Incorporation of Adenine Nucleotide into Ribonucleic Acid by Cytoplasmic Enzyme Preparations of Chick Embryos*

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The incorporation of labeled precursors into ribonucleic acid with cell-free preparations of animal tissues has been observed in a number of laboratories (1–10). Further studies with partially purified preparations indicate that the proximate precursors are nucleoside tri- rather than diphosphates (2–4). These enzyme preparations, unlike deoxyribonucleic acid polymerase (11–13) or polynucleotide phosphorylase (14), incorporate adenine nucleotide into RNA almost exclusively at the terminal position of the polynucleotide chain adjacent to a cytosine monophosphate residue (2). The final product, adenosine—CMP— CMP—RNA is the amino acid acceptor required for the transfer of aminoacyl residues to the site of their ultimate incorporation into proteins (15–18). Edmonds and Abrams (3), however, have provided some evidence for a separate enzyme, responsible for the incorporation of the adenine nucleotide moiety of ATP into internucleotide linkages in RNA. Herbert (10) has reported that this type of reaction is enhanced if cytoplasmic enzyme preparations are supplemented by nuclei.¹

In the course of studies on the possible occurrence of polynucleotide phosphorylase in extracts of tissues of chick embryos, we investigated the exchange of labeled inorganic phosphate and pyrophosphate with ribonucleoside di- and triphosphates. Although the rate of incorporation of phosphate into ribonucleoside diphosphates was rather low, that of inorganic pyrophosphate into ribonucleoside triphosphates appeared relatively high and independent of the presence of adenino acids, but dependent on the presence of polynucleotides (19–21). The present communication examines the reactions involved in more detail.

When the soluble fraction of homogenates of 14-day-old chick embryonic livers or hearts was purified by means of ammonium sulfate and calcium phosphate gel, an enzyme preparation was obtained which catalyzed the incorporation of the AMP moiety of ATP into internucleotide linkages in RNA. Herbert (10) found almost entirely at the terminal position of the polynucleotide chain. The reaction did not take place when ADP was substituted for ATP.

The incorporation of adenine nucleotide into the interior of the polynucleotide chain could be shown to take place in the presence of a crude enzyme preparation. This system required ATP as a substrate and was dependent on the presence of Mg++. Addition of Mn++ as well as Mg++ at its optimal concentration not only increased the extent of incorporation but also resulted in a marked increase in the relative amount of AMP appearing in internucleotide linkages. The reaction was also stimulated by the addition of other ribonucleoside triphosphates added either singly or in combination. Anionic polymers, including DNA, were found to be stimulatory at low but inhibitory at high concentration. Polyamines, such as spermidine and cadaverine, on the other hand, were inhibitory at low, but stimulatory at higher, concentrations.

EXPERIMENTAL

Materials

ATP 8 C¹⁴ and ADP 8 C¹⁴ as well as unlabeled adenosine sulfate, adenosine-HCl, 2' and 3'AMP, 2' and 3'GMP, 2' and 3'UMP, and 2' and 3'CMP were purchased from Schwarz Laboratories, Inc. 5'AMP, 5'GMP, 5'UMP, 5'CMP, 5'ADP, 5'GDP, 5'UDP, 5'CDP, 5'ATP, 5'GTP, 5'UTP, and 5'CTP were generous gifts supplied by the Sigma Chemical Company. Polyvinyl sulfate was synthesized according to the method of Nomura et al. (22). Heparin was obtained from Nutritional Biochemicals Company. Chitosan sulfate, chondroitin sulfate, and polyethylene sulfonates were gifts of Dr. Fokstra, Upjohn and Company. Cadaverine, spermidine-HCl, phosphopyruvate, and protamine sulfate, and streptomycin-H₂SO₄ were purchased from California Corporation for Biochemical Research, and aminosalicylic acid from British Drug House, Ltd. Crystalline α-amylase was purchased from Nutritional Biochemicals Company, crystalline ribonuclease from Sigma Chemical Company, and crystalline deoxyribonuclease from Worthington Biochemical Corporation.

Methods

Orthophosphate was estimated by the method of Fiske and SubbaRow (23) and pyrophosphate was measured as orthophosphate after hydrolysis in 1 N HCl at 100° for 10 minutes. Pentose was determined by the Mejbaum procedure (24) with crystalline ATP as a standard, deoxypentose by the Dische method (25) with deoxyadenylic acid as a standard, and protein by the Biuret method (26) with bovine serum albumin as a standard.

Labeled inorganic pyrophosphate was prepared by the method of Kornberg and Price (27). After pyrolysis of carrier-free labeled orthophosphate to pyrophosphate, carrier pyrophosphate was added and the labeled pyrophosphate was isolated by means of a Dowex 1 column.
Enzyme Assay. Routinely, enzyme activity was measured in two ways:

Assay Method I: To measure the incorporation of labeled pyrophosphate into ribonucleoside triphosphates, the standard incubation mixture in a final volume of 1.0 ml contained 10 μmoles of Tris buffer, pH 7.5; 100 μmoles of KF; 0.2 mg of cytoplasmic RNA (see below); 5 μmoles of unlabeled inorganic pyrophosphate, pH 7.5, containing labeled inorganic pyrophosphate of variable specific activity; 10 μmoles of MgCl₂; a mixture of the four nucleoside triphosphates (3.125 μmoles of ATP, 0.025 μmole each of GTP, UTP, and CTP) and 0.4 to 5.0 mg of enzyme protein. The mixture was incubated at 38° for 10 minutes.

The reaction was stopped by addition of 1.0 ml of 10% perchloric acid or 1 M perchloric acid. The supernatant solution after centrifugation was combined with the washings of the acid-insoluble precipitate (3 times with 3.0 ml of 5% perchloric acid, or 0.5 M perchloric acid). A 5-ml aliquot was mixed well with 250 mg of acid-washed Norit A (28). The charcoal was collected by centrifugation and washed once with 5 ml of cold 5% trichloroacetic acid or 0.5 M perchloric acid. A 5-ml aliquot was mixed well with 250 mg of acid-washed Norit A (28). The charcoal was collected by centrifugation and washed once with 5 ml of cold 5% trichloroacetic acid or 0.5 M perchloric acid, twice with water, and once each with 5 ml of cold 95% and 100% ethanol. During each washing, 0.2 ml of 95% ethanol was layered on top of the liquid to minimize the loss of charcoal. The washed charcoal was then dried in an oven at 90° overnight, suspended in 2 to 3 ml of 1 N HCl, and heated for 10 minutes at 100°. After removal of the charcoal by centrifugation or filtration, the supernatant or filtrate was neutralized. An aliquot was dried in a planchet and counted in a gas flow counter. The activities were expressed as counts per minute, as per cent of maximal incorporation (29), or as μmoles of pyrophosphate incorporated into acid-insoluble material, the basic reaction mixture being modified in the same medium. This was repeated twice.

Assay Method II: To measure the incorporation of adenine nucleotide into acid-insoluble material, the basic reaction mixture was modified in the same medium. This was repeated twice.

Procedure A:
1. The precipitate was finely suspended in 5 ml of cold 5% trichloroacetic acid or 0.5 M perchloric acid, centrifuged, and resuspended in the same medium. This was repeated twice.
2. The washed precipitate was dissolved in 2 ml of water by the addition of the minimal amount of 0.1 N NaOH and reprecipitated by the addition of 2 ml of 10% trichloroacetic acid or 1 M perchloric acid.
3. The acid-insoluble material was washed once with cold 100% alcohol.
4. After drying the material by inversion of the tubes, the precipitate was dissolved in 0.3 ml of dilute alkali. The solution was made up to 2.4 ml. An aliquot, 0.2 to 0.4 ml, was spread on a planchet, and dried, first in air and further in an oven at 60° for several hours.

Procedure B:
When measurement of incorporation of adenine nucleotides specifically into RNA rather than into acid-insoluble material was desired, or when the preparation contained water-insoluble particles (i.e. nuclei, mitochondria, or microsomes), Procedure A was, with the omission of Steps 2 and 4, followed by Steps 5 through 9 below. If it contained only water-soluble components, the complete Procedure A was then followed by Steps 6 through 9.

5. The precipitate after Step 3 was extracted with a 2:1 mixture of 95% ethanol and ether for 30 minutes at 50° to remove lipids.
6. The residue was washed twice with 95% ethanol.
7. Nucleic acids were extracted from the above residue with 4 ml of 10% NaCl at pH 7.0 and 100° for 30 minutes and centrifuged or filtered. For quantitative recovery this step was repeated once with an additional 2 ml of 10% NaCl at 100° for 15 minutes.
8. An aliquot of the NaCl extract was mixed with 2.5 volumes of cold 95% ethanol and left in a freezer at -20° for 12 hours or more. The precipitates were collected by centrifugation, dissolved in 2 to 3 ml of water and an aliquot was dried and counted as described in Step 4 above.
9. When DNA was present in the precipitate, the latter was dissolved in water, made 0.2 N in NaOH, and hydrolyzed at 80° for 3 hours. After cooling, the solution was neutralized and then made 0.5 M in perchloric acid. DNA was removed by centrifugation. An aliquot of the solution after neutralization was counted, and the RNA content was determined at 260 μm by the use of the extinction coefficient, 32.3 per mg of RNA (2, 31).

Enzyme activities were generally expressed in terms of the total counts incorporated in RNA under standard assay conditions. Occasionally, when no attempt was made to obtain complete recovery of RNA, results were expressed as counts incorporated per mg of RNA.

Column Chromatography—The acid-soluble nucleotides were separated by ion exchange chromatography on Dowex 1-formate resin columns (AG 1-X10, 200 to 400 mesh, 15 x 1 cm), with the use of the formic acid or the ammonium formate system of Hurlbert et al. (32). To obtain nucleoside 2'- and 3'-phosphates from the isolated RNA, the polynucleotide preparations were hydrolyzed in 0.1 N NaOH at 80° for 30 minutes. Carrier adenosine was added to the hydrolysate and the latter was chromatographed by use of the formic acid system by the method of Herbert (10). For complete hydrolysis to mixed nucleoside 2'- and 3'-phosphates, the samples were treated with 1 N NaOH at room temperature for 18 hours or with 0.1 N NaOH or KOH at 100° for 90 minutes.²

² Under these conditions there was essentially complete recovery of added nucleotides in control experiments. With some samples of chick embryo RNA we have occasionally found some hydrolysis (<10%) of AMP to adenine at 100°. Neither this reaction nor the conversion of adenine to hypoxanthine derivatives seemed to occur at 80°, the conditions most commonly employed by us. Hecht et al. used a 3-hour hydrolysis at this temperature and also observed no decomposition.
Paper Chromatography The isolated RNA preparations were hydrolyzed in 0.1 N KOH at 100° for 90 minutes or in 0.2 N KOH at 80° for 2 hours. After cooling, the hydrolyzates were neutralized with perchloric acid and the potassium perchlorate formed was removed. The solutions were concentrated, chilled, and any further precipitate formed was again removed. The resulting mixtures, with added carriers (usually adenosine and a mixture of 2'- and 3'-AMP), were applied to sheets of Whatman No. 1 filter paper. Routinely, a solvent system of isobutyric acid-NH₄OH-water (66:1:33) was used. Descending chromatograms were run at 25° for 16 to 18 hours. After development the ultraviolet quenching spots were visualized by means of a Minaralight lamp and compared with authentic samples run concurrently as standards. Elution was performed by completely immersing the excited spots in 0.5 M NH₄OH in small test tubes, which were covered by Parafilm to prevent any loss of the liquid, and incubating at 38° for 24 hours. An aliquot of the eluate was dried in a planchet and counted as usual.

Cell Fractionation—Fertilized eggs were incubated for 14 days. Livers and hearts excised from the embryos were cleared of connective tissue and washed in cold distilled water to remove as much blood as possible. Organs were stored in a deep freeze for several months. Organs were excised from embryos and fractionated as described in this paper a crude fraction, collected between 30 and 70% saturation. The solutions were concentrated, chilled, and any further precipitate formed was again removed. The combined eluate was then dialyzed overnight against 0.05 M Tris buffer, pH 7.5, with several changes of buffer.

Most of the enzyme activity could be found in the fractions obtained between 35 and 70% ammonium sulfate saturation. Occasionally, a considerable amount of activity still remained in solution at 70% saturation. In most of the experiments described in this paper a crude fraction, collected between 30 and 70% of saturation (Type I enzyme preparation), was employed.

A more highly purified preparation (Type II enzyme) was obtained by fractionation of Type I preparations directly from supernatant solutions. A typical protocol follows: A liver homogenate was freed of nuclei, mitochondria, and microsomes by high and low speed centrifugation as before. To 900 ml of supernatant fluid was added slowly, with stirring, 45 ml of 1 M NaCl. The mixture was kept at 0° for 2 hours, and the precipitate was removed by centrifugation. To the supernatant solution (885 ml) was added 88 ml of 1% protamine sulfate (1% streptomyacin sulfate may be substituted) and the preparation left in the cold room (4°) overnight; the precipitate formed was again removed by centrifugation. The supernatant solution (1050 ml) was treated with 600 ml of calcium phosphate gel (32.3 mg dry weight per ml) and the mixture stirred for 15 minutes. The gel was collected by centrifugation, washed twice by resuspension in 250 ml of distilled water, and the washings pooled with the original unadsorbed material. To this combined fluid (1640 ml) was then added 780 g of solid ammonium sulfate, and the resulting precipitate was dissolved in a minimal volume (about 50 ml) of 0.05 M Tris buffer, pH 7.5. The enzyme was exhaustively dialyzed against the same buffer, and 94 ml of the solution were then treated with 35.5 ml of the calcium phosphate gel described above. After stirring for 15 minutes the gel was collected by centrifugation and washed twice with 30-ml portions of 0.05 M phosphate buffer, pH 7.5; the enzyme was eluted with two 25-ml portions of the same buffer at 0.5 M. The combined eluate was then dialyzed overnight against 0.05 M Tris buffer, pH 7.5, with several changes of buffer.

Before purification the enzyme preparation contained approximately 14 g of protein, with an incorporating activity of 110 c.p.m. per mg under standard conditions. The final gel eluate contained approximately 300 mg of protein with an incorporating activity of 997 c.p.m. per mg under standard conditions.

Preparation of RNA—The RNA, which was routinely added to the incubation mixture, was prepared by a slight modification of the phenol method of Kirby (34, 35). Various cell fractions, separated by the Schneider and Hogeboom procedure (33), were separated by the Schneider and Hogeboom procedure (33).

More highly purified samples were obtained as follows: RNA isolated as above was dissolved in 0.01 M phosphate buffer, pH 7.0, 0.007 M NaCl, and was then treated with crystalline α-amylase (final concentration of 0.1 mg per ml) at 38° for 3 to 4 hours. RNA preparations from nuclei were also treated with deoxyribonuclease (0.05 mg per ml final concentration) and incubated for 8 hours or more. After amylase and deoxyribonuclease treatment the samples were then exposed to proteolytic enzymes (trypsin or bacterial protease, 0.05 mg per ml final concentration) for 3 hours at 37°. The RNA was isolated from the digestion mixture by addition of 2.5 volumes of alcohol. The precipitates were dissolved in water and RNA was again precipitated with alcohol. This process was then repeated once or twice more. The final preparations contained 85-95% RNA by dry weight.

DNA from nuclei was purified in a manner similar to that
RESULTS

Incorporation of Labeled Pyrophosphate into Ribonucleoside Triphosphates

Effect of Nucleotides and Amino Acids—As shown in Table I, preparations derived from chick embryonic tissues catalyzed the active incorporation of labeled pyrophosphate into nucleoside triphosphates, whereas its incorporation into nucleoside diphosphates was negligible (usually less than 10% of that observed with the triphosphates). The addition of a mixture of 18 amino acids to the nucleoside triphosphate system markedly decreased the activity. When added to the nucleoside diphosphate system, the amino acid mixture had almost no effect. The activity of the enzyme was not decreased by dialysis or by other manipulations such as salt fractionation, designed to lower the content of endogenous amino acids. Thus, the incorporation of pyrophosphate into nucleoside triphosphates appeared to be independent of the presence of amino acids and was, therefore, probably not catalyzed by amino acid activating enzymes. It is, perhaps, of interest that we have been completely unsuccessful in demonstrating the presence of these enzymes in our preparations by either the pyrophosphate exchange reaction or by hydroxamate formation. The preparations with which the data of Table I were obtained contained both the microsomal and the soluble portions of the cytoplasm. Either fraction alone was also active, but, on a protein basis, the latter had a higher specific activity. There also were present more interfering enzymes (nucleases, etc.) in the microsomal than in the supernatant fraction. Thus, when microsomes and supernatant were combined, the resulting activity was not additive, even in the presence of a high concentration of fluoride.

The incorporation of labeled orthophosphate into nucleoside diphosphates under the conditions outlined in Table I or with preparations obtained from either of the fractions separately was insignificant. All attempts at obtaining active preparations of polynucleotide phosphorylase from the embryonic cytoplasm were unsuccessful, even when the present assay methods were supplemented by that of Grunberg-Manago et al. (14).

Effect of Fluoride Ion—The results in Table I were obtained in the presence of high concentrations of fluoride. In its absence, the nucleotides added were rapidly destroyed by enzyme preparations containing both microsomes and supernatant fluid. Therefore the apparent activity was almost negligible, even when a very brief period of incubation was used. In the presence of fluoride at elevated levels, the initial activity was high, then reached a maximum and decreased rapidly with prolonged incubation.

Although required for the demonstration of pyrophosphate incorporation into nucleoside triphosphates with these crude preparations, fluoride was quite inhibitory to the enzyme. This is illustrated in Fig. 1, which shows the incorporation of pyrophosphate by a dialyzed 40 to 60% ammonium sulfate fraction of liver supernatant fluid in the absence and presence of fluoride. With an incubation time of 5 minutes the activity in the presence of fluoride was only half of that observed in its absence. On more prolonged incubation, however, the activity in the presence of fluoride continued to increase, whereas the rapid initial activity, observed in its absence, decreased.

As before, this decrease in activity was due to enzymatic hydrolysis of the organic polyphosphates, since the concentration of organic phosphates hydrolysable by acid (1 N HCl for 10 minutes at 100°) decreased rapidly in an incubation mixture devoid of fluoride.

RNA Dependence—All active enzyme preparations obtained from the soluble fraction of embryonic liver could be shown to be rich in RNA. Its concentration could be decreased by incubating the enzyme in 0.05 M pyrophosphate, pH 7.5, for 30 to 60 minutes. In a typical run the optical density ratio $A_{320}$ to $A_{260}$ was increased from 0.93 to 1.07 by this treatment. When the preincubated enzyme was precipitated with saturated ammonium sulfate and dialyzed, its enzymatic activity was lost. It could be restored completely, however, by the addition of RNA, isolated by the phenol method from a crude enzyme preparation not previously subjected to the pyrophosphate treatment. As shown in Fig. 2 the addition of increasing amounts of RNA to a reaction mixture containing an enzyme preparation low in endogenous RNA gave rise to enhanced incorporation. The curve of reaction rate plotted against RNA concentration appeared to be hyperbolic, since a double reciprocal plot gave a linear relation. With enzyme preparations rich in RNA, saturation appeared to have been already achieved, since little or no additional stimulation by added RNA was demonstrable.
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FIG. 1. Effect of fluoride on pyrophosphate incorporation. The reaction mixtures, in a final volume of 1.0 ml, contained 6.9 mg of protein of a 40 to 60% ammonium sulfate fraction of the supernatant fluid of 14-day-old chick embryonic liver; 100 μmoles of Tris buffer, pH 7.5; 10 μmoles of MgCl₂; 0.025 μmole each of CTP, UTP, and GTP; 3.125 μmoles of ATP; 5 μmoles of inorganic pyrophosphate containing 4.1 × 10⁴ c.p.m. of P³₂P³₂; O—O in the presence of 100 μmoles of KF; Δ—Δ in its absence. Incubation was carried out at 38°C. Assay Method I was used.

That the removal of RNA described above may have been due to pyrophosphorolysis, i.e. a reversal of the incorporation reaction, could be demonstrated by measuring the incorporation of pyrophosphate into ribonucleoside triphosphates on treatment of RNA with labeled pyrophosphate alone. Fig. 3 shows the formation of labeled charcoal-adsorbable material, presumably nucleoside polyphosphates, by incubating RNA with labeled pyrophosphate in the presence of Mg⁺⁺ and enzyme preparations from heart supernatant or microsomes, but in the absence of added ribonucleoside di- or triphosphates. The amounts formed were small, but clearly demonstrable by the tracer techniques described above. Fluoride added to the reaction mixture in these experiments was found to be strongly inhibitory.

Specificity for Different Nucleoside Triphosphates—The specificity of the pyrophosphate exchange reaction for individual nucleoside triphosphates is shown in Table II. If these substrates were added in equal concentration, and with short incubation periods, the activity was highest with ATP, next with UTP and least with GTP or CTP. The activity with the latter was nearly half that with ATP. With the four nucleoside triphosphates added simultaneously in equal concentrations (0.5 μmole total) the activity observed was just about the average calculated on the basis of that observed with each nucleoside triphosphate added individually.

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ATP but Not ADP as Precursor of Adenine Nucleotide of RNA—As a routine assay, the pyrophosphate exchange reaction was inadequate because the method was indirect and influenced by interfering enzymes. For example, Mg⁺⁺, which is required for enzyme activity, as will be seen later, was removed by pyrophosphate added in high concentration. At the resulting low Mg⁺⁺ levels the activity of the incorporating enzyme was decreased while that of a phosphodiesterase capable of destroying RNA and present in all our preparations was enhanced. With ATP-8-C¹⁴ as the tracer, the incorporation of adenine-C¹⁴ nucleotide into RNA could be demonstrated in the presence of the soluble fraction of the cytoplasm or more highly purified enzyme preparations obtained from this fraction. The rate of this incorporation was roughly linear with incubation time up to 20 minutes, provided that the amount of enzyme protein...
added was less than 0.5 mg per ml of the reaction mixture. This linear incorporation rate with ATP was observed either in the presence or absence of a mixture of the other three ribonucleoside triphosphates.

Dialyzed ammonium sulfate fractions without further treatment (Type I enzyme preparations) or calcium phosphate gel-treated enzymes (Type II enzyme preparations), catalyzed the incorporation of label into RNA with ATP C\textsuperscript{14} and a mixture of nucleoside triphosphates as substrates; this incorporation was negligible or absent with ADP-C\textsuperscript{14} and a mixture of nucleoside diphosphates. This is demonstrated in Fig. 4, where, with a Type II enzyme preparation freed of nucleoproteins, ADP-C\textsuperscript{14} plus the mixture of nucleoside diphosphates was completely inactive as a substrate during the first 20 minutes of incubation, while ATP-C\textsuperscript{14}, supplemented by the nucleoside triphosphate mixture, showed a high initial rate of activity. This indicates that ATP is the immediate precursor in the incorporation reaction. The slight incorporation of ADP-C\textsuperscript{14} observed after prolonged incubation was probably due to ATP-C\textsuperscript{14} generated from ADP-C\textsuperscript{14} by adenylic kinase present in the enzyme preparation.

Double reciprocal plots of activity incorporated into RNA during 10 minutes incubation, under the conditions outlined in Fig. 4, against the ATP concentration added initially, were found to be linear. Half saturation for ATP was observed at about 6 \times 10^{-5} M.

**Dependence of Adenine Nucleotide Incorporation on RNA**—Figs. 5 and 6 illustrate that the incorporation of adenine nucleotide into RNA with ATP-C\textsuperscript{14} as the substrate was dependent on the presence of RNA in the reaction mixture. In the absence of added RNA the activity was slight, especially when the preparations used were low in endogenous RNA or had been precipitated with pyrophosphate and precipitated by ammonium sulfate. With increasing amounts of RNA added to the reaction mixture, an increase in activity could be observed, indicating, in this case unambiguously,\textsuperscript{4} that the function of the RNA was that of a “primer” or acceptor of the adenosine-C\textsuperscript{14} nucleotide derived from C\textsuperscript{14-ATP}.

**Specificity for RNA of Different Sources**—The soluble cytoplasmic RNA’s from chick embryonic liver, rat liver, and Escherichia coli were equally active as acceptors of adenine nucleotide with Type I enzyme preparations. Similar results had previously been obtained by others with enzymes corresponding to our Type II enzyme preparation, i.e., one catalyzing incorporation into terminal positions (2, 15, 16). As shown in Fig. 6, nuclear and microsomal RNA, isolated from chick embryonic liver, were also active with our Type I enzyme preparations but were somewhat less effective than RNA isolated from the whole supernatant fraction. These results were in contrast to the observations reported by other workers who,

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\textsuperscript{4} In the ordinary pyrophosphate exchange reaction, i.e., one depending on occurrence of the reaction: mR-P-P\textsuperscript{*}P\textsuperscript{*} = (R-P)\textsubscript{n} + P\textsuperscript{*}P\textsuperscript{*} in both directions, the requirement for the polynucleotidase (R-P)\textsubscript{n} may be rationalized in terms of a cosubstrate, or cosubstrate generating system, not provided by individual components of the reaction mixture (e.g., if the reaction is slow from left to right as compared to its rate in the opposite direction, then the initial rate of (R-P)\textsuperscript{n} incorporation into R-P-P\textsuperscript{*}P\textsuperscript{*} will be slow in the absence of (R-P)\textsubscript{n}).

However, used “pH 5 enzyme,” which, as mentioned above, corresponds to our Type II enzyme preparation.

**pH Optimum**—In our earlier experiments, the reaction mixtures were incubated at pH 7.5. This pH was not optimal as disclosed by studies on the effect of pH on enzyme activity (Fig. 7). The optimum, with Tris as the buffer, was between 9.0 and 9.5 for both Type I and II enzyme preparations. The presence of M\textsuperscript{+} in addition to Mg\textsuperscript{+2} did not influence its position.

**Effect of Other Nucleoside Triphosphates**—Hochst et al. (2) reported that incorporation of ATP-C\textsuperscript{14} into RNA by “pH 5 enzyme” was stimulated greatly by added CTP, but only slightly by GTP and not at all by UTP. Under the same conditions,
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**FIG. 4.** Specificity of C\(^{14}\)-ATP and C\(^{14}\)-ADP as the source of RNA-adenine nucleotide. The reaction mixtures in a final volume of 1.1 ml contained 20 \(\mu\)moles of Tris buffer, pH 9.5; 0.4 mg of RNA; 0.42 mg of protein of calcium phosphate gel-treated enzyme (Type II enzyme preparation); 0.603 \(\mu\)mole of C\(^{14}\)-ATP (3.22 \(\times 10^4\) c.p.m. per \(\mu\)mole) and 0.28 \(\mu\)mole each of CDP, UDP, and GDP. Incubation was performed at 38\(^\circ\)C. Assay Method II, Procedure A, was used for activity determination.

CTP or UTP-C\(^{14}\) was incorporated into RNA, whereas GTP-C\(^{14}\) was not. In contrast to this, with our Type I enzyme preparation, the incorporation of ATP-C\(^{14}\) into RNA was stimulated by the addition of CTP, UTP, and GTP, singly or in combination. As shown in Table III, with a fresh Type I enzyme preparation, the stimulation was 200 to 300%: 300% by GTP, and 270% by UTP. The mixture of all three nucleoside triphosphates was slightly more effective than any single component at an equivalent concentration.

Preincubation of RNA and nucleoside triphosphates with Type I enzyme preparation did not increase the apparent activity. Any such stimulation may, however, have been obscured by the fact that preincubation of RNA with the enzyme alone, in the absence of any nucleoside triphosphates, already resulted in an increase (100%) in the uptake of adenine nucleotide into RNA, when compared to a control without preincubation. When the enzyme was tested after aging for 1 week in the frozen state with two or three thawings, a slight decrease in the relative stimulation exerted by GTP was observed with no change in stimulation by UTP or GTP. This aged enzyme when tested in the absence of any added nucleoside triphosphates was slightly more effective than any single component at an equivalent concentration.

**FIG. 5.** Effect of RNA on the time course of incorporation of adenine nucleotide into RNA. The reaction mixtures in a final volume of 1.0 ml consisted of 10 \(\mu\)moles of Tris buffer, pH 7.5; 5 \(\mu\)moles of MgCl\(_2\); 0.4 mg of RNA, isolated from liver supernatant; 0.25 \(\mu\)mole each of CTP, UTP, and GTP; 0.178 \(\mu\)mole of C\(^{14}\)-ATP, 1.36 \(\times 10^5\) c.p.m.; 7.7 mg of protein of a 60 to 100% ammonium sulfate fraction of heart supernatant fluid. Assay Method II, Procedure A was used.

for 15 minutes at 38\(^\circ\)C in the absence of added RNA or nucleoside triphosphates resulted in a decrease in its capacity to be stimulated by the addition of nucleoside triphosphates as compared to the aged but unpreincubated samples. This decrease may have been due to the destruction of the RNA present in the enzyme or to the denaturation of the enzyme.

The stimulation of activity by individual nucleoside triphosphates was quite variable. Some fresh or slightly aged preparations gave the best activity with CTP (results shown in Table III), whereas others exhibited their highest activity with UTP or GTP. With aged enzyme preparations often little or no stimulation by CTP or GTP could be observed, while a significant stimulation by UTP still persisted.

In Fig. 8, the effect of varying the concentration of individual nucleoside triphosphates on the adenine nucleotide incorporation is shown. The experiments were performed with the same enzyme preparation, but on three different days. Therefore quantitative differences in stimulation by different nucleotides are probably not significant, due to the continuous variation in stimulation by different nucleotides on aging the enzyme, as mentioned above. Maximal stimulation was observed with 0.5 \(\mu\)mole of CTP, 0.7 \(\mu\)mole of UTP, or 0.8 \(\mu\)mole of GTP present in a reaction mixture of 1.1 ml containing 0.178 \(\mu\)mole of C\(^{14}\)-ATP. Higher concentrations were inhibitory.

With Type II enzyme preparations (prepared by repeated adsorption on and elution from calcium phosphate gel of a Type I enzyme preparation from which ribonucleoproteins had been

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Fig. 4. Specificity of C\(^{14}\)-ATP and C\(^{14}\)-ADP as the source of RNA-adenine nucleotide. The reaction mixtures in a final volume of 1.1 ml contained 20 \(\mu\)moles of Tris buffer, pH 9.5; 0.4 mg of RNA; 0.42 mg of protein of calcium phosphate gel-treated enzyme (Type II enzyme preparation); 0.603 \(\mu\)mole of C\(^{14}\)-ATP (3.22 \(\times 10^4\) c.p.m. per \(\mu\)mole) and 0.28 \(\mu\)mole each of CDP, UDP, and GDP. Incubation was performed at 38\(^\circ\)C. Assay Method II, Procedure A, was used for activity determination.

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Preincubation of RNA and nucleoside triphosphates with Type I enzyme preparation did not increase the apparent activity. Any such stimulation may, however, have been obscured by the fact that preincubation of RNA with the enzyme alone, in the absence of any nucleoside triphosphates, already resulted in an increase (100%) in the uptake of adenine nucleotide into RNA, when compared to a control without preincubation. When the enzyme was tested after aging for 1 week in the frozen state with two or three thawings, a slight decrease in the relative stimulation exerted by GTP was observed with no change in stimulation by UTP or GTP. This aged enzyme when tested in the absence of any added nucleoside triphosphates was slightly more effective than any single component at an equivalent concentration.

**FIG. 5.** Effect of RNA on the time course of incorporation of adenine nucleotide into RNA. The reaction mixtures in a final volume of 1.0 ml consisted of 10 \(\mu\)moles of Tris buffer, pH 7.5; 5 \(\mu\)moles of MgCl\(_2\); 0.4 mg of RNA, isolated from liver supernatant; 0.25 \(\mu\)mole each of CTP, UTP, and GTP; 0.178 \(\mu\)mole of C\(^{14}\)-ATP, 1.36 \(\times 10^5\) c.p.m.; 7.7 mg of protein of a 60 to 100% ammonium sulfate fraction of heart supernatant fluid. Assay Method II, Procedure A was used.

for 15 minutes at 38\(^\circ\)C in the absence of added RNA or nucleoside triphosphates resulted in a decrease in its capacity to be stimulated by the addition of nucleoside triphosphates as compared to the aged but unpreincubated samples. This decrease may have been due to the destruction of the RNA present in the enzyme or to the denaturation of the enzyme.

The stimulation of activity by individual nucleoside triphosphates was quite variable. Some fresh or slightly aged preparations gave the best activity with CTP (results shown in Table III), whereas others exhibited their highest activity with UTP or GTP. With aged enzyme preparations often little or no stimulation by CTP or GTP could be observed, while a significant stimulation by UTP still persisted.

In Fig. 8, the effect of varying the concentration of individual nucleoside triphosphates on the adenine nucleotide incorporation is shown. The experiments were performed with the same enzyme preparation, but on three different days. Therefore quantitative differences in stimulation by different nucleotides are probably not significant, due to the continuous variation in stimulation by different nucleotides on aging the enzyme, as mentioned above. Maximal stimulation was observed with 0.5 \(\mu\)mole of CTP, 0.7 \(\mu\)mole of UTP, or 0.8 \(\mu\)mole of GTP present in a reaction mixture of 1.1 ml containing 0.178 \(\mu\)mole of C\(^{14}\)-ATP. Higher concentrations were inhibitory.

With Type II enzyme preparations (prepared by repeated adsorption on and elution from calcium phosphate gel of a Type I enzyme preparation from which ribonucleoproteins had been
Fig. 6. Specificity of RNA as acceptor of adenine nucleotide. The reaction mixtures in a final volume of 1.2 ml contained 4.62 mg of protein of a 40 to 70% ammonium sulfate fraction of liver supernatant fluid; 20 μmoles of Tris buffer pH 9.5; 15 μmoles of MgCl₂; 0.178 μmole of C⁴-ATP; 1.36 × 10⁶ c.p.m.; varying amounts of RNA from different chick embryonic liver fractions: RNA isolated from 30 to 40% ammonium sulfate fraction of chick embryonic liver supernatant (○), microsomes (▲), and nuclei (■). The reaction mixture was incubated at 38° for 20 minutes. Assay Method II, Procedure A, was used.

Requirement for Mg²⁺ and Mn²⁺—It was not possible to obtain a clear-cut demonstration of the effect of Mg²⁺ on the enzymatic activity by means of the pyrophosphate exchange reaction. The inhibition of ribonuclease by Mg²⁺ counter-balanced by complex formation of pyrophosphate with Mg²⁺ and fluoride probably interfered. By observing the incorporation of adenine nucleotide into RNA, however, it was not necessary to purify the enzyme very extensively to show a requirement of the enzyme system for Mg²⁺. In the presence of dialyzed Type I enzyme preparations (40 to 70% ammonium sulfate fraction), the activity was practically nil in the absence of added Mg²⁺, as shown in Fig. 9. On the addition of increasing amounts of Mg²⁺, the activity increased rapidly up to 4 × 10⁻⁴ M, and then a gradual increase in activity continued until 1.2 × 10⁻³ M was reached (Curve A). Further increase in Mg²⁺ concentration, above 1.5 × 10⁻³ M, was slightly inhibitory. Mn²⁺ could replace Mg²⁺. The optimal stimulation with Mn²⁺ was obtained at about 3 × 10⁻³ M, which was only 1/6 of the optimal Mg²⁺ concentration. The extent of activation exerted by Mn²⁺ was almost equal to that obtained with Mg²⁺ when each was measured at its optimal concentration. A further increase in Mn²⁺ concentration above 4 × 10⁻³ M decreased the activity (Curve B). In the presence of an optimal concentration of Mg²⁺, Mn²⁺ further stimulated the enzyme activity, with its own optimal concentration under these conditions shifted slightly toward lower values (between 1 × 10⁻³ M and 1.5 × 10⁻³ M) (Curve C and Curve D⁶).

The addition of Mn²⁺ in the presence of Mg²⁺ not only stimulated the total incorporation of adenine nucleotide into RNA, but also increased the proportion found in internucleotide linkages (Table IV). Other metals tested included Fe²⁺, Fe³⁺, Ca²⁺, Cu²⁺, Zn²⁺, and Co²⁺ at three different concentrations (8.23 × 10⁻³ M, 4.13 × 10⁻⁴ M, and 8.23 × 10⁻⁵ M), all in the presence of 1.25 × 10⁻³ M Mg²⁺. No stimulatory effects could be observed except with Co²⁺. A more complete investigation of this effect showed that this metal exerted its optimal effect at a very low concentration (8.33 × 10⁻⁵ M), but was less than 1/6 as effective as Mn²⁺.

In Curves A to C, the data are expressed as total activities found in the thoroughly washed acid-insoluble material, whereas in Curve D, which represents a separate experiment with a different, fresh enzyme preparation, the data are calculated in terms of total activities found in the RNA isolated. The apparent greater stimulation in this case is not due to this changed method of data presentation, but due to the nature of the enzyme used.
0.25 pmole each of CTP, UTP, and GTP; 0.178 pmole of \(^{14} \text{ATP} \), 1.36 \( \times 10^4 \) c.p.m.; 2.1 mg of protein of a 40 to 70% ammonium sulfate fraction of liver supernatant fluid. The tubes were incubated for 15 minutes after preincubation for 15 minutes. Assay Method II, Procedure A, was used.

### TABLE III

<table>
<thead>
<tr>
<th>Nucleoside triphosphate</th>
<th>Total c.p.m. per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td></td>
<td>Without pre-</td>
</tr>
<tr>
<td></td>
<td>incubation</td>
</tr>
<tr>
<td>(^{14} \text{ATP} ) alone</td>
<td>100</td>
</tr>
<tr>
<td>+ CTP</td>
<td>660</td>
</tr>
<tr>
<td>+ UTP</td>
<td>485</td>
</tr>
<tr>
<td>+ GTP</td>
<td>505</td>
</tr>
<tr>
<td>+ CTP + UTP + GTP</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) A freshly prepared enzyme was preincubated in the presence of the unlabeled nucleoside triphosphates and RNA; then ATP-\(^{14} \text{ATP} \) was added and the incubation continued.

\(^b\) The enzyme preparation was aged for 1 week in the frozen state and then preincubated in the absence of RNA or nucleoside triphosphates. \(^{14} \text{ATP} \), RNA, and nucleoside triphosphates were then added and the incubation continued.

Localization in RNA of the Adenine-\(^{14} \text{C} \) Incorporated—In addition to the findings with polynucleotide phosphorylase, two classes of data have been reported in the literature, viz. those dealing with terminal incorporation (1-4, 8, 10) and those describing the occurrence of some incorporation into internucleotide linkages, under rather special conditions (3, 10). For a consideration of the biosynthesis of extranuclear RNA in the cells of vertebrates the latter group of observations may be of greater pertinence.

Our Type II enzyme preparations, purified more than 10-fold from the original fraction, on the basis of total incorporating ability, could be shown to bring about incorporation almost entirely into the terminal position of the polynucleotide chain. This statement is based on studies of the RNA isolated from reaction mixtures and examined by the method described by Herbert (10).

In contrast to these findings, when the much cruder Type I enzyme preparations were tested under conditions previously found optimal for the Type II enzyme, the RNA isolated from these experiments contained newly incorporated adenylate in nonterminal positions\(^7\) to the extent of 10 to 30%. Furthermore, analysis of the acid-soluble fractions isolated from similar reaction mixtures indicated an even more extensive incorporation into internucleotide linkages, based on the demonstration of 2'- and 3'-AMP in alkaline hydrolysates of these fractions. This provides additional evidence for nonterminal incorporation of adenine nucleotide, since these entities presumably had their origin in RNA or oligonucleotides containing 3', 5'-phosphodiester bonds and not directly in ATP, ADP, or 5'-AMP.

In a typical experiment, a Type I enzyme preparation was added to a large scale incubation mixture and incubated for 20 minutes. Protein and RNA were removed by addition of perchloric acid, and the supernatant neutralized by the addition of KOH and chilled. After removal of a precipitate, the clear solution was concentrated, made 1 N in KOH, and incubated at room temperature for 18 hours. The hydrolysate was neutralized and 5 \( \mu \)moles of 5'-AMP, 5 \( \mu \)moles of 2'-AMP, and 2.5 \( \mu \)moles of 3'-AMP were added as carriers to an aliquot. The mixture was chromatographed on a Dowex 1 column by means of the formic acid system to separate the mixture of adenyllic acids. As can be seen in Fig. 10, the three peaks of 5', 2', and 3'-AMP were well separated and eluted in that order. The large amount of radioactivity in 5'-AMP formed from the initially added \(^{14} \text{ATP} \) by enzymatic as well as chemical hydrolysis obscured the exact amount of the radioactivity in the 2'-AMP by tailing. On the other hand, the radioactivity peak of 3'-AMP coincided exactly with its optical density peak. Out of a total of 1.904 \( \times 10^8 \) c.p.m. in the \(^{14} \text{ATP} \) added to the reaction mixture, only 1.2 \( \times 10^8 \) c.p.m. were in-
creasing amounts of Mg\(^{++}\) (0) for Curve A, Mn\(^{++}\) (0) for Curve 0.25 pmole each of CTP, UTP, and GTP; 0.178 pmole of CY-ATP, 3',5'-AMP on enzymatic hydrolysis and is stable under the conditions employed here.8

An alternative, though much less likely interpretation would be the possible occurrence of an enzyme capable of forming cyclic 3', 5'-AMP from ATP (36) in these preparations. This cyclic 3', 5'-AMP might then give rise to 3'-AMP either enzymatically or chemically under the conditions found in RNA. The reaction mixture in a total volume of 1.2 ml consisted of 20 \(\mu\)moles of Tris buffer, pH 9.5; 0.4 mg of RNA; 0.25 \(\mu\)moles each of CTP, UTP, and GTP; 0.178 \(\mu\)mole of CY-ATP, 1.36 \(\times\) 10\(^{-6}\) c.p.m. (0.79 c.p.m. per \(\mu\)mole); 2.1 mg of protein of a 40 to 70\% ammonium sulfate fraction of liver supernatant for Curve A, B, and C, and 4.1 mg of protein of a 45 to 60\% ammonium sulfate fraction of liver supernatant for Curve D; with increasing amounts of: Mg\(^{++}\) (\(\bigcirc\)) for Curve A, Mn\(^{++}\) (\(\bigcirc\)) for Curve B, Mn\(^{++}\) with 1.25 \(\times\) 10\(^{-6}\) M Mg\(^{++}\) for Curve C (\(\bigtriangleup\)) and Curve D (\(\bullet\)). The tubes were incubated at 38\°C for 20 minutes. Assay Method II, Procedure A, was used in preparing counting samples for Curves A, B, and C. RNA was isolated from samples by Assay Method II, Procedure B, and counts per minute per total RNA isolated for each sample were used for Curve D.

The results in Table IV show the extent of the incorporation of adenine-C\(^{14}\) nucleotide into RNA with a standard incubation mixture containing C\(^{14}\) ATP, a mixture of CTP, UTP, and GTP, and Type I enzyme preparations. In these experiments, the RNA, isolated from the reaction mixture after incubation, was hydrolyzed by dilute alkali, and the hydrolysate after addition of adenosine and 2'- and 3'-AMP was chromatographed on paper in the isobutanol-N\(_2\)OH-H\(_2\)O system described under "Methods." In the absence of Mg\(^{++}\), the total incorporation of adenine nucleotide into RNA was very small. Upon analysis of the isolated RNA, the radioactivity found in 2'- and 3'-AMP was, however, 28\% of the total incorporated. Even this relatively low nonterminal incorporation was much greater than that observed with Type II enzyme preparations tested under the standard conditions in the presence of optimal concentrations of Mg\(^{++}\). Addition of Mg\(^{++}\) (1.25 \(\times\) 10\(^{-2}\) \(\mu\) M) to the Type I system resulted in an increase in the radioactivity in 2'- plus 3'-AMP to 36\%. When the Mg\(^{++}\) was replaced by 8.23 \(\times\) 10\(^{-4}\) M Mn\(^{++}\), the optimal concentration, the activity in 2'- plus 3'-AMP was 32\%. When both Mg\(^{++}\) and Mn\(^{++}\) were added to the reaction mixture at their respective optima, the effect was additive (74\% of the activity in 2'- plus 3'-AMP). Further addition of spermidine or cadaverine to the above system led to a recovery in 2'- plus 3'-AMP of about 80\% of the activity added.

In a separate, large scale experiment with another Type I en-
Incorporation of Adenine Nucleotide into RNA

**TABLE IV**

Localisation of adenine-C14 nucleotide in alkaline hydrolysate of RNA

For the paper chromatograms, the basic reaction mixture in a final volume of 5.0 ml consisted of 60 μmoles of Tris buffer, pH 9.5; 1.6 mg of RNA; a mixture of CTP, UTP, and GTP, 1 μmole each; 0.712 μmole of C14-ATP, 5.44 × 10^4 c.p.m.; 20.4 mg of protein of a 30 to 60% ammonium sulfate fraction of liver supernatant fluid, plus other components as indicated. The tubes were incubated at 38° for 20 minutes. RNA was isolated, hydrolyzed in 0.1 × KOH for 60 minutes at 100°, and neutralised with perchloric acid. After removal of KClO4, the preparation was concentrated under reduced pressure. To each sample, 0.5 μmole of adenosine and 0.25 μmole each of 2′- and 3′-AMP were added and chromatographed (see text for details).

For the column chromatograms, the basic reaction mixture in a final volume of 10 ml contained 20 μmoles of NaF; 200 μmoles of Tris buffer, pH 9.5, 80 μmoles of creatine phosphate; 40 μmoles of phosphoenolpyruvate, 1.78 μmoles of C14-ATP, 1.36 × 10^6 c.p.m.; 21 mg of protein of 40 to 70% ammonium sulfate fraction of liver supernatant; 2 mg of RNA; with or without mitochondria and nuclei. Other procedures were the same as above. Assay Method II, Procedure B, was used.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Incubation period</th>
<th>Method of analysis</th>
<th>C.p.m. in 2′ and 3′-AMP</th>
<th>C.p.m. in adenosine</th>
<th>Per cent of total activity in 2′ and 3′-AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg2+ (1.25 × 10⁻² M) + Mn2+ (8.33 × 10⁻⁴ M)</td>
<td>0 min</td>
<td>Paper chromatogram</td>
<td>16</td>
<td>51</td>
<td>24</td>
</tr>
<tr>
<td>None</td>
<td>20 min</td>
<td>Paper chromatogram</td>
<td>26</td>
<td>67</td>
<td>28</td>
</tr>
<tr>
<td>Mg2+ (1.25 × 10⁻² M) + Mn2+ (8.33 × 10⁻⁴ M)</td>
<td>20 min</td>
<td>Paper chromatogram</td>
<td>416</td>
<td>757</td>
<td>36</td>
</tr>
<tr>
<td>Mn2+ (8.23 × 10⁻⁴ M)</td>
<td>20 min</td>
<td>Paper chromatogram</td>
<td>1320</td>
<td>1290</td>
<td>52</td>
</tr>
<tr>
<td>Mg2+ (1.25 × 10⁻² M) + Mn2+ (8.23 × 10⁻⁴ M)</td>
<td>20 min</td>
<td>Paper chromatogram</td>
<td>795</td>
<td>279</td>
<td>74</td>
</tr>
<tr>
<td>Same as above + cadaverine (4 × 10⁻⁴ M)</td>
<td>20 min</td>
<td>Paper chromatogram</td>
<td>878</td>
<td>164</td>
<td>84</td>
</tr>
<tr>
<td>Same as above + spermidine (3.5 × 10⁻⁴ M)</td>
<td>20 min</td>
<td>Paper chromatogram</td>
<td>640</td>
<td>110</td>
<td>82</td>
</tr>
<tr>
<td>Mg2+ (1.5 × 10⁻² M)</td>
<td>20 min</td>
<td>Column chromatogram</td>
<td>1506</td>
<td>388</td>
<td>82</td>
</tr>
<tr>
<td>Same as above + nuclei + mitochondria</td>
<td>20 min</td>
<td>Column chromatogram</td>
<td>822</td>
<td>324</td>
<td>72</td>
</tr>
</tbody>
</table>

Enzyme preparation, the basic incubation mixture contained NaF, phosphocreatine, and phosphoenolpyruvate in addition to the standard reaction mixture containing 1.5 × 10⁻² M Mg2+. After incubation, the RNA isolated from the sample contained after hydrolysis 80% of its total activity in the AMP fraction as determined by column chromatographic analysis. The addition of nuclei and mitochondria to another aliquot of the enzyme, and tested in the same system, slightly lowered the radioactivity incorporated.

**Effect of Anionic Polymers**—In attempts to inhibit phosphonono- and di-estersases interfering with the present assay system, the effect of adding anionic polymers was studied. The latter had been reported to inhibit ribonuclease (22, 37, 38) and phosphatases (39, 40). Polyvinyl sulfate, synthesized by the method of Nomura et al. (22), markedly inhibited the incorporation of adenine nucleotide into RNA. An 80% inhibition was observed at a concentration of 0.17 mg per ml, whereas 0.33 mg gave 100% inhibition. Heparin was less inhibitory. Reaction mixtures of 0.10 and 0.10 mg per ml resulted in 27 and 40% inhibition, respectively. One mg of heparin was necessary to reduce the enzyme activity to 50%, and 2 mg per ml were needed to cause 80% inhibition. No stimulatory effects could be observed with polyvinyl sulfate or heparin even at very low concentrations.

Other polymers tested included chitosan sulfate, chondroitin sulfate, chitosan N-sulfate, alginic acid, and three forms of polyethylene sulphonate with molecular weight of 5,700, 12,900, and 27,600. As shown in Table V, these compounds gave varying degrees of inhibition. In case of the polyethylene sulphonates, added at a concentration of 0.2 mg per ml, the extent of inhibition observed appeared to be a direct function of molecular weight. This group of polymers, when tested at a lower concentration (about 0.04 mg per ml), as shown in Table V, showed a slight stimulation (10 to 30% above the control activity) of adenine incorporation into RNA.

DNA was also found to be inhibitory to the enzyme. As can be seen in Table VI, 0.12 mg per ml gave 40% inhibition. The inhibition, when compared with the polymers mentioned above, was not very striking, especially considering the high molecular weight. At concentrations smaller than 0.007 mg per ml of reaction mixture, there was noted a distinct stimulation, much more pronounced than that manifested by any of the other polymers tested. Nearly 80% stimulation could be obtained at 0.003 mg of DNA per ml.

**Effect of Cadaverine and Spermidine**—It has been observed by one of us (41) that polyamines such as cadaverine or spermidine

<table>
<thead>
<tr>
<th>Anionic polymers</th>
<th>Per cent activity of the control at concentration of anionic polymers, mg per sample:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>Chitosan sulfate</td>
<td>100</td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>100</td>
</tr>
<tr>
<td>Chitosan N-sulfate (sulfato, oxidized)</td>
<td>100</td>
</tr>
<tr>
<td>Chitosan sulfate (sulfato, oxidized)</td>
<td>100</td>
</tr>
<tr>
<td>Alginic acid sulfate</td>
<td>100</td>
</tr>
<tr>
<td>Polyethylene sulphonate 17/4/e</td>
<td>100</td>
</tr>
<tr>
<td>1993/95</td>
<td>100</td>
</tr>
<tr>
<td>1947/A</td>
<td>100</td>
</tr>
</tbody>
</table>
exert a strong protective effect on urea-treated bacteriophage. Since these compounds are known to inhibit ribonuclease (42), spermidine and cadaverine were tested to determine whether they could protect the RNA added to the reaction mixture against denaturation or enzymatic degradation. As shown in Table VII, a stimulatory effect was observed at relatively high concentration. At 0.007 M, cadaverine stimulated 20 to 30%, and at 0.0039 M, spermidine gave over 90% stimulation. Concentrations greater than 0.01 M were inhibitory. In contrast to the amino acids, these polyamines were inhibitory at low concentrations (<3.9 × 10⁻⁴ M). Addition of these compounds (at 3.9 × 10⁻³ M) to the reaction mixture resulted in a slight increase in nonterminal incorporation of adenine nucleotide into RNA.

**DISCUSSION**

Four different types of soluble enzymes or enzyme systems responsible for the incorporation of mononucleotide units into polynucleotides have been demonstrated: polynucleotide phosphorylase (14), DNA polymerase (11–13), the enzyme (system) responsible for the formation of the proper terminal nucleotide sequence in (amino acid) transfer RNA (15–18), and the Type I system described here. The latter may be identical to the other two. The system under investigation differs from polynucleotide phosphorylase in that the system under investigation differs from polynucleotide phosphorylase in that the system under investigation differs from polynucleotide phosphorylase. The evidence for Point 1 and 2, and with AMP at least, for Point 5 is provided by experiments on the effect of aging on enzymatic activity. Although by no means as reproducibly as is desirable, it has nevertheless been possible to show on several occasions that stimulation by UTP is a great deal more resistant to this treatment than that by GTP.

<table>
<thead>
<tr>
<th>Table VI Effect of DNA on incorporating adenine nucleotide into RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (mg) per ml of reaction mixture</td>
</tr>
<tr>
<td>C.p.m. per mg of RNA</td>
</tr>
<tr>
<td>Per cent over the control</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>0.003</td>
</tr>
<tr>
<td>0.006</td>
</tr>
<tr>
<td>0.013</td>
</tr>
<tr>
<td>0.018</td>
</tr>
<tr>
<td>0.031</td>
</tr>
<tr>
<td>0.043</td>
</tr>
<tr>
<td>0.062</td>
</tr>
<tr>
<td>0.092</td>
</tr>
<tr>
<td>0.123</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table VII Effect of cadaverine and spermidine on adenine nucleotide incorporation into RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Cadaverine</td>
</tr>
<tr>
<td>0.0077 M</td>
</tr>
<tr>
<td>0.0039 M</td>
</tr>
<tr>
<td>0.00077 M</td>
</tr>
<tr>
<td>0.00039 M</td>
</tr>
<tr>
<td>Spermidine</td>
</tr>
<tr>
<td>0.007 M</td>
</tr>
<tr>
<td>0.0059 M</td>
</tr>
<tr>
<td>0.00077 M</td>
</tr>
<tr>
<td>0.00039 M</td>
</tr>
</tbody>
</table>

In this there is a striking difference from both polynucleotide phosphorylase and DNA polymerase, both of which appear to be single enzymes rather than complexes or systems of enzymes. The evidence for Point 1 and 2, and with AMP at least, for Point 4 appears to rest on a reasonably firm and direct experimental base. Evidence for Point 3 on the other hand is, at best, indirect: it depends on the demonstration that the incorporation of AMP into nonterminal positions of the polynucleotide chain was stimulated by GTP, UTP, and CTP added either singly or in combination, whereas incorporation into terminal positions was unaffected by these additions. Obviously a more direct demonstration, such as the incorporation of the other C¹⁴- or tritium-labeled ribonucleoside triphosphates would be desirable. These experiments are now in progress.

Evidence for Point 5 is provided by experiments on the effect of aging on enzymatic activity. Although by no means as reproducibly as is desirable, it has nevertheless been possible to show on several occasions that stimulation by UTP is a great deal more resistant to this treatment than that by GTP.
or CTP, and could thus be selectively retained. Contributory evidence is also provided by a study of the effects of Mn++ ions. This metal at a low concentration not only replaced Mg++ in its essential role for either terminal or nonterminal incorporation, but also markedly increased the total amount of adenylic incorporated. In its presence a high proportion of the incorporated activity was found in internucleotide linkages. This effect was especially striking in experiments with saturating levels of Mg++. There was then observed an added stimulation by Mn++, again suggesting the presence of more than one enzyme. At this stage an alternative or supplementary interpretation can, however, not be excluded: both Mn++ and Mg++ are known to inhibit pancreatic ribonuclease. If the phosphodiesterase(s), present in our preparations, were similarly affected, then addition of the metals would prevent the degradation of the newly formed RNA, containing labeled adenylate, and result in an apparent enhancement of the incorporation of the latter. The demonstration of a sizable pool of nonterminal, but acid-soluble AMP in the experiment of Fig. 10 (which, however, was performed before the requirement for Mn++ became known) may be due entirely to a similar, phosphodiesterase-catalyzed, breakdown of freshly formed RNA to the oligonucleotide level.

With the provisions indicated, the Type I enzyme system may then be visualized as catalyzing the following reaction:

\[ n(\text{NMP-PP}) + \text{RNA} \rightarrow (\text{NMP})_n - \text{RNA} + n\text{PP} - \]

Whether this enzyme system is actually responsible for the net synthesis of cytoplasmic RNA and whether its distribution extends to adult as well as embryonic tissues must remain moot at this time.

The effects exerted by the polyamines, cadaverine and spermidine, may be rationalized as follows: these compounds are known to inhibit ribonuclease. This mode of action may be invoked to account for both the stimulation of total incorporating activity, and the slight enhancement of the extent of internucleotide incorporation, observed at relatively elevated levels of these compounds. It cannot explain the inhibition of incorporating activity caused by either amine added at low concentrations. An alternative explanation would be to postulate binding of the amines by both the incorporating enzyme, relatively strongly and thus predominating at low amine concentrations, and the polyamidoesters primer, relatively weakly and important only at a more elevated concentration. If the last-named interaction were to lead to a changed configuration of the polymer, either more reactive towards the reaction under investigation, or more resistant to the action of lytic enzymes, then the strange biphasic response is explicable: at low concentrations of amine there is inhibition of the enzyme but at elevated levels this effect is more than compensated by a greatly enhanced activity of the substrate.

The more ordinary biphasic behavior of polyamines (stimulation at low, inhibition at higher, concentrations) on the other hand, may be due entirely to their structural similarity to, and competition with, polyribonucleotide for various enzymes. With small amounts of added polyamine, the latter is bound entirely to, and inactivates enzymes interfering with, the reaction proper, i.e. ribonuclease and other phospho-di- and monoesterases, since polyamines are known inhibitors of these enzymes (49). This inactivation would give rise to an apparent stimulation of the incorporating activity. As the amount of polyamine increases, it is able to compete with RNA not only for enzymes for which it has a relatively high affinity, such as the phosphoesterases, but also for the enzyme system under study, giving rise to net inhibition. DNA is a polyamine, more closely resembling RNA in its gross over-all structure than any of the other polymers tested. Its greater effectiveness as an activator may be due entirely to this close structural relationship. It is somewhat surprising, however, that this effect is not matched by a greater effectiveness as an inhibitor at high concentrations.

**SUMMARY**

Chick embryonic tissues contain an enzyme system catalyzing the incorporation of labeled pyrophosphate into nucleoside tri- but not diphosphates, in the presence of added cytoplasmic ribonucleic acid (RNA).

The same preparations were also capable of incorporating C4-adenosine triphosphate (ATP) into added RNA both in the presence or the absence of other nucleoside triphosphates. The crude enzyme (Type I enzyme preparation) yielded a more highly purified preparation (Type II enzyme preparation) by fractionation. Both Type I and II enzyme preparations required Mg++ for ATP-C4 incorporation and showed no incorporation of adenosine diphosphate-C4 even in the presence of mixtures of cytosine diphosphate (CDP), uridine diphosphate (UDP), and guanosine diphosphate (GDP). The pH optimum for ATP-C4 incorporation was between 9.0 and 9.5. With the Type II enzyme preparation, the incorporation of ATP-C4 into RNA was essentially unaffected by the addition of cytosine triphosphate (CTP), uridine triphosphate (UTP), guanosine triphosphate (GTP), or a mixture of the three, and could be shown to be mostly at the terminal position of the polynucleotide chain.

The ATP-C4 uptake into RNA catalyzed by the Type I enzyme preparations was stimulated 2- to 5-fold by the addition of CTP, UTP, and GTP, singly or in combination. In the presence of a mixture of the three triphosphates and of an optimal concentration of Mg++, the enzyme activity was further stimulated by the addition of Mn++. Under these conditions some 70 to 80% of the total incorporated activity could be demonstrated to be in internucleotide linkage.

The enzyme activity was inhibited by anionic polymers including deoxyribonucleic acid (DNA) at relatively high concentrations. At lower concentrations there was some stimulation of activity, with DNA more effective than the other agents tested.

Spermidine and cadaverine stimulated enzymatic activity at relatively high, but were inhibitory at lower concentration.

The significance of these findings with regard to the synthesis of RNA in the cytophism of animal cells is discussed.

**REFERENCES**

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