Studies on the Mechanism of Action of Polynucleotide Phosphorylase

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The bacterial enzyme, polynucleotide phosphorylase, catalyzes the synthesis of long chain polynucleotides from nucleoside diphosphates (Equation 1) (1-5), the phosphorolytic cleavage of polynucleotides (reverse of Equation 1), and also an exchange reaction between P₃ and the terminal phosphate of a nucleoside diphosphate (Equation 2) (1, 2).

\[ \text{n Nucleoside-P-P} \overset{\text{Mg}^{2+}}{\rightleftharpoons} \text{(nucleoside-P)}_n + \text{n P}_3 \] (1)

\[ \text{Nucleoside-P-P + P}_3 \rightleftharpoons \text{nucleoside-P-P}_n + \text{P}_3 \] (2)

It has been demonstrated that oligonucleotides with an unesterified, terminal, C-3' hydroxyl group serve as primers for the formation of polyadenylic acid, polyuridylic acid, polythymidylic acid, and polyguanylic acid (6, 7). Thus, in the presence of uridine diphosphate, a compound such as pApA is incorporated into newly formed polymer; the chains grow by successive additions of nucleoside monophosphate units to free C-3' hydroxyl groups and chains of the structure pApApApUpU . . . pU, for example, are formed (6, 7). Highly purified preparations of polynucleotide phosphorylase from Azotobacter agilis catalyze the formation of polyadenylic acid, polyuridylic acid, and polythymidylic acid after an initial lag period (6, 8), and the lag can be overcome by polyribonucleotides (8, 9), by oligonucleotides such as ApUp which do not serve as centers for chain proliferation, since they contain no unesterified C-3' hydroxyl group (6).

In contradistinction to the results with the other nucleoside diphosphates, earlier papers (1, 2) concerning polynucleotide phosphorylase reported that when guanosine 5'-diphosphate alone was used as the substrate, both polymer formation and the exchange reaction were either very slow or undetectable. Recent work (7) has shown that the polymerization of guanosine 5'-diphosphate does indeed occur, but only when an oligonucleotide with an unesterified 3'-hydroxyl group (pApA, for example) is present to initiate chain formation. This finding held, regardless of the enzyme preparation used (7).

It was, therefore, of interest to reinvestigate the exchange reaction (Equation 2) with guanosine 5'-diphosphate as a substrate. The data reported herein will show that under different conditions from those used previously (1, 2), the rate of exchange of guanosine 5'-diphosphate with P₃ is comparable to the rates observed with adenosine 5'-diphosphate and uridine 5'-diphosphate; oligonucleotides are not required to obtain such rates, although they afford some stimulation of the reaction. In addition, the oligonucleotides were found to stimulate the rates of adenosine 5'-diphosphate and uridine 5'-diphosphate exchange.

Earlier studies (10) showed that the series of oligonucleotides including ApUp, ApApUp, and ApApApUp, are not phosphorolyzed by polynucleotide phosphorylase. Because of the ability of these compounds to overcome the lag in the polymerization of adenosine 5'-diphosphate and uridine 5'-diphosphate (6) and their stimulatory effect on the nucleoside diphosphate-P₃ exchange (below), it was also of interest to test their influence on the phosphorolysis reaction, and experiments concerning this question are presented here.

Several experiments concerning the mechanism of the phosphorolysis reaction will also be presented in this report. These studies indicate that the phosphorolysis of susceptible oligoribonucleotides occurs in a stepwise fashion that is the reverse of the synthetic reaction.

EXPERIMENTAL PROCEDURE

Materials and Methods

Enzymes—Escherichia coli polynucleotide phosphorylase was prepared according to the procedure of Littauer and Kornberg (2) and was carried through the first alcohol precipitation. The dialyzed preparation used in these experiments had a specific activity of 13, determined by the "exchange" assay (2) and had been stored for almost 2 years at -20° with little loss in activity. A highly purified preparation of A. agilis polynucleotide phosphorylase was very generously supplied by Dr. Severo Ochoa. This fraction was the result of a protamine precipitation step (8) and it had a specific activity of 60 measured with the "exchange" assay (1). It is referred to in the text as SA 60. A second preparation from A. agilis was obtained in this laboratory according to the procedure of Mii and Ochoa (8)1 and is designated SA 55. Both preparations catalyzed the polymerization of UDP and ADP only after an initial lag period.

Nucleotides—The biosynthetic polymers were synthesized and isolated according to published procedures (1, 10). The methods used to prepare most of the oligoribonucleotides have been described by Heppel et al. (3, 4) and summarized by Singer (10). The preparation and characterization of pApApUp has also been reported (6). The nucleoside diphosphates were obtained from Sigma Chemical Company, except for thymine ribonucleotide.

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1 We are indebted to Dr. S. Mii and Professor S. Ochoa for making their unpublished data available to us and for helpful advice in the course of the fractionation experiments.
side pyrophosphate which was a gift from Professor Sir Alexander Todd.

Because the commercial GDP preparations were contaminated with GMP and GTP, the GDP content of each preparation was determined by chromatography and quantitative elution of the three components.

**Paper Chromatography**—Descending chromatography was carried out on Whatman No. 1 or No. 3 MM paper with the following solvent systems: Solvent 1, isoamyl alcohol-0.2 m ethylenediaminetetraacetic acid (100:60:0.8, volume for volume for volume) (11); Solvent 2, n-propanol-H2O-NH4OH (60:10:30, volume for volume for volume) (12). Nucleotides were visualized on the paper strips by means of an ultraviolet lamp, and photographs of the strips were made under ultraviolet illumination. Radioautograms were prepared by exposure of Kodak Type K X-ray film to paper strips containing radioactive materials. Compounds were eluted from filter paper with H2O unless indicated otherwise.

**Assay for Nucleoside Diphosphate-Pi Exchange and Phosphorolysis**—The method used was based on the procedure of Littauer and Kornberg (2). The labeled nucleoside diphosphate produced in the exchange reaction or by the phosphorolysis of a polynucleotide was separated from Pii by adsorption onto charcoal, and its radioactivity was measured. The contents of the reaction mixtures are described in the legends to the tables and figures. After incubation at 37°, the reaction was stopped by addition of 1.0 ml of 2.5% cold perchloric acid to the vessel. Acid-washed Norit A (0.1 ml of a 10% suspension, weight for weight) was added to adsorb the nucleotides. After 10 minutes in the cold, the suspension was centrifuged and the charcoal was washed three times with 2.5 ml portions of cold water. The charcoal was then suspended in 0.8 ml of 50% ethanol containing 0.3 ml of concentrated NH4OH per 100 ml. An aliquot of this suspension (usually 0.1 ml) was placed on a copper planchet, dried, and the radioactivity determined with a thin window gas flow counter. The total Cpm. incorporated into charcoal-adsorbable material was calculated, and from the specific radioactivity of the Pii the number of μmoles of phosphate incorporated was obtained. A self-absorption factor of 1.15 (2) was applied. A control incubation containing no enzyme was generally carried through the whole procedure with each experiment and the results presented have been corrected for the small amount of radioactivity adsorbed onto the charcoal from these samples. In some cases, enzyme was added to the control tube at the end of the incubation.

**RESULTS**

**Nucleoside Diphosphate-Pi Exchange Studies**

**Experiments with GDP**—The experiments of Grunberg-Manago et al. (1) were carried out in the presence of 0 mm MgCl2, 2 mm potassium phosphate, and 1 mm GDP (GDP:Pi = 0.5), whereas Littauer and Kornberg utilized 4 mm MgCl2, 0.52 mm potassium phosphate, and 0.8 mm GDP (GDP:Pi = 1.5). Analogous conditions gave good rates of exchange with UDP, ADP, and CDP, but the reaction was sluggish with GDP. A study of the rate of GDP-Pii exchange as a function of the ratio of GDP concentration to Pi concentration at 5 mm MgCl2 (Fig. 1) shows that the conditions described above do not afford the maximal reaction velocity. The maximal exchange rate occurs at a GDP:Pi, ratio of about 0.17. At the ratios used by the previous workers the rate is at best 1/5 of the maximum. Results similar to those shown in Fig. 1 were obtained with another lot of GDP and also with a GDP preparation that had been passed through a Dowex 50 column to remove extraneous metal ions. The experiment in Fig. 1 was carried out with E. coli enzyme; similar results were obtained with A. aquis polynucleotide phosphorylase (SA 80 and SA 55).

![Graph](http://www.jbc.org/)  
**Fig. 1.** The GDP-Pii exchange reaction as a function of the ratio, GDP:Pi. The reaction mixtures (0.1 ml) contained 3.9 μg of E. coli enzyme and the following in mm concentrations; Tri buffer, pH 8.2, 100; MgCl2, 5.0; ethylenediaminetetraacetic acid, 0.2; GDP, 4.0, and Pii as indicated (specific radioactivity, 220,000 c.p.m./μmole). Incubation time, 30 minutes.

The data in Table I indicate that 4 mm GDP represents an optimal GDP concentration at a GDP:Pi ratio of 0.17. At a GDP:Pi ratio of 1.0, an increase of the GDP concentration from 4 mm to 8 mm decreased the exchange rate by more than 50% (Experiment 2, Table I). The experiments of Fig. 1 were carried out at a GDP concentration of 4 mm.

At GDP:Pi ratios which were unfavorable for exchange at 5 mm MgCl2 stimulation of the reaction rate could be obtained by varying the MgCl2 concentration. Indeed, reaction rates were obtained similar to the maximum observed in the experiment of Fig. 1 (Fig. 2). In Fig. 2, the extent of reaction is plotted as a function of the MgCl2 concentration at three different GDP:Pi ratios. The MgCl2 concentration required for maximal exchange was 0.17 mm.

**TABLE I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>GDP:Pi</th>
<th>GDP concentration</th>
<th>μmoles incorporated into charcoal-adsorbable nucleotides/μg</th>
<th>μmoles/hr/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.17</td>
<td>0.8</td>
<td>4.2</td>
<td>27.1</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>4.0</td>
<td>1.86</td>
<td>0.62</td>
</tr>
</tbody>
</table>

The reaction mixtures (0.1 ml) contained 3.9 μg (Experiment 1) or 3.9 μg (Experiment 2) of E. coli enzyme, and the following in mm concentrations: Tri buffer, pH 8.2, 100; MgCl2, 5; ethylenediaminetetraacetic acid, 0.2; and GDP and sodium potassium phosphate as indicated. The specific activity of the Pi was 159,000 c.p.m./μmole in Experiment 1 and 300,000 c.p.m./μmole in Experiment 2. Incubation time was 30 minutes.
activity appears to decrease as the GDP:P ratio decreases.

This may be expressed in another way: with increasing inorganic phosphate concentration, the concentration of MgCl₂ required for maximal activity decreases. All the experiments were carried out at 4 mM GDP. Similar results have been obtained with *A. agilis* polynucleotide phosphorylase (SA 60 and SA 55) and with different samples of GDP.

With *E. coli* or *A. agilis* enzyme preparations, comparable rates of exchange for ADP, UDP, and GDP have now been observed (Table II). The experiments in Table II were carried out at a GDP:P ratio of 0.5 with 0.01 μg of *E. coli* enzyme; incubation time, 1 hour.

Data obtained with polynucleotide phosphorylase from *E. coli* or *A. agilis* enzyme preparations, comparable exchange rates for ADP, UDP, and GDP have now been observed (Table II). The experiments in Table II were carried out at a GDP:P ratio of 0.5 with 0.01 μg of *E. coli* enzyme; incubation time, 1 hour. Where GDP:P ratio = 0.5, they contained 8.0 μM Pi₃² (specific activity, 57,000 c.p.m./μmole) and 0.96 μg of *E. coli* enzyme; incubation time, 30 minutes. Where GDP:P ratio = 0.17, they contained 24 μM Pi₃² (specific activity, 151,000 c.p.m./μmole) and 0.96 μg of *E. coli* enzyme; incubation time, 30 minutes.

**Effect of Oligonucleotides on GDP-P₃ Exchange Reaction—**

Table III gives some representative data showing the stimulation of the GDP-P₃ exchange reaction by the addition of compounds such as pApA and pApApA. The compounds in this group contain unesterified, terminal C-3' hydroxyl groups and serve as primers for GDP polymerization (7). The experiments were carried out with a GDP:P ratio of 0.5, in the presence of different samples of GDP.

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**Comparison of exchange rates with GDP, ADP, and UDP**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>E. coli</th>
<th>A. agilis SA 55</th>
<th>A. agilis SA 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP</td>
<td>41</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>ADP</td>
<td>33</td>
<td>51</td>
<td>113</td>
</tr>
<tr>
<td>UDP</td>
<td>85</td>
<td>40</td>
<td>83</td>
</tr>
</tbody>
</table>


The complete reaction mixtures (0.1 ml) contained 6.3 μg of *A. agilis* polynucleotide phosphorylase (SA 60) per ml and the following in mM concentrations: Tris buffer, pH 8.2, 100; ethylenediaminetetraacetate, 0.2; Pi, 8 (specific activity, 60,000 c.p.m./μmole), and one of the following combinations: 4 mM GDP and 10 mM MgCl₂, 5.0 mM ADP and 5.0 mM MgCl₂, or 10 mM UDP and 5.0 mM MgCl₂. Enzyme additions were as follows (μg/ml): *E. coli*, 19; *SA 55*, 26; *SA 60*, 13. Incubation time, 1 hour.

<table>
<thead>
<tr>
<th>System</th>
<th>P₃ incorporated into charcoal-adsorbable nucleotides</th>
<th>Fold stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Complete + pApA (1.3)*</td>
<td>64</td>
<td>1.4</td>
</tr>
<tr>
<td>Complete + pApA (2.6)</td>
<td>64</td>
<td>1.4</td>
</tr>
<tr>
<td>Complete + pApApA (0.5)</td>
<td>160</td>
<td>3.2</td>
</tr>
<tr>
<td>Complete + pApApA (1.0)</td>
<td>170</td>
<td>3.0</td>
</tr>
<tr>
<td>No GDP, + pApA (0.5)</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>No GDP, + pApApA (1.0)</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate the mM concentrations of the oligonucleotides.
The data in Table IV show that oligonucleotides such as ApUp, which have phosphate monoesterified to the C-3' hydroxyl of the terminal nucleoside residue, also stimulate GDP exchange. These oligonucleotides are not phosphorolyzed by polynucleotide phosphorylase (10) and therefore no control incubations comparable to the ones described for pApApA were included.

In summary, a significant stimulation of the GDP-P$_{i}$ exchange reaction was obtained with pApApA and pApA, which promote GDP polymerization, and also by compounds such as ApUp which are not able to promote poly G$^2$ formation (7). Although in similar experiments the extent of stimulation varied somewhat (pApA, for example, failed to stimulate in certain experiments), the stimulation was never very large.

**Exchange Reaction with ADP and UDP**—The effect of various types of oligonucleotides on the rate of the exchange of P$_{i}$ with ADP and UDP was also investigated. The experiments in Table V show that a significant, although small, stimulation of the exchange rates was obtained in the presence of these oligonucleotides. These results confirm similar unpublished data obtained by Dr. Sanae Miyi. These experiments were carried out with an A. agilis polynucleotide phosphorylase preparation (SA 60) that catalyzes the polymerization of ADP or UDP only after a lag period (8). The concentrations of oligonucleotides used in these experiments were of the same order of magnitude as those required to overcome the lag in polymerization (8). In calculation of the stimulation by pApApA (Table V) the data were corrected for the amount of P$_{i}$ incorporated as a result of phosphorolysis of the oligonucleotide. The dinucleotide, pApA, and the compounds, ApUp, ApApUp, and ApApApUp, are not phosphorolyzed (10) and no such correction need be applied.

The lag in the polymerization of ADP and UDP found with certain A. agilis enzyme preparations (8) can be overcome by poly A and poly U, respectively (6, 8, 9). On the other hand, it was found that poly U inhibits poly A synthesis, and also that poly A inhibits poly U synthesis (8, 9). It was therefore of interest to study the effect of these polymers on the exchange reaction. In these studies it was necessary to consider the simultaneous phosphorolysis of the polymers, which, in the presence of P$_{i}$, leads to the formation of radioactive nucleotides. The data in Experiment 1, Table VI, indicate that when poly A and ADP are present in one reaction mixture, the total charcoal-adsorbable radioactive nucleotide formed is the sum of that obtained when each of the two substrates is present alone. Similar experiments were carried out for the combination of UDP with poly U (Table VI, Experiment 1). However, as shown in Table VI (Experiment 2), when poly A is added to UDP or poly U to ADP, the incorporation of P$_{i}$ into nucleotide material is greatly inhibited; the values obtained are lower than those noted for either the nucleoside diphosphate or polymer alone.

The experiments of Table VI were carried out with an A. agilis preparation (SA 60) that demonstrates the lag phenomenon in polymerization experiments. Similar results have been obtained with the E. coli enzyme that polymerizes ADP and UDP without a lag, but with which inhibition of polymerization has been demonstrated by mixing ADP and poly U or UDP and poly A.

GDP-P$_{i}$ exchange was not inhibited by the presence of either poly A or poly U.

The abbreviations used are: poly A, polyadenylic acid; poly U, polyuridylic acid; poly G, polyguanylic acid; poly I, polynosinic acid; and poly AGUC, mixed polymer of adenylc, uridylic, cytidylic, and guanylic acids.

* M. F. Singer, unpublished data.

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**Table IV**


The reaction mixtures (0.1 ml) contained 0.96 µg of E. coli enzyme and the following in mM concentrations: Tris buffer, pH 8.2, 100; MgCl$_2$, 5; ethylenediaminetetraacetate, 0.2; GDP, 4; and P$_{i}$, 24 (specific activity, in c.p.m./pmole, 10$^3$ in Experiment 1, and 92,000 in Experiment 2). Incubation time was 0.5 and 1.0 hour in Experiments 1 and 2, respectively. ADP, 5, or UDP, 10, and the compounds, ApUp, ApApUp, and ApApApUp, are not phosphorolyzed (10) and no such correction need be applied.

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**Table V**

**Effect of oligonucleotides on exchange between ADP or UDP and P$_{i}$**

Reaction mixtures (0.1 ml) contained the following in mM concentrations: Tris buffer, pH 8.2, 100; MgCl$_2$, 5; ethylenediaminetetraacetate, 0.2; GDP, 4; and P$_{i}$, 24 (specific activity, in c.p.m./pmole, 10$^3$ in Experiment 1, and 131 X 10$^3$ c.p.m./pmole in Experiment 2). Incubation time was 1 hour for Experiment 1, and 1 hour and 10 minutes for Experiment 2.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Nucleotides added</th>
<th>P$_{i}$ incorporated into acid-soluble nucleotides</th>
<th>Fold stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>20</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>ApUp (2.3)*</td>
<td>43</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>ApApUp (1.7)</td>
<td>34</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>ApApApUp (2.1)</td>
<td>43</td>
<td>2.2</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>23</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>ApUp (1.3)</td>
<td>50</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>ApApUp (1.7)</td>
<td>39</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>ApApApUp (2.1)</td>
<td>43</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate the mM concentrations of the oligonucleotides.
Thymine Ribonucleoside Pyrophosphate Exchange—It is of interest from the point of view of the specificity of polynucleotide phosphorolysis that the enzyme also catalyzes the synthesis of a polymer of thymine ribonucleotide units from thymine ribonucleoside pyrophosphate (14). We have now shown that the enzyme catalyzes an exchange between P_{1}\textsuperscript{32} and thymine ribonucleoside pyrophosphate. Thus, a ratio of thymine ribonucleoside diphosphate to P_{1}\textsuperscript{32} of 0.9, in the presence of 3.6 mmol of E. coli polynucleotide phosphorylase, the rates of exchange were 17 and 9 mmol per h per mg protein with the E. coli and A. agilis (SA 60) enzymes, respectively.

Phosphorolysis of Polynucleotides

Effect of ApUp and its Homologues on Phosphorolysis—In the presence of an excess or equimolar concentration of ApApUp, the phosphorolysis of pApApApA is inhibited about 20% (Table VII). The tetranucleotide ApApApUp is a more effective inhibitor of pApApApA phosphorolysis (Table VII) than is ApApUp. On the other hand, the phosphorolysis of pApApApA is not inhibited by a 4-fold excess of ApApUp and is only slightly inhibited by a 6-fold excess of ApApApApU phosphorolysis. This mechanism of phosphorolysis was also studied with a preparation of poly G that was synthesized with pApApA as a primer (7). This poly G sample had an average chain length of 114, indicating 84 guanine acid residues, on the average, to every molecule of pApApA. The details of its preparation and characterization have been described (7). Poly G (1.0 mg per ml) was incubated in the presence of 8.8 mM phosphate buffer, pH 7.4 (200,000 c.p.m./pmole), 3 mmol MgCl\textsubscript{2} and 39 pg per ml of E. coli polynucleotide phosphorylase. At 1, 2, 3, 4, 5, and 6 hours, 0.1 ml samples were assayed for P_{1}\textsuperscript{32} incorporation into mononucleotides. In this experiment, the mononucleotides were separated by elution from charcoal, and the area containing them was eluted and rechromatographed in Solvent 2 (Fig. 3B) in order to determine their relative concentrations. The various materials were eluted, their amounts determined quantitatively (Table VIII) and their identity confirmed by rechromatography in Solvent 2 (Table VIII). The major initial products of pApApApPu phosphorolysis are UDP and pApA. At the first time interval (1 h), only small amounts of pApA and ADP are formed. At the second time interval (3 h), the quantities of both pApA and ADP have increased, and in equimolar amounts. These results suggest that the first bond cleaved is the one between the uridine residue and its neighboring adenylic acid unit. The resulting pApA is then phosphorolyzed to give pApA and ADP. The dinucleotide pApA accumulates as an end product since it is not phosphorolyzed by polynucleotide phosphorylase (10).

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FIG. 3. Stepwise phosphorolysis of \( \text{dApApApU} \). The incubation mixture contained 25 \( \mu \text{g} \) of \( E. \text{coli} \) polynucleotide phosphorylase per ml and the following in mM concentrations: Tris buffer, pH 8.2, 50; MgCl\(_2\), 5; NaKPO\(_4\), pH 7.2, 30; and pApApApU, 2.2. At 0 time a 0.02 ml aliquot was applied to Whatman No. 3 MM paper and the paper was dried and stored in the cold. Incubation was at 37\(^\circ\). After 1 and 3 hours of incubation, additional aliquots (0.09 ml) were quantitatively applied to the paper which was chromatographed in Solvent 1 (A). The areas marked pApApA + pApA in A were eluted with H\(_2\)O and rechromatographed in Solvent 2 (B).

TABLE VIII


The incubation mixture is described in the legend to Fig. 3. The areas corresponding to the listed compounds were eluted with H\(_2\)O from the chromatogram shown in Fig. 3 and their concentrations were estimated from the absorbancy of the eluates at suitable wave lengths with known extinction coefficients.\(^4\) The absorbancy of each eluate was corrected for the absorbancy of the eluate of a corresponding area of blank paper. The eluates were concentrated and rechromatographed in Solvent 2. Each substance had the same \( R_d \) (\( R_f \) relative to that of ADP) in this solvent as the respective known markers. Solvent 2 also afforded the separation of pApA and pApApA (See Fig. 3) and the yieldds of these substances were determined after elution from the Solvent 2 chromatogram.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_d ) in Solvent 2</th>
<th>Amount recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>pApApApApU</td>
<td>0.18</td>
<td>1.7</td>
</tr>
<tr>
<td>UDP</td>
<td>0.77</td>
<td>0.0</td>
</tr>
<tr>
<td>ADP</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>pApApA</td>
<td>0.45</td>
<td>0.0</td>
</tr>
<tr>
<td>pApA</td>
<td>0.84</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^4\) It was assumed for this work that the molar extinction coefficient of an oligonucleotide is approximated by the sum of the molar extinction coefficients of its constituent nucleotides. For example, the concentration, \( C \), of pApApApU in \( \mu \text{moles} \) per ml, when the absorbancy is measured at pH 2, is given by \( C = \frac{\text{Abs}_{\text{meq}}}{3(15.1) + 10.0} \), where the molar extinction coefficients for AMP and UMP are 15,100 and 10,000, respectively. The approximation does not account for any hypochromic effect.
DISCUSSION

On the basis of the data concerning GDP-Pi exchange presented above and that concerning GDP polymerization (7), it appears likely that polynucleotide phosphorylase can efficiently utilize GDP, as well as ADP, UDP, CDP, and thymine ribonucleoside pyrophosphate as substrates. This conclusion holds for enzyme preparations from E. coli and A. agilis and is consistent with the findings of Ochoa et al. (13, 15). The latter investigators have shown that the relative activities of ADP, UDP, CDP, and GDP in the exchange reaction remain constant with the purification of the A. agilis enzyme (13). Also pertinent is the isolation (1) and characterization (4) of poly AGUC and the demonstration that all four nucleotides are bound together, at random, in poly AGUC (15). However, the work of Ohnsted and Lowe (16, 17) has supported the notion that separate enzymes for each of the nucleoside diphosphates are present in Micrococcus lysodeikticus.

Some of the experiments discussed above are pertinent to the question of the relation of the exchange reaction (Equation 2) to the mechanism of polymerization. Grunberg-Manago (18) has pointed out two hypotheses concerning this relation: (a) the exchange represents the reversible formation of a nucleoside monophosphate-enzyme complex; and (b) the exchange results from the occurrence of the over-all reversible reaction (Equation 1) under approximately equilibrating conditions. Earlier data bearing on this question have been reviewed by Grunberg-Manago (18). The observation that polynucleotide phosphorylase catalyzes an exchange between GDP and P₄ in the absence of any oligonucleotide primer suggests that the exchange reaction is independent of polymerization since the latter is dependent on the presence of a primer (7). The stimulation of GDP exchange by compounds like pApApA and ApApUp would appear to be unrelated to the effect of oligonucleotides on GDP polymerization since ApApUp and its homologues do not prime poly G formation. This conclusion is also supported by the exchange experiments with ADP and UDP, with enzymes which form poly A and poly U only after a lag period. No lag has been observed with the exchange reaction. In addition, the stimulation of the rate of ADP and UDP polymerization, when oligonucleotides are used to overcome the lag period (6), is of much greater magnitude than the increase in the rate of ADP and UDP exchange caused by such compounds.

The inhibition of ADP and UDP exchange by poly U and poly A, respectively, apparently reflects a reaction common to both exchange and polymerization since completely analogous inhibitions are observed with ADP and UDP polymerization (8). This specific inhibition of both exchange and polymerization by polymers has recently been confirmed by Heppel, who has extended the observation to several nucleoside diphosphate-polymer pairs, e.g., IDP and poly C, CDP and poly I.

A stepwise mechanism for the phosphorylization of oligoribonucleotides is indicated by the experiments on pApApApU and poly G phosphorylation. Thus, phosphorylization of pApApApU might have occurred (a) with random cleavage of the susceptible diester bonds, yielding equivalent amounts of pApA, pApApA, UDP, and pApU as initial products; or (b) in stepwise fashion from the end of the chain bearing a uridine residue, yielding equivalent amounts of pApApA and UDP as the initial products. The data obtained are consistent with the stepwise mechanism. This is confirmed by the fact that GDP was the only product observed during the phosphorylization of a short chain length preparation of poly G. None of the oligonucleotides that would have resulted from a random type of cleavage were detected. Razzell and Khorana (11) have recently demonstrated that snake venom diesterase hydrolyzes deoxyribo-oligonucleotides in a stepwise manner from the end of the chain bearing the free 3' hydroxyl group. Thus, polynucleotide phosphorylase and venom diesterase appear to have a common mode of action.

SUMMARY

Polynucleotide phosphorylase catalyzes an exchange between guanosine 5'-diphosphate and inorganic orthophosphate-P₄ at a rate similar to the analogous reactions with adenosine 5'-diphosphate and uridine 5'-diphosphate. Optimal conditions for this reaction were determined. Previous reports indicating that the enzyme does not utilize guanosine 5'-diphosphate as a substrate for the exchange reaction apparently resulted from the unfavorable Mg⁺⁺ and inorganic orthophosphate-P₄ concentrations that were used. When guanosine 5'-diphosphate, adenosine 5'-diphosphate, or uridine 5'-diphosphate is the substrate, oligonucleotides (including pApA, pApApA, ApApUp, ApApUp, and ApApApUp) stimulate the exchange reaction. Although polyadenylic acid does not inhibit the exchange reaction when adenosine 5'-diphosphate is the substrate, polyuridylic acid strongly inhibits uridine 5'-diphosphate exchange and polyuridylic acid inhibits adenosine 5'-diphosphate exchange. The significance of these results with respect to the mechanism of the exchange reaction is discussed.

Data are also presented that indicate that the polynucleotide phosphorylase catalyzed phosphorylization of relatively short chain length polyribonucleotides occurs in a stepwise fashion. Cleavage starts at that terminal nucleoside residue that has an unesterified 3'-hydroxyl group.

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