed.

Several different preparations of highly purified polynucleotide phosphorylase were tested for both polymerization and kinase activities. These preparations, purified up to but not including the usual final step (4), contained one contaminant protein band as judged by polyacrylamide gel electrophoresis. They were assayed at this stage of purity in order to follow the phosphorylase and kinase activities through the last purification step. The results of this experiment are shown in Table II. In all cases, the ratio of polymerization to kinase was virtually identical.
Fig. 2. Time course of dAMP' and dATP formation from dADP. Individual reactions, as described under "Materials and Methods," were incubated for the indicated times. A zero time control was carried out by the addition of EDTA prior to adding enzyme. $[^{14}C]dADP$ (7.5 x $10^4$ cpm/nmole) was used. Each assay contained 22 μg of enzyme in 0.016 ml. dATP and dAMP were separated on PEI-cellulose plates as described under "Materials and Methods." • • , dAMP and • • o, dATP.

Attempted Separation of Polynucleotide Phosphorylase and Deoxyadenylate Kinase: Several attempts were made to separate the deoxyadenylate kinase from the polynucleotide phosphorylase. One of the purified enzyme preparations described above was applied to a Sepharose 6B column (Fig. 3A). Fractions were collected and monitored for both phosphorylase and kinase activities. The enzyme was also centrifuged in a sucrose gradient (Fig. 3B). Fractions were collected and analyzed as above. In both of these procedures, gel filtration and density gradient centrifugation, the kinase
TABLE II. Comparison of Various Enzyme Preparations for Polynucleotide Phosphorylase and Deoxyadenylate Kinase.\(^a\)

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Polymerization (+AP(_3)A)</th>
<th>Deoxyadenylate Kinase</th>
<th>Ratio(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>382</td>
<td>10.0</td>
<td>153,000</td>
</tr>
<tr>
<td>2</td>
<td>317</td>
<td>8.0</td>
<td>159,000</td>
</tr>
<tr>
<td>3</td>
<td>305</td>
<td>8.6</td>
<td>142,000</td>
</tr>
<tr>
<td>4</td>
<td>326</td>
<td>9.8</td>
<td>133,000</td>
</tr>
</tbody>
</table>

\(^a\) Various preparations of polynucleotide phosphorylase, purified up to but not including the final step, were assayed for polymerization of ADP in the presence of primer, Ap\(_3\)A, and kinase. The results are expressed as units per mg of protein.

\(^b\) Ratio of \(\mu\)moles Pi liberated/min. in polymerization reaction at pH 9.0 to \(\mu\)moles dATP formed/min. in kinase reaction at pH 6.5.

activity eluted slightly ahead of the phosphorylase, however, the two activities were not fully resolved. The enzyme was applied to a DEAE-Sephadex column and eluted with a linear salt gradient. As shown in Fig. 4 a small amount of the kinase (approximately 20%) was eluted as a shoulder just prior to the major protein peak which contained all of the phosphorylase and the bulk of the kinase activity. The enzyme was applied to a hydroxylapatite column and eluted with a linear gradient of \((\text{NH}_4)_2\text{SO}_4\) as previously described (4). Fractions containing A\(_{280}\)-absorbing material were dialyzed vs. 0.01 M Tris-HCl, pH 8.2 - 0.1 M NaCl - 1 mM MgCl\(_2\) - 1 mM mercaptoethanol and were assayed for both kinase and phosphorylase activities. The results are shown in Fig. 5. The pattern of elution is similar to that observed on DEAE-Sephadex. Approximately 20% of the kinase is eluted prior to the elution of the major protein peaks. It should be noted that this activity does not correspond with the contaminant protein which is also eluted prior to the phosphorylase. The bulk of the kinase and the phosphorylase activities are eluted in two peaks, the first of which has the same ratio of phosphorylase to kinase as the starting material and the second of which has a ratio twice that of the starting material. When polyacrylamide gels were run on the various fractions and stained for both protein and polymerization activity (4), it was observed that fractions 46-60 were virtually free of any contaminant protein.
Fig. 3. Distribution of polynucleotide phosphorylase and deoxyadenylate kinase following chromatography on Sepharose 6B or centrifugation on sucrose gradient.

A. One milliliter of highly purified polynucleotide phosphorylase (2.2 mg) was applied to a Sepharose 6B column (0.9 x 57 cm) equilibrated with 0.01 M Tris-HCl, pH 8.2 - 0.1 M NaCl - 1 mM MgCl₂ - 1 mM mercaptoethanol. The enzyme was eluted with the above buffer and fractions (1 ml) were monitored for A₂₈₀, phosphorylase, and kinase.

B. One-tenth of a milliliter of highly purified polynucleotide phosphorylase (0.22 mg) was applied to a 4.8 ml 5-20% linear sucrose gradient in 0.01 M Tris-HCl, pH 8.2 - 0.1 M NaCl - 1 mM MgCl₂ - 1 mM mercaptoethanol. Centrifugation was carried out at 49,000 rpm in an SW 50.1 rotor (Beckman) for 7 hours. Fractions (5 drops) were collected and monitored for A₂₈₀, phosphorylase, and kinase.
Table III contains a summary of the attempts to resolve the two activities. In each case, the initial ratio of activities (calculated as moles of phosphate released during polymerization at pH 9.0 to moles of dATP formed by the kinase reaction at pH 6.5) is approximately 15U,UUU. Gel filtration or sucrose gradient centrifugation increases this value by 15-20%, whereas DEAE-Sephadex or hydroxylapatite chromatography yields preparations with double this value.

Detection of Adenylate Kinase Activity: The enzyme preparations were tested for their ability to catalyze an adenylate kinase reaction. The procedure was identical to that used in the deoxyadenylate kinase reaction.

Fig. 4. Distribution of polynucleotide phosphorylase and deoxyadenylate kinase following chromatography on DEAE-Sephadex. Purified polynucleotide phosphorylase (2.2 mg) was applied to a DEAE-Sephadex A-50 column (0.9 x 14 cm) which had been equilibrated with 0.05 M Tris-HCl, pH 8.2 - 0.05 M NaCl - 1 mM MgCl₂ - 1 mM mercaptoethanol. The column was eluted with a 50 ml gradient of 0.05-0.4 M NaCl in 0.05 M Tris-HCl, pH 8.2 - 1 mM MgCl₂ - 1 mM mercaptoethanol. Fractions (1.0 ml) were collected and monitored for A₂₈₀, phosphorylase, and kinase. The A₂₈₀ absorbing material in fractions 22, 26, and 40 was due to a small amount of DEAE-Sephadex which had leaked through the column and was not observed in a subsequent DEAE-Sephadex chromatography run on the same enzyme preparation.
Fig. 5. Distribution of polynucleotide phosphorylase, deoxyadenylate kinase, and nuclease following chromatography on hydroxylapatite. Highly purified fractions of polynucleotide phosphorylase (33 mg) were dialyzed against 0.01 M Tris-HCl, pH 7.5 - 0.1 M NaCl - 1 mM mercaptoethanol and were applied to a hydroxylapatite column (2.6 x 7.5 cm) which had been equilibrated with the above buffer. The enzyme was eluted with a linear (NH₄)₂SO₄ gradient (0.2-1.0 M) in the above buffer. Fractions (6.0 ml) were collected as described (1), concentrated to 1-2 ml on an Amicon PM-10 ultrafilter and dialyzed vs. 0.01 M Tris, pH 8.2 - 0.1 M NaCl - 1 mM MgCl₂ - 1 mM mercaptoethanol, and assayed for protein, phosphorylase, kinase, and nuclease.

except that ADP (0.25 mM) was substituted for dADP and the MgCl₂ concentration was 5 mM. Under these conditions, at pH 6.5, ATP was formed in amounts similar to those found for dATP formation in the deoxyadenylate kinase reaction; however, much of the ADP appeared to be converted to AMP (9.4 nmoles/hr. as compared to 0.2 nmoles of ATP formed/hr.) Since the data in
### TABLE III. Summary of Attempts to Resolve Polynucleotide Phosphorylase and Deoxyadenylate Kinase.

<table>
<thead>
<tr>
<th>Treatment (initial ratio x 10^-3)</th>
<th>% of Recovered Kinase</th>
<th>Ratio x 10^-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepharose 6B (133) (Fig. 3)</td>
<td>100</td>
<td>171 (125-211)</td>
</tr>
<tr>
<td>Sucrose Gradient (133) (Fig. 4)</td>
<td>100</td>
<td>168 (121-250)</td>
</tr>
<tr>
<td>DEAE Sephadex (133)</td>
<td>13</td>
<td>58</td>
</tr>
<tr>
<td>DEAE Sephadex (133) (Fig. 5)</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>Hydroxylapatite (182)</td>
<td>8</td>
<td>63</td>
</tr>
<tr>
<td>Hydroxylapatite (159) (Fig. 6)</td>
<td>19</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*The values represent the percentage in each fraction of the total kinase recovered in the procedure. The overall recovery is between 90% and 100% in the first four procedures. The hydroxylapatite procedures yielded approximately 50% recovery of the applied protein, kinase and phosphorylase activities.*

*b The values in parentheses represent the range of ratios observed.

*c DEAE-Sephadex peak chromatographed on hydroxylapatite.

Table I and Fig. 2 suggest the absence of phosphatase, we considered the possibility that the AMP produced was the result of polymerization by polynucleotide phosphorylase followed by nuclease attack on the newly synthesized Poly(A). In order to test this possibility, the enzyme preparation was assayed for nuclease as described in "Materials and Methods." The results, shown in Fig. 6, indicate that the nuclease activity measured under these conditions is approximately 50 times as great as that of the kinase (with dADP as substrate). Since no detectable oligonucleotides were observed in this assay, this activity represents either an exonuclease or a limiting amount of endonuclease plus an excess of exonuclease.

In an attempt to remove the nuclease, the enzyme was chromatographed on hydroxylapatite (Fig. 5). As can be seen, the elution pattern of the
Fig. 6. Time course of nuclease reaction. Individual reactions were carried out as described under "Materials and Methods" for the indicated time periods. Reaction products were separated on PEI-cellulose plates, and both the AMP spot and material at the origin [residual Poly(A)] were counted. o o, AMP and • •, Poly(A).

nuclease activity is indistinguishable from the polynucleotide phosphorylase activity.

Thus, although it appears that the enzyme is capable of carrying out an adenylate kinase reaction, the results are clouded by the presence of nuclease activity.

Enzymatic Properties of Deoxyadenylate Kinase: In order to compare the kinase and nuclease activities with those of polynucleotide phosphorylase more accurately, the enzyme was assayed at pH 6.5, 8.0, and 8.9. The data, presented in Table IV, show that the kinase, which is most active at pH 6.6, retains about 40% of its activity at pH 8 (the optimum for phosphorolysis) and about 25% of its activity at pH 8.9 (the optimum for polymerization). The nuclease, also most active at pH 6.6, has virtually no activity at the
### TABLE IV. Measurement of Polynucleotide Phosphorylase, Deoxyadenylate Kinase, and Nuclease at Various pHs.

<table>
<thead>
<tr>
<th>pH</th>
<th>6.5</th>
<th>8.0</th>
<th>8.9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kinase</td>
<td>Nuclease</td>
<td>Polymerization-Primer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>11.3 (100)</td>
<td>182 (100)</td>
<td>52 (16)</td>
</tr>
<tr>
<td></td>
<td>4.3 (38)</td>
<td>5 (3)</td>
<td>223 (68)</td>
</tr>
<tr>
<td>8.0</td>
<td>3.0 (26)</td>
<td>9 (5) C</td>
<td>326 (100)</td>
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</tbody>
</table>

The four activities were measured as described under "Materials and Methods." Reactions at pH 6.5 were run in Na cacodylate and at pH 8.0 and 9.0 in Tris-HCl. Values are expressed as units per mg protein. The values in parentheses are expressed as percentage of activity with 100% assigned to the activity at the pH at which maximum activity was observed.

- Final pH of reaction mixture was 6.6.
- Final pH of reaction mixture was 8.8.

Figure 7 shows the effect of Mg²⁺ concentration on the enzymatic activity of the kinase at two dADP concentrations. The data are plotted in terms of total MgCl₂ added to the system and not in terms of free Mg²⁺ or Mg·dADP complexes. As can be seen, the routine assays (0.2 mM dADP and 0.9 mM MgCl₂) are almost, but not quite, at the maximum activity obtainable at this dADP concentration. The data also show that the level of dADP in our assays is well below that required for maximum enzymatic activity; however, in order to reduce the amount of [¹⁴C]dADP required for routine assays, this level of dADP is routinely used in the assay.

The kinase was assayed for the ability to catalyze the reverse reaction (i.e., the formation of dADP from dATP and dAMP). Experiments were carried out in which either dATP or dAMP was labeled. When enzyme which contained 7.2 kinase units per mg was used, the amount of dADP formed from either [¹⁴C]dATP or [¹⁴C]dAMP was 11.2 nmoles per hour per mg protein under our experimental conditions (Na cacodylate buffer, pH 6.6; dATP, 0.2 mM; dAMP, 0.2 mM; and MgCl₂, 1.5 mM).

The enzyme was tested for its ability to catalyze the kinase reaction...
Figure 7. The effect of MgCl₂ and dADP concentrations on deoxyadenylate kinase activity. The reactions were carried out as described under "Materials and Methods," except that only 3 μl of enzyme was used per 20 μl assay and MgCl₂ concentrations are as shown. • •, 0.2 mM dADP and o o, 2.0 mM dADP.

using either dCDP or TDP as substrate. In these experiments, products were separated on PEI-cellulose plates using 1.0 M LiCl. The results showed that the enzyme could synthesize 1.8 nmoles of TTP and 3.3 nmoles of dCTP per hour per mg protein. In both cases, although the respective monophosphates were synthesized, exact quantitation was difficult due to the high monophosphate content of the diphosphate substrate (2.5% dCMP in dCDP and 4.5% TMP in TDP).

DISCUSSION

The results presented in this paper indicate that a deoxyadenylate kinase activity is associated with a variety of preparations of polynucleotide phosphorylase from M. luteus either as an integral part of the enzyme or as a separate activity which we were unable to separate from the phosphorylase. We have chosen to call this activity deoxyadenylate kinase because of the relative ease of deoxyadenylate kinase activity assay as opposed to the more complex assay with ADP as substrate in which both
polymerization and nuclease activities complicate the interpretation of the data. The fact that a partial separation of the activities may be achieved, as seen in Figs. 3-5, may be related to the fact that polynucleotide phosphorylase itself exhibits a multiple-band pattern when analyzed by polyacrylamide gel electrophoresis (3, 4) and that the various species may possess different kinase and phosphorylase activities. It should be pointed out that in none of these experiments (Figs. 3-5) did the primer stimulation of polymerization vary to a significant extent.

The possibility that the kinase activity is due to adenylate kinase contamination is unlikely in view of the fact that most known kinases have molecular weights (5, 6) considerably lower (usually about 24,000) than that of polynucleotide phosphorylase (270,000-250,000) (4). The data in Fig. 3 suggest that the deoxyadenylate kinase activity which we observe is, if anything, slightly larger than that of the bulk of the phosphorylase activity.

The nuclease activity, which appears to be associated with the phosphorylase (see Fig. 6), may be explained either as a contaminant activity or by the possibility that H2O may be able to substitute (to an extent of approximately 0.5% at pH 6.5) for phosphate in the phosphorolysis reaction.

It is interesting to note that Luria et al. (7) have reported that the adenylate kinase temperature-sensitive mutant CR31T28 cannot initiate transcription at non-permissive temperature, and these authors speculate on the role of adenylate kinase in the initiation of transcription. Miller and Wells (8) have found that a nucleoside diphosphokinase activity is associated with highly purified preparations of DNA polymerase from M. luteus and E. coli and with partially purified preparations of DNA polymerase from avian myeloblastosis virus although these authors do not suggest a particular functional role for this activity.

Wunderli et al. (9) have described an ATPase from T2L infected E. coli which, when functioning in conjunction with polynucleotide phosphorylase, produced Poly(A) from ATP. These two activities are separated on Bio-Gel A-5m in the absence of Mg2+. Ramanarayanan and Srinivasan (10) have described a Poly(A) polymerase from E. coli PR7 (lpp, RNase 1+ which could incorporate either ATP or ADP into Poly(A). The latter paper shows that both activities reside on the same protein as judged by in situ assays.
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on polyacrylamide gels. Although the two papers indicate that polynucleotide phosphorylase may utilize ATP as a substrate for polymerization (either in conjunction with an ATPase in the former report or as an integral part of the enzyme in the latter report), these observations do not appear to be related to the deoxyadenylate kinase activity which we have observed.

We can speculate on a possible role for the deoxyadenylate kinase reaction in the overall mechanism of polynucleotide phosphorylase. In order to achieve the synthesis of a 5'-monophosphate terminus in Poly(A) as observed in previous experiments (1), two moles of phosphate must be released, one from each of two ADP molecules, during the formation of the first internucleotide bond. In one possible mechanism, a 5'pyrophosphate terminus could be formed initially followed by the removal of the β-phosphate at a later point in the reaction. Another possible mechanism would involve the transfer of the β-phosphate of ADP such that the AMP product formed can be positioned on the enzyme as the 5'-monophosphate terminus of the nascent Poly(A) chain. This transfer could depend on the observed kinase activity.

We are currently undertaking further studies to determine whether or not there is a relation between the kinase activity and the overall reaction catalyzed by polynucleotide phosphorylase.

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REFERENCES