REVERSIBLE SYNTHESIS OF POLYRIBONUCLEOTIDES WITH AN ENZYME FROM ESCHERICHIA COLI*

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Studies on nucleotide and coenzyme synthesis in this laboratory led to an attempt to convert nucleoside 5'-polyphosphates to polyribonucleotides. We were able to show that extracts of *Escherichia coli* convert C^{14}-adenine-labeled ATP[1] to an acid-insoluble nucleotide, and that the addition of adenylate kinase increased the rate of the reaction (1, 2). The discovery of Ochoa and coworkers (3, 4) that polyribonucleotides are formed from nucleoside diphosphates in a reaction catalyzed by an enzyme from *Azotobacter vinelandii* made it clear that ADP rather than ATP was the reactive component. In preliminary reports (1, 2) we have described briefly an enzyme from *E. coli* which converts nucleoside diphosphates to polynucleotides according to the following general equation:

$$n \text{ nucleoside-PP} \rightleftharpoons (\text{nucleoside-P})_n + nP_i$$

A similar enzyme was found in an extract from *Micrococcus lysodeikticus* by Beers (5). The purpose of this report is to describe the purification of the *E. coli* enzyme, its properties, and the stoichiometry of the reversible phosphorolysis which it catalyzes.

Materials

5-Phosphoribosyl pyrophosphate was prepared from ribose 5-phosphate and ATP with a pigeon liver enzyme (6). C^{14}-A5P was prepared from adenine-8-C^{14} (Isotopes Specialties Company, Inc.) and 5-phosphoribosyl pyrophosphate with yeast A5P pyrophosphorylase (7). C^{14}-ATP was prepared from the labeled A5P by the combined action of yeast adenylate

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[1] The abbreviations used are adenosine 5'-phosphate, A5P; adenosine diphosphate, ADP; adenosine triphosphate, ATP; cytidine diphosphate, CDP; deoxyribonuclease, DNAase; deoxyribonucleic acid, DNA; guanosine diphosphate, GDP; inorganic orthophosphate, P_i; P^{32}-labeled P_i, P_i^{32}; ribonuclease, RNAase; ribonucleic acid, RNA; thymidine diphosphate, TDP; tobacco mosaic virus, TMV; turnip yellow mosaic virus, TYMV; tris(hydroxymethyl)aminomethane, Tris; uridine 5'-phosphate, U5P; uridine diphosphate, UDP.

kinase (8) and pyruvate phosphokinase with phosphopyruvate, and was purified on a Dowex 1 column (9). C^{14}-ADP was prepared from C^{14}-ATP and glucose by the action of purified yeast hexokinase and isolated by chromatography on a Dowex 1 column (9). Labeled U5P was prepared from uracil-2-C^{14} (Isotopes Specialties Company) and 5-phosphoribosyl pyrophosphate with U5P pyrophosphorylase obtained from *L. bifidus*.[2] C^{14}-UDP was prepared from labeled U5P by the action of yeast nucleoside monophosphate kinase (8) and pyruvate phosphokinase with phosphopyruvate. UDP was separated from U5P and UTP by Dowex 1 column chromatography (8). Thymidine diphosphate was prepared by the method of Hall and Khorana as described for the uridine nucleotides (10). Unlabeled ADP, UDP, CDP, and GDP were obtained from the Sigma Chemical Company.

Protamine sulfate was generously supplied by Eli Lilly and Company. Purified potato starch was obtained from the Fisher Scientific Company. Crystalline RNAase and DNAase were Worthington Biochemical Corporation products. Venom phosphodiesterase free from monoesterase was generously given to us by Dr. L. A. Heppel and Dr. L. Shuster

Methods

A5P was assayed with Schmidt's deaminase by Kalckar's method (11); ADP and ATP were determined enzymatically as previously described (12, 13); U5P, UDP, and UTP were separated by ion exchange chromatography and determined spectrophotometrically (8). Orthophosphate was estimated by the method of Fiske and Subbarow (14); acid-labile phosphate was the orthophosphate liberated after a 10 minute hydrolysis in 1 N HCl at 100°, and total phosphate was measured as orthophosphate after being ashed with a sulfuric-nitric acid mixture. Pentose was determined by the Mejbaum procedure (15), with a 45 minute heating period and A5P as a standard. Proteins were determined by the phenol method of Lowry *et al.* (16). Ion exchange chromatography was carried out at 2° with an automatic fraction collector on Dowex 1 columns (2 per cent cross-linked, 200 to 400 mesh, chloride form) (9). P^{32} was measured as a thin, dried layer on metal disks under a Geiger-Müller tube. C^{14}-containing samples were plated as thin layers on metal disks and measured in a gas flow counter. Self-absorption corrections were applied as indicated.

Enzyme Assays—The enzyme activity was determined in three ways.

Assay A: Incorporation of Labeled Nucleoside Diphosphate into Acid-Insoluble Precipitate—The incubation mixture (0.25 ml.) contained 0.05 ml. of glycylglycine buffer (1 M, pH 7.4), 0.02 ml. of ADP (0.04 M), 0.02 ml. of 8-C^{14}-ADP (0.00227 M, 7.8×10^5 c.p.m. per μmole), 0.01 ml. of

[2] See Crawford, *et al.* (22).

$MgCl_2$ (0.1 M), and less than 0.3 unit of enzyme. A similar procedure was used for studying C^{14}-UDP incorporation. After incubation at 37° for 10 minutes, the reaction was stopped by immersing the tubes in an ice bath; 0.5 ml. of carrier nucleic acid in the form of a 1:20 dilution of crude *E. coli* extract and 0.25 ml. of 7 per cent perchloric acid were then added. After 10 minutes in the cold, the precipitate was centrifuged, washed twice with 1.0 ml. portions of 1 per cent perchloric acid, and once with 1.0 ml. of 0.01 N HCl. The precipitate was dissolved in 0.4 ml. of 0.05 M KOH. A 0.10 ml. aliquot was removed to a planchet, dried, and assayed for radioactivity (self-absorption correction factor, 1.55). 1 unit of enzyme was defined as the amount catalyzing the incorporation of 1.0 μmole of ADP in 1 hour, and the specific activity was expressed as units per mg. of protein. Under these assay conditions, the radioactivity in the acid-insoluble precipitate was proportional to the enzyme concentration. Thus, with use of 0.01, 0.03, 0.06, and 0.12 ml. of a crude enzyme fraction, 0.005, 0.017, 0.027, and 0.048 μmole of ADP, respectively, were incorporated in the polynucleotide.

Assay B: 8-C^{14}-ATP Incorporation in Presence of Myokinase—The incubation mixture (0.25 ml.) contained 0.02 ml. of Tris buffer (1 M, pH 8.0), 0.02 ml. of ATP (0.05 M), 0.01 ml. of 8-C^{14}-ATP (0.0087 M, 3.8×10^5 c.p.m. per μmole), 0.04 ml. of ADP (0.006 M), 0.01 ml. of $MgCl_2$ (0.1 M), 0.02 ml. of yeast adenylate kinase (heated ethanol fraction (8)), and less than 0.25 unit of enzyme. The mixture was incubated for 10 minutes at 37°. The reaction was stopped and the precipitate treated as in Assay A.

An enzyme unit was defined as in Assay A, and equally good proportionality of the values obtained to the amounts of enzyme added was observed.

Assay C: Nucleoside Diphosphate Exchange with P^{32}—This assay was based on that of Grunberg-Manago *et al.* (4). The incubation mixture (0.5 ml.) contained 0.10 ml. of glycylglycine buffer (1 M, pH 7.4), 0.10 ml. of nucleoside diphosphate (0.004 M), 0.05 ml. of P_i^{32} in potassium phosphate buffer, pH 7.4 (0.0052 M, 5.2×10^6 c.p.m. per μmole), 0.02 ml. of $MgCl_2$ (0.1 M), and less than 0.3 unit of enzyme. The mixture was incubated for 20 minutes at 37°. The reaction was stopped by immersing the tubes in an ice bath, adding 0.5 ml. of 5 per cent perchloric acid to acidify the solution, and 0.10 ml. of an acid-washed Norit A suspension (10 per cent dry weight) to adsorb the nucleotides. After 10 minutes in the cold, the Norit was centrifuged and washed three times with 2.5 ml. portions of water. The precipitate was suspended in 0.8 ml. of 50 per cent ethanol containing 3 ml. of concentrated NH_4OH per liter. An aliquot (0.2 ml.) of the above Norit suspension was dried on a planchet and the radioactivity measured (self-absorption correction factor 1.15). 1 unit of enzyme was defined as the amount causing the incorporation of 1.0 μmole of P^{32}

into ADP per hour, and the specific activity was expressed as units per mg. of protein. The amount of P_i incorporated into the terminal phosphate of ADP was calculated from the equation:

$$\mu\text{moles phosphate incorporated} = \frac{\text{total c.p.m. in ADP}}{\text{initial specific activity of } P_i}$$

These values are somewhat low since no correction was made for the decrease in specific radioactivity of the P_i by the contribution of the terminal phosphate of ADP. However, since the initial rates were determined when the exchange was still less than 10 per cent of completion, this correction would be very small. With 0.01, 0.03, 0.06, and 0.08 ml. of enzyme (manganese supernatant fluid), 0.0028, 0.011, 0.021, and 0.028 μmoles of the P_i were found to be incorporated into the ADP.

Growth of Cells and Preparation of Cell-Free Extracts—*E. coli* strain B was grown in a medium (pH 6.8 to 7.0) containing 1 per cent yeast extract (Difco, dehydrated), 1 per cent glucose, 2.18 per cent K_2HPO_4, 1.70 per cent KH_2PO_4, and about 20 mg. per liter of Antifoam A (Dow-Corning); glucose was autoclaved separately and added to the cooled medium. 15 liters of the medium in a 20 liter Pyrex bottle were inoculated with 1.5 liters of a 14 to 16 hour culture and incubated at 37° with vigorous forced aeration until the end of the logarithmic phase of growth (3 to 4 hours). The cells were harvested in a Sharples supercentrifuge (8 gm. of wet cells per liter) and washed with 4 volumes of cold 0.9 per cent KCl. The cells (170 gm., wet weight) were suspended in 0.05 M glycylglycine buffer, pH 7.4, to a final volume of 680 ml. and placed for 10 minutes in a Raytheon 10-kc. oscillator at 6–8°. The residue was collected by centrifugation for 20 minutes at 10,000 $\times$ g in a Servall centrifuge and the supernatant fluid (10 minute sonic extract) was discarded. The residue was suspended in the same buffer at a final volume of 680 ml., subjected again to sonic oscillation for 30 minutes, and centrifuged for 20 minutes as before. The turbid supernatant fluid (sonic extract of the residue) was used for further purification steps. The cells were treated in the oscillator immediately after harvesting in order to obtain most of the enzyme activity in the residue of the 10 minute sonic extract. This residue, as well as the extract of it, was stored at −15° for over 2 months without loss of enzyme activity.

Results

Purification of Enzyme

All operations were carried out at 0–3° except as indicated.

Mn^{++} and Protamine Steps—To 615 ml. of the sonic extract of the residue (Table I), 31 ml. of 1 M $MnCl_2$ were added slowly with mechanical

stirring; the stirring was continued for 30 minutes. Insoluble material was removed by centrifugation for 20 minutes at 10,000 × *g* (manganese supernatant fluid). (For assays at this stage this fraction must be dialyzed overnight against 0.9 per cent KCl.) To the undialyzed, clear yellow supernatant fluid, 61 ml. of 1 per cent protamine sulfate were added with mechanical stirring during a period of 10 minutes. (The amount of protamine sulfate needed to precipitate over 90 per cent of the activity was determined for each run.) The precipitate formed was collected by centrifugation, suspended in 200 ml. of 0.05 M potassium phosphate buffer, pH 7.5, and recentrifuged. The supernatant fluid was dialyzed overnight against 6 liters of 0.9 per cent KCl and became slightly turbid (protamine eluate). This fraction was stored at −15° for no longer than 1 week.

TABLE I

Purification of Enzyme

Step	Units per ml.*	Total units*	Protein	Specific activity	λ280:λ260
			mg. per ml.	*units per mg. protein*	
10 min. sonic extract	1.1	670	10.9	0.1	0.55
Sonic extract of residue	3.0	1860	6.9	0.5	0.62
Protamine eluate	9.8	1960	0.88	11	1.75
Ethanol I	13.1	870	0.76	17	1.75
" II	4.8	320	0.17	28	1.75

* Assay A was used with ADP (8-C^{14}).

Ethanol Fractionation—To 200 ml. of the protamine eluate fraction were added 4.0 ml. of 1 M potassium acetate (pH 5.5) and then 1.2 ml. of 0.5 M $ZnCl_2$ (adjusted to pH 5.5 with acetic acid). After standing for 10 minutes, any precipitate which formed was removed by centrifugation for 3 minutes at 10,000 × *g*. To the supernatant fluid, 44 ml. of 50 per cent ethanol (−15°) were added over a 7 minute interval, during which time the mixture was chilled to −2°. The precipitate was removed by centrifugation for 3 minutes at 10,000 × *g*, and to the supernatant fluid 52 ml. of 50 per cent ethanol were added as described above, the temperature being maintained at −2° to −4°. The precipitate was collected by centrifugation and dissolved with 0.05 M Tris buffer, pH 8.0, to a final volume of 67 ml. (Ethanol I).

To 66 ml. of the Ethanol I fraction were added 0.5 N acetic acid to pH 5.5 (approximately 2.5 ml.) and then 0.40 ml. of 0.5 M $ZnCl_2$. After 10 minutes the precipitate was centrifuged as before, and to the supernatant fluid 13.5 ml. of 50 per cent ethanol (−15°) were added during a 7 minute period; the mixture was chilled to −2° during this interval. The precipitate was centrifuged and dissolved in 66 ml. of 0.05 M Tris buffer, pH 8.0 (Ethanol II). The optical density as indicated by the $\lambda280:\lambda260$ ratio varied for different enzyme preparations from 1.5 to 1.75.

Purification of the Ethanol I fraction could also be achieved by starch column electrophoresis. The column (3.5×50 cm.) was washed first with water and then with 0.05 M Tris buffer, pH 8.0. Ethanol I fraction (4.0 ml.) was added and the column was washed twice with 5.0 ml. of the Tris buffer. Current was applied for 19 hours (500 volts, 8 to 10 ma.). The enzyme was eluted with 0.05 M Tris buffer, pH 8.0 (8.0 ml. per hour), and 4.0 ml. fractions were collected. The enzyme appeared between Fractions 19 and 27 with an over-all recovery of 83 per cent. Fractions 20 and 21 contained 18 per cent of the total activity with a 10-fold increase in specific activity over the Ethanol I fraction; the enzyme was very labile in this state and activity was lost rapidly upon freezing and thawing.

Stability of Enzyme—A purified enzyme fraction (Ethanol II) retained about 70 per cent of its activity after storage for 2 months at $-10°$, but, when diluted (1:10 in 0.10 M glycylglycine buffer, pH 7.4), the activity was rapidly lost. Heat inactivation of the enzyme (manganese supernatant fluid in 0.10 M glycylglycine buffer, pH 7.4) was observed by heating for 5 minutes at 60° or 70°; 70 and 98 per cent, respectively, of the original activity was lost.

Presence of Other Enzymes—DNAase activity was not detected in the purified enzyme (0.08 unit of Ethanol II degraded less than 0.10 per cent of P^{32}-labeled T_2 phage DNA (0.25 γ of DNA, 1.7×10^4 c.p.m.) after a 20 minute incubation period at 37°. Adenylate kinase activity was low (1.0 unit of Ethanol I catalyzed the formation of 0.09 μmole of ADP per hour from ATP and A5P when measured with the coupled pyruvate phosphokinase-lactic dehydrogenase system (13)). An amount of RNAase activity was present in 1.0 unit of enzyme (Ethanol I) sufficient to liberate 0.05 μmole of mononucleotide per hour when incubated with yeast sodium nucleate in the absence of phosphate buffer. The crude extract contained a nucleotide-*N*-ribosidase[3] which hydrolyzed A5P (and thus in the presence of adenylate kinase removed ADP from the reaction). However, this activity was absent in the purified fractions; 1.5 units of the Ethanol I fraction liberated less than 0.002 μmole of ribose 5-phosphate per hour.

Incorporation of Nucleoside Diphosphates into Polynucleotides

Balance Study of Reaction—For each micromole of acid-labile phosphate and ADP disappearing, 1 μmole of P_i was liberated, and approximately equivalent amounts of pentose and phosphate appeared in the acid-insolu-

[3] We are grateful to Dr. J. Hurwitz, Dr. L. A. Heppel, and Dr. B. L. Horecker for informing us of their unpublished work on this enzyme.

ble product (Table II). The isolated polyadenylate was hydrolyzed with 1 N KOH for 15 hours at 37°, and the quantity of adenosine 2'- and 3'-phosphates found matched the disappearance of an equivalent amount of ADP.

TABLE II

Balance Study of ADP, UDP, CDP, and GDP Incorporation into Polynucleotides

	ADP (1)		UDP (2)		CDP (3)		GDP (4)	
	Acid-soluble	Polymer	Acid-soluble	Polymer	Acid-soluble	Polymer	Acid-soluble	Polymer
Orthophosphate, Δ *μmole*	+0.42		+0.21		+0.44		0.0	
Acid-labile phosphate, Δ *μmole*	−0.38		−0.21		−0.44		0.0	
Total phosphate, Δ *μmole*		+0.41						0.0
Ultraviolet density, Δ *μmole*	−0.36	+0.39	−0.25	+0.28	−0.44	+0.43	0.0	0.0
Pentose, Δ *μmole*		+0.36						
Radioactivity, % incorporation		57		30				
Acid-labile phosphate, % decrease	52		32		60		0.0	

(1) At zero time, the P_i, acid-labile phosphate, and "adenosine" values were 0.07, 0.75, and 0.86 μmoles, respectively; after 60 minutes, the respective values were 0.49, 0.37, and 0.50 μmoles. (2) At zero time, the P_i, acid-labile phosphate, and "uridine" values were 0.10, 0.64, and 0.75 μmoles, respectively; after 60 minutes the respective values were 0.31, 0.43, and 0.50 μmoles. (3) At zero time, the P_i, acid-labile phosphate, and "cytidine" values were 0.06, 0.74, 0.87 μmoles, respectively; after 60 minutes the respective values were 0.50, 0.30, and 0.43 μmoles. (4) At zero time, P_i, acid-labile phosphate, and guanine values were 0.11, 0.64, and 0.80 μmoles, respectively; after 60 minutes the respective values were 0.10, 0.66, and 0.79 μmoles. The reaction mixture (0.25 ml.) was as described in Assay A (see the text), with 0.8 unit of Ethanol I and an incubation period of 60 minutes at 37°. In the ADP experiment, 0.04 ml. of ADP (8-C^{14}, 0.00147 M, 6.17 × 10^5 c.p.m. per μmole) was used; in the UDP experiment, 0.02 ml. of UDP (2-C^{14}, 0.00522 M, 1.22 × 10^6 c.p.m. per μmole) was used; in the CDP and GDP experiments, the substrates were not labeled. The reaction was stopped by immersing the tubes in an ice bath, adding 0.5 ml. of a cold solution of crystalline serum albumin (1.6 mg. per ml.) and 0.25 ml. of 7 per cent perchloric acid. After 10 minutes in the cold, the precipitate was removed by centrifugation. The supernatant fluid is the "acid-soluble" fraction. The precipitate, washed twice with 1.0 ml. portions of 1 per cent perchloric acid and once with 1.0 ml. of 0.01 N HCl, and dissolved in 0.4 ml. of 0.025 N KOH, is the "polymer" fraction. The optical density of the polymer fraction was determined after hydrolysis of an aliquot in 1 N KOH for 15 hours.

The fraction of the total radioactivity found in the product derived from the added C^{14}-ADP (57 per cent) was in agreement with the fraction of the acid-labile phosphate disappearing (52 per cent).

Similar results were obtained in balance studies carried out with C^{14}-UDP and with CDP, but with GDP no reaction was detected (Table II).

However, in GDP experiments with large amounts of enzyme (5.0 units) and a longer incubation time (3 hours), an acid-insoluble product was obtained which, upon perchloric acid hydrolysis, yielded guanine, as determined by paper chromatography. When the reaction was carried out in the presence of all four nucleoside diphosphates, the extent of GDP incorporation increased considerably, and approximately equivalent amounts of each of the four nucleotides were found in the polymer.

Extent of Reaction—In the crude extract only small amounts of product accumulated, and when the incubation was continued for longer periods the product disappeared (Fig. 1). On the other hand, when a purer en-

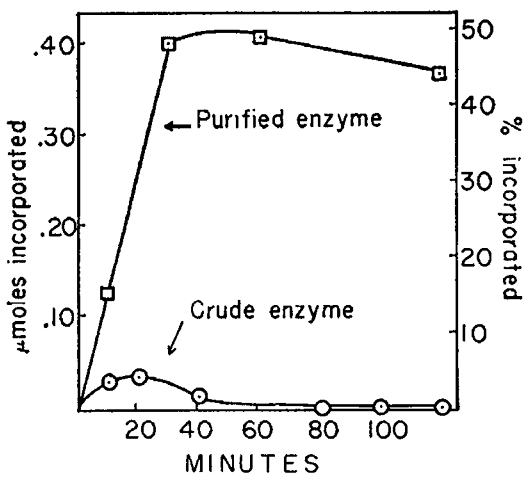

FIG. 1. The extent of ADP incorporation as a function of time. The reaction mixtures (0.25 ml.) were as described in Assay A. The incubation temperature was at 37° for the time indicated. The crude enzyme was 0.18 unit of a 30 minute sonic extract and the purified enzyme was 0.72 unit of an Ethanol II fraction.

zyme fraction (Ethanol I) was used, more than 50 per cent of the ADP was converted into polyadenylate, and this did not disappear on continued incubation. With sufficient amounts of enzyme and longer incubation periods, extensive polymerization of even low concentrations of ADP (*i.e.* 10^{-4} M) was observed.

Effect of Substrate and Mg^{++} Concentration—The K_m value for ADP was found to be 2.0×10^{-2} M. At this concentration of ADP, 1 mg. of the Ethanol II fraction polymerized 200 μmoles of ADP per hour. The reaction was found to require Mg^{++}. When this was omitted, no incorporation of ADP could be detected. Optimal Mg^{++} levels depended on the ADP concentration and were generally attained at an ADP:Mg^{++} ratio of 1.5; inhibiting effects were observed with lower ratios. At an ADP:Mg^{++} ratio of 0.475, the rate was only 46 per cent of the maximal rate.

Isolation of Products—Polyadenylate was isolated from an incubation mixture similar to that described for Assay A, with use of 0.016 M ADP (8-C^{14}) (2.7 × 10^4 c.p.m. per μmole) and 0.5 unit of enzyme. After 60 minutes at 37°, 0.20 ml. of 1.0 M sodium acetate buffer, pH 3.5, was added, and, after 30 minutes in the cold, a transparent, gel-like precipitate appeared. The precipitate was centrifuged for 10 minutes at 10,000 × g, washed twice with 1.0 ml. portions of cold water, and dissolved in 0.25 ml. of 1.0 M Tris buffer, pH 8.0. This gave a highly viscous solution from which threads could be drawn. The solution lost its viscosity when stored in the cold for 24 hours. When polyadenylate was precipitated with a stronger acid, such as 1.7 per cent perchloric acid, the precipitate obtained failed to give a viscous solution. The absorption spectrum of the polymer was different from that of AMP; the λ250:λ260 and λ280:λ260 absorption ratios (pH 8.0) were 0.93 and 0.32, respectively. When incubated with 1 N KOH for 15 hours at 37°, the polymer was rendered completely acid-soluble and the absorption spectrum was identical to that of adenosine 3′-phosphate. $MgCl_2$ (0.1 M) precipitated polyadenylate, and the precipitate could be redissolved with 1.0 M Tris buffer, pH 8.0. In the case of UDP and CDP, the polymerized products did not precipitate at pH 3.5, and with the use of stronger acid (1.7 per cent perchloric acid) the recoveries were poor. Although all the polymers were precipitated readily in the presence of 80 per cent alcohol, significant amounts of the nucleoside diphosphates were coprecipitated. Precipitation with streptomycin (17) provided the most satisfactory procedure: sodium acetate buffer (0.05 ml., 1 M, pH 3.5) and streptomycin sulfate (0.1 ml. of a 10 per cent solution) were added to the reaction mixture. After 10 minutes at 0°, the precipitate was centrifuged, washed twice with cold 1 per cent streptomycin, and dissolved in 0.40 ml. of 1.0 M Tris buffer, pH 8.0. The polymer solutions thus obtained were highly viscous.

Reversal of Reaction—The formation of polyadenylate was readily reversed by adding P_i to the incubation mixture. Table III shows the extent of polyadenylate (8-C^{14}) breakdown in the presence of graded phosphate concentrations.

Phosphorolysis of Nucleic Acids—The extent and rate of phosphorolysis of different nucleic acids are shown in Table IV.[4] Highly polymerized yeast RNA, prepared according to the procedure of Crestfield *et al.* (18),[5] and plant virus RNA were phosphorolyzed readily. On the other hand, a dialyzed commercial sample of yeast sodium nucleate (Schwarz Laboratories, Inc.) was decomposed at only about one-fourth the rate and to a more limited extent (18 per cent). Duplicate assays of each experiment

[4] These experiments were carried out together with Dr. L. A. Heppel in his laboratory at the National Institutes of Health.

[5] Kindly given to Dr. Heppel by Dr. F. W. Allen.

by paper chromatography (isopropanol-water 1:3, v/v, with NH_3 in the vapor phase (19)), showed an accumulation of nucleoside diphosphates which appeared to be approximately proportional to the amount of P_i^{32} incorporated into the acid-soluble nucleotides. The non-dialyzable limit polynucleotide obtained after exhaustive digestion of commercial yeast RNA with pancreatic ribonuclease was phosphorolyzed very little if at all. When phosphate was omitted from the reaction mixtures, no degradation of RNA was detectable.

TABLE III

Polyadenylate Phosphorolysis

The incubation mixture (0.25 ml.) contained 0.01 ml. of polyadenylate (8-C^{14}, containing 3.7 μmoles of adenine residues per ml. and 31,500 c.p.m. per ml.), 0.05 ml. of glycylglycine buffer (1 M, pH 7.4), P_i as indicated, 0.01 ml. of $MgCl_2$ (0.1 M), and 0.05 unit of Ethanol II. The mixture was incubated at 37° for 60 minutes. The reaction was stopped and the acid-insoluble fraction was separated and its radioactivity determined. Polyadenylate was prepared from ADP (8-C^{14}, 8.34 × 10^3 c.p.m. per μmole) as described in the text.

P_i concentration	Polyadenylate	
$M \times 10^4$	*c.p.m.*	*per cent breakdown*
0.64* (0 min.)	315	0
0.64* (60 min.)	272	14
4.40 (60 ")	223	29
19.0 (60 ")	13	96
38.0 (60 ")	3	99

* P_i concentration of the reaction mixture without added P_i.

Exchange of Inorganic Phosphate with Nucleoside Diphosphates

Specificity with Different Nucleoside Diphosphates—P_i^{32} exchanged with several nucleoside diphosphates, and this reaction was used for assaying the enzyme activity (Assay C). ADP, UDP, and CDP were found to react readily, both in the crude and in the purified enzyme fraction. However, with GDP the rate of exchange with P_i^{32} in the crude extract was considerably slower than with the other diphosphates, while in the purified fraction almost no activity relative to the other diphosphates was detected (Table V). Furthermore, when the extent of polymerization was measured with the purified enzyme, it was found that ADP, UDP, and CDP were polymerized to a considerable extent, but under these conditions no acid-insoluble polymer of GDP was observed. GTP plus G5P in the presence of purified yeast nucleoside monophosphate kinase gave the same low rate of exchange as did GDP alone. Thymidine diphosphate showed only a feeble activity; 0.06 ml. of Ethanol I incorporated 0.17 μmole of P_i into TDP as compared with 12 mμmoles into ADP.

Influence of Various Factors on Rate of Exchange

"Activating" Factor—When the enzyme fractions obtained during the course of purification were assayed for their P_i^{32} exchange rate with ADP,

TABLE IV

Phosphorolysis of Nucleic Acids

The incubation mixture (0.25 ml.) contained 0.02 ml. of Tris buffer (0.5 M, pH 8.0), 0.01 ml. of $MgCl_2$ (0.1 M), 0.06 ml. of potassium phosphate buffer (0.2 M, pH 7.2), 0.01 to 0.06 ml. of P_i^{32} solution, and amounts of RNA and enzyme (Ethanol I) as indicated. The mixture was incubated at 37°. The reaction was stopped and treated with Norit A as described in Assay C. The amounts of P_i^{32} added (expressed as 10^5 c.p.m.) were 2.1, 4.1, 4.5, 15, 15, and 17, in Experiments 1 through 6, respectively.

Experiment No.	Polymer	Amount polymer added	Amount enzyme added	Time of incubation	Phosphate incorporated	Extent of phosphorolysis of polymer	Rate of phosphorolysis of polymer*
		μmoles	*units*	*hrs.*	*μmole*	*per cent*	
1	Purified yeast RNA (17)†	0.37	0.60	24	0.41	110	
2	" " "	1.10	0.15	3	0.07	6.4	0.18
	" " "	1.10	0.60	3	0.26	24	
	" " "	1.10	1.50	3	0.62	56	
3	TYMV RNA	0.87	0.60	3	0.24	28	0.16
	Commercial yeast RNA	1.40	0.15	3	0.02	1.4	
	" " "	1.40	0.60	3	0.05	3.6	0.04
4	" " "	1.40	1.20	5	0.25	18	0.05
	" " "	1.40	2.40	5	0.26	19	
5	RNAase-limit polynucleotides	1.19	0.15	3	0.019	1.6	0.03
	RNAase-limit polynucleotides	1.19	0.60	3	0.027	2.3	
	Polyadenylate	1.02	0.075	3	0.59	58	3.10
	"	1.02	0.60	3	0.95	93	
	Polyuridylate	0.825	0.075	3	0.17	21	0.91
	"	0.825	0.60	3	0.31	37	
6	Polyadenylate	0.61	0.0075	3	0.056	9.2	2.39
	Polyuridylate	0.495	0.015	3	0.039	7.9	1.02
	TMV RNA	0.995	0.15	3	0.030	3.0	
	" "	0.995	0.60	3	0.15	15	0.10

* Micromoles of phosphate incorporated into the acid-soluble nucleotide fraction per mg. of protein per hour.

† In this experiment 0.05 M potassium phosphate buffer (0.6 ml.) was used.

it was found that the ratio of the rate of ADP incorporation into acid-insoluble nucleotide to the rate of ADP-P^{32} exchange was more than 4-fold greater in the purified fraction than in the crude sonic extract (Table V). Furthermore, it was found that, after precipitating the enzyme with protamine, the supernatant solution, although devoid of enzyme activity, increased the P_i^{32}-ADP exchange rate of a purified enzyme fraction (Ethanol II) by 3- to 4-fold (Table VI and Fig. 2). On the other hand, the activating factor did not affect the rate of ADP incorporation into the acid-insoluble fraction as measured with C^{14}-ADP. The factor was found to be

TABLE V

Specificity of P_i^{32} Exchange with Nucleoside Diphosphates

The exchange rate was measured as described in Assay C (see the text). No activator was added.

Substrate	Enzyme fraction			
	10 min. sonic extract		Ethanol I	
	units per ml.	*unit per mg. protein*	*units per ml.*	*units per mg. protein*
ADP	3.50	0.43	0.93	6.2
UDP	4.00	0.50	6.50	43.2
CDP	5.10	0.64	4.30	28.5
GDP	0.46	0.06	0.015	0.1

TABLE VI

P_i^{32} Exchange with ADP

The exchange rate was measured as described in Assay C (see the text).

	Exchange reaction specific activity, units per mg. protein			Ratio of polymer formation to P_i^{32} exchange	
	No activator	With activator*	Activation ratio	No activator	With activator
No enzyme	0.0	0.00			
10 min. sonic extract	0.20	0.20	1.0	0.5	0.5
Sonic extract of residue	0.44	0.44	1.0	1.1	1.1
Protamine eluate	3.3	7.9	2.5	3.3	1.4
Ethanol I	7.5	19.0	2.5	2.3	0.9
" II	12.3	46.5	3.8	2.3	0.6

* 0.1 ml. of the supernatant fluid obtained after precipitating the enzyme with protamine.

non-dialyzable, and more heat-stable than the enzyme; heating of the manganese supernatant solution in 0.10 M glycylglycine buffer, pH 7.4, for 5 minutes at 60° or 70° resulted in respective losses of 40 and 60 per cent of the original activity. The fraction heated at 70° was used for most of the experiments described; the λ280:λ260 absorption ratio was 0.50. The "activator" was resistant to DNAase and RNAase; its activity was slightly reduced by snake venom phosphodiesterase and more readily by the combined action of RNAase and phosphodiesterase. P_i^{32} exchange with UDP and CDP was not activated by this fraction when either Ethanol I or Ethanol II was used. With a fraction purified by starch column electrophoresis, UDP-P_i^{32} exchange was activated 2-fold, while the exchange with CDP was not affected.

Protamine—In the presence of 5 γ of protamine, the exchange rate of P_i^{32} with ADP was increased about 2-fold, but at higher concentrations the activation fell off (Fig. 2). It may be noted that the same enzyme

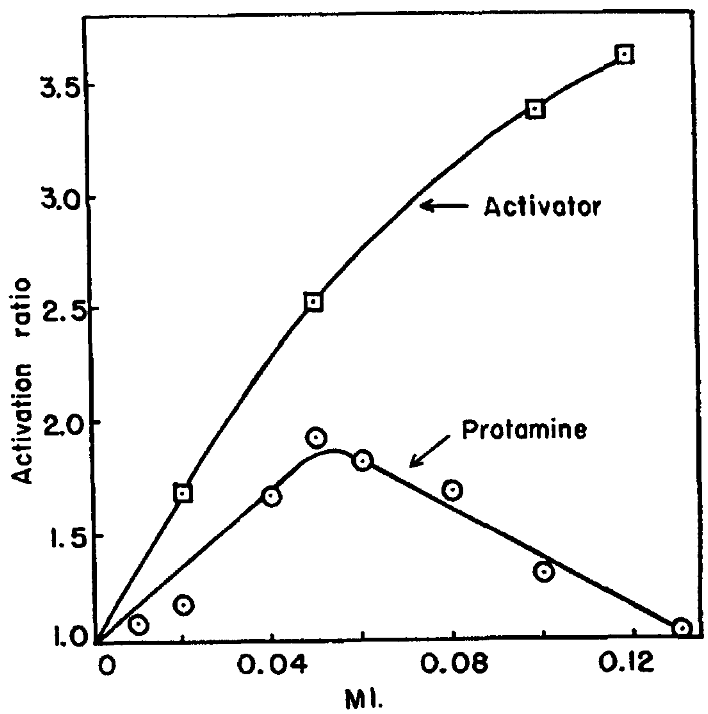

FIG. 2. P_i^{32}-ADP exchange activation. The reaction mixture was the same as described in Assay C with 0.01 ml. of Ethanol II. The activator was a protamine supernatant fluid fraction (see the text) and the protamine was a 0.01 per cent protamine sulfate solution.

fraction was activated 3.6-fold by adding the activating factor. The ADP incorporation rate was not increased by protamine, and at higher concentrations it was inhibited; 50 γ of protamine produced a 60 per cent inhibition.

Effect of RNAase—RNAase did not significantly affect the exchange rate of P_i^{32} with ADP, or the incorporation rate. Thus 1.0 ml. of enzyme (Ethanol I) incorporated 3.2 μmoles of P_i^{32} per hour in the presence of 0.10 mg. of crystalline RNAase as compared with 5.5 μmoles in its absence. The respective ADP incorporation rates (Assay A) were 7.8 and 5.7 μmoles. RNAase inhibited UDP incorporation almost completely, but had relatively little effect on the exchange with P_i^{32} (1.0 ml. of enzyme (Ethanol I) incorporated 13.2 μmoles of UDP per hour as compared with 0.47 μmole when 0.10 mg. of crystalline RNAase was added to the reaction mixture; the P_i^{32} exchange rates were 6.9 and 9.6 μmoles, with and without RNAase, respectively).

Effect of Mg^{++}, Mn^{++}, and Pyrophosphate—Mg^{++} was essential for the exchange activity. In its absence no exchange with P_i^{32} was observed. Under the conditions described for Assay C, the rate was half maximal at 3.7×10^{-4} M, and maximal at 1.2×10^{-3} M. Mn^{++} was inhibitory (1.4×10^{-4} M gave 83 per cent inhibition). Pyrophosphate (6.0×10^{-3} M) did not inhibit the reaction. In addition, no exchange could be found between P^{32}-labeled pyrophosphate and ATP or GTP.

Streptomycin—Streptomycin (0.014 per cent) inhibited the P_i^{32} exchange reaction by 50 per cent.

Heating—Heating the enzyme for 5 minutes at 70° destroyed over 98 per cent of the ADP-P_i^{32} exchange activity.

DISCUSSION

The numerous metabolic implications of the polyribonucleotide phosphorylase reactions have been previously discussed (4). Of overriding importance is the relevance of this reaction to the synthesis of the ribonucleic acids of the cell. Thus far this enzyme provides the only known way of making high molecular weight polymers of ribonucleotides, and it is abundant in a wide variety of microbial cells (20). On the other hand, it is not yet apparent how the relatively constant and perhaps specific nucleotide composition of the RNA of a given species is produced by an enzyme that appears to polymerize the available nucleoside diphosphates in a relatively random fashion. It is also of interest that the physiological concentrations of the nucleoside diphosphates appear to be far below those necessary for rapid rates of enzymatic polymer synthesis, and furthermore that the intracellular P_i values seem sufficient to inhibit polymer synthesis and to effect extensive phosphorolysis. It appears clear that, at this early stage in the development of this field of investigation, conclusions regarding the function of this reaction in the biosynthesis of RNA must remain tenuous.

Aside from the physiological significance of the polynucleotide phosphorylases, there are a number of complexities of the reaction itself which have not been resolved. For example, in the presence of RNAase, accumulation of polymer from UDP is hardly detectable but yet the rate of P_i^{32} exchange with UDP is not appreciably diminished. Also of interest is the observed stimulation of P_i^{32} exchange under conditions whereby the rate of polymer formation was not affected. It seems reasonable to consider the initial formation of a highly reactive di- or oligonucleotide which is very readily phosphorolyzed or can become the nucleus of a higher polymer. Thus far no evidence is available that an oligonucleotide primer is essential for polymer formation, but its presence as a trace contaminant in the enzyme preparation cannot be excluded.

Of considerable interest is the observed very slow rate of GDP polymerization as compared with that of other nucleoside diphosphates. Yet in the presence of other diphosphates the extent of GDP incorporation is at about the level of the other nucleotides. Similar findings were obtained by Grunberg-Manago, Ortiz, and Ochoa (4, 21). The formation of high molecular weight polymers in an enzyme reaction introduces certain kinetic problems and enormously complicates the questions of specificity and steric relationships of the enzyme and substrate, some of which may be at the root of the behavior of GDP in polymer formation with the phosphorylases.

Phosphorolysis of high molecular weight ribonucleic acids and of the polymers synthesized by the enzyme was extensive, but only limited and slow phosphorolysis of commercial samples of yeast RNA was observed and RNAase-limit polynucleotides were attacked very little if at all. It may be that 3′-phospho-ended polynucleotide chains which are relatively abundant in the partially degraded samples cannot serve as substrates and may even be inhibitory to the enzyme, and that only a 5′-phospho-ended polynucleotide chain is degraded. It is difficult to reconcile these results simply because of the size of the polymers.

SUMMARY

An enzyme has been partially purified from *Escherichia coli* which catalyzes the reversible polymerization of ribonucleoside diphosphates according to the equation:

$$n \text{ nucleoside-PP} \rightleftharpoons (\text{nucleoside-P})_n + nP_i \text{ (inorganic phosphate)}$$

This reaction is thus a further example of that described by Ochoa and coworkers for an enzyme obtained from *Azotobacter vinelandii.*

High molecular weight polymers of adenosine, uridine, and cytidine monophosphates were synthesized by the enzyme from the corresponding diphosphates.

The synthesized polymers and high molecular weight ribonucleic acids from yeast, turnip yellow virus, and tobacco mosaic virus were extensively phosphorolyzed; commercial samples of yeast ribonucleic acid were phosphorolyzed slowly and ribonuclease-limit polynucleotides were not phosphorolyzed to a significant extent.

An exchange of P^{32}-labeled P_i with the terminal phosphate of nucleoside diphosphates is catalyzed by the enzyme. A loss in the exchange activity with adenosine diphosphate which accompanied purification of the enzyme

was restored by the addition of a relatively heat-stable fraction removed in the purification; this fraction did not affect the rate of polymer formation.

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