Enzymatic Synthesis of Polynucleotides

IV. PURIFICATION AND PROPERTIES OF POLYNUCLEOTIDE PHOSPHORYLASE OF AZOTOBACTER VINELANDII*

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Several publications from this laboratory have dealt in a preliminary fashion with the preparation and properties of highly purified polynucleotide phosphorylase from Azotobacter vinelandii (2-4). A more detailed account of this work is presented in this paper. Although polynucleotide phosphorylase preparations can catalyze the synthesis of a variety of homopolymers (polyadenylic acid, polyuridylic acid, etc.) and copolymers (of adenylic and uridylic acids, of adenylic, guanylic, uridylic, and cytidylic acids, etc.), the evidence so far available indicates that a single enzyme is involved. The best preparations represent a 500-fold purification of the enzyme from the initial extracts and are largely, although not totally, devoid of nuclease activity. These preparations contain about 3% of a firmly attached, small polyribonucleotide. It has not been possible to remove this nucleotide without denaturing the enzyme, and it remains unsettled whether it is a prosthetic group or merely a tenacious contaminant. Although the oligonucleotide appears to act as a primer of polymer synthesis, the need for added oligonucleotide (5) or polynucleotide primers (2-4) can be shown readily under appropriate conditions.

EXPERIMENTAL PROCEDURE

Methods

Synthesis of polynucleotides was followed by measuring the release of P_i from nucleoside diphosphates. The final volume of the reaction mixture was usually 0.1 ml. At various times during the incubation, aliquots (0.01 to 0.02 ml) were withdrawn and added to enough cold 2.5% perchloric acid or 5% trichloroacetic acid to give a total volume of 0.5 or 1.0 ml. Precipitated protein or polymer was removed by centrifugation and an aliquot of the supernatant fluid was used for the determination of P_i. This was carried out by the method of Lohmann and Jendrassik (6) adapted to small amounts with use of the Beckman spectrophotometer. Ribose and deoxyribose were determined by the orcinol and diphenylamine reactions, respectively, as described by Dische (7). Alkaline hydrolysis was carried out in 0.32% KOH for 18 hours at 37°. The solution was neutralized with perchloric acid and the insoluble potassium perchlorate removed by centrifugation. Base analysis was done as in previous work (8) or according to Kleinschmidt and Manthey (9).

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Preparations

Nucleoside diphosphates were either obtained commercially (Sigma Chemical Company, Pabst Laboratories) or prepared synthetically (10, 11) and isolated and used as the lithium salts. We are indebted to Dr. R. W. Chambers for samples of synthetic ADP, GDP, UDP, and CDP. These were used for the experiments of Tables II and III and Figs. 4 and 6. P^32-labeled phosphate was obtained from the Oak Ridge National Laboratory on allocation from the Atomic Energy Commission. Polymers were prepared and isolated as previously described (12) in most cases with enzyme at step 4 of purification. Poly AGUC was made from equimolar amounts of ADP, GDP, UDP, and CDP. Triadenylic acid was a gift of Dr. L. A. Heppel, National Institutes of Health. Whole cell Azotobacter RNA and DNA, and rat liver RNA were gifts of Dr. R. M. S. Smellie. Yeast RNA and salmon sperm DNA were commercial preparations. Crystalline pyruvic kinase and lactic dehydrogenase and phosphonopyruvate (silver-barium salt) were purchased from C. F. Boehringer and Sons, Mannheim, Germany, DPNH from Sigma or Boehringer and Sons. Crystalline egg albumin and protamine sulfate were gifts of Dr. R. C. Warner and the Lilly Research Laboratories, Indianapolis, respectively. Calcium phosphate gel was purchased from the Sigma Chemical Company and processed as follows before use. Gel (1 kg of lot No. 47-240 and 3 kg of lot No. 57-240), was diluted to 12 liters with deionized water. The suspension was adjusted to pH 5.8 by addition of 4.5 ml of glacial acetic acid, with mechanical stirring, and the gel was allowed to settle. After decantation of the water, the residue was washed six times with each 12 liters of water and finally suspended in 4 liters of water to give 130 mg of Ca_3(P0_4)_2 per ml. The suspension was kept in the refrigerator.

Isolation of Enzyme

Assay—Two methods, based on Reaction 1, were used for the assay of polynucleotide phosphorylase. In one, the rate of exchange of P_i with ADP was measured as in previous work (12);

1 The abbreviations used are: poly A, poly G, poly U, and poly C, polyadenylic, polyguanylic, polyuridylic, and polycytidylic acid, respectively; poly AU and poly AGUC, the copolymers of adenylic and uridylic acid and those of adenylic, guanylic, uridylic, and cytidylic acid.

The exchange may represent the reversible formation of an AMP-enzyme complex thus, ADP + enzyme = AMP-enzyme + P_i, since there are indications (13) that it may occur in the absence of the over-all Reaction 1. Singer et al. (13) give the fol-
more recent, unpublished experiments, Dr. Singer (personal com-

fore, be the result of Reaction 1.

of 1,000 and 10,000 c.p.m. However, in

side-P-P + Pi32 ti nucleoside-P-P32 + Pi. However, in

the conditions of the assay: values above pH 7.5 were

obtained by substitution of Tris-HCl buffer of the desired pH

(final concentration, 0.1 M) for the glycylglycine buffer of the

standard assay. For other details, see text.

\[ n \text{ADP} \rightleftharpoons \text{poly} A + n \text{P}_1 \] (1)

in the other, the rate of formation of ADP by phosphorylation of

poly A was determined spectrophotometrically in a multi-

enzyme system.

\[ P, \text{exchange assay} - \text{this was carried out as previously}

described (12) with some modifications. The samples contained:

K_2HPO_4, 3.5 μmoles; ADP, 2.5 μmoles; enzyme (not over 0.35

unit), and 0.8 ml of a mixture of Tris-HCl buffer, pH 8.1, 100

μmoles; MgCl_2, 5 μmoles; EDTA, 2 μmoles; and KH_2PO_4 with

100,000 to 200,000 c.p.m. The final volume was made up to 1.0

ml with water. Enzyme dilutions were made in 0.1

M Tris-HCl buffer, pH 7.4. A blank without either ADP or enzyme

was always run with assay samples. Commercial samples of ADP

were routinely used for the assay. However, since different

batches of ADP frequently gave somewhat different rates of

exchange, probably due to variations in their degree of purity,

each batch was previously standardized against a sample of

synthetic ADP (10). After incubation for 15 minutes at 30°

the amount of exchange taking place was determined from the

amount of organically bound radioactive phosphate. This is

given by the radioactivity remaining in the protein-free filtrate

after quantitative removal of the inorganic phosphate through

conversion to ammonium phosphomolybdate and extraction with

isobutanol. The procedure was as described in the preceding

paper of this series (1). One unit was defined as the amount of

each stage of purification (Table I) protein was deter-

mined by the biuret method (16) or by the method of Lowry et al.

(17) because of the high nucleic acid content of the enzyme

fractions. Thereafter, it was determined spectrophotometrically

(18) with use of the table given by Layne (19) to correct for the

nucleic acid content. For correspondence of the values obtained

by the colorimetric and spectrophotometric methods, the protein

concentration of crystalline egg albumin solutions used as

standard for the former was determined spectrophotometrically.

Because of the development of turbidity and the catalysis of

by fresh DPNH solutions were prepared every few days, ad-

justed to pH 10 with sodium hydroxide, and kept frozen when

not in use.

Optical assay—In this assay, the rate of production of ADP

by phosphorylation of poly A was measured through coupling with

the reactions catalyzed by pyruvic kinase and lactate dehydro-

genase. In the presence of an excess of these enzymes, phos-

phoenolpyruvate and DPNH, ADP reacts with phosphoenol-

pyruvate to give ATP and pyruvate, and the latter reacts with

DPNH to give lactate and DPN+. The rate of oxidation of

DPNH is proportional to the rate of formation of ADP. The

reaction, which had a fairly sharp optimum around pH 7.4
(Fig. 1 A), was followed as the decrease in absorbancy at 340 μm

and was carried out in Corex or silica cells (b = 1.0 cm) in the

Beckman spectrophotometer. The cell compartment was filled

with a jacket, through which was circulated water at a tempera-

ture of 30°. The reaction mixtures contained the following com-

ponents in micromoles per ml: potassium phosphate, pH 7.4, 10;

glycylglycine buffer, pH 7.4, 5; MgCl_2, 5; EDTA, 1; crystalline

egg albumin, 2 mg; phosphoenolpyruvate, 1.6; excess of crystal-

line pyruvic kinase and lactate dehydrogenase (each in amount

sufficient to give a rate of DPNH oxidation of 0.3 μmole per ml

per minute at 30°, under the conditions of their respective optical

assays (14, 15)); DPNH, about 0.13 (initial absorbancy at

340 μm, about 0.8); poly A (aqueous solution), 0.06 mg (0.18

μmole as adenylic acid); and enzyme. Enzyme dilutions were

frequently made in 0.5% crystalline egg albumin. The final volume

was usually 1.0 ml and occasionally 0.4 ml. Suitable micro-

cells were used in the latter case. The reference cell contained

water. In practice, it was found to be convenient to use 0.75

ml per ml of a mixture containing the indicated amounts of phos-

phate, glycylglycine, MgCl_2, EDTA, crystalline egg albumin,

phosphoenolpyruvate, and lactate dehydrogenase. This mixture

was kept frozen and was thawed just before use. It often de-

veloped some turbidity on thawing which was removed by cen-

trifugation at 0°. Pyruvic kinase, DPNH, and either poly A

or enzyme were then added, and the mixture was equilibrated

in a bath at 30° for 1 to 2 minutes after which the absorbancy

at 340 μm was determined. If this remained constant and no

turbidity developed, the final addition (poly A or enzyme) was

made, and readings were taken at 30-second intervals for 3 to

5 minutes during which time the oxidation of DPNH proceeded

at an approximately constant rate. This was proportional to

the concentration of enzyme up to about 0.3 unit per ml (Fig.

1 B). One mole of ADP is produced per mole of DPNH

oxidized and, for correlation with the exchange assay unit, one

optical unit was taken as the amount of enzyme catalyzing the for-

mation of one μmole of ADP in 15 minutes at 30° and pH 7.4.

As determined experimentally this optical unit is equivalent to

approximately 1.2 P, exchange units. Specific activity is ex-

pressed as units per mg of protein.

Through stage 4 of purification (Table I) protein was deter-

mined by the biuret method (16) or by the method of Lowry et al.

(17) because of the high nucleic acid content of the enzyme

fractions. Therefore, it was determined spectrophotometrically

(18) with use of the table given by Layne (19) to correct for the

nucleic acid content. For correspondence of the values obtained

by the colorimetric and spectrophotometric methods, the protein

concentration of crystalline egg albumin solutions used as

standard for the former was determined spectrophotometrically.

Because of the development of turbidity and the catalysis of

polynucleotides. IV

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Fig. 1. Optical assay of polynucleotide phosphorylase. Ac-

tivity as a function of pH (A) and enzyme concentration at pH

7.4 (B); temperature, 30°; final volume, 0.4 ml; enzyme of specific

activity (optical) 140. In Experiment A, each sample had 0.9

μg of enzyme protein. pH values up to 7.5 were obtained with the

appropriate phosphate and glycylglycine buffers at the concen-

trations used in the standard assay; values above pH 7.5 were

obtained by substitution of Tris-HCl buffer of the desired pH

(final concentration, 0.1 M) for the glycylglycine buffer of the

standard assay. For other details, see text.
DPNH oxidation with crude enzyme solutions, the optical assay could only be used after stage 4 of purification (Table I). The assay results have been calculated throughout in terms of optical units.

**Purification**

**Growth of Cells**—*Azotobacter vinelandii* (strain O)* was carried on agar slants with frequent transfers (approximately once a month). Inoculations were made into 150 ml portions of Burk's medium (20) in 1-liter conical flasks and the cells were grown at 32° for 18 to 20 hours on a rotary shaker (New Brunswick Scientific Company, model V55370) set at maximal speed. The absorbancy of the cell suspension at 500 m\(\mu\) should not be lower than 2.5. Fresh 150 ml portions of medium were inoculated with 5 ml of the culture thus obtained, the cells were grown as above, and the new culture was used to inoculate fresh medium again. This operation was repeated several times. Approximately 1 liter of this culture was used to inoculate 150 liters of medium in a vat fermenter. Cells were grown at 28° with vigorous agitation for 18 to 20 hours and harvested, at about 0°, with an industrial type refrigerated Sharples centrifuge within 1 hour. The yield of wet cells was about 1.5 kg. After harvesting, the cells were frozen until used. Under these conditions, polynucleotide phosphorylase activity remained unchanged on storage for several months.

**Step 1. Extraction**—This was carried out in the cold room (4°) in a stainless steel Waring Blendor essentially as described by La Manna and Mallette (21) for the preparation of *Escherichia coli* extracts. Frozen cells, 1 kg, were milled in a 1-gallon blendor with 450 to 500 ml of ice-cold 0.01 m potassium phosphate buffer, pH 7.4, and 3 kg of glass beads, and extracted by first slow stirring for a few minutes until a thick slurry was produced, then for 15 minutes at about two-thirds maximal speed. Two liters of buffer were then added, and stirring was continued for 10 more minutes as before. After the beads had settled, the extract was poured off. The residue was re-extracted in the same way with 2 liters of buffer for 1 minute. The combined supernatants yielded approximately 4.5 liters of greenish brown, turbid extract with about 20 mg of protein per ml. The enzyme is somewhat unstable at this stage, and the next step was therefore started at once.

**Step 2. Ammonium Sulfate Fractionation**—After cooling to 0°, the extract (4.6 liters) was diluted with ice-cold 0.01 m potassium phosphate buffer, pH 7.4, to a protein concentration of 10 mg per ml with addition of enough 0.5 m EDTA to give a final concentration of 0.001 m, volume, 10 liters. To the diluted extract were added 2290 g of solid, finely powdered ammonium sulfate (to give approximately 0.33 saturation) over a period of about 60 minutes with mechanical stirring, the temperature being maintained at 0° and the pH kept at 7.4 (glass electrode) by occasional dropwise addition of from 50 to 60 ml of 6.0 m potassium hydroxide. Stirring was continued for another hour and, after adjustment of the pH to 7.4 if necessary, the mixture was allowed to stand at 0° overnight. The precipitate, containing over 80% of the protein and 30 to 40% of the units (specific activity, 0.0 to 0.7) was removed by centrifugation for 1 hour at 0° and maximal speed of the large rotors of the refrigerated Servall or Lourdes angle centrifuge and discarded. Solid ammonium sulfate, 910 g, was added as above to the ice-cold supernatant fluid (to give approximately 0.46 saturation) over a period of 30 minutes, maintaining the temperature at 0°, and the pH at 7.4. This required about 12 ml of 0.0 nitrogen phosphate hydroxide. After the mixture was stirred for a further 60 minutes, it was centrifuged as before, and the supernatant fluid was discarded. The precipitate was dissolved in 170 ml of ice-cold 0.01 m potassium phosphate buffer, pH 7.4, and dialyzed at 0° with stirring overnight against 6 liters of 0.033 m succinate buffer, pH 6.3, containing 0.5 \(\times 10^{-3}\) m cysteine. The clear, reddish brown dialyzed solution (470 ml) contained 86 mg of protein per ml. Usually, several batches of cells were worked up through Step 2, and the dialyzed solutions were stored in the frozen state (−18°) before proceeding to the next step. However, storage for periods longer than 3 months should be avoided. It was found that after storage for about a year, the enzyme retained its original activity but could no longer be successfully fractionated with ethanol.

**Step 3. Low Temperature Ethanol-Zinc Acetate Fractionation**—The solution from the previous step was diluted with 0.033 m succinate buffer, pH 6.3, containing 0.5 \(\times 10^{-3}\) m cysteine, to a protein concentration of 10 mg per ml and the diluted solution (8.1 liters) cooled to 0°. To this solution were added, in small alternating fractions, 570 ml of absolute ethanol (chilled to −15°) and 156 ml of 0.1 m zinc acetate (cooled to 0°) with vigorous mechanical stirring over a period of 40 minutes, the temperature being gradually lowered to −6°. The concentration of ethanol was approximately 15% by volume and that of zinc acetate 0.004 m. After being stirred for a further 30 minutes, the mixture was centrifuged for 2 hours at −10° and maximal speed of the large rotors of the refrigerated Servall or Lourdes angle centrifuge. The precipitate, containing 60 to 80% of the protein and 30 to 40% of the units (specific activity about 0.5) of the

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Units</th>
<th>Protein</th>
<th>Specific activity</th>
<th>280/260</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extract</td>
<td>4,600</td>
<td>101,360</td>
<td>10.4</td>
<td>1</td>
<td>0.60</td>
<td>100</td>
</tr>
<tr>
<td>2. (NH₄)₂SO₄ fractionation</td>
<td>470</td>
<td>58,262</td>
<td>31.1</td>
<td>2</td>
<td>0.60</td>
<td>58</td>
</tr>
<tr>
<td>3. Ethanol fractionation</td>
<td>235</td>
<td>42,287</td>
<td>5.4</td>
<td>8</td>
<td>0.60</td>
<td>42</td>
</tr>
<tr>
<td>4. Ca₃(PO₄)₂ gel eluate</td>
<td>115</td>
<td>17,850</td>
<td>0.4</td>
<td>45</td>
<td>0.65</td>
<td>18</td>
</tr>
<tr>
<td>5. Protamine and (NH₄)₂SO₄ fractionation</td>
<td>2</td>
<td>4,275</td>
<td>19</td>
<td>226</td>
<td>0.91</td>
<td>68</td>
</tr>
<tr>
<td>6. Chromatography</td>
<td>20</td>
<td>2,812</td>
<td>8</td>
<td>350</td>
<td>0.02</td>
<td>45</td>
</tr>
</tbody>
</table>

* P,⁴³ exchange assay used through Step 4; optical assay used thereafter. Specific activity expressed in terms of optical assay throughout (1.0 unit = 1.2 P,⁴³ exchange units).

† Ratio of light absorption at 280 m\(\mu\) to 260 m\(\mu\).

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* Obtained from the American Type Culture Collection, Washington, D. C. Listed as No. 9104 in the 6th (1956) Edition of the ATCC catalogue.

* Superbrite No. 100 (average diameter 200 µ) obtained from the Minnesota Mining and Manufacturing Company, St. Paul, Minnesota.
ammonium sulfate fraction, was discarded. To the supernatant fluid were added, as above, 420 ml of ethanol and 520 ml of 0.1 M zinc acetate over a period of 30 to 45 minutes, the temperature being lowered gradually to -8°. The concentration of ethanol was approximately 20% by volume, and that of zinc acetate was 0.014 M. The mixture was stirred for a further 30 minutes and centrifuged for 2 hours at -15° as before; the clear, pale pink supernatant was discarded. The precipitate was immediately dissolved in about 160 ml of ice-cold 0.1 M potassium phosphate buffer, pH 7.4, containing 0.01 M cysteine and 0.03 M EDTA and dialyzed with stirring at once against 6 liters of 0.01 M potassium phosphate buffer, pH 7.4, containing 0.001 M EDTA, at 0° overnight. The clear, reddish brown dialyzed solution (235 ml), containing 23 mg of protein per ml, could be stored in the frozen state with little loss of activity.

Step 4. Adsorption and Elution from Calcium Phosphate Gel—
The solution from the previous step (235 ml) was brought to 540 ml with 0.01 M potassium phosphate buffer, pH 7.4, giving a protein concentration of 10 mg per ml, cooled to 0°, and brought to pH 6.4 (glass electrode) by the dropwise addition of 1.0 N acetic acid (about 6 ml) with mechanical stirring. Calcium phosphate gel (130 mg of Ca₃(PO₄)₂ per ml), 25 ml, was then added; after the gel was stirred for 15 minutes, it was centrifuged off at 0° and discarded. To the supernatant (pH 5.6) were added 50 ml of the calcium phosphate gel, and the gel was discarded after stirring and centrifuging as above. The supernatant (pH 5.7) was brought to pH 5.5, with about 0.4 ml of 1.0 N acetic acid, and a further 50 ml of gel were added. After stirring for 15 minutes, the gel was collected by centrifugation, and the supernatant was discarded. This gel was eluted four times at 0°, each with 30 ml of 0.1 M potassium phosphate buffer, pH 6.0, and the eluates were combined to give a clear, pale yellow solution with 3.0 to 3.5 mg of protein per ml. It was dialyzed overnight at 0° against 3 liters of 0.02 M potassium phosphate buffer, pH 6.8, containing 0.001 M EDTA. The dialyzed eluate (115 ml) could be stored in the frozen state for periods up to 2 or 3 months with little loss of activity.

Step 5. Protamine and Ammonium Sulfate Fractionation—A freshly prepared 2% solution of protamine sulfate was added dropwise to 41 ml of the dialyzed eluate at 0° with mechanical stirring. Just enough protamine sulfate solution, in this case 0.1 ml, was added; after the gel was stirred for 15 minutes, the gel was collected by centrifugation, and the supernatant was discarded. The yellow precipitate, which became gummy on stirring, was washed with 20 ml of 0.02 M phosphate buffer, pH 6.8, containing 0.001 M EDTA, and dissolved in 6.5 ml of a solution containing 0.1 M glycine and 20% saturated ammonium sulfate, the pH of which had been adjusted to 6.3 with potassium hydroxide. A small amount of insoluble residue was removed by centrifugation. To the clear yellow supernatant (7.4 ml), cooled to 0°, were added 5.0 ml of saturated ammonium sulfate with magnetic stirring, bringing the ammonium sulfate concentration to approximately 0.43 saturation. After being stirred for 10 minutes, the precipitate, which had little activity, was removed by centrifugation for 15 minutes at 0° and 15,000 X g and discarded. A further 2.0 ml of saturated ammonium sulfate were now added to the supernatant as above, bringing the ammonium sulfate concentration to approximately 0.52 saturation. The precipitate was collected by centrifugation, washed twice with each 5 ml of 60% saturated ammonium sulfate containing 0.001 M EDTA, and dissolved in 1.5 ml of 0.02 M phosphate buffer, pH 6.8, containing 0.001 M EDTA. A light precipitate formed whenever the dark yellow solution was brought to 0°. From previous trials, this precipitate was known to carry down much of the enzyme as the activity of the supernatant diminished considerably, and enzyme could be recovered from the precipitate by extraction with the 0.1 M glycine-20% saturated ammonium sulfate solution. Since it was found that the material responsible for precipitate formation could be removed with charcoal, a pinch of acid-washed Norit A was added to the solution of the ammonium sulfate precipitate at about 10°, at which temperature the solution was quite clear. Addition of too much Norit leads to substantial losses of enzyme and should be avoided. After a few minutes of stirring, the Norit was removed by centrifugation at 0° and washed with 0.5 ml of the 0.02 M phosphate-0.001 M EDTA buffer, pH 6.8. The combined supernatants were dialyzed overnight at 0° against the same buffer, yielding 2.0 ml of dark yellow solution containing 9.5 mg of protein per ml.

Step 6. Chromatography on Hydroxylapatite—
Hydroxylapatite, prepared by the method of Tiselius et al. (22), was packed by gravity into a 1 X 40-cm column and washed overnight in the cold room (34°) with 0.02 M phosphate buffer, pH 6.8, containing 0.001 M EDTA. The flow rate was adjusted to roughly 20 ml per hour by applying slight pressure (20 to 50 mm Hg).

The enzyme solution from the previous step was passed through the column whereby all the protein was retained by the gel. Elution was carried out stepwise with each 30 ml of 0.02, 0.04, and 0.06 M and each 60 ml of 0.11 and 0.2 M sodium phosphate buffer, pH 6.8, containing 0.001 M EDTA. The volume of the individual fractions collected in a fraction collector at 10-minute intervals in the cold room varied between 2.5 and 3.5 ml. The elution of protein was followed spectrophotometrically by determining the absorption of light at 280 mp and that of enzyme by optical assay. As shown in Fig. 2, small amounts of inactive protein were released by phosphate buffer up to 0.06 M and the first half of the 0.11 M buffer. The enzyme was eluted as a sharp band with 0.11 M buffer; these eluates were colorless. A nonfluorescent yellow protein, responsible for the color of the protamine fraction, was eluted with 0.2 M buffer. Fractions with specific activities of 300 or higher were combined to give 20 ml of solution with 0.4 mg of protein per ml. This solution was dialyzed overnight at 0° against 3 liters of 0.5 X 10⁻³ M EDTA, adjusted to pH 7.4, and concentrated by lyophilization to about 1.0 ml. The concentrated enzyme was dialyzed for 5 hours at 0° against 1 liter of 0.02 M sodium phosphate buffer, pH 6.8, containing 0.001 M EDTA.

The dialyzed enzyme was rechromatographed as above. Fractions of specific activity above 400 were combined (9.5 ml), and the enzyme was precipitated at 0° by addition of 3.2 g of solid ammonium sulfate. The precipitate was washed with 5 ml of 50% saturated ammonium sulfate containing 0.001 M EDTA, dissolved in 1.5 ml of 0.01 M glycylglycine buffer, pH 6.8, con-
taining 0.001 M EDTA, and dialyzed at 0° against 1 liter of the same buffer overnight. Two ml of enzyme solution containing 5 mg of protein were obtained. This solution was stored at -20°. The enzyme is relatively stable under these conditions. A summary of the purification procedure is given in Table I.

The above procedure was carried out several times with similar results during 1958 and 1959, and a total of 10 kg of cells was worked up in this manner. More recently, the reproducibility of Steps 3 and 4, as regards both purification and yield, has been poor. The following average specific activities and percentages of over-all yields (given in parentheses) were obtained in several small scale runs between June and December, 1960; Step 2, 2.0 (60%); Step 3, 4.5 (25%); Step 4, 17.0 (5%); Step 5, 65.0 (2.5%). These values are to be compared with the corresponding values in Table I. The cells were grown from agar slants that had been kept in the laboratory with occasional transfers for over a year, and bacterial variation may have occurred. It should be pointed out that, in any case, polynucleotide phosphorylase preparations of specific activity 40 to 60 are quite suitable for the preparation of polyribonucleotides.

Evidence for Single Enzyme—Although preparations of Azotobacter polynucleotide phosphorylase react with individual nucleoside 5'-diphosphates to give the corresponding homopolymers or with mixtures of nucleoside diphosphates to form different copolymers, a single enzyme appears to be involved. This is indicated by the fact that when preparations at different stages of purification are assayed by P32 exchange with individual nucleoside diphosphates, the degree of purification at each step is approximately the same for five different substrates, namely ADP, GDP, UDP, CDP, and IDP. Earlier results of such a study for purification Steps 1 through 4 have been presented (2). The results for Steps 4 and 6b are presented below.

The rate of the P32 exchange reaction with different nucleoside diphosphates is affected to a varying extent by such factors as the concentration of P and magnesium ions and, as previously pointed out (12), by the ratio of the concentration of nucleoside diphosphates to that of P. We have, therefore, determined the optimal P and magnesium ion concentrations for the P32 exchange assay with each of the substrates ADP, GDP, UDP, CDP, and IDP with the results shown in Table II. The assays were carried out as described in the legend to the table, the incorporation of P32 into nucleoside diphosphates being measured after their adsorption on charcoal (23). With 2 x 10^{-5} M

### Table II

<table>
<thead>
<tr>
<th>Additions</th>
<th>Specific activity*</th>
</tr>
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<tbody>
<tr>
<td>P1</td>
<td>Mg+</td>
</tr>
<tr>
<td>-----</td>
<td>----</td>
</tr>
<tr>
<td>2 x 10^{-5} M</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
</tr>
</tbody>
</table>

* Micromoles of P32 incorporated per 15 minutes per mg of protein at 30°.
nucleoside diphosphate, maximal exchange rates were obtained for UDP with \(2 \times 10^{-4} \text{ M P}^+\) and \(4 \times 10^{-5} \text{ M Mg}^{++}\), for both CDP and IDP, with \(P_1\) and \(Mg^{++}\) each at \(4 \times 10^{-5} \text{ M}\), and for both ADP and GDP with \(4 \times 10^{-4} \text{ M P}^+\) and \(1.6 \times 10^{-4} \text{ M Mg}^{++}\) (Table II). A comparison of the polynucleotide phosphorylase specific activity with all of the five nucleoside diphosphates at Steps 4 and 6b of purification, under the exchange assay conditions of Table II, is given in Table III. It may be seen that the degree of purification between the two steps was about the same for each of them.

### Table III

<table>
<thead>
<tr>
<th>Nucleoside diphosphate</th>
<th>Specific activity</th>
<th>Ratio (A/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>50</td>
<td>528</td>
</tr>
<tr>
<td>GDP</td>
<td>45</td>
<td>450</td>
</tr>
<tr>
<td>UDP</td>
<td>77</td>
<td>600</td>
</tr>
<tr>
<td>CDP</td>
<td>48</td>
<td>425</td>
</tr>
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**Fig. 3.** Ultraviolet absorption spectrum of polynucleotide phosphorylase (Step 6b) and the nucleotide therefrom.

**Properties of Enzyme**

Preparations of specific activity 400 to 500 appeared to be essentially devoid of nuclease activity. However, when assayed viscosimetrically in cacodylate buffer, pH 7.0, with poly U as substrate in the absence of \(P_1\) and \(Mg^{++}\), 7 \(\mu \text{g}\) of enzyme brought about decrease in viscosity at about the same rate as 0.01 \(\mu \text{g}\) of crystalline pancreatic ribonuclease. 7

Under the conditions of the standard optical assay, the turnover number of the enzyme at Step 6b of purification for phosphorylase of poly A is about 3000 moles per minute per \(10^4 \text{ g}\) of protein. The value is a little higher, namely 4500 to 5000, for the synthesis of poly A from 0.02 \(\mu \text{M}\) ADP in Tris buffer, pH 8.0, at 30° under optimal conditions. Polymer synthesis from GDP is much slower than from the other nucleoside diphosphates; moreover, GDP markedly hinders the synthesis of copolymeric polynucleotides when present along with other diphosphates (12). To obtain similar rates, 20 to 30 times more enzyme must be used for synthesis of poly AGUC from equimolar concentrations of ADP, GDP, CDP, and UDP (each 0.005 M) than for poly A synthesis. The turnover number in this case is only in the neighborhood of 200.

As noted in Table I, the best preparations of *Azotobacter* polynucleotide phosphorylase have a ratio of light absorption at 280 \(\mu\text{m}\) to that at 260 \(\mu\text{m}\) (280:260 \(\mu\text{m}\) ratio) in the neighborhood of 0.9 to 1.0, indicating the presence of 3 to 4% nucleotide material. This material is not removed by activated charcoal, Dowex 1 resin, DEAE-cellulose chromatography, or by incubation with ribonuclease followed by dialysis. Denaturation of the protein by precipitation with perchloric acid or treatment with phenol releases the nucleotide into the supernatant fluid or the aqueous layer. As described below, this material has proved to be a small polyribonucleotide. The ultraviolet absorption spectrum of the enzyme and the nucleotide therefrom, as well as the difference spectrum for the enzyme minus the nucleotide, are shown in Fig. 3.

**Polynucleotide from Enzyme**—A solution of enzyme at purification Step 6b containing 53.7 \(\mu\text{g}\) of protein in 3.4 ml of 0.02 \(\mu\text{M}\) phosphate buffer, pH 8.0, was extracted twice with an equal volume of 85\% redistilled phenol. The aqueous layer was collected, residual phenol being removed by extraction with ether and residual ether by aeration. This yielded 2.3 ml of solution; \(A_{260} = 1.2\) after alkaline hydrolysis; 280:260 \(\mu\text{m}\) ratio, 0.54. Taking \(\varepsilon_{280}\) as 10\(^6\), from the absorbancy after alkaline hydrolysis the solution contained around 1.2 \(\mu\text{mol}\) of nucleotide per ml or a total of 2.8 \(\mu\text{mol}\). This would amount roughly to 0.9 mg or 1.7\% of the enzyme protein.

By colorimetric assay, the material was found to contain ribose but not deoxyribose. Alkaline hydrolysis yielded AMP, GMP, UMP, and CMP. The compound was cleaved by pancreatic ribonuclease and by polynucleotide phosphorylase. All these observations indicate that it is a polyribonucleotide. Base analysis gave the following molar ratios: adenine, 1.0; guanine, 1.2; uracil, 0.85; cytosine, 0.89. These values are close to those previously reported (8) for whole cell *Azotobacter* RNA and poly AGUC. End group assay indicated an average chain length around 12. On ultracentrifugation, the polynucleotide appeared polydisperse with an average sedimentation constant \(s_{20,w} = 0.678\). The fact that 10 to 15\% of the material was dialyzable is a further indication of its heterogeneity.

**Polynucleotide Synthesis**—It has been reported (2, 3) that the synthesis of polynucleotides by *Azotobacter* polynucleotide phosphorylase preparations of low nucleic acid content is slow.

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7 We are indebted to Dr. R. C. Warner for these measurements.
in the absence of added polynucleotide primers. More detailed investigation with preparations as Step 9b of purification showed that the requirement or lack of requirement for added primers may depend on such factors as the concentration of enzyme and Mg++. This is illustrated in Fig. 4. With small amounts of both enzyme and Mg++, synthesis of poly A was primed by poly A (Curves 1 and 2). As previously noted (2, 3), poly U was inactive or inhibitory of poly A synthesis (Curve 3). With either four times as much Mg++ (Curve 4) or three times as much enzyme (Curve 5) the rate of synthesis, in the absence of added poly A, was the same as or higher than in the presence of poly A with the smaller amounts of both enzyme and Mg++. These results suggest that the polynucleotide present in the enzyme preparations can prime polynucleotide synthesis. As shown by Singer et al. (5), oligonucleotides are good primers for polynucleotide phosphorylase.

As already reported (2, 3), the synthesis of homopolymers is primed by the corresponding polynucleotides (poly A by poly A, poly U by poly U, etc.). Poly AGUC or RNA prime the synthesis of either poly A or poly U. The synthesis of poly G is primed by poly C but not by RNA, poly A, or poly U, whereas, on the other hand, poly G primes the synthesis of several polynucleotides, e.g. poly A, poly U, poly G, poly AGUC. DNA does not prime polynucleotide synthesis by polynucleotide phosphorylase. Evidence for some of these statements has been presented previously (2, 3) and priming of poly G synthesis by poly C will be described in a forthcoming paper. Supplementary evidence on the priming features of homopolymer synthesis is presented in Fig. 5 with synthesis of poly U as a typical example. With UDP as substrate (Curve 1, no primer), the reaction rate was not affected by DNA (Curve 2), was inhibited by poly A (Curve 3), and was stimulated by poly U, yeast RNA, Azotobacter RNA and poly AGUC (Curves 4 through 7, respectively). In a separate experiment, under the same conditions but with a smaller amount (0.25 μg) of a different enzyme fraction (specific activity, 150; 280:260 μm ratio, 9.2), 6 μg; primer polynucleotide (when present), 0.2 (as mononucleotide). Incubation at 30°. The ordinate gives the percentage of acid-labile phosphate released as orthophosphate. Curve 1, no primer; Curve 2, Azotobacter DNA; Curve 3, poly A; Curve 4, poly U; Curve 5, yeast RNA; Curve 6, Azotobacter RNA; Curve 7, poly AGUC.

Of special interest are the priming requirements for poly AGUC synthesis. A requirement for primer was observed in this case even with high concentrations of enzyme and Mg++. Fig. 5 presents additional evidence for previous statements (2-4) that synthesis of poly AGUC is primed by RNA or poly C (Curve 2) but not by poly A or poly U (Curve 1). In a similar experiment, salmon sperm DNA was inactive as a primer. Singer et al. (5) found that oligonucleotides, such as di-, tri-, and tetramethylene acidi, serve as primers for the synthesis of poly A, poly U, and polyribothymidylic acid (24). That this is also true of poly AGUC synthesis is shown in Fig. 6 (Curve 5) for triadethylene acid (pApGpAe, see (5)). There were no indications in experiments similar to those of Fig. 6, but on a larger scale, that the nature of the added primer whether triadethylene acid, poly C, or rat liver RNA, had a significant influence on the base composition of the resulting poly AGUC. As in the case of polymers made with crude Azotobacter polynucleotide phosphorylase preparations and equimolar amounts of ADP, GDP, UDP, and CDP (8), their base composition was similar to that of whole cell Azotobacter RNA.
DISCUSSION

The results reported in this paper strongly suggest that, in A. vinelandii, polynucleotide phosphorylase is a single enzyme. The fact that the 500-fold purified enzyme contains a small amount of a low molecular weight polynucleotidophsphate, with adenine, guanine, uracil, and cytosine in similar ratios as in whole cell Azotobacter RNA, might be of functional significance. However, the material could simply be a partially degraded RNA contaminant tenaciously retained by the protein. We are inclined to consider the latter as the more likely alternative but, insofar as it has not been possible to remove the oligonucleotide without denaturing the enzyme, the matter remains unsettled.

Whereas synthesis of poly AGUC and poly G with the purified enzyme is markedly dependent on the addition of oligomeric ribonucleotide primers, synthesis of homopolymers (e.g. poly A) can occur rapidly in the absence of added primers. Nevertheless, the reaction can be accelerated by addition of primer when very small amounts of enzyme or Mg++ are used. It is possible, and indeed likely, that no polynucleotide synthesis whatsoever would occur without added primer if the enzyme were free of oligonucleotide. The results suggest that this oligonucleotide acts as a primer but the question whether the presence of primer is an absolute requirement for polynucleotide synthesis cannot be answered with the Azotobacter enzyme preparations available.

The mode of action of oligoribonucleotide primers of polynucleotide phosphorylase has been elucidated in elegant experiments by Singer, Heppel, and Hilmoe (5). The mechanism of priming by polynucleotides, on the other hand, remains unknown. Priming by oligonucleotides is nonspecific, whereas, as pointed out in this paper and elsewhere (2-4), priming by polynucleotides exhibits a certain specificity. However, the base composition of poly AGUC was not significantly affected by the primer used, whether triadenylic acid, poly C, or liver RNA. Some influence of the primer on the product may be indicated by the fact that the reaction mixture became visibly viscous during synthesis of poly AGUC with poly C as primer but not in the other cases. All the poly AGUC preparations showed polydispersity on ultracentrifugation. The following average sedimentation coefficients (S equals) were obtained: poly AGUC (primed with triadenylic acid), 9.8 S; poly AGUC (primed with poly C), 7.0 S; poly C, 5.8 S. DNA was inactive as primer.

From the above, it seems unlikely that a replication or copying mechanism is operative in RNA synthesis by polynucleotide phosphorylase unless such a mechanism was mediated by the oligonucleotide it contains. The protein may, therefore, be asked whether this enzyme has a synthesizing function in the cell.

The fact that it catalyzes polymerization of such compounds as thiorouridine diphosphate (25) and fluorouridine diphosphate but is inactive with azauridine diphosphate (26) and further footnotes could be in line with such a function since thiorouracil and fluorouracil are incorporated into RNA in vivo, whereas 4-azauracil is but poorly incorporated. On the other hand, the enzyme, which is widely distributed in bacteria (27) and appears to be present in liver nuclei (28), cannot be detected in extracts of Lactobacillus arabinosus (20, 29). If really absent from this organism, polynucleotide phosphorylase could hardly be involved in RNA synthesis. While the biological role of polynucleotide phosphorylase awaits clarification, search for enzymes of RNA synthesis other than polynucleotide phosphorylase has been intense in recent years and has led to the discovery of enzyme requiring all four ribonucleoside 5'-triphosphates and RNA for RNA formation (29-33). These enzymes may be concerned with the synthesis of specific RNA molecules.

SUMMARY

The relative activity toward different nucleoside diphosphates remains essentially unchanged after 500-fold purification of polynucleotide phosphorylase of Azotobacter vinelandii suggesting that a single enzyme is involved. The purified enzyme, virtually free of nucleases, contains small amounts of an oligoribonucleotide (with adenine, guanine, uracil, and cytosine in similar ratios as in whole cell Azotobacter RNA) which is released on denaturation of the protein. Although we do not know whether this oligonucleotide is a prosthetic group or a tightly clinging contaminant, it may act as a primer of polynucleotide synthesis by the enzyme. No primer addition is required for the synthesis of poladenylic acid when relatively large amounts of enzyme or Mg++ are used, but a requirement for added primer becomes apparent with small amounts of either component. Synthesis of polymers containing guanylic acid is always markedly dependent on addition of primer and requires much more enzyme. Under optimal conditions, the turnover number for poladenylic acid synthesis (0.02 M adenosine diphosphate, 30°C) is about 4,560 moles per minute per 10^6 g of protein, whereas that for synthesis of the copolymer of adenyl, guanylic, uridylic, and cytidylic acids (0.005 M each, adenosine diphosphate, guanosine diphosphate, uridine diphosphate, and cytidine diphosphate) is only in the neighborhood of 200.

Polymer synthesis can be primed by polynucleotides including ribonucleic acid and by such oligoribonucleotides as triadenylic acid, but not by deoxyribonucleic acid. Priming by polynucleotides has certain specific features of obscure nature and need not be considered here. The primary role of primers is to initiate RNA synthesis. However, since the enzyme continues to grow and increase in size, it may act as a primer of polynucleotide synthesis by the enzyme.
significance. Synthesis of the copolymers of adenylic, guanylic, uridylic, and cytidylic acids can be primed by triadenylic acid, polycytidylic acid, or ribonucleic acid from various sources but not by polyadenylic acid or polyuridylic acid. There is little or no influence of the primer on the base composition of the polymer. This is discussed in relation to the function of polynucleotide phosphorylase.

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