

ENZYMIC SYNTHESIS OF POLYNUCLEOTIDES

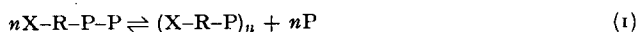
I. POLYNUCLEOTIDE PHOSPHORYLASE OF *AZOTOBACTER VINELANDII**

by

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As previously reported^{1,2}, an enzyme isolated from the microorganism *Azotobacter vinelandii* catalyzes the synthesis of highly polymerized polynucleotides from 5'-nucleoside diphosphates with release of orthophosphate. The reaction, which requires magnesium ions and is reversible, can be represented as in Equation (1) where R stands for ribose, P-P for pyrophosphate, P for orthophosphate, and X for one or more of the following bases: adenine, hypoxanthine, guanine, uracil or cytosine.



The reaction is analogous to the reversible synthesis and breakdown of polysaccharides catalyzed by phosphorylase and, for this reason, the name polynucleotide phosphorylase has been proposed¹ for the new enzyme. Chemical and enzymic degradation of the biosynthetic polynucleotides, carried out in part in collaboration with Dr. LEON A. HEPPEL of the National Institutes of Health, has shown that they are made up of 5'-nucleoside monophosphate units linked to one another through 3'-phosphoribose ester bonds as in ribonucleic acid². It has further been shown² that, from a mixture of ADP***, GDP, UDP and CDP, the *Azotobacter* enzyme catalyzes the synthesis of polynucleotides which, in the light of chemical and enzymic evidence, cannot be distinguished from natural RNA.

Recent experiments indicate that polynucleotide phosphorylase is widely distributed and suggest that this enzyme may be essential for the synthesis and

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*** The following abbreviations are used: Ribonucleic acid, RNA; deoxyribonucleic acid, DNA; 5'-diphosphates (pyrophosphates) of adenosine, inosine, guanosine, uridine and cytidine, ADP, IDP, GDP, UDP and CDP; 5'-monophosphates of the same nucleosides, AMP, IMP, GMP, UMP and CMP; adenosine- and inosine-5'-triphosphate, ATP and ITP; ultraviolet, U.V.; counts per minute, c.p.m.; ethylenediaminetetraacetic acid (versene), EDTA; and tris(hydroxymethyl)-aminomethane, Tris.

breakdown of RNA in the cell. In this connection it is of interest that POTTER and his collaborators³ have demonstrated the occurrence in tissues of 5'-mono-, di-, and triphosphates of adenosine, guanosine, uridine and cytidine. POTTER⁴ has recently suggested the nucleoside diphosphates as the most likely precursors of RNA.

The partial purification of the *Azotobacter* enzyme, the properties of the system, and the preparation and isolation of various polynucleotides, are described in this paper. Other papers of this series will be concerned with further studies of the structure of biosynthetic polynucleotides, their physical properties, the distribution and the isolation of polynucleotide phosphorylase from various sources.

EXPERIMENTAL

Preliminary experiments

Polynucleotide phosphorylase was discovered in the course of a study of biological phosphorylation mechanisms⁵. The aim of this work was to look for soluble enzyme systems catalyzing the incorporation of radioactive orthophosphate in ATP. On incubation of dialyzed *Azotobacter* extracts with ATP, in the presence of Mg^{++} , there was a rapid "exchange" or incorporation of the labeled phosphate. Since the extracts contained adenylic kinase (myokinase)⁶, the labeled phosphate was incorporated both in ATP and ADP. The early experiments were carried out with amorphous preparations of ATP which contained ADP as a contaminant. However, when crystalline ATP was used there was little or no "exchange". Eventually, it was found that the "exchange" was promoted by ADP, prepared from crystalline ATP through reaction with glucose in the presence of hexokinase, and not all by ATP or AMP. It was

TABLE I
PHOSPHATE "EXCHANGE" AND LIBERATION WITH VARIOUS NUCLEOTIDES

Nucleotide*	Purity	P incorporation in 15 min c.p.m.	P liberation in 30 min μmoles
AMP	Crystalline	0	0
ATP	Amorphous	5000	
ATP	Crystalline	256	0.07
ADP	Amorphous	41000	0.35
ADP	Crystalline**	41000	0.35
ADP + boiled extract***	Amorphous	42000	
ATP + AMP	Crystalline	17200	0.20
ADP (no Mg^{++})	Amorphous	0	0
ITP	Amorphous	195	0.06
IDP	Amorphous	57000	0.52

Samples contained (in μmoles), Tris buffer, 100; $MgCl_2$, 10; and EDTA, 1. Final volume, 1.0 ml; incubation at 30°. In addition, the samples for phosphate incorporation (pH, 8.1) contained potassium phosphate buffer, 2.3, with ^{32}P -labelled orthophosphate (142,000 c.p.m.); nucleotide as indicated, 3; and enzyme of specific activity 1.94 (assay 1) with 0.83 mg of protein (1.6 units). The samples for phosphate liberation (pH, 7.4) contained nucleotide as indicated, 5; and enzyme of specific activity 1.7 (assay 1) with 0.35 μg of protein (0.6 unit).

* All nucleotides are the 5'-mono- or polyphosphates.

** Prepared from crystalline ATP by reaction with glucose in the presence of crystalline hexokinase, and isolation of the ADP by ion-exchange chromatography⁷.

*** Initial *Azotobacter* extract (9.7 mg of protein per ml) heated for 3 minutes at 100°. 0.2 ml of supernatant fluid used for experiment.

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further found that nucleoside diphosphates other than ADP, for example IDP, promoted the "exchange" reaction and that, simultaneously with the incorporation of labeled phosphate, there was a liberation of orthophosphate (*cf.* Reaction 1) which also required magnesium ions. These experiments were performed with enzyme preparations in which the activity had been partially purified employing the "exchange" with ADP as an assay.

Typical data with partially purified enzyme preparations are shown in Table I. With the exception of amorphous ATP, only the nucleoside diphosphates were active in giving rise to either incorporation of phosphate or phosphate liberation; the mono- and triphosphates had little or no activity. Positive results were also obtained with ATP plus AMP owing to the presence of adenylic kinase. This enzyme catalyzes the reaction $ATP + AMP \rightleftharpoons 2ADP$. Similar results are shown in Fig. 1. These experiments were carried out with an enzyme preparation largely freed of adenylic kinase by precipitation with acetone. After incubation of the nucleotides with the enzyme, in the presence of Mg^{++} , and deproteinization of the reaction mixture the nucleotides were separated by paper chromatography in the solvent system of KREBS AND HEMS⁸ followed by autoradiography to localize the radioactive spots containing ^{32}P . It will be seen that on incubation of mixtures of ADP or IDP with other nucleotides only ADP and IDP became labeled. The fact that AMP was not labeled when ADP and AMP were incubated together indicated that the radioactive phosphate was incorporated in the terminal phosphate of ADP. On incubation of IDP and ADP together both became labeled although, in this experiment, the labeling of ADP was weak. Fig. 2 shows the time course of incorporation of ^{32}P -orthophosphate in ADP as well as that of liberation of orthophosphate from ADP.

A number of experiments were carried out with IDP as substrate to determine the stoichiometry of the reaction. IDP, rather than ADP, was used because adenylic kinase is not active on IDP. When the incubated reaction mixtures were deproteinized

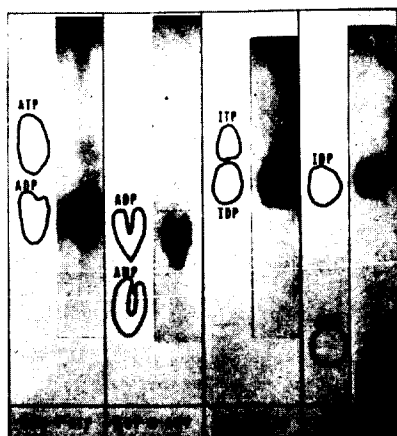


Fig. 1. Enzymic incorporation of ^{32}P -orthophosphate in ADP and IDP. Conditions as in phosphate incorporation experiments of Table I. Paper chromatography by the method of KREBS AND HEMS⁸. Spots located with U.V. lamp. Corresponding autoradiograms to the right of sketch of each chromatographic strip.

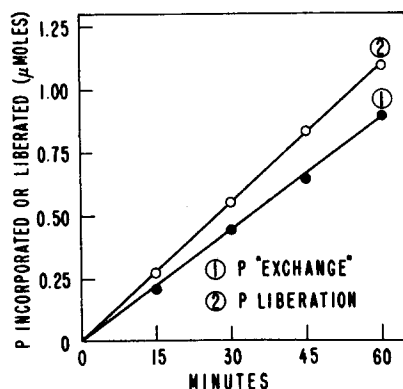


Fig. 2. Enzymic incorporation of ^{32}P -orthophosphate in ADP (Curve 1) and liberation of orthophosphate from ADP (Curve 2) as a function of time. The conditions were those of the standard assays described in the text. 20.5 μg of enzyme of specific activity 10.6 (0.218 unit) were used for Curve 1, and 61.6 μg of enzyme of specific activity 13.0 (0.8 unit) for Curve 2. The specific activities are expressed in terms of assay 1.

with trichloroacetic acid, there was a disappearance of total nucleotide (determined by measurement of U.V. absorption) from the supernatant fluid corresponding to the amount of IDP disappearing and that of orthophosphate liberated. On the other hand, there was no disappearance of total nucleotide when the reaction mixture was deproteinized by heating for 1 or 2 minutes at 100° (Table II). However, the reaction product could not be eluted from Dowex-1 (formate) columns with formic acid at concentrations much higher than those required to elute the most acidic mononucleotides. A typical experiment is shown in Table II. In this experiment 2.0 ml aliquots of the protein-free supernatant fluid were used for the separation of nucleotides by ion-exchange chromatography. The samples were brought to 50 ml with water and made alkaline by addition of 0.5 ml of concentrated ammonia. The solution was chromatographed on a Dowex-1- (formate) column (4 × 1 cm). The resin used was Nalcite SDR, 200-400 mesh, 2% cross-linkage. After placing the sample on the column, it was washed with water. Then it was eluted successively with 0.1 *M* sodium formate (which removes the orthophosphate), 0.15 *M* sodium formate with 0.01 *M* formic acid (which elutes the IMP), 0.2-0.3 *M* sodium formate with 0.01 *M* formic acid (which elutes the IDP) and, finally, with 0.4 *M* sodium formate with 0.01 *M* formic acid (which elutes ITP). It may be seen in the table that, on incubation for 20 and 60 minutes, the disappearance of IDP and the liberation of orthophosphate were equimolar. It may also be seen from the Δ values of the last column that there was a deficit in the nucleotide recovered from the column of the same order of magnitude as the IDP disappearance and orthophosphate liberation. As already reported², it was further found that the product of the reaction of IDP or ADP, in the presence of the enzyme and magnesium, was non-dialyzable against distilled water or dilute salt solutions. It could be quantitatively precipitated with trichloroacetic acid or alcohol in the cold; either of these two methods could be used for its isolation. The product was soluble in water, yielding relatively viscous solutions with a typical nucleotide ultraviolet absorption spectrum, and it remained at the origin of paper chromatograms whichever the solvent system used. All these facts indicated that the product was a polynucleotide.

TABLE II
REACTION WITH IDP

<i>Incubation min</i>	<i>Orthophosphate</i>	<i>IMP</i>	<i>IDP</i>	<i>Nucleotide recovered from column</i>
0	1.32	0.6	10.7	11.3
20	3.80	0.6	8.6	9.2
Δ	+ 2.48	0	- 2.1	- 2.1
60	6.40	0.6	6.5	7.1
Δ	+ 5.08	0	- 4.2	- 4.2

The reaction mixture contained the following components (in μ moles per ml), Tris buffer, pH 8.1, 36; MgCl_2 , 4.8; potassium phosphate, pH 8.1, 1.3; IDP, 11.5; and enzyme of specific activity 4.6 (assay 1) with 0.54 mg of protein (2.5 units). Final volume, 8.5 ml. 2.5 ml aliquots removed at time zero and after incubation for 20 and 60 minutes at 30°. After heating for 2 minutes at 100°, cooling, and removing the precipitated protein by centrifugation, aliquots of the supernatant fluid were taken for the determination of orthophosphate (0.1 ml) and nucleotides (2.0 ml). Values expressed in μ moles per ml of reaction mixture. The small amount of IMP was present as a contaminant of the IDP preparation. There was no change in the amount of total nucleotide (determined by U.V. absorption at wave-length 247 $m\mu$) during incubation.

Isolation of enzyme

Enzyme assays. The enzyme has been assayed in either of two ways: (1) by determining the rate of incorporation of radioactive orthophosphate into nucleoside diphosphates (phosphate "exchange" assay) and (2) by determining the rate of liberation of orthophosphate from nucleoside diphosphates. Either ADP or IDP, but mostly the former, has been used for assay purposes.

Assay 1. The reaction mixture contains the following components (in μ moles): Tris buffer, pH 8.1, 100; $MgCl_2$, 5; ADP (or IDP), 2.5; potassium phosphate, pH 8.1, with 100,000–200,000 c.p.m. ^{32}P , 3.6 (or 2.5); and enzyme. Final volume, 1.0 ml. If ADP is used, the molar ratio ADP to orthophosphate should be 0.7; if IDP is used, the ratio IDP to orthophosphate should be 1.0. It is convenient to add a small amount of EDTA for assay of the enzyme during the initial steps of the purification. The reaction mixture is incubated for 15 minutes at 30° . With small amounts of enzyme the reaction is linear for at least 60 minutes. One unit of enzyme is defined as the amount which catalyzes the incorporation of 1.0 μ mole of $^{32}PO_4^{=}$ under the conditions of the assay in 15 minutes at 30° . The radioactivity in the organic phosphate fraction, determined as described in the next section, is used to calculate the amount of orthophosphate incorporated from the expression:*

$$\mu\text{moles phosphate incorporated} = \frac{\text{c.p.m. incorporated} (\mu\text{moles phosphate} + \mu\text{moles ADP})}{\text{c.p.m. phosphate}}$$

The specific activity of the enzyme is expressed as units per mg of protein. Protein was determined either by the biuret method⁹ with crystalline ovalbumin, previously dialyzed free of salt, as a standard, or spectrophotometrically by measuring the absorption of light at wave-lengths 280 and 260 $m\mu$, with a correction for the nucleic acid content from the data given by WARBURG AND CHRISTIAN¹⁰. The first method was used to determine the protein content of the extract and the first ammonium sulfate fraction, with high nucleic acid content, the second method was used for the later steps in purification.

Determination of orthophosphate incorporation. This procedure follows that described by ROSE AND OCHOA¹¹ with some modifications. The orthophosphate incorporated is given by the radioactivity remaining in the protein-free filtrate after removal of the orthophosphate. The procedure is based on the quantitative conversion of orthophosphate to ammonium phosphomolybdate and the removal of the latter by extraction with *isobutanol*, according to BERENBLUM AND CHAIN¹². The reaction is stopped by addition of 0.1 ml of 40% trichloroacetic acid per 1.0 ml of reaction mixture. After centrifugation, aliquots are taken for determination of the specific radioactivity of the orthophosphate and the radioactivity fixed. For removal of the orthophosphate, usually 1.0 ml of the protein-free filtrate is made up with water to 3.0 ml. To this solution are added 0.3 ml of 10.0 M H_2SO_4 , 1.5 ml of 5% ammonium molybdate, and 5.0 ml of *isobutanol*. A slow stream of air is bubbled through the mixture, usually for one minute, to obtain good mixing. After allowing the liquid phases to separate by standing, the upper *isobutanol* layer is removed by aspiration and discarded. The aqueous phase, which must be clear**, is washed with 3.0 ml of ether; the ether is removed and discarded. An aliquot (usually 1.0 ml) of the aqueous phase, is taken for determination of the radioactivity of the organically-bound phosphate. When the procedure is performed on a control reaction mixture to which the enzyme is added after trichloroacetic acid, it is found that over 99% of the radioactivity (*i.e.*, of the orthophosphate) is removed. Such a control is included with every experimental run and the radioactivity of this blank sample is subtracted from that in the experimental ones to correct for the small amount of orthophosphate not removed. This should always be very small.

Assay 2. The rate of liberation of orthophosphate from ADP is used in this assay. For economy reasons the assay is carried out with concentrations of ADP, which although higher than those used in Assay 1, are insufficient to saturate the enzyme as far as the rate of liberation of phosphate is concerned. At saturation levels of ADP, the rate of liberation of phosphate is some three times faster than obtained in the standard assay. The reaction mixture contains the following components (in μ moles), Tris buffer, pH 8.1, 70–100; $MgCl_2$, 5; ADP, 10; and enzyme. Final volume, 1.0 ml. The amount of enzyme should be such that the reaction rate remains

* This is not an accurate calculation of the absolute amount of phosphate incorporated and is used only as a convenient expression of the activity of the enzyme under the conditions of Assay 1.

** Occasionally the aqueous phase may not be clear after one extraction with *isobutanol*; when this happens, the removal of orthophosphate is incomplete. A second extraction usually eliminates the remaining phosphate satisfactorily.

essentially linear with time during the incubation; this amount is usually below 1.5 units, as determined by Assay 1. Usually, two samples are run for 5 and 15 minutes, respectively. The incubation is carried out with shaking at 30°. One unit of enzyme is defined as the amount which catalyzes the liberation of 1 μ mole of orthophosphate per 15 min at 30°. The specific activity is defined as units per mg of protein. The reaction is stopped with trichloroacetic acid as in Assay 1 and orthophosphate determined by the method of LOHMANN AND JENDRASSIK¹³. The rate of phosphate liberation is proportional to the concentration of enzyme between the limits of 0 and 1.5 (Assay 1) units. When the enzyme is free or almost free of adenylic kinase, the rate of phosphate liberation remains constant for at least 1 hour. In the presence of adenylic kinase it may be necessary to calculate the reaction rate from the 5 minute incubation value.

As shown in Fig. 3, the reaction rate, under the conditions of either Assay 1 or 2, is proportional to the concentration of enzyme over a fairly wide range of enzyme dilutions. It may also be seen that the ratio of phosphate incorporation (Assay 1) to that of phosphate liberation rate (Assay 2) is approximately 2.5–2.8. Assay 1 has been the one routinely used for purification of the enzyme. Unless otherwise stated, units and specific activities of the enzyme preparations throughout this paper are expressed in terms of Assay 1.

Purification of enzyme

Azotobacter vinelandii (strain original)* was grown as described by HYNDMAN *et al.*¹⁴. The cells were harvested after 15 hours of growth; at this time a 1:5 dilution of the medium should read about 115 in a Klett colorimeter with filter No. 540. The yield was around 6 g of wet cells per liter of medium.

Step 1. Extraction. The enzyme, which is easily soluble, can be extracted from fresh, acetone-dried, or lyophilized cells. Fresh or dried cell preparations retain their activity for a long time when stored at –18°.

The fresh cells are ground in a mortar with double their weight of alumina A301 (325 mesh, Aluminum Company of America) at 0°, then extracted with 4.5 volumes of 0.15 *M* KCl and centrifuged for 30 minutes at 3000–4500 *g*. The residue of cells and alumina is re-extracted with 2.0 volumes of 0.15 *M* KCl for 30 min and the mixture is centrifuged as before. The supernatant extracts are combined and centrifuged for 30 minutes at 0° and 16,000 r.p.m. in the high speed head of the International Centrifuge. The supernatant fluid is then dialyzed against 0.01 *M* phosphate buffer, pH 7.0, for 2 hours with stirring. In the case of dried cell preparations, the extraction is carried out in the same way, except that an equal weight of alumina and 22.5 and 10.0 volumes, respectively, of 0.15 *M* KCl are used. The extract contains from 8–15 mg of protein per ml. An amount of extract containing 0.5–1.0 mg of protein is used per assay (Assay 1). The specific activity of the extract varies from 0.3 to 0.5.

Step 2. First ammonium sulfate fractionation. The extract is diluted, if necessary, with 0.01 *M* phosphate buffer, pH 7.0, to contain 10 mg of protein per ml and cooled to 0°. Solid ammonium sulfate is added with mechanical stirring to make 0.35 saturation. After stirring for 5 minutes, the mixture is centrifuged in the cold room at top speed in the Servall angle centrifuge (20,000 *g*). The precipitate, which contains β -hydroxybutyric dehydrogenase, has been used for the purification of this enzyme; this preparation will be described elsewhere. Solid ammonium sulfate is added to the supernatant solution as above to make its degree of saturation 0.46. The precipitate contains most of the polynucleotide phosphorylase activity. This fraction, which contains from 20 to 25 % of the protein of the extract, is dissolved in 0.01 *M* potassium phosphate buffer, pH 7.4, and dialyzed with stirring in the cold for 2 hours against the same buffer. An amount containing about 0.2 mg of protein is used per assay. The specific activity of this fraction is about 2.0.

Step 3. Adsorption and elution from calcium phosphate gel. The dialyzed solution of the 0.35–0.46 ammonium sulfate precipitate is diluted, if necessary, with 0.01 *M* potassium phosphate

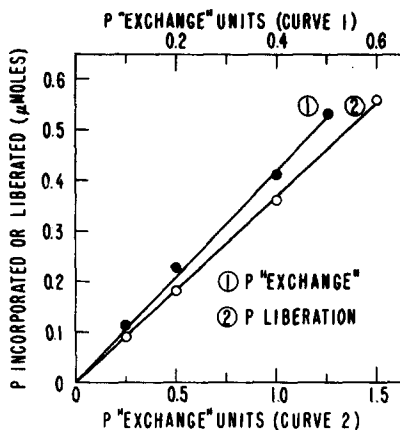


Fig. 3. Rate of incorporation of ³²P-orthophosphate in ADP (Curve 1) and of phosphate liberation from ADP (Curve 2) as a function of enzyme concentration. Curve 1 was obtained with a solution of enzyme (1.02 mg of protein per ml) of specific activity 10.6; Curve 2, with a solution of enzyme (1.08 mg of protein per ml) of specific activity 22.5. Specific activities are expressed in terms of assay 1. The ordinate represents μ moles of phosphate incorporated or liberated in 15 minutes at 30°.

* We are indebted to Dr. P. W. WILSON, Department of Bacteriology, School of Agriculture, University of Wisconsin, Madison, Wisconsin, for a culture of the organism.

buffer, pH 7.4, to contain 9–10 mg of protein per ml and cooled to 0°. To 95 ml of the enzyme solution is added 0.9 ml of 1.0 *M* acetate buffer, pH 5.1. The final pH of this solution is 5.8 and the salt concentration about 0.02 *M*. 15 ml of calcium phosphate gel¹⁵ (dry weight, 20 mg per ml) are added to 96 ml of the above solution. After stirring for 20 minutes at 0° the gel, which contains about 9% of the protein of the fraction, and 5% of the activity, is centrifuged off and discarded. 5.0 ml of calcium phosphate gel are now added to the supernatant solution and the mixture is stirred and centrifuged as above. The gel is eluted first with 30–40 ml of 0.1 *M* potassium phosphate buffer, pH 6.0, at 0° and, after centrifugation in the cold, with 15 ml of 0.01 *M* potassium phosphate buffer, pH 7.3. The buffers used for elution are at 0° but the elutions are carried out at room temperature. The eluates are combined and dialyzed at 0° against 0.01 *M* potassium phosphate buffer pH 7.4, for 2 hours with stirring. An amount of eluate containing about 0.05 mg of protein is used per assay. The specific activity of the eluate, which contains about 18% of the protein of the fraction with 76% of the activity, is 8.0 to 10.0. The overall recovery of enzyme in the supernatant fluid and gel eluates is about 90%.

Step 4. Second ammonium sulfate fractionation. The dialyzed eluate, containing about 2.5 mg of protein per ml, is fractionated at 0° with saturated ammonium sulfate adjusted to pH 7.4 with ammonium hydroxide. Three fractions are obtained: fraction 1, between the limits of 0 and 0.45 ammonium sulfate saturation, fraction 2 between 0.45 and 0.55 saturation, and fraction 3 between 0.55 and 0.60 saturation. The ammonium sulfate precipitates are collected by centrifugation in the cold room (about 5°) at high speed in the Servall Angle centrifuge, dissolved in a small volume of 0.01 *M* potassium phosphate buffer, pH 7.4, and dialyzed at 0° with stirring against the same buffer for several hours. Fraction 1, which contains about 27% of the protein and 15% of the activity, and fraction 3 containing 10% of the protein and 11% of the activity of the eluate, are discarded. Fraction 2, which contains 22% of the protein and 73% of the activity of the eluate is the best preparation of the enzyme so far obtained. An amount of fraction containing from 0.02 to 0.03 mg of protein is used per assay; its specific activity is from 18 to 22. The overall recovery of activity in the three ammonium sulfate fractions amounts to 99%.

A summary of the purification procedure, starting with acetone-dried cells, is given in Table III. This run was particularly successful both as regards purification and yield. In two large-scale preparations from fresh cells, carried out subsequently*, the specific activity of the enzyme at stage 4 was 14.6 and 21.6, with a yield of 28 and 24%, respectively. The ammonium sulfate steps are generally reproducible but the degree of success with the calcium phosphate gel step depends on the particular gel preparation or batch used and the procedure finally utilized at this stage may have to deviate from the one described here.

TABLE III

PURIFICATION OF POLYNUCLEOTIDE PHOSPHORYLASE OF *Azotobacter vinelandii*

Step	Volume ml	Units (Assay 1)	Protein mg	Ratio* 280/260	Specific activity units/mg protein	Yield per cent
1. Dialyzed extract	376	1840	3450	0.5	0.5	100
2. (NH ₄) ₂ SO ₄ (0.35–0.46)	95	1750	810		2.2	95
3. Ca ₃ (PO ₄) ₂ gel eluate	58	1500	142	0.7	10.6	82
4. (NH ₄) ₂ SO ₄ (0.45–0.55)	5	1020	45	1.1	22.5	56

15.0 g of acetone-dried cells (*A. vinelandii*, strain original) extracted with an equal weight of alumina and 400 ml of 0.15 *M* KCl.

* Ratio of light absorption at λ 280 to that at λ 260 μ .

In the run of Table III, besides Assay 1, on which the specific activities given in the table are based, Assay 2 was also used at each step. The specific activities based on this assay at steps 1, 2, 3, and 4, respectively, were 0.17, 0.95, 3.30, and 8.30 (*cf.* Table III). Thus, the ratios of rate of phosphate incorporation to rate of phosphate liberation, *i.e.*, the ratios of specific activities determined by Assay 1 to those determined by Assay 2, at each step, were 2.9, 2.3, 3.2, and 2.7, for steps 1 through 4, respectively, the average value of the ratio being 2.76 (*cf.* also Fig. 3). Thus, throughout the purification so far achieved of the *Azotobacter* enzyme, the ratio of the rate of phosphate incorporation to that of phosphate liberation remained constant.

* By Dr. M. STAHELIN and Mr. M. C. SCHNEIDER.

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Properties of enzyme

Stability. Preparations of the enzyme at any step of purification remain active for periods as long as one year if kept in the frozen state at -18° . However, the enzyme loses activity on thawing, especially on repeated freezing and thawing. Although the enzyme is relatively stable at an acid pH (4.5 to 5.5), it cannot be stored frozen at this pH and loses about 70% of its activity on thawing under these conditions. Heating for 5 minutes at 50° and pH 5.8 destroys 50% of the enzyme activity. However, only 10 to 20% is lost if this heating is carried out at pH 6.4. The enzyme is completely destroyed by heating for 5 minutes at 60° and pH 5.6, or for 1 minute at 100° and pH 7.4.

pH Optimum. Under the conditions of Assay 1 the phosphate incorporation reaction has a rather sharp optimum at pH 8.1. At pH 6.4 the activity is 28% and at pH 10.0, 50% of that at pH 8.1. Under the conditions of Assay 2, the phosphate liberation reaction has a plateau of maximum activity between pH 7.5 and pH 9.0; at pH 6.4 the activity is 30% lower.

Affinity constants. Under the conditions of Assay 1 the system is saturated with concentrations of ADP of the order of $2.5 \cdot 10^{-3}M$ and concentrations of $MgCl_2$ between 1 and $2 \cdot 10^{-3}M$. The corresponding half saturation concentrations were $2.5 \cdot 10^{-4}M$ and $5 \cdot 10^{-4}M$, respectively. Much higher concentrations of nucleoside diphosphates are required to saturate the system in the case of Assay 2. Thus, saturating concentrations of ADP and IDP were of the order of $10^{-1}M$ and $5 \cdot 10^{-2}M$, respectively. The half saturating concentrations were $2.7 \cdot 10^{-2}M$ and $10^{-2}M$, respectively. The concentrations of $MgCl_2$ required to saturate the system of Assay 2 were about the same as those in Assay 1, namely between 1 and $2 \cdot 10^{-3}M$; the half saturation concentration for $MgCl_2$ was $4 \cdot 10^{-4}M$. Whereas small variations in the ratio of the concentration of nucleoside diphosphate to that of orthophosphate do not appear to influence significantly the reaction rate in the case of Assay 2, in that of Assay 1 the rate of incorporation of orthophosphate is markedly dependent on the above concentration ratio. In this case, the optimum concentration ratio of nucleoside diphosphate to orthophosphate was 0.7 for ADP and 1.0 for IDP. The reaction rates decrease rather sharply for values of the concentration ratio above or below the optimum.

TABLE IV
EFFECT OF INHIBITORS

Addition to assay system		Per cent inhibition	
Test substance	Concentration (molar)	Assay 1	Assay 2
Potassium arsenate	0.004	7	0
Potassium arsenate	0.01	44	0
Potassium fluoride	0.04	13	0
p-chloromercuribenzoate	0.00015	27	18
2,4-dinitrophenol	0.0005	0	
AMP	0.01	10	0
ATP	0.002	10	
Sodium pyrophosphate	0.01	18	

Enzyme of specific activity 16 with 0.037 mg of protein (0.6 unit) used for the first five experiments. Enzyme of specific activity 12 with 0.048 mg of protein (0.58 unit) used for the last three experiments. Conditions of standard assays.

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Inhibitors. As shown in Table IV, the enzyme is relatively insensitive to poisons being essentially unaffected by fluoride and by low concentrations of arsenate. Higher concentrations of the latter (0.01 *M*) inhibited 44% the incorporation reaction, although they had no effect on the liberation of orthophosphate. *p*-Chloromercuribenzoate had relatively little effect; hence, SH groups do not seem to be essential for the activity of the enzyme. The incorporation reaction (Assay 1) was relatively little affected by the presence of AMP, ATP or pyrophosphate. On the whole this reaction was more sensitive to inhibitors than the liberation of orthophosphate.

Synthesis of polynucleotides

The incorporation of orthophosphate into nucleoside diphosphates and the liberation of phosphate therefrom occur not only with the diphosphates so far considered, namely ADP and IDP, but also with any of the other diphosphates such as GDP, UDP, and CDP, or with mixtures of two or more nucleoside diphosphates. Table V illustrates the incorporation of ^{32}P -orthophosphate, measured as described for Assay 1,

TABLE V
 ^{32}P "EXCHANGE" WITH NUCLEOSIDE DIPHOSPHATES SINGLY AND MIXED

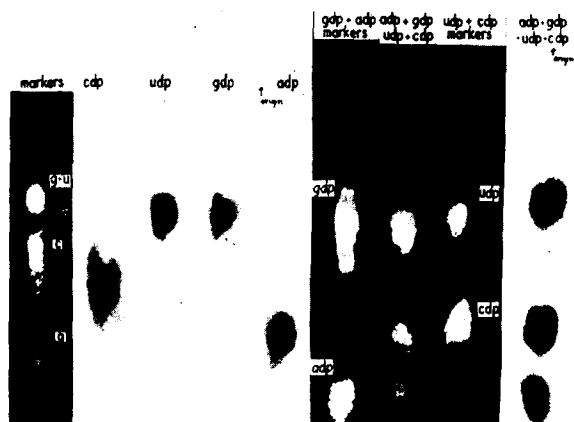
Nucleoside diphosphate μmoles	^{32}P incorporated	
	Expt. 1 c.p.m.	Expt. 2 c.p.m.
ADP, 2.0	16,600	108,000
GDP, 1.0	8,000	29,000
UDP, 4.0	22,500	142,300
CDP, 2.0	10,200	79,600
ADP + GDP + UDP + CDP*	1,270	5,800
ADP + GDP + UDP + CDP**		13,600

The samples contained the following components (in μmoles per ml), Tris buffer, pH 8.0, 100; MgCl_2 , 5; potassium phosphate (pH 8.0) with 95,000 c.p.m. ^{32}P in experiment 1 and 690,000 c.p.m. ^{32}P in experiment 2; enzyme of specific activity 18 with 0.063 mg of protein (1.12 units) in experiment 1 and of specific activity 14 with 0.046 mg of protein (0.7 unit) in experiment 2; and nucleoside diphosphates as indicated. Other conditions as in standard assay 1.

* Each in same amount as when tried singly.

** Each in half the amount as when tried singly.

Fig. 4. Enzymic incorporation of ^{32}P -orthophosphate in nucleoside diphosphates singly and in combination. Conditions as in phosphate incorporation experiments of Table V. Paper chromatography by the method of KREBS AND HEMS⁸. (A) Strip to the left, U.V. print of markers of GDP (G), UDP (U), CDP (C), and ADP (A). GDP and UDP are not separated by the isobutyric acid-ammonia solvent system; strip to the right, autoradiograms of separate experiments with single nucleoside diphosphates. (B) Strip to the right, autoradiogram of experiment with simultaneous incubation of all four nucleoside diphosphates; strip to the left, U.V. print of simultaneous incubation experiment (center) and of markers of GDP and ADP or UDP and CDP, respectively, on either side.



in the presence of ADP, GDP, UDP, and CDP, or a mixture of the four nucleoside diphosphates. The autoradiograms of Fig. 4 show that the radioactive phosphate is incorporated into each of the diphosphates whether incubated singly or in combination. These results suggested that the enzyme is able to catalyze the synthesis of either "single" polynucleotides, *i.e.*, polynucleotides containing a single basic unit, or "mixed" polynucleotides, *i.e.*, polynucleotides containing two or more basic nucleotide

units. As already reported² this was confirmed by subsequent work.

On incubation of purified polynucleotide phosphorylase with nucleoside diphosphates singly or in combination, in the presence of Mg^{++} , there is a liberation of orthophosphate. The reaction reaches equilibrium and comes to a standstill when 60 to 80% of the acid labile phosphate has been released as orthophosphate. The time course of the reaction with ADP, IDP, GDP, UDP and CDP, or with mixtures of either ADP and UDP, or ADP, GDP, UDP, and CDP, is shown in Fig. 5. Addition of more nucleoside diphosphate when the liberation of orthophosphate has stopped disturbs the equilibrium and causes further liberation of phosphate until a new equilibrium position is reached. The polynucleotides which accumulate in the reaction mixtures are usually isolated by precipitation with cold ethanol and purified by solution in a small volume of water, reprecipitation with ethanol, solution, and exhaustive dialysis against distilled water. They are then recovered from the dialyzed solution by lyophilization, and obtained in the form of white powders.

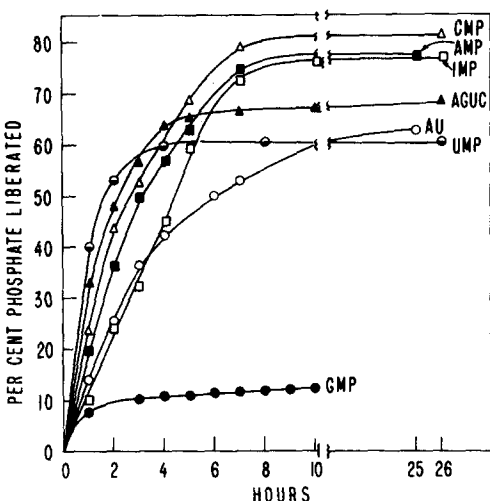


Fig. 5. Orthophosphate liberation during synthesis of polymers. The reaction mixtures contained Tris buffer, pH 8.1, 50, and $MgCl_2$, 10 μ moles per ml. For "single" polymers 50 mg per ml were used of each of the following nucleoside diphosphates, ADP, IDP, UDP and CDP, and 25 mg per ml of GDP. The sample for A-U polymer contained 25 mg of each ADP and UDP per ml; that for A-G-U-C polymer contained 7 mg per ml of each ADP, UDP and CDP, and 3.5 mg per ml of GDP. The samples for GMP and A-G-U-C polymers contained, per ml, 2.5 mg of enzyme of specific activity 9.5 (24 units); all others contained 0.5 mg of the same enzyme (4.75 units). A drop of toluene was added to each sample as a bacteriostat. Final volume, 0.5 ml. Incubation with shaking at 30°. 0.02 ml aliquots were removed as indicated for orthophosphate determination. The ordinate gives the percentage of acid-labile phosphate released as orthophosphate.

It may be seen in Fig. 5 that all nucleoside diphosphates except GDP react readily. In the case of GDP the reaction is much slower and often comes to a standstill when a small fraction of the diphosphate has reacted. A precipitate, containing both polynucleotide and protein, is frequently obtained at this point. Addition of more enzyme brings about further reaction which, however, soon stops. So far, we have only been able to obtain small amounts of the GMP polynucleotide. It is because of this behavior that GDP is used at lower concentration than other nucleoside diphosphates and that much more enzyme is utilized for the preparation of polymers containing GMP.

Preparation and isolation of polymers. Single polymers have been prepared from

ADP, IDP, GDP, UDP, or CDP, and are referred to as the AMP, IMP, GMP, UMP, or CMP polymers, respectively. To date, two "mixed" polymers have been prepared, one from approximately equimolar mixtures of ADP and UDP and one from mixtures of ADP, GDP, UDP, and CDP, in approximate molar proportion, 1:0.5:1:1; these polymers are referred to as the A-U, and the A-G-U-C polymers, respectively. The composition of the samples per ml of the reaction mixture is, in general, as indicated in the legend to Fig. 5. Since the incubation periods are of 24 hours or longer, a small amount of toluene is added to the reaction mixtures to prevent bacterial growth.

The polymers are isolated as follows:

Any precipitate which may be present in the samples at the end of the incubation is removed by centrifugation at low temperature and low speed. The clear solution is then cooled to 0°, two volumes of ice-cold absolute ethanol are added, and the mixture is allowed to stand at 0° for one hour. The precipitated polymer is centrifuged off for 10 minutes at 12,000 *g* and 0°; the supernatant fluid is decanted and the precipitate is dissolved in a minimum amount of distilled water. The polymer is reprecipitated once with two volumes of ice-cold absolute ethanol and the precipitate collected and dissolved in water as above. Any insoluble material at this point is removed by centrifugation and discarded. The clear, colorless polymer solution is dialyzed against several changes of distilled water in the cold room (about 5°) for 48 hours and the dialyzed solution dried from the frozen state.

Detailed information on individual preparations, including composition of the reaction mixtures, the extent to which the reaction proceeded as judged from the liberation of orthophosphate, the yield of lyophilized material and the percentage recovery as polynucleotide of the diphosphates disappearing, are recorded in Table VI.

It will be noted that in order to avoid degradation of the polymers by heat or acid, protein is not removed prior to the precipitation of the polynucleotides with alcohol. Protein determinations in the solutions of polymers after the second alcohol precipitation indicated that about 70% of the protein had been removed. Thus, the

TABLE VI
DATA ON SYNTHESIS OF VARIOUS POLYMERS

No.	Reaction mixture					Extent of reaction* per cent	Yield of lyophilized material mg	Recovery** as polynucleotide per cent
	Nucleoside diphosphate mg	Tris buffer pH 8.1 μmoles	MgCl ₂ μmoles	Enzyme units	Total vol. ml			
1	ADP, 1000	1000	200	80	20	60	356	82
2	UDP, 450	450	90	40	9	40	75	65
3	UDP, 200	200	40	20	4	59	43	56
4	ADP, 180 UDP***, 180	360	72	32	7	46	88	87
5	ADP, 100 UDP***, 100	200	40	19	4	44	62	90-100
6	IDP, 200	200	40	19	4	80	82	80
7	CDP, 150	150	30	16	3	70	40	63
8	ADP, 200 GDP, 100 UDP, 200 CDP, 200	1400	280	725	28	64	154	64

Incubation with shaking at 30°, 40 to 46 hours.

* As determined from orthophosphate liberation.

** Calculated for composition of nucleoside diphosphates as follows: ADP, NaH₂ADP·2H₂O; GDP, Na₃GDP·3H₂O; UDP, Na₃UDP·3H₂O; CDP, Na₃CDP·4H₂O; IDP, Na₃IDP·4.4H₂O.

*** Correction was made for an inorganic impurity present in this UDP sample.

amount of protein remaining and contaminating the polymer preparations is insignificant, except in the case of the AGUC polymer in which the amount of enzyme used was much larger than in the others.

Phosphorolysis

The reaction catalyzed by polynucleotide phosphorylase is reversible. In the presence of the enzyme, orthophosphate and Mg^{++} , the biosynthetic polynucleotides undergo phosphorolysis to yield the corresponding 5'-nucleoside diphosphates. There is no reaction in the absence of orthophosphate. Quantitative data on the stoichiometry of the reaction with IDP in both directions have been presented previously¹. We have as yet no accurate data on the position of the equilibrium. In the direction of polynucleotide synthesis and under our usual experimental conditions (pH 8.1, 30°), the reaction comes to a standstill when the ratio of the concentration of orthophosphate to that of nucleoside diphosphate is from 1.5 to 2. This is the case with ADP or IDP. Under these conditions the reaction favors polynucleotide synthesis, as would be expected from the fact that the pyrophosphate bonds of nucleoside diphosphates are converted to the phosphodiester bonds of the polynucleotide.

TABLE VII
PHOSPHOROLYSIS OF BIOSYNTHETIC POLYMERS AND NUCLEIC ACIDS

Experiment No.	Polymer	Source of polymer	³² P added c.p.m. · 10 ⁻⁴	Enzyme units*	³² P incorporated c.p.m.	Ortho- phosphate disappearance μmoles
1	AMP	Synthetic	3.65	9.3	1,380,000	2.75
	RNA	<i>A. vinelandii</i>			16,000	0
	AMP + RNA				320,000	1.85
2	AMP	Synthetic	1.68	9.3	860,000	1.50
	RNA	<i>A. vinelandii</i>			25,600	0
	RNA + IDPase				11,600	0
	AMP + RNA				276,000	0.40
3	AMP	Synthetic	0.31	4.6	71,000	0.80
	UMP	Synthetic			85,000	0.70
	A-U	Synthetic			28,000	
4	IMP	Synthetic	0.35	6.2	82,000	0.58
	RNA	<i>A. vinelandii</i>			7,600	
	RNA followed by IDPase				3,850	
	IMP + RNA				60,500	0.50
5	AMP	Synthetic	0.55	6.2	200,000	1.10
	RNA	<i>E. coli</i>			11,700	0
	AMP + RNA				100,000	
	RNA	<i>Str. pyogenes</i>			11,700	0
6	AMP	Synthetic	0.82	4.6	380,000	
	RNA	Yeast			18,800	
	RNA	Ox liver			9,300	
	RNA	Wheat germ			5,800	

Samples contained (in μmoles per ml), Tris buffer, pH 8.1 in experiments 1 to 3, pH 7.0 in experiments 4 to 6, 100; $MgCl_2$, 5; potassium phosphate, 7 to 9 with ³²P as indicated; polymer, 0.5 to 3.0 mg; and enzyme (specific activity 15 to 20) as indicated. Final volume, 1.5 ml. Incubation 90 minutes at 30°. When two different polymers were present, equal amounts of each were added. Values expressed per ml of reaction mixture.

* Assay 1.

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Phosphorolysis of "single" polymers can be readily detected and measured by the disappearance of orthophosphate. The biosynthetic A-U and A-G-U-C polymers are also phosphorolyzed although less readily than "single" polymers. Moreover, the enzyme can catalyze the phosphorolysis of natural RNA from *Azotobacter vinelandii* as well as from other sources. However, with the exception of turnip yellow mosaic virus RNA*, nucleic acids, including that from *Azotobacter*, do not seem to be phosphorolyzed easily. Under these conditions one may be unable to detect disappearance of orthophosphate. Nevertheless, the occurrence of phosphorolysis can be readily detected by the use of ^{32}P -orthophosphate which is incorporated into the nucleoside diphosphates, and these can be identified chromatographically and autoradiographically. Calf thymus DNA and a sample of yeast RNA "core" i.e., the fraction of RNA which following exhaustive digestion with pancreatic ribonuclease is non-dialyzable against distilled water, were not attacked². Thus, as far as phosphorolysis is concerned, the *Azotobacter* polynucleotide phosphorylase does not appear to be specific for *Azotobacter* RNA.

Table VII shows data on the phosphorolysis of biosynthetic polymers and natural ribonucleic acids by the *Azotobacter* enzyme. Both the incorporation of labeled phosphate (determined as described for Assay 1) and the disappearance of orthophosphate were measured in most cases. Only in the case of biosynthetic polynucleotides was there a measurable disappearance of orthophosphate although incorporation of labeled phosphate occurred throughout. It may further be seen (Experiments 1, 2, 4 and 5) that RNA inhibited to a greater or lesser extent the phosphorolysis of the AMP or the IMP polymer. Attention is called to the RNA phosphorolysis of Experiments 2 and 4 in which a separate aliquot contained, or was subsequently incubated with, inosine diphosphatase (IDPase). This enzyme¹⁶ catalyzes the hydrolysis of IDP, GDP and UDP to the corresponding 5'-nucleoside monophosphates but is inactive on ADP and CDP. Where IDPase was used, the radioactivity incorporated was halved indicating that the enzyme hydrolyzed the GDP and UDP (produced by phosphorolysis) with release of the terminal radioactive phosphate as orthophosphate. This conclusion is supported by the autoradiograms of Fig. 6. Comparison with the markers shown on the central strip suggests that the two upper spots on the first strip correspond to GDP and UDP, whereas the two lowest spots on this strip correspond to CDP



Fig. 6. Phosphorolysis of RNA. Samples of RNA from *Azotobacter vinelandii* (first two autoradiograms from left), *Streptococcus pyogenes* and *Escherichia coli* (last two autoradiograms) were used. Aliquots of the corresponding samples of experiments 4 and 5 of Table VII, following deproteinization, were chromatographed on paper by the method of KREBS AND HEMS⁸ and autoradiograms obtained. The center strip is the sketch of a chromatographic strip with markers (from top to bottom) of GDP, UDP, CDP and ADP. The second strip from left was derived from a sample incubated with IDPase (see text) before chromatography.

* L. A. HEPPEL and J. D. SMITH, unpublished experiments.

and ADP. The middle spot is probably labeled ATP formed from labeled ADP by adenylic kinase contaminating the *Azotobacter* enzyme preparation. It may be seen (second strip from left) that the two upper spots largely disappeared from the sample treated with IDPase following incubation with RNA. The last two strips in Fig. 5 suggest the formation of radioactive GDP, UDP, CDP and ADP by phosphorolysis of RNA from *Escherichia coli* or *Streptococcus pyogenes*. They correspond to two of the samples of Experiment 5 in Table VII.

DISCUSSION

The results of work already published^{1,2} and of further work to be reported in detail in subsequent papers of this series, show that the polynucleotides synthesized by polynucleotide phosphorylase consist of 5'-nucleoside monophosphate units linked to one another through 5'-3'-phosphoribose diester bonds. The chains are terminated by a phosphate group esterified at carbon 5' of the nucleoside moiety*. The biosynthetic polynucleotides of Table VI have average chain lengths ranging from 30 for the A-G-U-C polymer to 230 for the AMP polymer*. Ultracentrifugal studies by Dr. R. C. WARNER in this laboratory suggest average molecular weights ranging from 70,000 to 350,000 for these polynucleotides. Higher values, namely 570,000 and 800,000 for an AMP and an IMP polymer, respectively, were obtained by the method of light scattering**. However, during the preparation of the latter polymers the equilibrium was shifted at various time intervals by supplementary additions of nucleoside diphosphate to promote further synthesis. It is possible that polymers of larger size are obtained by multiple additions of nucleoside diphosphates. Some of the biosynthetic polymers can be stretched into fibres¹⁷ from which Dr. ALEXANDER RICH has obtained X-ray diffraction patterns suggesting a high degree of orientation***. "Single" polymers, such as the AMP polynucleotide, give X-ray diffraction patterns which are very similar, although not identical to those given by natural RNA. On the other hand, biosynthetic polynucleotides containing both purine and pyrimidine bases, such as the A-U and the A-G-U-C polymer, give patterns virtually identical to those of natural RNA. Thus, the information at present available on structure, base ratios, size, X-ray diffraction patterns, and behavior toward different enzymes² indicates that the "mixed" polynucleotides synthesized by polynucleotide phosphorylase from 5'-nucleoside diphosphates are closely related to RNA. Indeed, the A-G-U-C polymer, *i.e.*, the one containing adenylic, guanylic, uridylic and cytidylic acid residues, appears to be indistinguishable from natural RNA.

It cannot as yet be decided whether the reaction catalyzed by polynucleotide phosphorylase represents a general biological mechanism for the synthesis of ribonucleic acids. Wide distribution of the enzyme would favor such a possibility. Recent work⁸ indicates that the distribution of the enzyme is rather wide. It has been found in extracts of a number of bacterial cells, irrespective of whether the organisms are aerobic or anaerobic, gram positive or gram negative. The enzyme has also been found in yeast extracts and in extracts of green plants (spinach). However, no unequivocal evidence has yet been obtained for the presence of the enzyme in extracts

* J. D. SMITH and L. A. HEPPEL, unpublished end-group assays.

** L. F. CAVALIERI and M. ROSOFF, unpublished experiments.

*** A. RICH, unpublished experiments.

§ D. O. BRUMMOND, M. STAEHELIN and M. GRUNBERG-MANAGO, unpublished experiments.

of animal tissues. It may be that polynucleotide phosphorylase is present in all cells although in widely different amounts. Larger amounts would be expected in cells which have high rates of proliferation.

Polynucleotide phosphorylase may prove useful for gaining further knowledge of the chemistry and the physico-chemical properties of nucleic acids as well as in studies on the mechanism of action and specificity of nucleases. Since the enzyme can catalyze the synthesis of polynucleotide chains containing either a single mononucleotide or two or more different nucleotides (some of which may not occur naturally in nucleic acids), a number of non-naturally occurring polynucleotides can be obtained for studies of various kinds. It has been found*, for example, that "single" polynucleotides containing only adenylic or guanylic acid rapidly interact in aqueous solution to form a new polymer of much higher molecular weight than that of each of the initial reactants. This polymer migrates as a homogeneous compound in an electric field.

Little is as yet known of the mechanism of action of polynucleotide phosphorylase. Studies aimed at elucidating this point may have to await further purification of the enzyme. The question of whether the enzyme, in analogy with muscle phosphorylase, can only add nucleotide units to a pre-existing polynucleotide chain, *i.e.*, whether the enzyme requires a primer, or whether it can build a polynucleotide chain starting only from a mixture of nucleoside diphosphates is of obvious interest. No definite answer can yet be given to this question. In unpublished experiments, Dr. L. A. HEPPEL has so far failed to find evidence for accumulation of small polynucleotides on brief incubation of the enzyme with nucleoside diphosphates. The possibility that a nucleoside monophosphate, for example adenosine-5' monophosphate, reacts initially with a nucleoside diphosphate, such as uridine-5'-diphosphate, to give a dinucleotide has been eliminated by Dr. HEPPEL by end-group assays of the polynucleotide synthesized.

Azotobacter polynucleotide phosphorylase shows considerable lack of specificity since it can act on single nucleoside diphosphates, whether naturally or non-naturally occurring, or on mixtures of two or more of these. It also lacks specificity in the direction of phosphorolysis as it can attack ribonucleic acids from various sources. However the possibility that, in the presence of adequate concentrations of the four naturally occurring nucleoside diphosphates, the enzyme could synthesize a polynucleotide chain with a determined sequence of nucleotides, *i.e.*, a specific RNA, is indeed intriguing. It is conceivable that RNA bound on the enzyme might act as a prosthetic group and a template for the reproduction of its own nucleotide pattern. Answers to this question might be obtained through studies now under way of the polynucleotides synthesized by phosphorylases from different sources.

Finally, an interesting corollary of the reversibility of the reaction catalyzed by polynucleotide phosphorylase is that the free energy of hydrolysis of the phosphodiester bonds in polynucleotides may not be much lower than that of the pyrophosphate bonds of nucleoside diphosphates.

METHODS AND PREPARATIONS

The analytical methods used have been described or referred to in the experimental sections. For the determinations of radioactivity, aliquots of deproteinized reaction mixtures, before and

* R. C. WARNER, unpublished experiments.

after removal of orthophosphate, were counted directly as liquid samples with use of a thin-window-Geiger-Müller counter. Since the radioactivity of the orthophosphate was determined in every experiment under exactly the same conditions as that of other experimental samples, no corrections were applied for self-absorption or decay.

Azotobacter RNA was prepared from the residue (particles) obtained by high speed centrifugation of the bacterial extract during the first step of isolation of polynucleotide phosphorylase. The material was kept frozen until used. 100 g of residue were extracted in a Waring blender for 15 minutes at 3–4° with 10 volumes of 0.14 *M* sodium chloride. The mixture was centrifuged at 3–4° at high speed in a Servall angle centrifuge (20,000 *g*). The sediment was re-extracted as above with 2 volumes of 0.14 *M* sodium chloride and the extracts were combined. The combined extracts were heated for 1 hour at 85° and the protein precipitate was removed by centrifugation after cooling. To the supernatant fluid was added 40% trichloroacetic acid to give a final concentration of 5%. The precipitate was collected by centrifugation, stirred in 0.1 *M* Tris buffer, pH 7.35, and the insoluble residue centrifuged off and discarded. The supernatant fluid was treated once more with trichloroacetic acid as above. The precipitated nucleic acid was dissolved in Tris buffer, pH 7.35, leaving no insoluble residue, and the solution was dialyzed with stirring against distilled water at 0° for 48 hours. Based on U.V. absorption at wave-length 260 $m\mu$, the yield of nucleic acid at this point was 39.0 mg. The solution was adjusted to pH 7.0 and the nucleic acid was precipitated with 3 volumes of ethanol. The precipitate was collected by centrifugation, washed twice with ethanol and once with ether, and dried in vacuum over P_2O_5 . An aqueous solution of the powder after dialysis contained 26% DNA as determined by the DISCHE reaction¹⁸. Before use for the experiments of Table VII, the solution of nucleic acid was incubated for 1 hour at 30° with an excess of crystalline deoxyribonuclease, in the presence of $MgCl_2$, and dialyzed against water. The DNA content was reduced to 18%.

ATP and ADP were obtained commercially from the Sigma Chemical Co. and the Pabst Laboratories. The crystalline disodium salt of ATP was obtained from the Sigma Chemical Laboratories.

We are deeply indebted to Mr DAN BROIDA, Sigma Chemical Co., St. Louis, Mo., for generous gifts of large amounts of UDP, GDP, IDP and other nucleotides. We are also much indebted to Dr. ALEXANDER FRIEDEN and Dr. SAMUEL A. MORELL, Pabst Laboratories, Milwaukee, Wisc., for generous gifts of UDP and CDP. The present work would not have been possible without the above help. Our thanks are due to Dr. A. W. BERNHEIMER, Dept. of Microbiology, New York University College of Medicine, for samples of bacterial, wheat germ and ox liver RNA, to Dr. J. R. FRESCO, Dept. of Pharmacology, New York University College of Medicine, for help with the preparation of *Azotobacter* RNA and for samples of yeast RNA and calf thymus DNA, and to Dr. L. A. HEPPEL, National Institutes of Health, Bethesda, Md., for a sample of yeast RNA "core". IDPase was kindly supplied by Dr. G. W. E. PLAUT. We are indebted to Dr. M. KUNITZ, Rockefeller Institute for Medical Research, New York, N.Y., for a gift of crystalline yeast hexokinase.

SUMMARY

The isolation, partial purification and some properties of polynucleotide phosphorylase of *Azotobacter vinelandii* are described. The enzyme catalyzes the synthesis of highly polymerized ribonucleic acid-like polynucleotides from 5'-nucleoside diphosphates with release of orthophosphate. The reaction requires magnesium ions and is reversible. Thus, the enzyme also catalyzes the phosphorolysis of polynucleotides to yield the corresponding 5'-nucleoside diphosphates. The preparation and isolation of a number of polynucleotides containing one or several kinds of mononucleotide units is described. The scope, mechanism and significance of the reaction are discussed.

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