The Purification and Characterization of Adenosine Triphosphate-Ribonucleic Acid Adenylyltransferase from Pseudomonas putida*

K. J. PAYNE* and J. A. BOEZI

From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823

SUMMARY

ATP-RNA adenylyltransferase was isolated from the 80,000 × g pellet of Pseudomonas putida. The enzyme specifically catalyzed the incorporation of AMP from ATP into a polymeric product. The \( K_m \) for ATP was \( 3 \times 10^{-4} \text{M} \). The rate of incorporation of CMP from CTP was about 7% that of AMP, while GTP, UTP, dATP, and ADP were not utilized by the enzyme. The reaction was dependent upon the cofactor, magnesium ion (\( K_m = 3 \times 10^{-3} \text{M} \)), which could neither be replaced nor supplemented by manganese ion.

The polymerization reaction, which was accompanied by the stoichiometric release of inorganic pyrophosphate, was dependent upon exogenous RNA. This requirement could be satisfied by ribosomal RNA, soluble RNA, poly C, and (Ap)₅A while DNA, poly A, poly I, poly U, (Ap)₅A, ApA, and (Ap)₅ were ineffective. The \( K_m \) for ribosomal RNA was \( 6 \times 10^{-4} \text{M} \) and the \( K_m \) for soluble RNA was \( 1 \times 10^{-3} \text{M} \). Ribosomal RNA functioned in the reaction by acting as a primer upon which adenylate residues were added. The polymeric product was shown to be a chain of adenylate residues, greater than 100 nucleotides in length, covalently attached to the ribosomal RNA. In addition to its role as a primer in the reaction, ribosomal RNA also appeared to act as a kinetic effector.

Numerous enzymes have been reported in the past few years which catalyze the addition of the nucleoside monophosphate portion of a nucleoside triphosphate onto the 3'-end of oligo- or polynucleotides. The terminal addition of deoxyribonucleotides to oligo- or polynucleotides has been well documented. Krakow, Coutsogeorgopoulos, and Canellakis (1) reported the isolation of a terminal deoxyribonucleotide transferase from the nuclear fraction of calf thymus gland which utilized each of the four common deoxyribonucleoside triphosphates and, in the presence of \( \text{Mg}^{2+} \), added the corresponding monodeoxyribonucleotides onto the 3' end of denatured DNA.

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The enzyme, which was distinct from DNA polymerase, synthesized short chains of deoxyribonucleotide product (less than 4 residues in length). Referred to as the “nuclear terminal addition enzyme” (2), it was also capable of incorporating the common ribonucleoside triphosphates, but only a single ribonucleotide residue could be added to each primer molecule (2, 3).

The isolation of another terminal deoxyribonucleotide transferase has been reported by Bollum (4), who utilized the soluble fraction of calf thymus gland. This enzyme, distinct from the nuclear enzyme and referred to as “polydeoxynucleotide synthetase” (5), incorporated the mononucleotide residues of each of the four common deoxyribonucleoside triphosphates onto the 3'-end of a denatured DNA or oligodeoxyribonucleotide primer. Variable chain lengths (one to 1000 nucleotides), random copolymerization of deoxyribonucleotides, and synthesis de novo of polydeoxyribonucleotides could be achieved depending upon incubation conditions (5-7).

Enzymes which catalyze the terminal incorporation of ribonucleotides from ribonucleoside triphosphates into RNA have been shown in a wide variety of systems. One of these enzymes is the well characterized and ubiquitous ATP(CTP)-tRNA nucleotidyltransferase, which synthesizes the pCpCpA sequence on the 3'-end of transfer RNA (8-12). This enzyme is specific for transfer RNA and produces a product of well defined length and sequence, factors which distinguish it from other terminal ribonucleotide-incorporating activities.

The remainder of these terminal ribonucleotide-incorporating activities, which have been isolated from mammalian (13-25), plant (26), avian (27-29), and bacterial (30-33) sources, have not been as well characterized. Nevertheless, all require a ribonucleoside triphosphate as substrate, a divalent metal ion as cofactor, and an oligo- or polynucleotide primer. In those cases in which sufficient data are available, the reactions appear to proceed, with one possible exception (see below), by the addition of monoribonucleotide residues onto the 3'-end of the primer. The resulting products are homoribopolymer chains of varying lengths (one to 200 nucleotides), covalently attached to the primer molecule.

Within these basic characteristics, however, a great deal of diversity exists among the various ribonucleotide incorporating activities. Enzymes which are reasonably specific for each of the four common ribonucleoside triphosphates have been prepared from Escherichia coli (ATP), calf thymus (CTP), rat liver (UTP), and spinach (GTP), while an enzyme fraction...
from Landsehr[t ascites tumor cells will utilize any of the ribonucleoside triphosphates. It is not known whether the latter enzyme fraction contains one enzyme which will incorporate each ribonucleoside triphosphate or an enzyme for each one.

Among the best characterized of the ribonucleoside-incorporating activities are those from E. coli. In 1952, two groups of investigators reported the isolation of specific ATP-incorporating activities from this source. These enzymes were isolated by Gottesman, Canellakis, and Canellakis (30) using the soluble fraction and August, Ortiz, and Hurwitz (31) using the ribosomal pellet. In the presence of the divalent metal ions, Mg"+ and Mn**, the enzyme from the soluble fraction was reported to catalyze the terminal addition of adenylic residues onto the 3'-end of an RNA primer. It should be pointed out that the reason for the discrepancy between the results of August, Ortiz, and Hurwitz and Hardy and Kurland is not known. Perhaps there are two different ATP-incorporating enzymes present in E. coli ribosomes. Possibly there is a single enzyme which, depending on the conditions, is able to catalyze the synthesis of a new of poly A or the addition of adenylic residues to the 3'-end of an RNA primer. It should be pointed out that the purification procedures used by the two groups were different, the specific activities of the partially purified enzymes varied, and neither enzyme was free of endogenous RNA.

The purpose of this report is to present the purification and characterization of an enzyme, designated ATP-RNA adenyltransferase, from the ribosomal pellic of Pseudomonas putida. The enzyme catalyzes the incorporation of adenylic residues from ATP into a polymeric product, with the concomitant release of inorganic pyrophosphate. The reaction is completely dependent upon exogenous RNA and requires the cofactor, magnesium ion. The product is a homopolymer of adenylate residues, greater than 100 nucleotides in length, which is covalently attached to the 3'-end of the added ribosomal RNA primer.

**EXPERIMENTAL PROCEDURE**

Materials and Methods—NADP+, UDPG, glucose 6-phosphate dehydrogenase, 2',3'-dATP, and all unlabeled 5'-phosphate derivatives of the ribonucleosides were purchased from P-L Biochemicals. Sephadex G-100 and DEAE-Sephadex (A-25) were obtained from Pharmacia and Whatman cellulose phosphate (P-floc) from Reeve Angel. Calf thymus DNA, poly A, poly U, poly C, poly I, and phosphoglomucitate were purchased from Sigma. Labeled nucleotides were obtained from Schwarz BioResearch. P. putida bacteriophage gh-1 DNA (35, 36) was purfied by the method of Thomas and Abelson (37). The gh-1 and calf thymus DNA were denatured by heating at 100° for 10 min followed by quick cooling. UDPG pyrophosphorylase was isolated from calf liver (38) and recrystallized twice. The oligoribonucleotides, (Ap)4(Ap)2A, (Ap)2A, and ApA, were prepared according to the procedure of Rottman and Nirenberg (39). We are indebted to Mr. Joseph Abbate and Mr. Richard Jagger of this department for gifts of Xenopus laevis ribosomal RNA and (Pi), respectively.

Protein concentrations were determined by the method of Lowry et al. (40) with bovine albumin Fraction V powder (Nutritional Biochemicals) as a standard. The concentrations of gh-1 and calf thymus DNA and ribosomal and soluble RNA were determined spectrophotometrically based on the extinction coefficient Document Issue of March 25, 1970 1379

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
being collected by centrifugation and discarded. Soluble RNA was then precipitated with ethanol, redissolved, and dialyzed extensively against 10 mM succinate-NaOH (pH 5.3). The resulting soluble RNA, which was free of detectable high molecular weight RNA as determined by sucrose density gradient centrifugation, was stored at -20°C.

**Enzyme Assays**—The incubation temperature for all reactions was 37°C. The standard assay measured the conversion of 3H-ATP into an acid-insoluble product. Unless otherwise indicated, the incubation mixture (0.5 ml) contained 30 mM glycine-NaOH (pH 9.5), 0.8 mM 3H-ATP, 20 mM MgCl₂, saturating amounts of *P. putida* ribosomal RNA, and an appropriate amount of enzyme. Samples of 100 μl were withdrawn at various times and 5 ml of cold 10% trichloracetic acid were added to each sample. The acid-insoluble material was collected on membrane filters (Schleicher and Schuell, type B-6) and washed three times with 0.5 ml of 0.1 M sodium hydroxide (pH 10.6), 0.8 mM UDPG, 0.2 mM NADP⁺, a suitable amount of enzyme and excess phosphoglucomutase, glucose 6-phosphate dehydrogenase, and UDPG pyrophosphorylase. The assay as described by Johnson et al. (42) couples the formation of inorganic pyrophosphate from ribonucleoside triphosphate polymerization to NADP⁺ reduction which is measured at 340 μm in a spectrophotometer.

For the determination of inorganic pyrophosphate, the reaction mixture (0.5 ml) contained 30 mM glycine-NaOH (pH 9.5), 0.8 mM 3H-ATP, 20 mM magnesium acetate, 17 μg of *P. putida* ribosomal RNA, 0.2 mM UDPG, 0.2 mM NADP⁺, a suitable amount of enzyme and excess phosphoglucomutase, glucose 6-phosphate dehydrogenase, and UDPG pyrophosphorylase. The assay as described by Johnson et al. (42) couples the formation of inorganic pyrophosphate from ribonucleoside triphosphate polymerization to NADP⁺ reduction which is measured at 340 μm in a spectrophotometer. Calculation of the number of nmoles of NADPH formed was made with the molar extinction coefficient of 6.22 x 10³ (43).

Inorganic pyrophosphatase was measured by the disappearance of inorganic pyrophosphate with the coupled UDPG pyrophosphorylase assay system. Adenylyl kinase and ATPase were measured by the conversion of [3H-ADP and [3H-ATP, respectively, to other adenosine derivatives, which were separated by means of paper chromatography in isobutyric acid-NH₄OH-H₂O (66:1:33). Polynucleotide phosphorylase was measured under the same conditions as described for the standard assay except that [3H-ADP was used in place of [3H-ATP. (These conditions were found to be near optimal for the ADP-incorporating activity isolated from the 80,000 g pellet of *P. putida*.) Ribonuclease was assayed by measuring the loss of acid-insoluble radioactivity and the change in the sucrose density gradient profile of enzyme-treated ¹³C-ribosomal RNA.

**Alkaline Hydrolysis of Polymeric Product**—After incubation of the mixture for 60 min, the reaction was terminated by the addition of 2 volumes of cold 5% HClO₄. The resultant precipitate was collected by centrifugation at 12,000 x g for 10 min and the supernatant solution was discarded. The pellet was washed once with 2 ml of cold 5% HClO₄ and twice with 2 ml of cold 1% HClO₄. After the addition of 2.0 ml of 0.3 N KOH, the dissolved pellet was incubated at 37°C for 18 hours. The solution was then neutralized with Dowex 50 (H⁺), which was then removed by filtration through Whatman No. 42 filter paper and washed three times with 0.5 ml of 0.1 N NaOH. The combined filtrates were lyophilized, dissolved in H₂O, and spotted with the appropriate standards on Whatman No. 3MM paper for electrophoresis and Whatman No. 41 paper for solvent chromatography. Electrophoresis was carried out in 0.05 M ammonium formate (pH 3.6) at 36 volts per cm for 100 min. Descending solvent chromatography was performed in isobutyric acid-NH₄OH-H₂O (66:1:33). The radioactive lanes were cut out and counted in a liquid scintillation spectrometer.

**RESULTS**

**Purification of Enzyme**

The entire purification procedure was performed at 0-4°C.

**Preparation of Initial Extract**—Frozen *P. putida*, 40 g of cells which had been grown to the early stationary phase, was homogenized in a Servall Omni-Mixer with 100 g of cells washed with 100 ml of 10 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.1 mM EDTA. After 5 min of homogenization, 40 ml of the same buffer were added and the entire suspension was centrifuged at 12,000 x g for 10 min. The supernatant solution was decanted and recentrifuged at 12,000 x g for 10 min. The resulting supernatant solution was carefully decanted and diluted to 100 ml with the above buffer (glass bead extract).

**Sedimentation**—The glass bead extract enzyme fraction was centrifuged at 80,000 x g for 120 min. The supernatant solution, which was devoid of detectable ATP-incorporating activity, was poured off and discarded, and the pellet was stored at -20°C overnight. (No difference was observed if the purification was continued immediately without freezing the high speed pellet.)

**Solubilization**—The high speed pellet was thawed and homogenized in 100 mM glycine-NaOH (pH 10.6) by means of a glass tube fitted with a Teflon-tipped pestle. The resulting homogenate was centrifuged at 80,000 x g for 120 min and the supernatant solution was poured off and saved. The pellet was rehomogenized with 70 ml of the same buffer followed by centrifugation at 80,000 x g for 120 min. The supernatant solution was poured off and combined with the first (pH 10.6 supernatant, 194 ml).

**pH Fractionation**—Solid KCl was slowly added with stirring to the pH 10.0 supernatant enzyme fraction to make the solution 1 M with respect to KCl. Hydrochloric acid (1 M) was then added dropwise with stirring until a pH of 5.0 was reached. A flocculent white precipitate formed during this procedure. After the suspension was stirred for 4 hours, the precipitate, which was devoid of ATP-incorporating activity, was removed by centrifugation at 12,000 x g for 10 min and discarded. The supernatant solution was dialyzed overnight against 4 liters of 10 mM Tris-HCl (pH 8.0)-(pH 5 supernatant, 142 ml).

**pH Concentration**—The pH 5 supernatant enzyme fraction was adjusted to pH 3.2 by the slow addition of 1 M hydrochloric acid. A precipitate formed which, after stirring for 15 min, was collected by centrifugation at 12,000 x g for 10 min. The supernatant solution, which was devoid of ATP-incorporating activity, was poured off and discarded. The precipitate was redissolved in 22 ml of 100 mM glycine-NaOH (pH 10.1) by gentle stirring for 3 hours. The resulting solution was then dialyzed overnight against two successive 4-liter volumes of 10 mM Tris-HCl (pH 8.0)-(pH 3.2 precipitate, 25 ml).

**Sephadex G-100 Gel Filtration**—Sephadex G-100, previously chromatographed according to the procedure of Brown and Lardy (20), was found to be unsuitable for the purification of this enzyme. The results of the Sephadex G-100 gel filtration experiment are shown in Table I.
Fig. 1. Sephadex G-100 gel filtration of pH 3.2 precipitate enzyme fraction. A 5-ml aliquot of the pH 3.2 precipitate enzyme fraction was applied to a column, 43 × 2.5 cm, of Sephadex G-100 equilibrated with 10 mM Tris-HCl (pH 8.0) containing 1 M KCl. The column was developed at a flow rate of 0.5 ml per min by the continual addition of the same buffer. Thirty milliliters of the 66-ml void volume were collected, followed by the 40-ml fractions presented in the figure. 

### Table I

| Enzyme fraction          | Specific activity | Total units |
|--------------------------|-------------------|-------------|
| Glass bead extract       | 5                 | 9,900       |
| pH 10.6 supernatant      | 37                | 11,500      |
| pH 5 supernatant         | 97                | 9,100       |
| pH 3.2 precipitate       | 79                | 7,400       |
| Sephadex G-100           | 146               | 3,500       |
| DEAE-Sephadex            | 344               | 1,200       |
| DEAE-Sephadex (dialysis) | 125               | 450         |
| Cellulose phosphate      | 1,800             | 486         |

enzyme fraction was dialyzed overnight against the same phosphate buffer (DEAE-Sephadex (dialysis), 20 ml). As a result of this procedure, approximately two-thirds of the ATP incorporating activity was lost with the concomitant appearance of a very fine precipitate. The entire suspension was applied to the cellulose phosphate column. Elution was achieved with a 100-ml linear gradient of 0 to 1 M KCl in 10 mM phosphate buffer (pH 7.1). The enzyme eluted as a single symmetrical peak at approximately 0.7 M KCl. The peak enzyme fractions were pooled (cellulose phosphate, 9 ml) and stored at 2°C.

### Comments on Purification Procedure

A summary of the purification procedure is presented in Table I. The data describe approximately a 360-fold purification with an apparent 5% recovery of initial enzyme activity. For the experiments described in this report, either the DEAE-Sephadex or the cellulose phosphate enzyme fractions were used.

### Association with Macromolecules

An important aspect of the purification is the association of the enzyme with macromolecular cellular components. Following the preparation of the glass bead extract and removal of cellular debris, high speed centrifugation of the enzyme extract was performed. Although most cellular enzymes, including DNA-dependent RNA polymerase, remain in the supernatant solution during this procedure, the ATP-incorporating activity was detected only in the pellet. Solubilization of the enzyme was accomplished by homogenization of the high speed pellet in alkaline buffer, pH 10.6. At pH 10.1 only about 50% of the enzyme was released into the supernatant fraction, while less than 10% was solubilized at pH 9.8. Even after solubilization from the high speed pellet, the enzyme was found to aggregate with other cellular components. The pH 5 fractionation and Sephadex G-100 gel filtration steps were ineffective unless carried out in the presence of 1 M salt.

### Removal of Contaminating Nucleic Acid

Even though the majority of the A260-absorbing material was separated from the ATP-incorporating activity in the gel filtration step (Fig. 1), the A320/A260 ratio of the enzyme fractions remained at 0.5. At this point in the purification, the residual nucleic acid was still in sufficient quantity to prevent any stimulation of the ATP-incorporating activity by the addition of exogenous RNA. The next step, DEAE-Sephadex chromatography, was utilized to remove most of the remaining A260-absorbing material. The A320/A260 ratio of the eluate fractions in the region of the enzyme was approximately 1.0. Following this step, the ATP-
incorporating activity was completely dependent upon added RNA.

Stability of Enzyme—The stability of the enzyme fractions, up to and including the DEAE-Sephadex fraction, was such that, when stored at 2°C, no loss in activity could be detected for at least 1 month. However, dialysis of the DEAE Sephadex enzyme fraction led to a considerable loss in activity. The cellulose phosphate enzyme fraction was also unstable, losing about 50% of its activity in 1 week.

Contaminating Activities—When examined at the concentration used in the activity assay, the cellulose phosphate enzyme fraction was shown to be free of detectable adenylyl kinase, ATPase, inorganic pyrophosphatase, primer-dependent or primer-independent (44) polynucleotide phosphorylase, and ribonuclease activities. Polynucleotide phosphorylase activity was removed by gel filtration on Sephadex G-100 (Fig. 1). The DEAE-Sephadex enzyme fraction was free of ribonucleate activity, but contained small amounts of adenylyl kinase, ATPase, and inorganic pyrophosphatase activities.

Characteristics of Reaction

General Properties—Both the rate and extent of ribonucleotide incorporation into an acid-insoluble product increased with temperature and were optimal at 37°C. Higher temperatures, up to 45°C, produced an equally efficient initial reaction but the total incorporation was significantly inhibited.

The optimal pH for the reaction was 9.5 with either 30 mM glycine-NaOH buffer or Tris adjusted to that pH with HCl. Ribonucleotide incorporation equal to approximately 50% of the optimal activity was observed with Tris-HCl buffer at pH 8.5 and with glycine-NaOH buffer at pH 8.8 and 10.2.

The rate of the reaction was directly proportional to the amount of enzyme added. The addition of 0.30, 1.5, and 3.0 µg of the cellulose phosphate enzyme fraction to the standard reaction mixture resulted in the incorporation of 0.41, 1.8, and 3.8 nmoles of AMP, respectively, in 20 min. Similar proportionality was obtained with the less purified fractions.

The kinetics of the reaction was consistently biphasic (Fig. 2), independent of the purity of the enzyme or the pH of the reaction mixture. All of the studies presented in this report were carried out within the initial linear range of the reaction.

**Table II**

| Additions | Activity (nmole/20 min) | Per cent of 3H-ATP |
|-----------|------------------------|---------------------|
| Experiment 1 | | |
| 3H-ATP | 18.5 | 100 |
| 3H-CTP | 1.2 | 6.5 |
| 3H UTP | <0.1 | <1 |
| 3H-GTP | <0.1 | <1 |
| Experiment 2 | | |
| 3H-ATP + UTP | 13.5 | 75 |
| 3H-ATP + CTP | 12.9 | 72 |
| 3H-ATP + GTP | 11.5 | 64 |
FIG. 4. AMP incorporation plotted against ATP concentration. Conditions were the same as those described in Fig. 5 except that 20 mM MgCl₂ and varying amounts of ATP were present in each reaction mixture. The inset is a Lineweaver-Burk plot of the data.

**TABLE III**

Stimulation of AMP incorporation by polynucleotides

| Additions                  | Amount of polynucleotide added (μg) | AMP incorporation (nmole/20 min) |
|---------------------------|------------------------------------|---------------------------------|
| Experiment 1              |                                    |                                 |
| None                      |                                    | <0.1                            |
| P. putida ribosomal RNA   | 84                                 | 24.7                            |
| P. putida soluble RNA     | 78                                 | 15.8                            |
| Poly C                    | 64                                 | 6.7                             |
| Poly A                    | 84                                 | 2.4                             |
| Poly I                    | 64                                 | 0.7                             |
| Poly U                    | 84                                 | 0.1                             |
| gh-1 DNA                  | 41                                 | 2.1                             |
| calf thymus DNA           | 80                                 | 1.7                             |
| gh-1 denatured DNA        | 41                                 | 0.2                             |
| calf thymus denatured DNA | 80                                 | 1.1                             |
| (pT)                      | 84                                 | 0.3                             |
| Experiment 2              |                                    |                                 |
| None                      |                                    | <0.1                            |
| P. putida ribosomal RNA   | 84                                 | 6.3                             |
| E. coli ribosomal RNA     | 84                                 | 5.7                             |
| X. laevis ribosomal RNA   | 85                                 | 8.3                             |

**Metal Ion Requirement**—The enzyme requires the presence of magnesium ion for activity (Fig. 3). There was no detectable activity when Mg²⁺ was omitted from the assay. The activity increased with increasing Mg²⁺ until a maximum between 15 and 30 mM, followed by considerable inhibition between 30 and 40 mM. The apparent Kₘ for Mg²⁺ as determined from the Lineweaver-Burk plot shown in the inset of Fig. 3 was 3 × 10⁻² M. Manganese ion was ineffective as a substitute for Mg²⁺ in this reaction.

**RNA Requirement**—Incorporation of AMP into a polymeric product by the enzyme was dependent upon exogenous RNA. In the absence of added RNA, no acid-insoluble product was detected even after incubation for 3 hours (see Fig. 2). Ribosomal RNA, relatively independent of its source, soluble RNA, and poly C were capable of fulfilling this requirement (Table II).
Fig. 5. ATP incorporation plotted against soluble RNA concentration. Conditions were the same as those described in Fig. 5 except that varying amounts of P. putida-soluble RNA were present in each reaction mixture in place of ribosomal RNA. The inset contains a plot of the reciprocal reaction velocity against the reciprocal of the soluble RNA concentration.

Fig. 6. AMP incorporation plotted against soluble RNA concentration. Conditions were the same as those described in Fig. 5 except that varying amounts of P. putida-soluble RNA were present in each reaction mixture in place of ribosomal RNA. The inset contains a plot of the reciprocal reaction velocity against the reciprocal of the soluble RNA concentration.

Table IV

| Reaction | Distribution of radioactivity |
|----------|-------------------------------|
|          | AMP | Adenine | 2'(3')-AMP-adenosine |
| 1        | 1200 | 20670 | 190 |
| 2        | 1000 | 20310 | 120 |

Characterization of Reaction Products

Stoichiometry of Reaction Products—As measured by means of the production of NADPH in the coupled assay with UDPG pyrophosphorylase (see “Experimental Procedure (‘‘Enzyme Assays’’))", inorganic pyrophosphate was shown to be a product of the reaction. The amount of inorganic pyrophosphate produced was equivalent to the amount of AMP incorporated into acid-insoluble product. In two separate experiments, when 11.4 and 8.2 nmoles of inorganic pyrophosphate were produced in 40 min, 10.8 and 7.4 nmoles of AMP were incorporated into polymeric product, respectively. When ribosomal RNA was omitted from the reaction mixture, neither acid-insoluble product nor inorganic pyrophosphate was formed.

Chain Length Determination of Polymeric Product—Alkaline hydrolysis of the acid-insoluble product formed when "H-ATP was used as substrate in the standard reaction mixture, followed by paper chromatography of the hydrolysate in isobutyric acid-NH₄OH-H₂O and the chromatogram was analyzed for radioactivity. No radioactivity was detected other than in the spots indicated in the table.

a straight line (Inset a of Fig. 5). A Hill plot (45, 46) of these data yielded a straight line with a slope of 1.8. A plot of 1/v against 1/rRNA₁₈ is presented in Inset b of Fig. 5. Assuming an average molecular weight of 8.2 × 10⁵ daltons for the two species of ribosomal RNA, the apparent Kₐ was 6 × 10⁻⁷ M.

The oligoribonucleotide, ApApApA, could also function in this reaction. At the saturating concentration of 50 μg per ml, ApApApA, after a lag of about 20 min, was capable of stimulating the incorporation of AMP into acid-insoluble product (Fig. 7). The rate was approximately 50% of that observed with ribosomal RNA. Shorter oligoribonucleotides of adenylic acid, ApApA or ApA, did not function in this reaction. The oligoribonucleotide, ApApApA, which lacks the free 3'-OH due to the presence of a phosphate group, was also ineffective. When added to the standard reaction mixture which contained ribosomal RNA, ApApApA inhibited the incorporation of AMP by approximately 50%, whereas the addition of an equivalent amount of ApApApA to the standard reaction mixture had no inhibitory effect.
to 96% of the radioactivity cochromatographed with 2'(3')-AMP (R_f = 0.66), while 0.5 to 0.7% moved with adenosine (R_f = 0.83). The remaining radioactivity chromatographed as a single peak (unknown) with an R_f of 0.46. Upon electrophoresis of the alkaline hydrolysate, this material migrated slightly ahead of 2'(3')-AMP and was well separated from the adenosine di-, tri-, and tetraphosphate regions. Since it is known that, under the conditions used, the hydrolysis of poly A may be only 95% complete (47), this radioactive material most probably represented unhydrolyzed oligomer(s) of adenyl acid.

The length of the average polymeric product can be approximated by dividing the amount of 2'(3')-AMP, which represents the internal residues of the chain, by the amount of adenosine, which represents the 3'-external residue. The results presented in Table IV indicate a length in the range of 100 to 200 adenylate residues per chain. Incomplete hydrolysis of the product and the slow conversion of 2'(3')-AMP to adenosine (48) imply that this approximation is a minimum value. The absence of any radioactive material which might represent a 5'-external residue that this approximation is a minimum value. The absence of any radioactive material which might represent a 5'-external residue suggests that the adenylate chain probably was not synthesized de novo.

**Nearest Neighbor Analysis of Polymeric Product—When α-32P-ATP was used as substrate and the resultant product was subjected to alkaline hydrolysis and analysis by paper electrophoresis, 94% of the radioactivity migrated with 2'(3')-AMP (Table V). A small amount of radioactivity (about 3%, data not shown in Table V) migrated slightly ahead of 2'(3')-AMP, corresponding to the unhydrolyzed oligomer(s) of adenyl acid observed in the chain length analysis. The remainder of the radioactivity was divided among the other three common 2'(3')-ribonucleoside monophosphates. Some of the radioactivity in the 2'(3')-CMP region undoubtedly resulted from trailing of 2'(3')-AMP which migrates just in front of 2'(3')-CMP in this system. These data, along with the results of the chain length analysis, are consistent with a polymeric product composed of long chains of adenylate residues which are attached to the 3'-end of the added ribosomal RNA.

**Sucrose Density Gradient Analysis of Polymeric Product—**Reaction products, prepared in standard assay mixtures containing ribosomal RNA and 3H-ATP, were analyzed by SDS-sucrose density gradient centrifugation as described in the legend to Fig. 8. The data presented in this figure show that the major portion of the product sedimented in the region of the 23 and 16 S ribosomal RNA species. Chains of adenylate residues, 100 to 200 nucleotides in length, would not be sufficient to produce these large sedimentation values. Since noncovalent binding of poly A to ribosomal RNA does not occur even in the presence of divalent cations (49, 50), it was concluded that the polymeric product was covalently attached to the ribosomal RNA. These results also show that both species of ribosomal RNA can fulfill the requirement for exogenous RNA in this reaction. Taking into account that 23 S ribosomal RNA has twice the molecular weight of 16 S ribosomal RNA, the amount of radioactive product associated with the smaller species is slightly greater (1.3 to 1.6 times) than that associated with the larger species.

**DISCUSSION**

The ATP-incorporating activity described in this report, which was isolated from the 80,000 × g pellet of P. putida, was purified from major contaminating activities which might interfere in the assay. These activities included ATPase, adenylate kinase, polynucleotide phosphorylase, RNase, and inorganic pyrophosphatase. Furthermore, the purified enzyme was essentially free of endogenous RNA. In the presence of magnesium ion and exogenous RNA, the enzyme specifically catalyzed
ized the incorporation of adenylate residues from ATP into a polymeric product, with the concomitant release of an equimolar amount of inorganic pyrophosphate. Based on this reaction scheme we have chosen to call the enzyme ATP-RNA adenyllytransferase.

The exogenous RNA, upon which the reaction is totally dependent, could be utilized by the enzyme in the following ways: (a) by acting as a template in a reiterative copying process similar to that described for the production of poly A from denatured DNA by the DNA-dependent RNA polymerase (51); (b) by acting as a primer providing a free 5'-hydroxyl end upon which to add adenylate residues; and (c) by acting as a kinetic effector.

Since the reiterative copying process requires the use of a base-pairing mechanism, the stimulation by ApApApAp and poly C and the lack of stimulation by poly U argue against this possibility. These results, however, are consistent with the second possibility, the use of the exogenous RNA as a primer. This role of RNA as a primer is substantiated by the lack of stimulation by ApApApAp, the absence of radioactive material corresponding to the 5' terminus of the polynucleotide product following alkaline hydrolysis, and the transfer of 32p from Ap32p to other nucleotides in the nearest neighbor analysis. Finally, the results of the sucrose density gradient centrifugation experiments leave no doubt that the polymeric product is covalently attached to the primer RNA.

The unusual response of the enzyme to the addition of ribosomal RNA (see Fig. 5), however, suggests that, in addition to its role as primer, ribosomal RNA may also be acting as a kinetic effector. The data presented in Fig. 5 are consistent with a model involving a cooperative effect of ribosomal RNA on the enzyme, involving more than one binding site. Other possible explanations of the phenomenon, e.g. the incapacity of the primer at low concentrations due to the presence of a minor contaminating activity, have not, however, been ruled out.

Further study, with purified 16 and 23 S ribosomal RNA, must be undertaken in order to determine whether or not this effect is restricted to one of the two species. The effect of other kinds of high molecular weight RNA, such as viral RNA, should also be studied. The response of the enzyme to the addition of soluble RNA (see Fig. 6) suggests that soluble RNA did not act as a kinetic effector.

Although the ATP-RNA adenyllytransferase of *P. putida* was initially isolated from the 80,000 × g pellet, which consists primarily of ribonucleoprotein particles, a direct association of this enzyme with ribosomes has not yet been established. Studies on the ATP-incorporating activity isolated from similar high speed pellets of extracts from *E. coli* (52, 53) have shown that the enzyme from this source is associated with ribosomes, although only with a small fraction of the total ribosomes of the cell. Hardy and Kurland (53), using several criteria, have shown that the enzyme is probably not a structural component of the ribosomes. Nevertheless, Smith and August (52) have concluded that the association with ribosomes may be physiologically significant.

Except for the ATP(CTP)-RNA nucleotidyltransferase, the function in vivo of terminal ribonucleotide-incorporating activities is presently unknown. A possible role for the "poly A polymerase" of *E. coli* in the synthesis of RNA was suggested by Ortiz et al. (34). These authors reported that "poly A polymerase" activity diminished rapidly after infection of *E. coli* by T5, T4, T6, and T2 bacteriophage, while it was unaffected after infection by T7 or λ-virulent or after induction of *E. coli* K12 (λ). In *T4*-infected *E. coli*, the inhibition of "poly A polymerase" was temporarily related to the inhibition of host-directed RNA synthesis. The inhibition was apparently produced by the rapid synthesis of an inhibitor. A similar inhibitor has been observed in extracts of *T4*-infected *E. coli* and *Shigella dysenteriae* (33).

Several "A-rich RNA" fractions have been isolated from cellular and viral sources (34–59). Although the involvement of other enzymes, e.g. RNA polymerase or polynucleotide phosphorylase, cannot be ruled out, the terminal riboadenylate incorporating activities might play some role in the production of these "A-rich RNAs."

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K. J. Payne and J. A. Boezi

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