A KINETIC AND STRUCTURAL CHARACTERIZATION OF ADENOSINE 5' - TRIPHOSPHATE: RIBONUCLEIC ACID ADENYLYLTRANSFERASE FROM PSEUDOMONAS PUTIDA

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ABSTRACT

A KINETIC AND STRUCTURAL CHARACTERIZATION OF ADENOSINE 5'-TRIPHOSPHATE: RIBONUCLEIC ACID ADENYLYLTRANSFERASE FROM *PSEUDOMONAS PUTIDA*

By

Robert William Blakesley

This dissertation describes the research conducted on ATP:RNA adenylyltransferase isolated from two types of cells. The major portion of this work represents a kinetic and structural study of ATP:RNA adenylyltransferase from the bacterium *Pseudomonas putida*. This is followed by a brief investigation of the effect of 3'-deoxyadenosine 5'-triphosphate on the AMP incorporation by ATP:RNA adenylyltransferase from Novikoff hepatoma cells. As an appendix to this dissertation there is presented a simple, rapid staining technique for proteins separated by polyacrylamide gel electrophoresis.

ATP:RNA adenylyltransferase (EC 2.7.7.19) catalyzes the polymerization of ATP into the homopolymer, polyadenylic acid. The activity of this enzyme requires a divalent metal ion and a polyribonucleotide as primer. The enzymatic activity has been detected in a wide variety of sources,

such as mammals, plants, avia, yeast and bacteria. The ubiquity of this enzymatic activity in nature suggests an importance for the activity in a cell. However, no direct evidence for the *in vivo* function of ATP:RNA adenylyltransferase has been presented.

Although there were many reports of the isolation of ATP:RNA adenylyltransferase from various sources, few kinetic or structural characteristics of an enzyme of this type were known at the time this research began. Thus, a kinetic and structural study of ATP:RNA adenylyltransferase from the particulate fraction of the bacterium *Pseudomonas putida* was initiated. During the large-scale purification of this enzyme, designated adenylyltransferase B, a previously undetected ATP-incorporating activity, designated adenylyltransferase A, was observed. Adenylyltransferases A and B were indistinguishable catalytically. Both of these ATP-specific, primer-dependent activities were optimally active at pH 9.5, were inhibited by inorganic pyrophosphate but not by orthophosphate, and were found to utilize effectively the same polynucleotides as primers. The divalent metal ion requirement for both activities was satisfied by Mg^{2+}, with an optimum at 20 m*M*. Within experimental error, both adenylyltransferases had identical values for the Km for ATP ($1\text{-}2 \times 10^{-4}$ *M*) and the Km for ribosomal RNA ($2\text{-}4 \times 10^{-4}$ *M*; expressed, by convention, in terms of nucleotide phosphate).

Adenylyltransferases A and B were distinguishable by their chromatographic and sedimentation properties. Adenylyltransferases A and B were resolved by chromatography on phosphocellulose or poly(U)-Sepharose. When chromatographed on Bio-Gel P-100, adenylyltransferase A was excluded, while adenylyltransferase B penetrated the gel. By sedimentation through glycerol gradients, adenylyltransferases A and B were determined to have sedimentation coefficients ($s^{\circ}_{20,w}$) of 9.3 S and 4.3 S, respectively. The molecular weight of adenylyltransferase A was estimated to be 185,000 and that of adenylyltransferase B to be 50,000 to 60,000. Apparently, adenylyltransferase A was generated from adenylyltransferase B during the purification.

Two derivatives of the antibiotic rifamycin, AF/013 and AF/DNFI, inhibited the incorporation of ATP into acid-insoluble form catalyzed by adenylyltransferases A and B. The polymerization rate by either adenylyltransferase was inhibited 50% at 5 μg per ml AF/013 or 10 μg per ml AF/DNFI. Poly(U) and poly(dT) were competitive inhibitors of the ribosomal RNA-primed polymerization reaction. The Ki for poly(U) or poly(dT) was 4-10 x 10^{-6} *M* (in terms of nucleotide phosphate) for adenylyltransferases A and B. The inhibition was a result of the competition between the non-priming poly(U), or poly(dT), and ribosomal RNA for the primer-binding site. The 5'-triphosphate derivative (3'-dATP) of the drug cordycepin (3'-deoxyadenosine) was a competitive inhibitor with TP for both adenylyltransferases. The Ki for 3'-dATP was 6-10 x

10^{-4} *M*. The activity of adenylyltransferase A or B was not affected by several other analogs of ATP; namely, 2'-dATP, 2'-*O*-methylATP, or 3-β-*D*-ribofuranosylimidazo [2, 1-i] purine 5'-triphosphate.

The second research problem was undertaken to determine the *in vitro* effect of 3'-deoxyadenosine 5'-triphosphate on the AMP incorporation by a eukaryotic ATP:RNA adenylyltransferase. The *in vivo* synthesis of the polyadenylate segment found covalently linked to eukaryotic messenger RNA or to its precursor, heterogeneous DNA-like RNA of the nucleus, is sensitive to the drug cordycepin (3'-deoxyadenosine). If the eukaryotic adenylyltransferase is responsible for the posttranscriptional synthesis of the polyadenylic acid of heterogeneous DNA-like RNA as has been suggested, the adenylyltransferase should also be sensitive to the drug. The AMP incorporation by the adenylyltransferase purified from calf thymus nuclei or from rat liver nuclei, however, was not affected by the presence of cordycepin. The triphosphate derivative of cordycepin (3'-dATP) is probably the form in which cordycepin would inhibit adenylyltransferase. To test this hypothesis ATP:RNA adenylyltransferase was partially purified from the cytoplasm of Novikoff hepatoma cells. This enzyme was similar to the eukaryotic Mn^{2+}-dependent adenylyltransferases previously reported. The activity was primer-dependent, ATP-specific ($Km = 2 \times 10^{-5}$ *M*) and was sensitive to the sulfhydryl reagent, *p*-hydroxymercuribenzoate. The sedimentation

coefficient ($s^{\circ}_{20,w}$) was 4.1 S. Optimal activity for the enzyme was at pH 8. The preferred primers were polyadenylic acid and yeast transfer RNA. Rifamycin AF/013 inhibited this adenylyltransferase; 50% of the activity remained at 20 µg per ml AF/013. When 3'-dATP was examined, it was shown to be a competitive inhibitor of the activity of adenylyltransferase. The Ki for 3'-dATP was 2×10^{-5} *M*. Also, in agreement with the results previously reported, cordycepin itself was ineffective in inhibiting the synthesis of polyadenylate by adenylyltransferase.

In the appendix there is presented a new staining procedure. It was developed in order to provide a simpler, quicker staining technique for the identification of proteins separated by polyacrylamide gel electrophoresis. The staining solution was composed of Coomassie Brilliant Blue G250 suspended in a mixture of sulfuric acid and trichloroacetic acid. The staining technique was rapid (results as quick as 30 minutes), required little or no destaining, and had a sensitivity of at least 1 µg of protein per band. The clear, uniform background obtained was well-suited for densimetric scanning. In addition, a linear relationship was observed between absorbance and protein concentration in the concentration range of 1 µg to at least 10 µg per band. The staining technique can be used with sodium dodecyl sulfate as well as conventional standard polyacrylamide gel electrophoresis.

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By

Robert William Blakesley

A DISSERTATION

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DEDICATION

To my entire family who provided confidence and moral support, and through whose sacrifice made possible the opportunity for this work.

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Publications

1. Boezi, J. A.; Lee, L. F.; Blakesley, R. W.; Koenig, M.; and Towle, H. C. "Marek's Disease Herpesvirus-Induced DNA Polymerase." Journal of Virology (in press) 1974.

2. Blakesley, R. W.; and Boezi, J. A. "A Kinetic and Structural Characterization of ATP:RNA Adenylyltransferase from *Pseudomonas putida*," to be submitted for publication.

3. Blakesley, R. W., and Boezi, J. A. "The Effect of 3'-Deoxyademosine 5'-Triphosphate on the Reaction Catalyzed by ATP:RNA Adenylyltransferase from Novikoff Hepatoma," to be submitted for publication.

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GENERAL INTRODUCTION

The past two decades could be called the era of molecular biology. It was ushered in by the famous Watson and Crick model for DNA. The presentation of this model demonstrated that the chemical structure of a molecule could explain biological phenomena. In this case, the base sequence of the DNA represented genetic information. Since then there has been a great deal of research interest in how the genetic information of DNA is replicated, and how the transmission of this information to various parts of the cell is controlled. The study of the enzymes that polymerize nucleic acids is an integral part to the understanding of these processes.

This dissertation covers research conducted on a nucleic acid polymerizing enzyme from two types of cells. The major portion of this work represents a structural and kenetic study of ATP:RNA adenylyltransferase from the bacterium *Pseudomonas putida*. This is followed by a brief investigation of the effect of 3'-deoxyadenosine 5'-triphosphate on the activity of ATP:RNA adenylyltransferase from the eukaryotic cell, Novikoff hepatoma. In addition, a literature review of the adenylyltransferases isolated from

a wide variety of cells in nature, with emphasis on those from prokaryotes, is also presented.

The *in vitro* synthesis of homopolynucleotides can be catalyzed by several types of enzymes, including DNA-dependent RNA polymerase, terminal nucleotidyl transferase and polynucleotide phosphorylase. If these enzymes are presented nucleotides of more than one base-type, then they will catalyze the formation of heteropolynucleotides. On the other hand, there are some nucleic acid polymerizing enzymes which only catalyze the synthesis of homopolyribonucleotides *in vitro*. These homopolyribonucleotide polymerases are found throughout nature and examples preferentially utilizing CTP, UTP, GTP, or ATP as substrate have been described. Their ubiquity in nature would seem to indicate their necessity to the biology of the cell. However, none of the investigations have provided direct evidence for the *in vivo* role of these enzymes.

From a separate line of investigation, messenger RNA, the transcription product of DNA-dependent RNA polymerase, isolated from a wide variety of eukaryotic sources was recently found to contain a terminal homopolyribonucleotide sequence, polyadenylic acid. This homopolymer was not coded for by the template, but, in fact, synthesized on the end of the messenger RNA molecule after transcription had taken place. The presence of the polyadenylic acid sequence is presently thought to be necessary for the proper

processing of messenger RNA from its precursor and for the functionality of messenger RNA in translation. It has been suggested that a homopolyribonucleotide polymerase is responsible for ths synthesis of the terminal polyadenylate sequence. Until the presentation of this hypothesis, the homopolyribonucleotide polymerases were little-known curiosities. Now some of these enzymes are being considered for an integral part in the genetic transfer of information within a cell.

Of the four types of homopolyribonucleotide polymerases, that which synthesizes polyadenylic acid from ATP has been studied in the greatest detail. The many reports of this activity in nature are discussed, with emphasis on those from prokaryotes, in the literature survey which comprises section one. The accepted systematic name for this enzyme is ATP:RNA adenylyltransferase (EC 2.7.7.19); however, more often the trivial name poly A (Adenylate) polymerase is used. When the research for this dissertation began, the enzymatic activity of adenylyltransferase had been known for about ten years. The enzyme had been partially purified, the basic requirements of the reaction had been determined, but there was neither information on the structure of the protein nor detailed kinetics of the enzymatic reaction. The objectives of this research then were to elucidate many of the structural and kinetic

characteristics of this enzyme, which, in turn might provide for an understanding of its *in vivo* role.

A short time before this work began, the enzyme from the bacterium *Pseudomonas putida* had been partially purified on a small scale in this laboratory. Scaling up and improving this already established purification procedure was necessary in order to carry out the proposed characterization of adenylyltransferase. During the course of this investigation a previously undetected adenylyltransferase activity was observed. It was identical catalytically, but was different in size and chromatographic behavior from the enzyme previously studied. The second section of this dissertation describes the kinetic and structural characterization of both of these adenylyltransferases. This section is to be submitted for publication in a scientific journal.

The third section of this dissertation presents a brief investigation of a eukaryotic ATP:RNA adenylyltransferase. As previously described, eukaryotic messenger RNA contains polyadenylic acid, proposed to be posttranscriptionally synthesized by an adenylyltransferase. The *in vitro* polyadenylation of messenger RNA can be inhibited by the drug cordycepin. If adenylyltransferase is responsible for the polyadenylation of messenger RNA, this drug should inhibit the activity of the enzyme *in vitro*. Adenylyltransferase was purified from Novikoff hepatoma, then briefly characterized. To test the hypothesis, the effects

of the drug and its triphosphate derivative on the reaction catalyzed by adenylyltransferase were examined. The results of this study are presented in the format of a brief scientific journal article, which also will be submitted for publication.

SECTION I

THE ATP:RNA ADENYLYLTRANSFERASES OF PROKARYOTES, EUKARYOTES, AND VIRAL CORES

LITERATURE SURVEY

Introduction

The *in vitro* formation of homopolymers of nucleic acid can be catalyzed by a variety of enzymes. DNA-dependent RNA polymerase can, by the reiterative copying of short sequences of thymidylic acid residues in the DNA template, produce a homopolymer (1). Without templates, terminal deoxynucleotidyl transferase can polymerize deoxynucleoside triphosphates into homopolydeoxynucleotides (2); and polynucleotide phosphorylase, which proved instrumental in the early deciphering of the genetic code, can catalyze the formation of homopolyribonucleotides from ribonucleoside diphosphates (3). However, when presented nucleotides of more than one base-type, these enzymes will produce heteropolymers. Unlike these enzymes, there exists one group of polymerases which synthesize only homopolymers, regardless of the type of nucleotide bases available for reaction. These unusual enzymes can be distinguished by their production of polymers, up to 200 residues in length, consisting of only one of the four common ribonucleoside 5'-triphosphates. The enzymatic activity requires a divalent metal ion and a primer. The primer is a polyribonucleotide with a free 3'-hydroxyl end to act as an initiation point for the polymerization. Homopolyribonucleotide polymerizing

enzymes which are specific for each of the ribonucleoside triphosphates, UTP (4,5), CTP (6,7), GTP (8,9), and ATP (9-62) have been isolated. Examples from a wide variety of sources, such as, bacteria (10-24), mammals (5-7, 25-45), plants (9, 46-50), avia (4, 51, 52), and viruses (57-62) have been reported. Of these enzymes those which polymerize ATP into polyadenylic acid have received the greatest attention.

Nearly fifteen years ago Edmonds and Abrams (25) reported the characteristics of an enzyme partially purified from the particulate fraction of calf thymus nuclei. This activity polymerized ATP into long chains of AMP residues. The activity required Mg^{2+}, but did not require exogenous polynucleotide as primer. The specificity for ATP as substrate, the inhibition of AMP incorporation into acid-insoluble form by the presence of the other three common ribonucleoside 5'-triphosphates or inorganic pyrophosphate, and the product length of 25 to 100 AMP residues distinguished this activity from the activities of several other enzymes that could synthesize homopolymers. Later, by eliminating contaminating RNA from their preparation, these investigators found that this enzymatic activity required a polynucleotide to prime the reaction. The primer requirement was satisfied by polyadenylic acid, but not by yeast RNA or by calf thymus DNA (26).

A second group of investigators, August *et al.* (10), also made a major early contribution to the characterization of a polyadenylate synthesizing activity. In 1962 (10), they reported the purification of the enzyme from the particulate fraction of *Escherichia coli*. Through a detailed analysis, the enzyme was found to utilize only ATP as substrate (Km = 6×10^{-5} *M*) and to be Mg^{2+}-dependent. The enzyme, optimally active at pH 10.5, could polymerize up to 200 AMP residues in chain length. Specific AMP incorporation by the enzyme was inhibited 20 to 30% in the presence of the other three ribonucleoside 5'-triphosphates. In contrast to the calf thymus enzyme, the *E. coli* enzyme could be stimulated by many different natural RNAs, for example, ribosomal RNA, transfer RNA, and the *in vitro* transcription product of *E. coli* DNA-dependent RNA polymerase. However, the synthetic homopolyribonucleotides, polyuridylic acid, polycytidylic acid or polyadenylic acid would not function as primers.

The general reaction catalyzed by an ATP-specific polyadenylate synthesizing enzyme was:

$$n\ ATP + (nucleotide)_m \xrightleftharpoons{Mn^{2+}\ or\ Mg^{2+}} (nucleotide)_{m+n} + n\ pyrophosphate.$$

The accepted systematic name, ATP:RNA adenylyltransferase (EC 2.7.7.19), is descriptive of this reaction. More

frequently, the trivial name, poly A (Adenylate) polymerase, is given to an enzyme that catalyzes this type of reaction and displays properties similar to those already mentioned.

The reports from calf thymus and *E coli* stimulated many workers to search for poly A polymerase activity in other organisms. The ubiquity of the activity was thereby well established; however, little new information was obtained. A majority of the subsequent reports were limited characterizations of partially purified enzymes. They confirmed the distinctive characteristics of the enzyme, but failed to provide a functional role for the activity in the biology of the cell.

The Enzymes from Prokaryotes

The investigators of the prokaryotic poly A polymerase made several attempts to determine its physiological role. Those that utilized the purification procedure of August *et al.* (10) found that the enzymatic activity was tightly associated with the particulate fraction of *E. coli*. This suggested that the enzyme might be an integral part of the ribosomes and, thereby, be providing a role in the translational process. Smith and August found that the activity was bound to the ribosomes of *E. coli* upon purification and speculated that the enzyme might be involved in protein synthesis. However, the activity could be released by suspension of the ribosomes in 20 m*M* glycylglycine,

pH 9.5 (12). Because this treatment was known to release electrostatically bound proteins from ribosomes (82), they stressed that the observed binding need not restrict the enzyme to a similar location *in vivo*. Hardy and Kurland elaborated on this last point by noting the failure of the enzyme-ribosome relationship to meet four criteria. The enzyme did not affect protein synthesis; it was not specific for either the 30 S or 50 S ribosomal subunit; the number of binding sites on the ribosome was not well-defined; and, as before, the enzyme was not associated with the ribosome when subjected to conditions which removed absorbed contaminants (13,14). Thus, the observed association of the enzyme was probably an artifact generated by an electrostatic attraction to the ribosomes upon cell rupture.

Another method used to determine the physiological significance of poly A polymerase was analysis of the enzyme's activity following bacteriophage infection. Investigators of prokaryotic systems were accustomed to this technique for discovering many aspects in the control of DNA and RNA metabolism in a cell. Thus, if poly A polymerase had a function essential to this metabolism, its activity might be altered upon infection. In fact, upon infection by a T-even phage, the *E. coli* particulate poly A polymerase was irreversibly inhibited. The inhibition was specific only for this enzyme, as other nucleic acid enzymes seemed to be unaffected. Appearance of the

inhibition was chloramphenicol-sensitive, indicating that a protein might be responsible (15). Later, the inhibitor was localized in the non-particulate fraction of T4-infected *E. coli* and *Shigella dysenteriae*. The inhibitor from either source could inhibit the particulate poly A polymerase from *E. coli* or from *S. dysenteriae* (16). The concomitant inhibition of the particulate enzyme and the host RNA metabolism suggested the possibility that this enzyme had some important physiological role in the RNA metabolism of the host cell (15). However, the lack of further evidence left this hypothesis undeveloped.

The last approach was a familiar method used by microbiologists for deducing the function of an enzyme. The technique was to isolate a temperature-sensitive mutant of the enzyme, then determine what function was affected at the non-permissive temperature. Terzi *et al.* (18) attempted to demonstrate that an observed thermo-sensitivity in the RNA metabolism of an *E. coli* mutant was the result of a temperature-sensitive poly A polymerase activity. In crude extracts, DNA, RNA and protein synthesis, in addition to poly A polymerase activity were arrested at the non-permissive temperature. Upon purification of the poly A polymerase, the supposed temperature sensitivity of the activity was lost. Thus, no conclusive results were obtained from their experiments. As part of the investigation, though, these workers compared the poly A polymerase and *E. coli*

DNA-dependent RNA polymerase by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. They concluded that the two enzymes "have some common subunits," and that "poly A polymerase seems to be just a special form of RNA polymerase, special in having the same polymerase core but with a different head." This statement, based upon a partially purified enzyme, raised the question of the uniqueness of poly A polymerase.

To determine whether or not the structures of poly A polymerase and RNA polymerase were related, some laboratories carried their characterization of poly A polymerase further. Based upon a previous report that RNA polymerase could synthesize a polyadenylic sequence (1) and their own SDS polyacrylamide electrophoresis data, Ohasa *et al.* concluded that the *E. coli* poly A polymerase and the α-subunit of the RNA polymerase complex ($\alpha_2\beta\beta'\sigma$) were the same polypeptide (19,20). However, this argument was severely weakened by their poor purification of the enzyme, as evidenced by the lack of conformity of the characteristics of their activity to those known for poly A polymerase. For instance, the activity was inhibited by Mn^{2+} or Mg^{2+}, and by inorganic orthophosphate. In addition, the activity lacked substrate specificity as CTP, UTP and GTP were incorporated nearly as well as ATP.

The reports in support of equating poly A polymerase and a subunit of RNA polymerase (18-20) suffered from a

failure to purify the enzyme. The first report was based on finding RNA polymerase subunits in poly A polymerase preparations (18), while the second report rested on locating poly A synthesizing activity in RNA polymerase preparations (19,20). In order to best answer the question, it was necessary to purify poly A polymerase to homogeneity. In 1973, Sippel reported the extensive purification of a poly A polymerase from *E. coli*. SDS polyacrylamide gel electrophoresis revealed the enzyme to be a single polypeptide of approximately 50,000 molecular weight, well-differentiated from any of the subunits of RNA polymerase. By Sephadex gel chromatography, the native molecular weight was estimated to be 58,000. Two inhibitors of RNA polymerase, rifampicin and streptolydigin, were ineffective against the ATP-incorporating activity of this enzyme (22). This evidence together with the distinctive kinetic characteristics already established contradicted the claim that this poly A polymerase and the α-subunit of RNA polymerase were the same. However, as will be demonstrated, there are possibly several distinct poly A polymerases in prokaryotes. Each will have to be examined for its possible relationship to RNA polymerase in order to definitively prove or disprove the hypothesis.

Poly A polymerase has been isolated from the particulate fraction of sources other than *E. coli*. The enzyme was partially purified from *S. dysenteriae* by the procedure

of August *et al.* (10). From a brief characterization, this enzyme was found to be slightly different than that from *E. coli*. Optimal activity occurred at pH 9 and at Mg^{2+} concentrations of less than 1 m*M* (16). On the other hand, pH 10.5 and 20 to 30 m*M* Mg^{2+} were optimal for the *E. coli* enzyme (10). A poly A polymerase activity has also been detected in the particulate fraction of *Pseudomonas putida*. Optimal activity occurred at pH 9.5 and at concentrations of 20 to 25 m*M* Mg^{2+}. The Km for ATP as substate was determined to be 3×10^{-4} *M*. The activity was entirely dependent upon exogenous RNA. This primer requirement could be satisfied by ribosomal RNA, transfer RNA or $Ap(Ap)_2A$, but not by DNA, polyadenylic acid or $(Ap)_4$. The product was in excess of 100 AMP residues in length covalently attached to the 3'-end of the primer molecule (24).

A comparison of these reports with those of *E. coli* demonstrate the close correspondence between prokaryotes of the characteristics of the particulate poly A polymerase. However, there are several other reports of polyadenylate synthesizing activities in *E. coli* whose characteristics are sufficiently different to suggest that there are several distinct enzymes. An early report (11) of a partially purified enzyme from *E. coli* had two features that were taken to be significant. First, the *in vitro* reaction mixture contained Mn^{2+} in addition to Mg^{2+}. No evidence was presented to determine whether either or both divalent

metal ions were required for activity. Second, the activity was detected in the non-particulate (100,000 x *g* supernatant) fraction of *E. coli*. The localization upon purification, though, was not a sufficient criterion for establishing the activity as a unique enzyme. In addition, the investigators did not examine their particulate fraction for polyadenylate synthesizing activity. Thus, it is not possible to determine that this activity was different from that detected in the particulate fraction.

The effect of bacteriophage infection upon polyadenylate synthesizing activity stimulated Gross and Alberty (17) to investigate the effect on the "non-particulate enzyme." They concluded that T4 infection resulted in a 2.5-fold increase in the non-particulate activity and in a physical modification of that enzyme. The particulate poly A polymerase was unchanged by T4 infection. While observing the changes in poly A polymerase activity upon infection, the activity of another enzyme, ATP(CTP)-transfer RNA nucleotidyltransferase, was to serve as a control. The assay conditions for these two activities were similar so the workers used only those of the latter enzyme in their analysis of the modification on poly A polymerase. The failure to assay specifically for poly A polymerase activity generates considerable doubt in the validity of their conclusion. This work also suffered from the false assumption that the determination of the cellular fraction of this

activity upon purification was sufficient evidence for a different polyadenylate synthesizing enzyme.

Modak and Srinivasan (23) have reported the purification of a primer-independent ATP-incorporating enzyme from the non-particulate fraction of *E. coli*. The activity was either Mg^{2+}- or Mn^{2+}-dependent and optimally active at pH 7.9 to 8.9. The Km for ATP as substrate was determined to be 1.4×10^{-3} *M*. The incorporation of AMP could be inhibited by streptolydigin, but not by rifampicin or α-amanitin. A sedimentation coefficient ($s^{\circ}_{20,w}$) of 8-9 S was determined for the enzyme. These characteristics are different from those already described for the particulate poly A polymerase, but the fact these workers emphasized most was that the enzyme did not require exogenous RNA for AMP incorporation. However, the activity was stimulated by the addition of ribosomal RNA and transfer RNA, an indication that their preparation might not be free of nucleic acid. Their best evidence that the enzyme did not require an exogenous primer was the sucrose gradient centrifugation of a reaction mixture incubated with exogenous primer. The ^{3}H-labeled AMP incorporation product was separated from ^{14}C-labeled exogenous primer by the centrifugation, indicating no covalent linkage between the two and, therefore, the activity's primer-independence. However, if contaminating nucleic acid were present in their enzyme preparation, it could preferentially prime the reaction and be undetected by this procedure. Thus,

their claim that exogenous primer was not required for this enzyme's activity is questionable. But the other characteristics which were established do distinguish this ATP-incorporating enzyme from the particulate ATP:RNA adenylyltransferase.

Recently, Sippel (22) has characterized a highly purified "adenyltransferase" from the particulate fraction of *E. coli*. This enzyme is similar in respect to ATP-specificity and primer-dependency to the particulate poly A polymerases already described. The Km for ATP as substrate was 5×10^{-5} *M*. Qβ RNA and *E. coli* transfer RNA satisfied the requirement for polynucleotide as primer. The activity of this enzyme is distinguished from that of other prokaryotic poly A polymerases by its preference for Mn^{2+} over Mg^{2+}, and by a pH optimum of 8. This adenylyltransferase was also inhibited by inorganic orthophosphate as well as by pyrophosphate, but not by rifampicin or streptolydigin. In addition, enzymatic activity was stimulated approximately 2.5-fold in the presence of 0.35 *M* KCl. From SDS polyacrylamide gel electrophoresis and from Sephadex G-100 gel filtration, the enzyme appeared to be a single polypeptide of approximately 58,000 molecular weight.

Finally, one report (21) of an ATP-incorporating activity from *E. coli*, designated RNA-primed polynucleotide-pyrophosphorylase, clearly pointed out the distinction of this activity from poly A polymerase. The activity

incorporated CTP and UTP as well as ATP, had pH optima at 7 and 10, was RNA-primed, and was inhibited by actinomycin. Structurally, the enzyme was about 40,000 molecular weight, composed of two 20,000 molecular weight subunits. The enzyme had a sedimentation coefficient ($s^{\circ}_{20,w}$) of 3.1 S. Even though polyadenylate can be synthesized, this enzyme is distinct from ATP:RNA adenylyltransferase.

In summary, there are several ATP-incorporating activities in prokaryotes. How many of these reported activities represent truly distinct enzymes is difficult to determine from the limited amount of data available. In general, ATP: RNA adenylyltransferase utilizes almost exclusively ATP as substrate. The divalent metal ion requirement with one exception (22) is preferentially satisfied by Mg^{2+}. Several synthetic and natural RNAs with a free 3'-hydroxyl end can satisy the requirement for exogenous polynucleotide (primer). The optimum pH is alkaline, varying from pH 8.0 to 10.5.

The product is composed almost entirely of adenylic acid, generally greater than 100 residues in length. The activity is not inhibited by rifampicin, actinomycin or streptolydigin. And, in one case (22), the enzyme is probably a single polypeptide of approximately 60,000 molecular weight.

The Enzymes from Eukaryotes

Only a brief review of the more important facts about eukaryotic enzymes will be presented. Polyadenylate

synthesizing activities have been detected in a wide variety of eukaryotic sources, including mammals (25-45), plants (9, 46-50), avia (51-52), yeast (53,54), and echinoderms (55,56). Poly A polymerase can be isolated from many parts of the cell, for example, the nucleus (25-28, 46, 47, 53, 55, 56), ribosomes (54), mitochondria (43-46), chloroplasts (9,46), or the soluble fraction (34-42, 48, 49, 51). Unlike prokaryotes, the characteristics of these activities permit a subclassification into three groups; the Mg^{2+}-dependent activities, predominantly located in the nucleus, the Mn^{2+}-dependent activities, isolated outside the nucleus, and the activities from organelles, such as mitochondria and chloroplasts. Generally, they are all ATP-specific; incorporation yields products significantly less than 100 residues in length; the activity is dependent on a RNA primer and either Mn^{2+} or Mg^{2+}; and optimal activity occurs near pH 8.

Unfortunately, most of the work on the eukaryotic enzymes have been confined to brief reports of these basic characteristics of reaction from crude preparations. The trend has been away from purification to homogeneity or extensive characterization of these enzymes, but rather toward identification of new sources. In addition, little research into the function of this activity has been made compared to the attempts with the prokaryotic enzyme.

The Mg^{2+}-specific poly A polymerase isolated from calf thymus nuclei in 1960 (25) was further purified and

characterized by Winters and Edmonds (27). They found that the primer requirement could now be satisfied by RNAs other than polyadenylic acid (28). Transfer RNA proved to be especially effective as a primer. The native molecular weight determined from gel chromatography was estimated to be 140,000 to 160,000. Incorporation of ATP into acid-insoluble form was not affected by α-amanitin, actinomycin D or cordycepin (27). These drugs also failed to inhibit the enzyme isolated from rat liver nuclei (31, 32). However, one derivative of rifamycin, AF/013, which was shown to be singularly effective against a variety of nucleic acid polymerizing enzymes (81), inhibited the rat liver Mg^{2+}-specific poly A polymerase (32).

The Mn^{2+}-specific poly A polymerase has been best purified and characterized by a group of workers headed by Bollum (34). This enzyme from the cytoplasm of calf thymus electrophoresed as a single polypeptide of 60,000 molecular weight in polyacrylamide gels containing SDS. Upon electrophoresis in the absence of SDS, the enzyme appeared as a single, coincident band of protein and enzymatic activity. The enzyme was determined to have a sedimentation coefficient ($s^{\circ}_{20,w}$) of 3.5 S from sucrose gradient centrifugation analysis. Bollum's group also has isolated this enzyme from the cytoplasm of human lymphocytes (35). The activity was similar to that of the calf thymus enzyme, including the preference for oligoadenylic acid as primer. The molecular

weight of the enzyme was 60,000. Interestingly, upon stimulation of these cells by phytohemaglutinin, the poly A polymerase activity increased three- to six-fold. This was the first demonstration of the enzyme from a "mammalian, non-viral system in which increases in . . . activity were observed with changes in the physiological state of the cell" (35). The Mn^{2+}-specific enzyme is not restricted to the cytoplasm, as this type of activity has also been detected in the nuclei of rat brain (29), yeast (53), sea urchin embryo (55, 56) and guinea pig, and ox cerebral gray and white matter (30). The characterizations of these activities, although limited, have shown that they are catalytically similar to the enzyme from the cytoplasm of calf thymus.

The enzyme from the mitochondria of rat liver was not purified sufficiently to allow many significant characterizations. The activity used Mg^{2+} and Mn^{2+}, but no primer requirement was established. In contrast to the other poly A polymerases, the activity was not inhibited by the presence of the other three ribonucleoside 5'-triphosphates (43). The activity from wheat chloroplasts has been better characterized. The divalent metal ion requirement was satisfied equally well by Mn^{2+} or Mg^{2+}. Many synthetic and natural RNAs primed the reaction. AMP incorporation was inhibited by the other three ribonucleoside triphosphates, but not by actinomycin D or α-amanitin (9). No characterization of the structure of either of these two enzymes has been made.

In addition to normal cells, there have been several reports of polyadenylate synthesizing activities in transformed cells. The activity from HeLa cytoplasm was Mn^{2+}-dependent and used polyadenylic acid or transfer RNA as primer. The drugs rifamycin AF/AP and AF/ABDMP could inhibit AMP incorporation when added at very high concentrations. It was not determined, however, whether the nucleotide incorporating activity was specific for only ATP (38). An ATP-specific polymerizing activity was also detected in the mitochondria of rat liver tumors. This activity used Mn^{2+} and Mg^{2+} and was primer-dependent. Polyadenylic acid satisfied the requirement for primer. AMP incorporation into acid-insoluble form was not inhibited when the other three ribonucleoside 5'-triphosphates were present. Compared to the mitochondrial activity of normal tissue, the specific activity of the mitochondrial enzyme from rat liver tumors was nearly the same (44, 45). Probably one of the best purified and characterized poly A polymerases of transformed cells was reported from the cytoplasm of mouse ascites cells. This Mn^{2+}-dependent enzyme had an activity optimum at pH 8.1 to 8.5. The primer requirement was fulfilled by transfer RNA, ribosomal RNA or polyadenylic acid. The enzyme had a sedimentation coefficient ($s^{\circ}_{20,\omega}$) of 4.8 S and an isoelectric point of pH 6 (36, 37).

The Enzymes from Viral Cores

The investigation into viruses as a source of poly A polymerase activity is a relatively new and, therefore, limited field. Polyadenylic acid synthesizing activity has been detected in cells infected with vesicular stomatitis virus (57,58). Samples from a crude cytoplasmic extract of virus-infected cells were assayed. However, proper controls were not included, so the observed synthesis of polyadenylic acid cannot be taken as proof of a viral poly A polymerase. On the other hand, there are several reports of poly A polymerase activity in purified virions. For example, in reovirus there is an ATP-specific incorporating activity. The activity utilized Mn^{2+} and had a pH optimum of 7.5 to 8.0. Due to a partial purification, the activity was not primer-dependent (59).

The best evidence for a viral poly A polymerase has been the study of the activity from purified cores of vaccinia virus (60-62). The incorporation was ATP-specific, Mn^{2+}-dependent, and inhibited in the presence of the other three ribonucleoside 5'-triphosphates. Enzymatic activity was stimulated upon addition of several synthetic homopolymers, for example, oligoadenylic and polyadenylic acid, however, primer-dependency was not established (60,62). SDS polyacrylamide gel electrophoresis of the partially purified vaccinia enzyme revealed polypeptides of 35,000 and 50,000 molecular weights (62).

A Summary of the ATP:RNA Adenylyltransferases

Poly A polymerase activity has been detected in a large number of biological sources. For a majority of the cases the enzyme was partially purified and its activity was minimally characterized. Because of these qualities and the repetitiveness of the data, it was not practical for many of the literature reports to be represented heretofore. A table to summarize all of the reported poly A polymerase activities is now presented (Table I). It is readily apparent from the table that poly A polymerase activity is ubiquitous in nature. Enzymatic activity has been reported in eukaryotes: nuclei, cytoplasm, mitochondria, chloroplasts, and ribosomes; in prokaryotes: particulate, and non-particulate; and eukaryotic viruses. It is also evident that the enzyme is ATP-specific; utilizes Mn^{2+} or Mg^{2+} to satisfy the divalent metal ion requirement; has optimal activity at alkaline pH; and is not inhibited by α-amanitin, actinomycin, striptolydigin or rifampicin. Many of the activities are found not to be primer-dependent, but primer-stimulated. This is a feature of characterizing impure enzyme preparations.

Although the variation between reports for some characteristics can be explained by enzyme impurity or different experimental technique, there are several trends that may be of significance. For instance, with one exception (22), the prokaryotic enzymes utilize only Mg^{2+}, while the eukaryotes have numerous examples using Mn^{2+}, Mg^{2+} or both.

TABLE I.--A General Characterization of ATP:RNA Adenylyltransferase.

Sources	ATP Specific ?	Exogenous Primer Required ?	Inhibited by Other 5 NTPs ?	Optimum pH	Metal Ion	Product Length	Protein Molecular Weight	Antibiotic Sensitivity	References
EUKARYOTES									
Nuclei									
Calf thymus	+[a]	+	+	8.2	Mg^{2+}	25-100	150,000	ama[b], cor, act :(-)	25-28
Rat liver	+	-	+		Mn^{2+},Mg^{2+}	9-31		act, cor, rif :(-); 013 :(+)	31, 32
Guinea pig brain	+	-	+	8.0	Mn^{2+}			act :(-)	30
Ox brain	+	-	+	8.0	Mn^{2+}			act :(-)	30
Rat brain		-	+	8.5	Mn^{2+}	7-59		act :(-)	29
Landschutz ascites	+	-			Mg^{2+}				33
Sea urchin embryo	+	+	+		Mn^{2+}	3-4		act :(-)	55, 56
Tobacco leaf	+	-			Mg^{2+}			act :(-)	46
Wheat leaf	+	+			Mn^{2+}	18		ama, act :(-)	9
Wheat seed	+		+	8.0	Mg^{2+}, Mn^{2+}			act :(-); rif :(+)	47
Yeast	+	+	+		Mn^{2+}		100,000	ama, act :(-)	53
Cytoplasm									
Calf thymus	+	+		8.3	Mn^{2+}		60,000		34
Human lymphocytes		-			Mn^{2+}		60,000	ama, act, rif :(-)	35
Mouse embryo	+	+	+		Mn^{2+}				40
Rat liver	+	+	+		Mn^{2+}	20-50			41, 42
HeLa		+			Mn^{2+}			AP, ABIMP :(+)	38
Mouse ascites	+	+		8.3	Mn^{2+}	30-40	(4.8 S)[c]		36, 37[d]
Novikoff hepatoma	+	+	+	7.8	Mn^{2+}		(4.1 S)[c]	ama, act :(-); 013 :(+)	
Chick embryo		-		9-9.5	Mg^{2+}				51, 52
Maize seed	+	-	+	8.4	Mn^{2+}	30			48-50
Yeast	+	+	+	8.5	Mn^{2+}, Mg^{2+}	10-20			54

Mitochondria									
Tobacco leaf	+	-			Mg^{2+}			act :(-)	46
Rat liver	+	-	-		Mn^{2+}, Mg^{2+}			rif :(-)	43
Morris hepatoma	+	+	-		Mn^{2+}, Mg^{2+}				44, 45
Chloroplasts									
Tobacco leaf	+	-			Mg^{2+}			act :(-)	46
Wheat leaf	+	+			Mn^{2+}, Mg^{2+}	27		ama, act :(-)	9
Ribosomes									
Yeast	+	+	+	8.5	Mn^{2+}, Mg^{2+}	10-20			54
PROKARYOTES									
Particulate									
E. coli	+	+	+	10.5	Mg^{2+}	40-200	(3 S)[c]	act :(-)	10, 12-16
E. coli	+	+	+	8.0	Mn^{2+}	300	58,000	rif, str :(-)	22
S. dysenteriae				9.0	Mg^{2+}				16
P. putida	+	+	+	9.5	Mg^{2+}	100	55,000	act, rif, str :(-); 013 :(+)	24,[d]
Non-Particulate									
E. coli	+	-	+	7.9-8.9	Mn^{2+}, Mg^{2+}	30-130	(8-9 S)[c]	ama, rif, str :(-)	11, 17, 23
VIRUSES									
Vesicular stomatitis		-			Mg^{2+}	80-120			57, 58
Reovirus	+	-	+	7.5-8.0	Mn^{2+}	10-20			59
Vaccinia	+	-	+		Mn^{2+}			act :(-)	60-62

[a]Symbols used; +, yes; -, no; and blank, not determined.

[b]Abbreviations used; ABDMP, rifamycin AF/ABDMP; act, actinomycin D; ama, α-amanitin; AP, rifamycin AF/AP; cor, cordycepin (3'-deoxyadenosine); rif, rifampicin; str, streptolydigin; and 013, rifamycin AF/013.

[c]Sedimentation coefficient ($s^{\circ}_{20,w}$).

[d]Data taken from elsewhere in this dissertation.

The enzymes of prokaryotes generally are optimally active at pHs greater than pH 8, the optimum for the enzymes of eukaryotes. And, comparing chain lengths of polyadenylate synthesized, the poly A polymerases from prokaryotes generally polymerize greater than 100 AMP residues, while those from eukaryotes polymerize significantly less than 100 AMP residues. The enzyme from viruses seems to be similar to the eukaryotic poly A polymerases in pH optimum and in the divalent metal ion utilized. Finally, the poly A polymerase from mitochondria could be unique in the failure of UTP, CTP and GTP together to inhibit AMP incorporation.

Polyadenylic Acid

Poly A polymerase has been found in many different types of cells throughout nature. Despite the importance of this enzyme as suggested by its ubiquity in nature, investigators have failed to deduce the role the enzyme plays in the physiology of a cell. Recently, a separate line of investigation has led to a hypothesis for the function of the eukaryotic poly A polymerase. This investigation has involved the identification of polyadenylic acid *in vivo* and the processing of functional messenger RNA from a transcriptional precursor. The purpose of this section of the survey is only to briefly cover the facts from this investigation that are relevant to an understanding of the hypothesis. For a more thorough review of the proposed

biogenesis of eukaryotic messenger RNA the reader is directed to articles by Darnell *et al.* (63), and Brawerman (80).

Polyadenylic acid was discovered *in vivo* many years ago as ribonuclease-resistant segments in RNA digests. An adenine-rich polymer was found associated with the messenger RNA of rabbit reticulocytes (64); the cytoplasm of rat liver microsomes contained polyadenylic acid sequences (65); and Edmonds and Abrams discovered that *in vivo* polyadenylic acid was responsible for priming the calf thymus nuclear poly A polymerase (26). But no significance was attached to these discoveries. The rediscovery several years later of naturally occurring polyadenylic acid in the messenger RNA isolated from vaccinia virus-infected cells (66), sparked a wide spread interest in the isolation of polyadenylate-rich RNA from a variety of sources. Of the RNA species of eukaryotic cells, it was found that only messenger RNA (mRNA) and the large, non-descrete RNA of the nucleus, called heterogeneous nuclear RNA (HnRNA), contained long polyadenylic acid sequences. These sequence were nearly 200 residues in length and located at the 3'-hydroxyl end of the molecule.

These discoveries were made with RNA isolated by new techniques that made use of the unusual properties of polyadenylic acid. Polyadenylate containing polynucleotides can be separated from other nucleic acids by extraction in aqueous phenol at pH 9 (67), or by the selective binding to "Millipore" filters at high salt (68), or by binding

to polyuridylate- (69) or polydeoxythymidylate-cellulose (70). The significance of this work was two-fold. First, a possibility existed that mRNA had a transcriptional precursor, HnRNA, that now could be identified and whose *in vivo* processing could be studied, as had been done with ribosomal RNA. The polyadenylic acid would provide a "handle" for the selective isolation and identification of these species. Second, the methods of selective isolation of polyadenylate-containing RNA could be taken advantage of for the isolation of "pure" eukaryotic message, a heretofore difficult task.

From the studies on the precursor-product relationship of HnRNA and mRNA, it was postulated that the polyadenylic acid sequence might be necessary for the proper processing, transport and functionality of mRNA (for a review, see LaTorre and Perry [71]). This hypothesis was suggested from the results of *in vivo* studies on the effect of two antibiotics on HnRNA and polyadenylic synthesis. Actinomycin blocked the synthesis of HnRNA, but had almost no effect on polyadenylic acid synthesis. Cordycepin, 3'-deoxyadenosine, preferentially inhibited polyadenylate synthesis. In either case, little functional mRNA on polyribosomes was found. The selective inhibition by cordycepin and the knowledge that the transcription was from non-polydeoxythymidylate containing DNA suggested that the polyadenylic acid sequence was a posttranscriptional product. That is, the polyadenylate

was not coded for in the template read by DNA-dependent RNA polymerase, but synthesized in the nucleus after the transcription had taken place. These qualities suggested an enzyme, separate from DNA-dependent RNA polymerase, that could synthesize long polyadenylic acid sequences on the 3'-hydroxyl end of nuclear transcription products was responsible. Of course, this description fit poly A polymerase. Thus, the enzymatic activity that had earlier been a mere curiosity was implicated as having an important physiological function in the biogenesis of mRNA.

Even though this is an attractive hypothesis, there are several additional facts about polyadenylate-containing RNA which need to be accounted for. For instance, the eukaryotic poly A polymerases are found not only in the nucleus, but also in the cytoplasm. A trivial explanation for the enzyme in the cytoplasm could be that some of the enzyme leaked out of the nucleus. However, as has already been demonstrated there are some catalytic differences for distinguishing between the enzymes isolated from the nucleus and the cytoplasm. A different explanation for the activity in the cytoplasm involves some additional findings about polyadenylic acid. The mRNA of certain viruses that replicate only in the cytoplasm contain polyadenylic acid (72). There is also evidence for the polyadenylate sequence of mRNA becoming shorter with time when in the cytoplasm (73). It is possible that the addition of ribonuclease-resistant

polyadenylic acid to mRNA provides a mechanism by which a cytoplasmic poly A polymerase could regulate certain classes of mRNA. The extent of polyadenylation would then be correlated to the effective lifetime of that mRNA. In fact, the lifetime of histone mRNA, the only mRNA found not to contain polyadenylic acid, is very short in comparison to other mRNA (69,74).

The ubiquity of poly A polymerase in nature suggests an importance for this activity in the biology of a cell. If the hypothesized *in vivo* function for the enzyme is correct, one might expect to find polyadenylated-mRNA also throughout nature. This certainly seems to be true for the nuclear and cytoplasmic fractions from the many types of eukaryotic cells studied (63-65,69,71,73). Viral (66,75) and tumor (68,70) mRNA also contain these polyadenylic acid sequences. Even mitochondrial DNA transcripts have a polyadenylic acid tract about 50 residues in length at their 3'-hydroxyl end (76).

Not all of the characteristics of the polyadenylation of nuclear mRNA, though, can be expected to apply to mitochondrial "mRNA." For example, in mitochondria there is no analogous nuclear to cytoplasmic transport for which polyadenylation could be associated. Also, cordycepin was found to inhibit the transcription of HeLa mitochondrial DNA, but did not effect the posttranscriptional synthesis of polyadenylic acid on the messenger-like RNA of mitochondria

(77). This is just opposite the effect that was observed in the nucleus (69,71). Thus, the enzymes responsible for transcription and posttranscriptional polyadenylation in mitochondria could be expected to be catalytically different than those from the nucleus. Indeed, it has already been shown that the poly A polymerases are different. Since polyadenylate synthesis probably occurs subsequent to the processing of the mitochondrial DNA transcript (77), only the imparting of functionality for translation or regulating the turnover of mRNA from the proposed model remains applicable to mitochondria.

Based upon the frequently used analogy between prokaryotes and mitochondria, one would expect to find in bacteria polyadenylated-mRNA and probably the same limitations on the model as has been described for mitochondria. Two groups of investigators attempted to demonstrate the existance of polyadenylated-mRNA in *E. coli*. Edmonds and Kopp (78) reported the isolation of polyadenylic acid segments, similar in size to those from mammalian cells, from the steady state-labeled total RNA of *E. coli*. The relative content of polyadenylic acid from those cells, though, was one-tenth of that found in mammalian cells. Perry *et al.* (79) found fault with their analysis on the basis that rather inaccurate results for estimating the polyadenylate content were obtained by using steady state-labeled RNA. In addition, Edmonds and Kopp had failed to demonstrate that the

polyadenylate was actually part of polyribosomal RNA. In order to obtain a more satisfactory answer, Perry and his coworkers isolated pulse-labeled polyribosomal mRNA. Based upon a variety of the usual techniques used for the analysis of mammalian mRNA, the polyribosomal mRNA of *E. coli* was found to contain no appreciable polyadenylic acid. Even though there are limitations to the resolution of these techniques, Perry and coworkers demonstrated that prokaryotic mRNA does not contain polyadenylate tracts even to the extent found in mitochondria.

The lack of polyadenylated-mRNA in bacteria suggests that the prokaryotic poly A polymerase might not mediate the same process *in vivo* as has been suggested for its eukaryotic counterpart. This presents a paradox; the many *in vitro* catalytically similar polyadenylate synthesizing enzymes do not have the same unique function in all levels of evolutionary development. Of course, it is also very probable that the true *in vivo* function for any poly A polymerase has not yet been discovered. Gaining a better understanding of these unusual and important enzymes will provide a basis for an understanding of their role *in vivo*.

REFERENCES

1. Chamberlin, M., and Berg, P. (1964) *J. Mol. Biol. 8*, 708-726.

2. Kato, K.; Gonclaves, J. M.; Houts, G. E.; and Bollum, F. J. (1967) *J. Biol. Chem. 242*, 2780-2789.

3. Klee, C. B. (1971) in *Procedures in Nucleic Acid Research*. Edited by S. L. Cantoni and D. R. Davies. Vol. II. New York: Harper and Row. pp. 896-911.

4. Straus, D. B., and Goldwasser, E. (1961) *J. Biol. Chem. 236*, 849-856.

5. Klemperer, H. G., and Kammen, H. O. (1961) *Biochem. Biophys. Res. Commun. 6*, 344-348.

6. Cory, J. G.; Benson, A. W.; and Girgenti, A. J. (1971) *Biochem. Biophys. Res. Commun. 42*, 778-783.

7. Edmonds, M. (1965) *J. Biol. Chem. 240*, 4621-4628.

8. Federoff, N. V., and Zinder, N. D. (1971) *Proc. Nat. Acad. Sci.* U.S.A. *68*, 1838-1843.

9. Burkard, G., and Keller, E. B. (1974) *Proc. Nat. Acad. Sci.* U.S.A. *71*, 389-393.

10. August, J. T.; Ortiz, P. J.; and Hurwitz, J. (1962) *J. Biol. Chem. 237*, 3786-3793.

11. Gottesman, M. E.; Canellakis, Z. N.; and Canellakis, E. S. (1962) *Biochim. Biophys. Acta 61*, 34-42.

12. Smith, I., and August, J. T. (1966) *J. Biol. Chem. 241*, 3525-3532.

13. Hardy, S. J. S., and Kurland, C. G. (1966) *Biochemistry 5*, 3668-3676.

14. Hardy, S. J. S., and Kurland, C. G. (1966) *Biochemistry 5*, 3676-3684.

15. Ortiz, P. J.; August, J. T.; Watanabe, M.; Kaye, A. M.; and Hurwitz, J. (1965) *J. Biol. Chem. 240*, 423-431.

16. Colvill, A. J. E., and Terzi, M. (1968) *Biochim. Biophys. Acta 155,* 394-400.

17. Gross, H. J., and Alberty, H. (1962) *Biochem. Biophys. Res. Commun. 46,* 1581-1587.

18. Terzi, M.; Cascino, A.; and Urbani, C. (1970) *Nature 226,* 1052-1054.

19. Ohasa, S., and Tsugita, A. (1972) *Nature New Biol. 240,* 35-38.

20. Ohasa, S.; Tsugita, A.; and Mii, S. (1972) *Nature New Biol. 240,* 39-41.

21. Schäfer, R.; Zillig, W.; and Priess, H. (1972) *Fed. Eur. Biochem. Soc. Lett. 25,* 87-90.

22. Sippel, A. E. (1973) *Eur. J. Biochem. 37,* 31-40.

23. Modak, M. J., and Srinivasan, P. R. (1973) *J. Biol. Chem. 248,* 6904-6910.

24. Payne, K. J., and Boezi, J. A. (1970) *J. Biol. Chem. 245,* 1378-1387.

25. Edmonds, M., and Abrams, R. (1960) *J. Biol. Chem. 235,* 1142-1149.

26. Edmonds, M., and Abrams, R. (1962) *J. Biol. Chem. 237,* 2636-2642.

27. Winters, M. A., and Edmonds, M. (1973) *J. Biol. Chem. 248,* 4756-4762.

28. Winters, M. A., and Edmonds, M. (1973) *J. Biol. Chem. 248,* 4763-4768.

29. Dravid, A. R.; Pete, N.; and Mandel, P. (1971) *J. Neurochem. 18,* 299-306.

30. Kato, T., and Kurokawa, M. (1970) *Biochem. J. 116,* 599-609.

31. Niessing, J., and Sekeris, C. E. (1972) *Fed. Eur. Biochem. Soc. Lett. 22,* 83-88.

32. Niessing, J., and Sekeris, C. E. (1973) *Nature New Biol. 243,* 9-12.

33. Burdon, R. H. (1963) *Biochem. Biophys. Res. Commun. 11,* 472-476.

34. Tsiapalis, C. M.; Dorson, J. W.; DeSante, D. M.; and Bollum, F. J. (1973) *Biochem. Biophys. Res. Commun. 50,* 737-743.

35. Coleman, M. S.; Hutton, J. J.; and Bollum, F. J. (1974) *Nature 248,* 407-409.

36. Giron, M. L., and Huppert, J. (1972) *Biochim. Biophys. Acta 287,* 438-447.

37. Giron, M. L., and Huppert, J. (1972) *Biochim. Biophys. Acta 287,* 448-455.

38. Mans, R. J. (1973) *Fed. Eur. Biochem. Soc. Lett. 33,* 245-246.

39. Edmonds, M., and Abrams, R. (1957) *Biochim. Biophys. Acta 26,* 226-227.

40. Hadidi, A., and Sagar Sethi, V. (1974) *Fed. Proc. Fed. Amer. Soc. Exp. Biol. 33,* 1485.

41. Klemperer, H. G. (1963) *Biochim. Biophys. Acta 72,* 416-420.

42. Klemperer, H. G. (1965) *Biochim. Biophys. Acta 95,* 251-261.

43. Jacob, S. T., and Schindler, D. G. (1972) *Biochem. Biophys. Res. Commun. 48,* 126-134.

44. Jacob, S. T.; Schindler, D. G.; and Morris, H. P. (1972) *Science 178,* 639-640.

45. Rose, K. M., and Jacob, S. T. (1974) *Fed. Proc. Fed. Amer. Soc. Exp. Biol. 33,* 1485.

46. Sachar, R. C. (1968) *Biochim. Biophys. Acta 169,* 58-66.

47. Sasaki, K., and Tazawa, T. (1973) *Biochem. Biophys. Res. Commun. 52,* 1440-1449.

48. Mans, R. J., and Walter, T. J. (1971) *Biochim. Biophys. Acta 247,* 113-121.

49. Walter, T. J., and Mans, R. J. (1970) *Biochim. Biophys. Acta 217,* 72-82.

50. Mans, R. J. (1971) *Biochem. Biophys. Res. Commun. 45,* 980-983.

51. Chung, C. W.; Mahler, H. R.; and Enrione, M. (1960) *J. Biol. Chem. 235,* 1448-1461.

52. Venkataraman, P. R., and Mahler, H. R. (1963) *J. Biol. Chem. 238*, 1058-1067.

53. Haff, L. A., and Keller, E. B. (1973) *Biochem. Biophys. Res. Commun. 51*, 704-710.

54. Twu, J. S., and Bretthauer, R. K. (1971) *Biochemistry 10*, 1576-1582.

55. Hyatt, E. A. (1967) *Biochim. Biophys. Acta 142*, 246-253.

56. Hyatt, E. A. (1967) *Biochim. Biophys. Acta 142*, 254-262.

57. Galet, H., and Prevec, L. (1973) *Nature New Biol. 243*, 200-203.

58. Reichman, M. E.; Villareal, L. P.; Kohne, D.; Lesnaw, J.; and Holland, J. J. (1974) *Virology 58*, 240-249.

59. Stoltzfus, C. M.; Morgan, M.; Banerjee, A. K.; and Shatkin, A. J. (1974) *J. Virol. 13*, 1338-1345.

60. Brown, M.; Dorson, J. W.; and Bollum, F. J. (1973) *J. Virol. 12*, 203-208.

61. Kates, J., and Beeson, J. (1970) *J. Mol. Biol. 50*, 19-33.

62. Moss, B.; Rosenblum, E. N.; and Paoletti, E. (1973) *Nature New Biol. 245*, 59-62.

63. Darnell, J. E.; Jelinek, W. R.; and Molloy, G. R. (1973) *Science 181*, 1215-1221.

64. Lim, L., and Canellakis, E. S. (1970) *Nature 227*, 710-712.

65. Hadjivassiliou, A., and Brawerman, G. (1966) *J. Mol. Biol. 20*, 1-7.

66. Kates, J. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35*, 743-752.

67. Brawerman, G.; Mendecki, J.; and Lee, S. Y. (1972) *Biochemistry 11*, 792-798.

68. Lee, S. Y.; Mendecki, J.; and Brawerman, G. (1971) *Proc. Nat. Acad. Sci.* U.S.A. *68*, 1331-1335.

69. Adesnik, M.; Salditt, M.; Thomas, W.; and Darnell, J. E. (1972) *J. Mol. Biol. 71,* 21-30.

70. Edmonds, M., and Caramela, M. G. (1969) *J. Biol. Chem. 244,* 1314-1324.

71. LaTorre, J., and Perry, R. P. (1973) *Biochim. Biophys. Acta 335,* 93-101.

72. Yogo, Y., and Wimmer, E. (1972) *Proc. Nat. Acad. Sci.* U.S.A. *69,* 1877-1882.

73. Sheiness, D., and Darnell, J. E. (1973) *Nature New Biol. 241,* 265-268.

74. Adesnik, M., and Darnell, J. E. (1972) *J. Mol. Biol. 67,* 395-406.

75. Armstrong, J. A.; Edmonds, M.; Phillips, B. A.; and Vaughan, M. H. (1972) *Science 176,* 526-528.

76. Hirsch, M., and Penman, S. (1973) *J. Mol. Biol. 80,* 379-391.

77. Hirsch, M., and Penman, S. (1974) *J. Mol. Biol. 83,* 131-142.

78. Edmonds, M., and Kopp, D. W. (1970) *Biochem. Biophys. Res. Commun. 41,* 1531-1537.

79. Perry, R. P.; Kelley, D. E.; and LaTorre, J. (1972) *Biochem. Biophys. Res. Commun. 41,* 1593-1600.

80. Brawerman, G. (1974) *Annu. Rev. Biochem. 43,* 621-642.

81. Gerard, G. F.; Gurgo, C.; Grandgenett, D. P.; and Green, M. (1973) *Biochem. Biophys. Res. Commun. 53,* 194-201.

82. Petermann, M. L. (1964) *The Physical and Chemical Properties of Ribosomes.* New York: Elsevier, p. 174.

SECTION II

A KINETIC AND STRUCTURAL CHARACTERIZATION OF ATP:RNA ADENYLYLTRANSFERASE FROM *PSEUDOMONAS PUTIDA*

ABSTRACT

A catalytic and structural study of ATP:RNA adenylyltransferase (EC 2.7.7.19) from the particulate fraction of *Pseudomonas putida* was made. During the large-scale purification of this enzyme, designated adenylyltransferase B, a previously undetected ATP-incorporating activity, designated adenylyltransferase A, was observed. Adenylyltransferases A and B were indistinguishable catalytically. The incorporation of AMP from ATP into a polymeric product by the adenylyltransferases required Mg^{2+} and a polyribonucleotide as primer. The rate of incorporation of CTP was about 12% that of AMP, while GTP, UTP, 2'-dATP or ADP were not utilized by these adenylyltransferases. The Km for ATP was 1-2 x 10^{-4} *M*. The primer requirement was satisfied by transfer RNA, ribosomal RNA, poly (C), $Ap(Ap)_2A$, and ApApA. DNA, Qβ RNA, poly(A), poly(U), poly(dT), or ApA were not effective primers. The Km for ribosomal RNA, in terms of nucleotide phosphate, was 2-4 x 10^{-4} *M*. The polymerization reaction catalyzed by the two adenylyltransferases gave the fastest rate at pH 9.5, and was inhibited by inorganic pyrophosphate but not orthophosphate.

Adenylyltransferases A and B were distinguishable by their chromatographic and sedimentation properties.

Adenylyltransferases A and B were resolved by phosphocellulose and by poly(U)-Sepharose chromatographies. When chromatographed on Bio-Gel P-100, adenylyltransferase A was excluded, while B penetrated the gel. By sedimentation through glycerol gradients, adenylyltransferase A was determined to have a sedimentation coefficient ($s^{\circ}_{20,w}$) of 9.3 S and B of 4.3 S. The molecular weight of adenylyltransferase A was estimated to be 185,000 and that of adenylyltransferase B to be 50,000 to 60,000. Apparently, adenylyltransferase A was generated from adenylyltransferase B during the purification.

Two derivatives of the antibiotic rifamycin, AF/013 and AF/DNFI, inhibited the AMP incorporation catalyzed by adenylyltransferases A and B. The polymerization rate by either enzyme was inhibited 50% at 5 μg per ml of AF/013 or 10 μg per ml AF/DNFI. The 5'-triphosphate derivative (3'-dATP) of the drug cordycepin (3'-deoxyadenosine) was a competitive inhibitor with ATP for both adenylyltransferases. The Ki for 3'-dATP was 6-10 x 10^{-4} *M*. Several other analogs of ATP, 2'-dATP, 2'-*O*-methylATP, or the fluorescent 3-β-*D*-ribofuranosylimidazo [2, 1-i] purine 5'-triphosphate did not affect the activity of adenylyltransferase A or B. Poly(U) and poly(dT) were competitive inhibitors of the ribosomal RNA-primed polymerization reaction. The Ki for poly(U) or poly(dT), in terms of nucleotide phosphate, was 4-10 x 10^{-6} *M* for adenylyltransferases A and B. The inhibition was a result of the competition between the non-priming

poly(U), or poly(dT), and ribosomal RNA for the primer-binding site on the enzyme.

INTRODUCTION

ATP:RNA adenylyltransferase (EC 2.7.7.19) catalyzes the polymerization of ATP into polyadenylic acid. This enzyme preferentially utilizes ATP as substrate. Its activity depends upon a divalent metal ion and a primer. The primer is a polyribonucleotide with a free 3'-hydroxyl end to act as an initiation point for the polymerization. Because of the nature of the product it polymerizes, this enzyme is commonly referred to by the trivial name, poly A (Adenylate) polymerase. Poly A polymerase is ubiquitous in nature, having been isolated from such a wide variety of sources as mammals (1-6), plants (7), avia (8), yeast (9,10), echinoderms (11,12), bacteria (13-16) and viruses (17,18).

It has been widely observed that polyadenylic acid (poly[A]) sequences are covalently linked to the heterogeneous DNA-like RNA in the nucleus (HnRNA) and to messenger RNA (mRNA) in the cytoplasm of eukaryotic cells (for a review, see Darnell *et al.* [19], and Brawerman [20]). Polyadenylation appears to be important for the proper processing of HnRNA to mRNA and for the transport of functional mRNA to the cytoplasm. The poly(A) sequences are believed to arise by a posttranscriptional addition to the 3'-hydroxyl end

of HnRNA. This synthesis is speculated to be catalyzed not by a DNA-dependent RNA polymerase, but by a poly A polymerase-type enzyme. The mRNA of eukaryotic mitochondria also contain 3'-terminal poly(A) sequences (21,22). An examination of *E. coli* mRNA, however, failed to detect any similar poly(A) tracts (23,24).

The ubiquity of poly A polymerase in nature certainly suggests an importance for this activity in the biology of the cell. In eukaryotes this function might be the poly(A) addition on DNA transcripts. However, none of the investigations on this enzyme have provided direct evidence of its *in vivo* role. The reports on the enzymes from prokaryotes have presented many different structural and catalytic properties for poly A polymerase, making for a confusing situation. August *et al.* reported the first purification of a particulate poly A polymerase from *E. coli* (13). Recently, Sippel purified a particulate enzyme from *E. coli*, different from the August enzyme in its pH optimum and preference for metal ion (14). From the non-particulate fraction of *E. coli* there was isolated an apparent primer-independent enzyme, also with unique pH optimum, metal ion preference and molecular size (15). Another ATP-incorporating activity, RNA-primed polynucleotide pyrophosphorylase, was detected in *E. coli* by Schäfer *et al.* (43). However, this enzyme was distinguishable from poly A polymerase, since other nucleotides were incorporated as efficiently as ATP.

It is difficult to determine from the data whether or not there are several distinct poly A polymerase activities in a prokaryotic cell. Such a decision must await the further characterization of the reported poly A polymerases.

When the work for this report was initiated, no structural and few kinetic properties of poly A polymerase from prokaryotes was known. It was the objective of this project to further purify the particulate enzyme from the bacterium *Pseudomonas putida* first reported by Payne and Boezi (16), then obtain structural and further kinetic characteristics of it. During the course of the purification, a previously undetected ATP:RNA adenylyltransferase activity was observed. This new activity and that previously described (16) were indistinguishable catalytically, but they differed in molecular size and in chromatographic properties. That which follows is the structural and kinetic characterization of these adenylyltransferases.

EXPERIMENTAL PROCEDURE

Materials

DEAE-Sephadex (A-25), Sepharose 4B and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals, Inc. and Whatman cellulose phosphate (P-1) was from H. Reeve Angel, Inc. Bio-Gel P-100 (100-200 mesh) and P-200 (50-100 mesh) were obtained from Bio-Rad Laboratories. Calf thymus DNA, herring sperm DNA, poly(A), poly(C), poly(G), poly(I), poly(U), ApA, ApApA, PMSF,[1] hemoglobin (type I), cytochrome c (type VI), ribonuclease A (type I-A), pepsin, and bovine serum albumin were from Sigma Chemical Co. Poly(dA), poly(dT), and Qβ viral RNA were obtained from Miles Laboratories, Inc. Pancreatic DNase I and *E. coli* alkaline phosphatase (both electrophoretically purified) were from Worthington Biochemical Corp. Dithiothreitol, $Ap(Ap)_2A$, 2'-dATP, ε-ATP, and all unlabeled 5'-triphosphate derivatives of the ribonucleotides were obtained from P-L Biochemicals, Inc. Nitrocellulose membrane filters (type B-6) were obtained from Schleicher and Schuell, Inc. ^{3}H-labeled nucleotides were purchased from Schwarz/Mann. Rifamycin derivatives were the

[1]The abbreviations used that are not listed in the *Journal of Biological Chemistry 249*, 1 (1974) are: PMSF, phenylmethylsulfonyl flouride; ε-ATP, 3-β-*D*-ribofuranosyl-imidazo [2, 1-i] purine 5'-triphosphate; 3'-dATP, 3'-deoxyadenosine 5'-triphosphate; poly(Am), poly(2'-*O*-methyl adenylic acid); poly(Um), poly(2'-*O*-methyluridylic acid); AmTP, 2'-*O*-methyladenosine 5'-triphosphate; and SDS, sodium dodecyl sulfate.

gifts of Dr. Luigi Silvestri, Gruppo Lepetit, Inc., Milan, Italy. ^{3}H-labeled R17 viral RNA was a gift from B. Hall, University of Washington, Seattle. Chromatographically pure 3'-dATP was a figt from R. Desrosiers of this department. *B. subtilus* ribosomal RNA, yeast transfer RNA, *E. coli* ribosomal and transfer RNA, poly(Am), poly(Um), and AmTP were all gifts from F. Rottman also of this department. *P. putida* ribosomal RNA was prepared according to a procedure previously described (16). Ribosomal RNA and transfer RNA were chromatographed electrophoretically on polyacrylamide gels and found to be homogeneous.

Analytical Methods

Protein concentration was determined by the method of Lowry *et al.* (25) with bovine serum albumin as the standard. Calf thymus DNA was denatured by heating to 100° for 10 min followed by quick cooling in ice. Qβ RNA was purified by sedimentation through a sucrose gradient before use. The concentrations of Qβ RNA, oligoadenylic acid and calf thymus DNA were determined spectrophotometrically based on the extinction coefficient $E^{1\%}_{260} = 200$. The molar extinctions, $\varepsilon(P)$, used to determine the concentration of ribosomal and transfer RNA at 260 nm were 7.4×10^3 (26) and 7.2×10^3 (27), respectively. The molar extinctions, $\varepsilon(P)$, for polyribonucleotides were: 10.5×10^3 at 257 nm, 9.2×10^3 at 260 nm and 6.5×10^3 at 267 nm for poly(A), poly(U), and poly(C), respectively (28) and 10.2×10^3 and 9.4×10^3 at 259 nm for poly(Am) and poly(Um), respectively

(29). The molar extinctions, ε(P), for polydeoxyribonucleotides were 8.1×10^3 and 9.7×10^3 at 260 nm for poly(dT) and poly(dA), respectively (26).

Growth of Pseudomonas putida

P. putida (ATCC 12633) was grown in a medium which contained the following in grams per liter: 6, Na_2HPO_4; 3, KH_2PO_4; 8, NaCl; 5, bacto yeast extract (Difco); 5, bacto-tryptone (Difco); and 5, glucose. *P. putida* was grown in 100-liter volumes in a New Brunswick Fermacell, model F-130, at 32° with aeration at 140 liters per min. After entering early stationary phase, the cells were harvested in a Sharples centrifuge. The 1000 to 1300 g (wet weight) of packed cells were stored at -20°.

Assay of ATP:RNA Adenylyltransferase

The standard assay measured the conversion of [^{3}H] ATP into acid-insoluble product. Unless otherwise indicated, the reaction mixture (0.25 ml) contained 30 m*M* glycine-NaOH (pH 9.5), 20 m*M* $MgCl_2$, 0.8 m*M* [^{3}H]ATP (about 2×10^3 cpm per nmol), saturating amounts of *P. putida* ribosomal RNA, 100 m*M* KCl and an appropriate amount of enzyme. After incubation at 37° for 10 min, the reaction mixture was mixed with 0.1 ml of 0.1% SDS. Cold 10% trichloroacetic acid -1% sodium pyrophosphate (5ml) and 250 μg of herring sperm DNA were added. After 10 min at 0-4°, insoluble material was collected on a nitrocellulose filter. The

filter was washed with three 5 ml portions of cold 10% trichloroacetic acid -1% sodium pyrophosphate, dried and then monitored for radioactivity using liquid scintillation spectrometry. The scintillation fluid (5 ml) contained 4 g of 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)] thiophene per liter of toluene. One unit of enzyme activity is defined as that amount of enzyme which catalyzes the incorporation of one nmol of AMP into an acid-insoluble product in 10 min at 37°. Specific activity is expressed as units per mg of protein. The amounts of enzyme activity which were added to the reaction mixture were within the activity range that gave direct proportionality to the rate of AMP incorporation.

Preparation of Poly(U)-Sepharose

Poly(U)-Sepharose was prepared by a procedure similar to that of Poonian *et al.* (30). After activation by cyanogen bromide, the Sepharose 4B was thoroughly washed by filtration with 25 volumes of 100 m*M* potassium phosphate (pH 7.5). A slurry of activated Sepharose 4B was obtained by mixing it with an equal volume of the above buffer. To this slurry was added an equal volume of poly(U), 2.4×10^{-4} *M* nucleotide phosphate, and the entire volume was stirred slowly for 18 hours at 4°. The final product was 2×10^{-7} mol nucleotide phosphate of poly(U) bound per ml of packed Sepharose as determined by the method of Failla and Santi (31).

RESULTS

Purification of ATP:RNA Adenylyltransferases A and B

The entire purification was performed at 0-4°.

Preparation of Initial Extract.--A block of frozen *Pseudomonas putida* cells (500 g wet weight) was cut into small pieces, then 500 ml of buffer (10 m*M* Tris-HCl [pH 8.0],[2] 10 m*M* $MgCl_2$, 0.1 m*M* EDTA, 5 m*M* 2-mercaptoethanol and 0.1 m*M* PMSF) was added. The mixture was stirred until a smooth suspension was obtained. The cell suspension was passed thrice through the pressure chamber of a Manton-Gaulin lab homogenizer, which was maintained at 7000 p.s.i. Pancreatic DNase I (0.5 µg per ml) was added to the cell extract and the extract was stirred for 15 min. Following centrifugation at 16,000 x *g* for 60 min, the resulting supernatant fraction was carefully decanted and diluted to 840 ml with the above buffer (Initial Extract).

Sedimentation.--The initial extract fraction was centrifuged at 80,000 x *g* for 4 hours. The supernatant fraction was carefully removed by suction and discarded, and the fluffy pellet was stored at -20° overnight (80,000 x *g* Pellet).

Solubilization.--The 80,000 x *g* pellet fraction was thawed and homogenized in buffer (100 m*M* glycine-NaOH [pH

[2]The pH of buffers used in the purification were measured at 0-4°.

10.6], 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF) using a Potter-Elvejnem homogenizer. After adjusting the volume to 420 ml, the suspension was centrifuged at 80,000 x *g* for 2 hours. The supernatant fraction was carefully decanted (pH 10.6 Supernatant).

pH Fractionation.--Solid KCl was added slowly to the pH 10.6 supernatant fraction to a final concentration of 1 *M*. Cold 1 *M* HCl was then added dropwise with stirring until pH 5.0 was reached. The suspension was stirred for 2 hours, then centrifuged at 16,000 x *g* for 20 min. The supernatant fraction was dialyzed overnight against 14 liters of 10 m*M* Tris-HCl (pH 8.0), 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF (pH 5 Supernatant).

pH Concentration.--Cold 1 *M* HCl was added dropwise with stirring to the pH 5 supernatant fraction until pH 3.2 was reached. After stirring for 15 min, the suspension was centrifuged at 16,000 x *g* for 15 min. The supernatant fraction was poured off and discarded. The pellet was dissolved in 100 m*M* glycine-NaOH (pH 10.1), 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF by stirring for 4 hours. After the volume of the solution was adjusted to 300 ml, solid KCl was added to a final concentration of 1 *M*. The solution was dialyzed overnight against 12 liters of 10 m*M* Tris-HCl (pH 8.0), 1 *M* KCl, 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF (pH 3.2 Precipitate).

Bio-Gel P-200 Gel Filtration.--Bio-Gel P-200, previously equilibrated with 10 m*M* Tris-HCl (pH 8.0), 1 *M* KCl, 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF, was used to prepare a column 10 x 87 cm. The column was rigged for ascending flow and thoroughly washed before use. The pH 3.2 precipitate fraction was applied to the column and eluted by the addition of the same buffer. The effluent pattern of the column, Figure 1, shows good resolution between ATP-incorporating activity and the majority of the A_{280}-absorbing material. Some ATP-incorporating activity, however, did elute with the A_{280}-absorbing material. The amount varied up to 15% of the total enzyme activity recovered from the column, depending upon the purification trial. The peak fractions of ATP-incorporating activity, corresponding to elution volumes from 3.6 to 4.3 liters, were pooled and dialyzed against 28 liters of 10 m*M* Tris-HCl (pH 9.1), 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF (Bio-Gel P-200).

DEAE-Sephadex Chromatography.--A column (2.5 x 20 cm) of DEAE-Sephadex was prepared, then washed thoroughly with 10 m*M* Tris-HCl (pH 9.1), 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF. The Bio-Gel P-200 enzyme fraction was applied to the column. The column was then washed with 150 ml of the above buffer. Upon application of a 500 ml linear gradient from 0 to 1 *M* KCl in the above buffer, the ATP-incorporating activity was eluted as a single peak at 0.2 *M*

KCl, separated from A_{260}-absorbing material. The peak fractions of the ATP-incorporating activity were pooled and dialyzed against 12 liters of 10 m*M* potassium phosphate (pH 7.1), 250 m*M* KCl, 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF (DEAE-Sephadex).

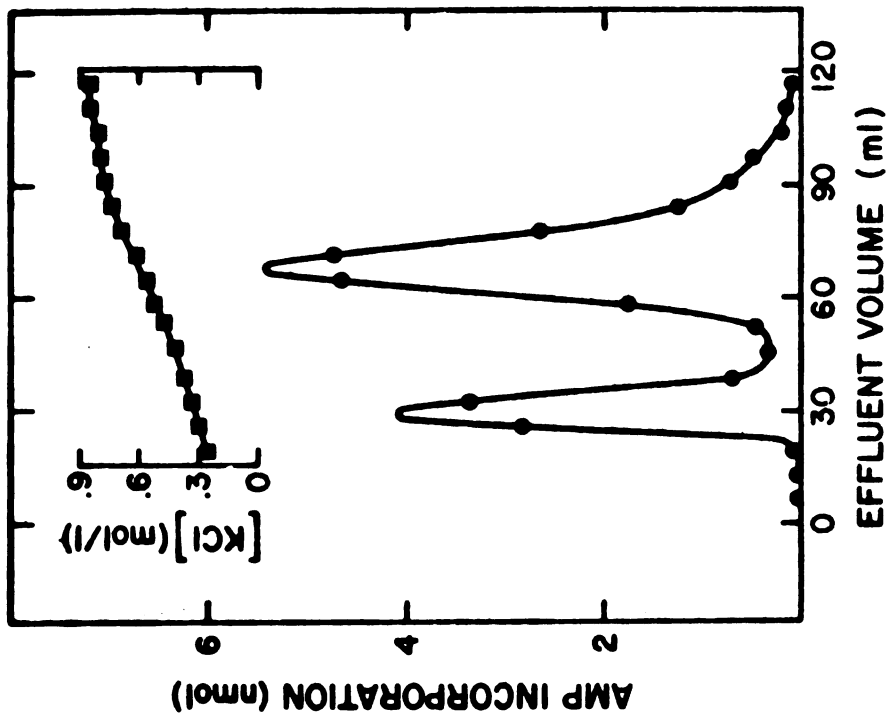
Phosphocellulose Chromatography.--A column (1.2 x 8 cm) of phosphocellulose was prepared and washed thoroughly with 10 m*M* potassium phosphate (pH 7.1), 250 m*M* KCl, 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF. After applying the DEAE-Sephadex enzyme fraction to the column, the column was washed with 25 ml of the above buffer. ATP-incorporating activity was eluted from the column by application of a 100 ml linear gradient from 0.25 to 1 *M* KCl in the above buffer. As shown in Figure 2, two peaks of ATP-incorporating activity were eluted from the column, one at 0.3 *M* KCl, designated A, and the other at 0.6 *M* KCl, designated B. This elution pattern was reproducible, but depending upon the purification trial, varying amounts of peak A activity were obtained, ranging from 5 to 50% of the total activity eluted from the column. The fractions containing the activity of peaks A and B were pooled separately and dialyzed against 4 liters of 10 m*M* potassium phosphate (pH 7.1), 250 m*M* KCl, 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF (Phosphocellulose A and B).

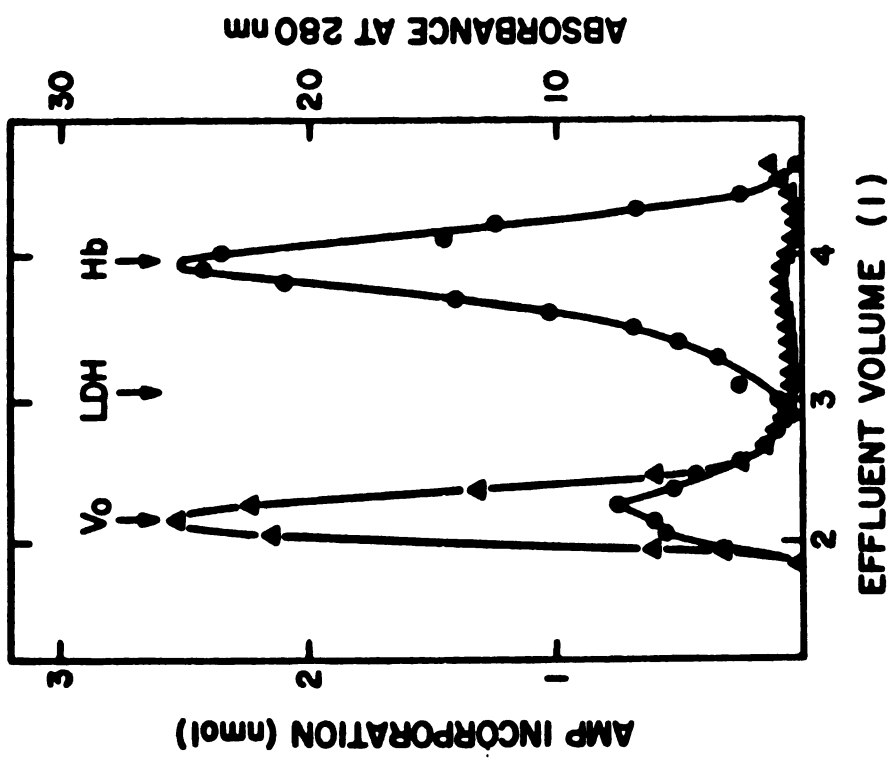
Summary and Comments on Purification Through Phosphocellulose Chromatography.--A summary of a typical

Figure 1.--(left panel) Bio-Gel P-200 gel filtration of fraction pH 3.2 precipitate. The pH 3.2 precipitate enzyme fraction was applied to a column (10 x 87 cm) of Bio-Gel P-200 equilibrated with 10 m*M* Tris-HCl (pH 8.0), 1 *M* KCl, 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF. The column was developed under conditions of ascending flow at a rate of 1.25 ml per min by the continual addition of the same buffer. Then, 1750 ml of the 2200 ml void volume were collected, followed by 140 21-ml fractions. For the ATP-incorporating activity assay, 25 µl samples of the fractions were assayed in the reaction mixture described under EXPERIMENTAL PROCEDURE. Absorbance measured at 280 nm (▲); and nmol AMP incorporated (●).

In order to calibrate the column for molecular size, a solution of Blue Dextran (V_o), lactate dehydrogenase (LDH), and hemoglobin (Hb) was chromatographed on the same column under identical conditions. The detection of Blue Dextran and hemoglobin in the effluent was made spectrophotometrically at 650 and 410 nm, respectively. Lactate dehydrogenase was assayed spectrophotometrically at 340 nm using the substrates NADH and pyruvate (40).

Figure 2.--(right panel) Phosphocellulose chromatography of fraction DEAE-Sephadex. The DEAE-Sephadex enzyme fraction was applied at 0.75 ml per min to a column (1.2 x 8 cm) of phosphocellulose equilibrated with 10 m*M* potassium phosphate (pH 7.1), 250 m*M* KCl, 5 m*M* 2-mercaptoethanol, 0.1 m*M* PMSF. Following application of the enzyme fraction, the column was washed with 25 ml of the same buffer. A 100 ml linear gradient from 0.25 to 1.0 *M* KCl in 10 m*M* potassium phosphate (pH 7.1), 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF was used to elute the enzyme. The column was developed at 1 ml per min and 54 fractions of 2.2 ml each were collected. For the assay of ATP-incorporating activity, 5 µl samples of the fractions were assayed in the reaction mixture described under EXPERIMENTAL PROCEDURE. The KCl concentration as determined from conductivity measurements (■); and nmol AMP incorporated (●).


AMP INCORPORATION (nmol)
6
4
2
[KCl] (mol/l)
0
3
6
9
0
30
60
90
120
EFFLUENT VOLUME (ml)


ABSORBANCE AT 280 nm
30
20
10
AMP INCORPORATION (nmol)
3
2
1
Vo
LDH
Hb
2
3
4
EFFLUENT VOLUME (l)

purification is presented in Table I. The purification procedure used was similar to that described by Payne and Boezi (16). The scale of the purification, however, was increased over ten-fold, from 40 g to 500 g of cells. Gel filtration was on Bio-Gel P-200 rather than Sephadex G-100; all buffers used after DEAE-Sephadex chromatography contained at least 250 m*M* KCl; and 5 m*M* 2-mercaptoethanol and 0.1 m*M* PMSF were present in all buffers throughout the purification. The activity of enzyme fraction phosphocellulose B, but not of fraction phosphocellulose A, was detected by Payne and Boezi (16). Those authors described some of the catalytic properties of fraction phosphocellulose B, but did not examine its molecular structure.

TABLE I.--Purification of ATP:RNA Adenylyltransferases A and B.

Enzyme Fraction	Specific Activity	Total Units
Initial Extract	14	260,000
pH 10.6 Supernatant	16	96,000
pH 5 Supernatant	60	70,000
pH 3.2 Precipitate	120	70,000
Bio-Gel P-200	440	58,000
DEAE-Sephadex	990	30,000
Phosphocellulose A	2,000	5,500
Phosphocellulose B	6,900	8,300

Bio-Gel P-100 Gel Filtration.--Bio-Gel P-100 gel filtration was used as the last step in the purification procedure. When enzyme fraction phosphocellulose A or B was chromatographed on a Bio-Gel P-100 column, however, most of the enzyme activity was lost. Furthermore, the protein concentrations of the peak fractions that contained the enzyme activities A and B which were recovered after gel filtration were not measureable by the method of Lowry *et al.* (25). Consequently, specific enzymatic activities for A and B through Bio-Gel P-100 gel filtration cannot be reported. Significant purifications of the enzymes, however, were undoubtedly achieved by gel filtration. For example, as analyzed by SDS polyacrylamide gel electrophoresis, polypeptide chains of molecular weights in the 10,000 to 20,000 range, which were present in fraction phosphocellulose B and which amounted to 60% (by weight) of the protein (see Figure 8), were removed from enzyme activity B by Bio-Gel P-100 gel filtration.

If Bio-Gel P-100 gel filtration was performed in the presence of 100 µg per ml bovine serum albumin, most of the enzyme activities of A and B were recovered after gel filtration. When this procedure was to be used in the purification, fractions phosphocellulose A and B were made 100 µg per ml with respect to bovine serum albumin and concentrated 20- to 40-fold to approximately 1 ml using

an Amicon Ultrafiltrator, Model 8 MC with PM-10 filter (Concentrated Phosphocellulose A and B).

Bio-Gel P-100, previously equilibrated with 10 m*M* potassium phosphate (pH 7.1), 1 *M* KCl, 5 m*M* 2-mercaptoethenol, 0.1 m*M* PMSF and 100 µg per ml bovine serum albumin, was used to prepare a column 0.9 x 90 cm. The concentrated phosphocellulose A enzyme fraction (1 ml) was applied to the column and eluted by the addition of the above buffer. In a subsequent run of the column, the concentrated phosphocellulose B enzyme fraction (1 ml) was chromatographed. Enzyme activities A and B eluted as single peaks of ATP-incorporating activity, as seen in Figure 3. The peak fractions of ATP-incorporating activity for A and B were separately pooled and concentrated by dialysis against 50% glycerol, 10 m*M* potassium phosphate (pH 7.1), 250 m*M* KCl, 5 m*M* 2-mercaptoethanol, and 0.1 m*M* PMSF (Adenylyltransferase A and B).

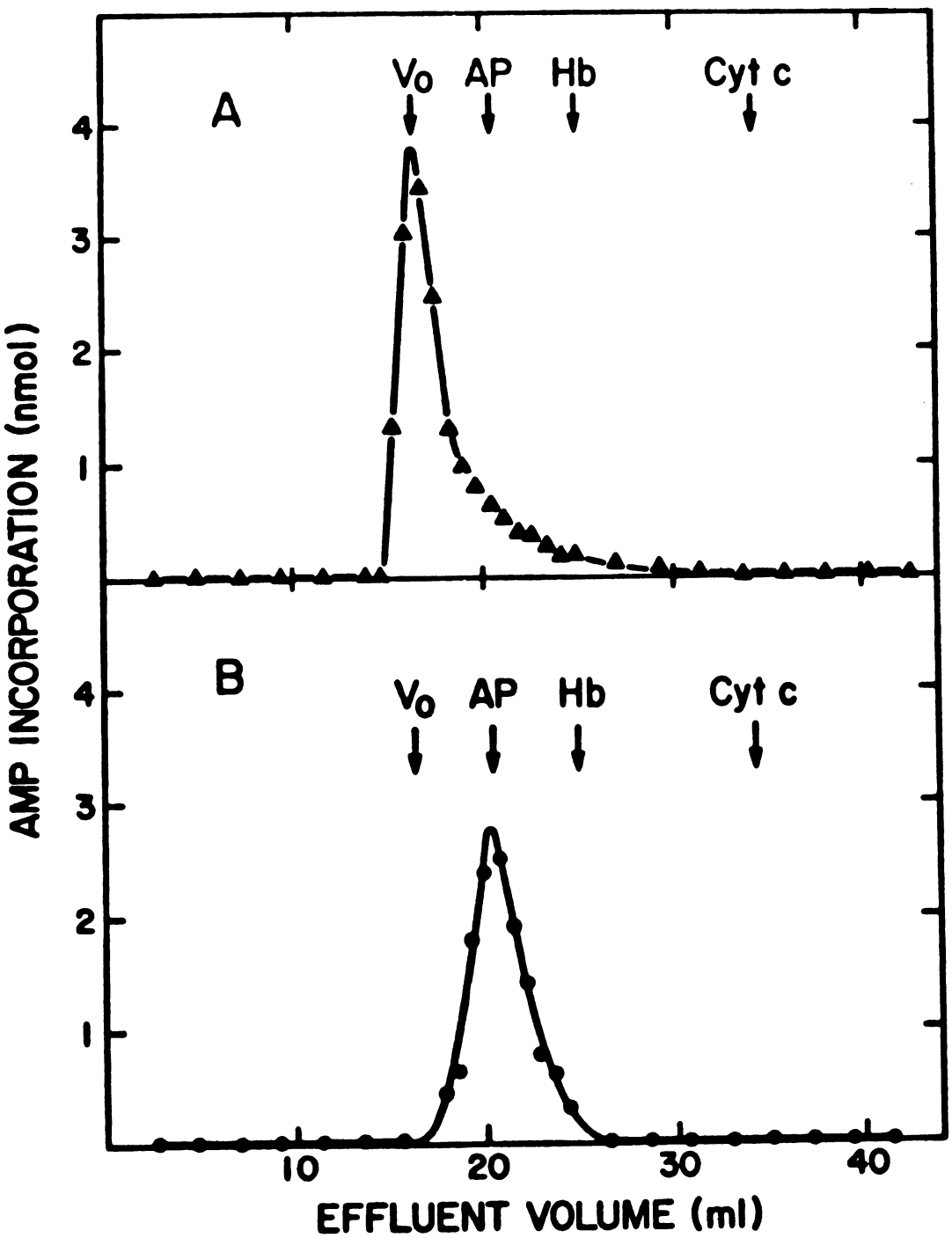
The adenylyltransferase A and B enzyme fractions could be stored at -20° for at least three months without significant loss of enzymatic activity. Fractions adenylyltransferase A and B were free of ribonuclease activity. The sedimentation of ^{3}H-labeled R17 RNA through sucrose gradients remained unchanged following incubation of the labeled RNA with either fraction adenylyltransferase A or B.

Properties of the Reaction Catalyzed by Adenylyltransferases A and B

Characteristics of the Reaction Catalyzed by Adenylyltransferase A.--Some of the characteristics of the reaction

Figure 3.--Bio-Gel P-100 gel filtration of fractions phosphocellulose A and phosphocellulose B. One ml of the concentrated phosphocellulose A enzyme fraction was applied to a column (0.9 x 90 cm) of Bio-Gel P-100 equilibrated with 10 m*M* potassium phosphate (pH 7.1), 1 *M* KCl, 5 m*M* 2-mercaptoethanol, 0.1 m*M* PMSF, and 100 µg per ml bovine serum albumin (panel A). The column was developed at a flow rate of 0.1 ml per min by the continual addition of the same buffer and sixty 0.7 ml fractions were collected. For the assay of ATP-incorporating activity (▲), 25 µl samples of the fractions were assayed in the reaction mixture described under EXPERIMENTAL PROCEDURE. Similarly, one ml of fraction concentrated phosphocellulose B was applied to the same Bio-Gel P-100 column, then developed as described above (panel B). For the assay of ATP-incorporating activity (●), 5 µl samples of the fractions were assayed in the reaction mixture described under EXPERIMENTAL PROCEDURE.

In order to calibrate the column for molecular size, a solution of Blue Dextran (V_o), alkaline phosphatase (AP), hemoglobin (Hb), and cytochrome c (Cyt c) was chromatographed on the same column under identical conditions. The detection of Blue Dextran, hemoglobin, and cytochrome c in the effluent was made spectrophotometrically at 650, 410 and 410 nm, respectively. Alkaline phosphatase was assayed spectrophotometrically by measuring the appearance of *p*-nitrophenol at 410 nm (41).


A
B
V_O
AP
Hb
Cyt c
AMP INCORPORATION (nmol)
EFFLUENT VOLUME (ml)
1
2
3
4
10
20
30
40

catalyzed by adenylyltransferase A are presented in Table II. Incorporation of ATP into a trichloroacetic acid-insoluble form was dependent on added enzyme, an exogenous RNA primer, and Mg^{2+}. Mn^{2+} could substitute for Mg^{2+}. The rate of AMP incorporation into acid-insoluble form at 1 m*M* Mn^{2+} was about 15% of that observed in the presence of 20 m*M* Mg^{2+}. These concentrations of the divalent metal ions were those that gave the optimal rates of AMP incorporation. If 10 m*M* sodium pyrophosphate was added to the reaction mixture, no AMP was incorporated. On the other hand, the addition of 10 m*M* sodium phosphate to the reaction mixture had no effect on the rate of AMP incorporation. Adenylyltransferase A was relatively specific in its requirement for ATP as the substrate. The rate of reaction using CTP as the substrate was about 12% of that for ATP. GTP, UTP, 2'-dATP or ADP did not serve as substrates. For comparison, the characteristics of the reaction catalyzed by adenylyltransferase B are also presented in Table II. These characteristics are in agreement with those reported by Payne and Boezi (16).

The results of a study of the primer requirements for adenylyltransferase A are presented in Table III. Ribosomal RNA and transfer RNA, but not Qβ RNA or denatured calf thymus DNA were effective primers. Of the synthetic polyribonucleotides tested, poly(A) and poly(C) were minimally effective as primers. Poly(U) and poly(dT) did not function as primers. The oligonucleotides, $Ap(Ap)_2A$

TABLE II.--Characteristics of the Reaction Catalyzed by Adenylyltransferase A.*

Components of the Reaction Mixture	Relative Incorporation of Mononucleotides by	
	Adenylyltransferase A	Adenylyltransferase B
Complete	100 (2.61 ± 0.01)	100 (7.78 ± 0.04)
minus enzyme	0	0
minus ribosomal RNA	0	0
minus Mg^{2+}	0	0
minus Mg^{2+}, plus 1 m*M* Mn^{2+}	15	15
plus 10 m*M* sodium pyrophosphate	0	0
plus 10 m*M* sodium phosphate	100	100
minus ATP, plus CTP	12	12
minus ATP, plus GTP	0	0
minus ATP, plus UTP	0	0
minus ATP, plus 2'-dATP	0	0
minus ATP, plus ADP	0	0

*The complete reaction mixture (0.25 ml) contained 30 m*M* glycine-NaOH (pH 9.5), 20 m*M* $MgCl_2$, saturating amounts of *P. putida* ribosomal RNA, 100 m*M* KCl, 20 µl adenylyltransferase A or 10 µl adenylyltransferase B, and 0.8 m*M* labeled nucleoside di- or triphosphate as indicated. The labeled nucleoside di- or triphosphates were of the following specific radioactivities (cpm per nmol): [^{3}H]ADP, 1.0 x 10^3; [^{3}H]2'-dATP, 1.0 x 10^3; [^{3}H]GTP, 1.9 x 10^3; [^{3}H]UTP, 2.3 x 10^3; and [^{3}H]CTP, 2.0 x 10^3. The nmol of [^{3}H]nucleotide monophosphate incorporated into acid-insoluble product after 10 min of incubation at 37° is given in parentheses.

TABLE III.--Primer Specificity for Adenylyltransferase A.*

Primer	Relative Incorporation of AMP by	
	Adenylyltransferase A	Adenylyltransferase B
P. putida ribosomal RNA	100 (6.82 ± 0.03)	100 (5.11 ± 0.03)
E. coli ribosomal RNA	114	114
B. subtilus ribosomal RNA	80	92
E. coli transfer RNA	288	361
S. cerevisiae transfer RNA	441	436
Qβ RNA	11	30
Denatured calf thymus DNA	3	0
Poly(A)	18	12
Poly(C)	15	26
Poly(U)	1	2
Poly(dT)	0	0
$A_p(A_p)_2A$	78	111
A_pA_pA	38	47
A_pA	0	0
None	0	0

*Reaction mixtures (0.25 ml) contained 30 m*M* glycine-NaOH (pH 9.5), 0.8 m*M* [^{3}H]ATP (1.8 x 10^3 cpm per nmol), 20 m*M* $MgCl_2$, 100 m*M* KCl, 20 µl adenylyltransferase A or 10 µl adenylyltransferase B and a saturating amount of primer. The nmol of [^{3}H]AMP incorporated into acid-insoluble product after 10 min of incubation at 37° is given in parentheses.

and ApApA, but not ApA functioned as primers for adenylyltransferase A. In parallel experiments, similar results were obtained for adenylyltransferase B.

In additional experiments designed to characterize the reaction catalyzed by adenylyltransferase A, the pH optimum was found to be 9.5. The Km[3] for ATP was found to be $1 \pm 0.5 \times 10^{-4}$ *M* and Km for *P. putida* ribosomal RNA, in terms of nucleotide phosphate, was $2 \pm 1 \times 10^{-4}$ *M* (P). In parallel experiments, results identical within experimental error were obtained with adenylyltransferase B. The characteristics of adenylyltransferase B were identical to those reported for the less highly purified enzyme fractions used by Payne and Boezi (16).

The Effect of ATP Structural Analogs on Adenylyltransferases A and B.--Four structural analogs of ATP were tested for their effect on AMP incorporation by adenylyltransferase A and B. The rate of AMP incorporation was measured at various concentrations of ATP in the absence and in the presence of each analog which was at a concentration of 0.7 m*M*. The data were then analyzed using the Lineweaver-Burk double reciprocal plots. Three of the structural analogs of ATP, ε-ATP, a fluorescent derivative of ATP, and 2'-dATP and AmTP, analogs of ATP altered at the

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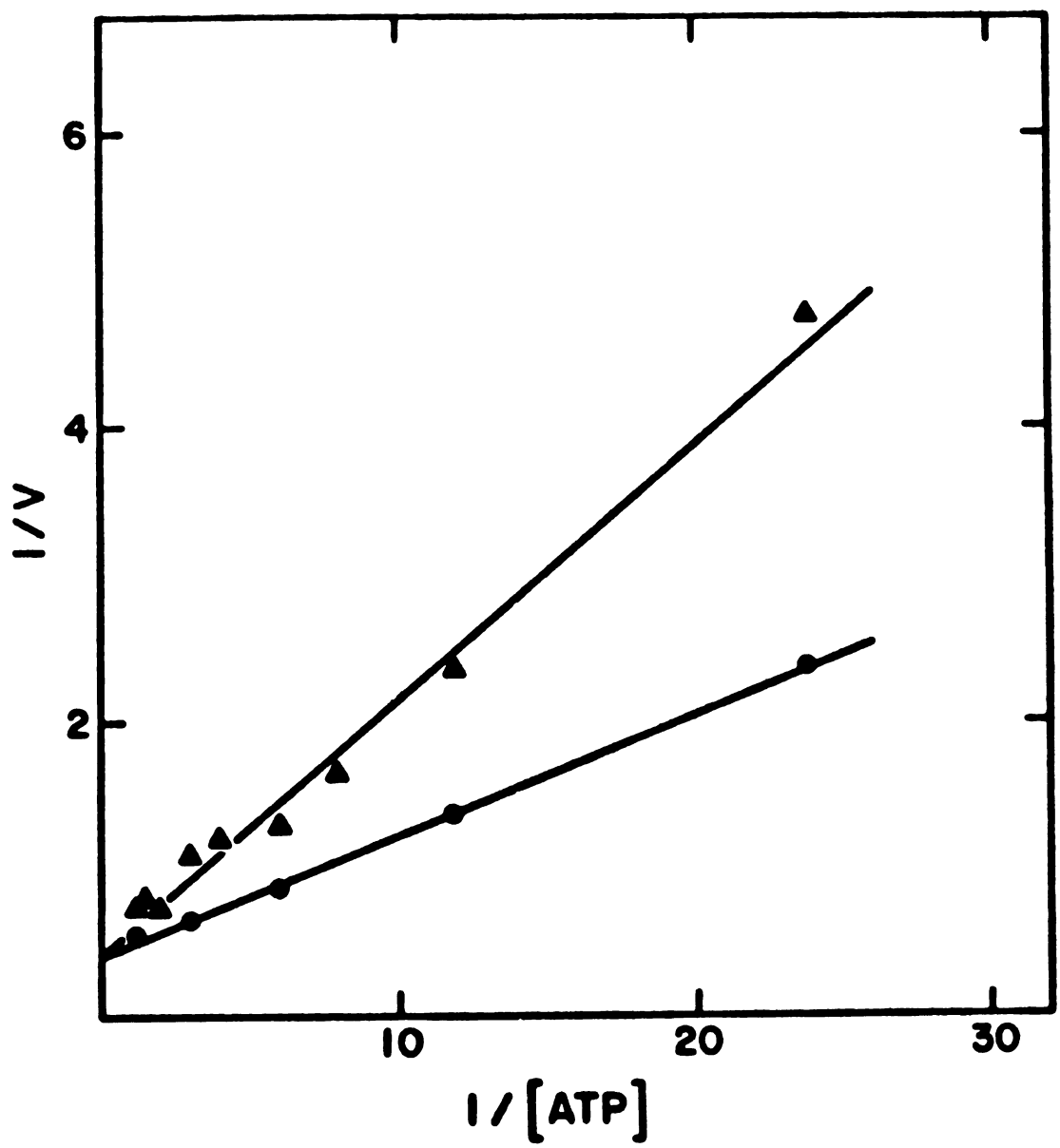
[3]A linear least squares analysis of the data provided the source for the reported values of Km or Ki. The variances following the values represent the error from multiple determinations.

2'-position of the ribose moiety, had no effect on AMP incorporation by either adenylyltransferase A or B. The fourth analog of ATP, 3'-dATP, the triphosphate derivative of the drug cordycepin, was an effective competitive inhibitor of ATP for adenylyltransferase A and B. The data for adenylyltransferase B is presented in Figure 4. The Ki for 3'-dATP with adenylyltransferase A was $1 \pm 0.1 \times 10^{-3}$ *M* and for adenylyltransferase B was $6 \pm 1 \times 10^{-4}$ *M*. The Km for ATP was $1 \pm 0.5 \times 10^{-4}$ *M* for adenylyltransferase A and $2 \pm 0.5 \times 10^{-4}$ *M* for adenylyltransferase B.

The Effect of Rifamycin Derivatives on Adenylyltransferases A and B.--The rifamycin family of antibiotics are effective inhibitors of bacterial RNA polymerases (32-34). Only one derivative, rifampicin, has been tested and was found inactive as an inhibitor of bacterial poly A polymerases (14,15,35). In the present study fourteen derivatives of rifamycin, all of them effective inhibitors of *P. putida* DNA-dependent RNA polymerase,[4] were tested for their effects on AMP incorporation by adenylyltransferase A and B. The rifamycin derivatives were dissolved in dimethyl sulfoxide and added to the reaction mixtures at a concentration of 40 µg per ml. As a consequence of these additions, the concentration of dimethyl sulfoxide in the reaction mixtures was 16% (v/v). When this concentration

[4]H. C. Towle, personal communication.

Figure 4.--Inhibition of the ATP-incorporating activity of adenylyltransferase B in the presence of 3'-dATP. The reaction conditions were the same as those described under EXPERIMENTAL PROCEDURE, except that varying amounts of [^{3}H]ATP (2.4×10^3 cpm per nmol) were present in each reaction mixture. AMP incorporated in the complete reaction mixture (●), or in the complete reaction mixture plus 0.7 m*M* 3'-dATP (▲). Velocity is in nmol AMP incorporated per 10 min; and substrate is in m*M* ATP.


6
4
2
1/V
10
20
30
1/[ATP]

of dimethyl sulfoxide was added to the standard reaction mixture, AMP incorporation by adenylyltransferase A or B was increased 2.6-fold. In other experiments in which the effect of the concentration of dimethyl sulfoxide on AMP incorporation was examined, 16% was found to be the concentration which produced the greatest increase in the rate of AMP incorporation.

Of the fourteen rifamycin derivatives tested at 40 μg per ml, two derivatives, AF/013 and AF/DNFI, were effective inhibitors of AMP incorporation by adenylyltransferase B (Table IV). As determined in other experiments, a concentration of 5 μg per ml AF/013 inhibited AMP incorporation 50% by either adenylyltransferase A or B. AF/DNFI, at a concentration of 10 μg per ml, inhibited 50%.

The Effect of Synthetic Polynucleotides on AMP Incorporation by Adenylyltransferases A and B with Ribosomal RNA as the Primer.--A variety of synthetic polynucleotides were tested for the effects that they would have on the rate of AMP incorporation by adenylyltransferase A and B with ribosomal RNA as the primer. The addition to the standard reaction mixture of poly(A), poly(dA), poly(Am), or poly(Um), at a concentration of about 1×10^{-4} *M* (P), had no effect on the rate of AMP incorporation by adenylyltransferase A or B. The addition to the standard reaction mixture of poly(C) or poly(I) resulted in a 30% lower rate of AMP incorporation by either enzyme. Little or no AMP

TABLE IV.--The Effect of Rifamycin Derivatives on Adenylyltransferase B.*

Rifamycin Derivative Added to the Reaction Mixture	Relative Incorporation of AMP
None	100 (6.31 ± 0.03)
Rifamycin AG	113
Rifamide	106
PR/14	105
PR/19	100
4-Desossi SV	83
AF/APR	97
AF/DEI	97
AF/BO	92
AF/AP	100
AF/DA-AMP	92
AF/AOP	87
AF/ABDP-CIS	70
AF/DNFI	2
AF/013	0

*Reaction mixtures (0.25 ml) contained 30 m*M* glycine-NaOH (pH 9.5), 20 m*M* $MgCl_2$, 100 m*M* KCl, 0.8 m*M* [^{3}H]ATP (1.9 x 10^3 cpm per nmol), a saturating amount of *P. putida* ribosomal RNA, 1 µl of adenylyltransferase B, 16% (v/v) dimethyl sulfoxide and the additions noted. The final concentration of a rifamycin derivative in the reaction mixture was 40 µg per ml. The nmol of [^{3}H]AMP incorporated into acid-insoluble product after 10 min of incubation at 37° is given in parentheses.

incorporation was observed when poly(U), poly(dT), or poly(G) was added to the standard reaction mixture.

The mechanism by which poly(U) and poly(dT) inhibit AMP incorporation was examined. As shown in Table III, poly(U) and poly(dT) did not function as primers for adenylyltransferase A or B. These polynucleotides inhibit AMP incorporation by functioning as competitive inhibitors of the ribosomal RNA primer (Figure 5). In these experiments, the rates of AMP incorporation by adenylyltransferase A and B were measured at various concentrations of ribosomal RNA in the absence and in the presence of either poly(U) or poly(dT). The data were then analyzed using the Lineweaver-Burk double reciprocal plots. The Km for the ribosomal RNA primer was $2 \pm 1 \times 10^{-4}$ *M* (P) for adenylyltransferase A and $4 \pm 1 \times 10^{-4}$ *M* (P) for adenylyltransferase B. The Ki for poly(U) or poly(dT) for adenylyltransferase A was $4 \pm 2 \times 10^{-6}$ *M* (P). The Ki for poly(U) or poly(dT) for adenylyltransferase B was $1 \pm 0.2 \times 10^{-5}$ *M* (P).

Just as adenylyltransferase A and B bind poly(U), the enzymes can bind to a poly(U)-Sepharose column (Figure 6). Upon application of a linear KCl gradient, adenylyltransferase A eluted at 0.19 *M* KCl, while in a separate run of the column, adenylyltransferase B eluted at 0.38 *M* KCl. In both experiments, nearly 100% of the enzyme activity applied to the column was recovered upon elution.

Figure 5.--Inhibition of the ATP-incorporating activity of adenylyltransferase B in the presence of poly(U) or poly(dT). The reaction conditions were the same as those described under EXPERIMENTAL PROCEDURE, except that varying amounts of *P. putida* ribosomal RNA were present in each reaction mixture. AMP incorporated in the complete reaction mixture (●), in the complete reaction mixture plus 16 μ*M*(P) poly(dT) (■), or in the complete reaction mixture plus 39 μ*M* (P) poly(U) (▲). Velocity is in nmol AMP incorporated per 10 min; and substrate is in m*M* (P) *P. putida* ribosomal RNA.

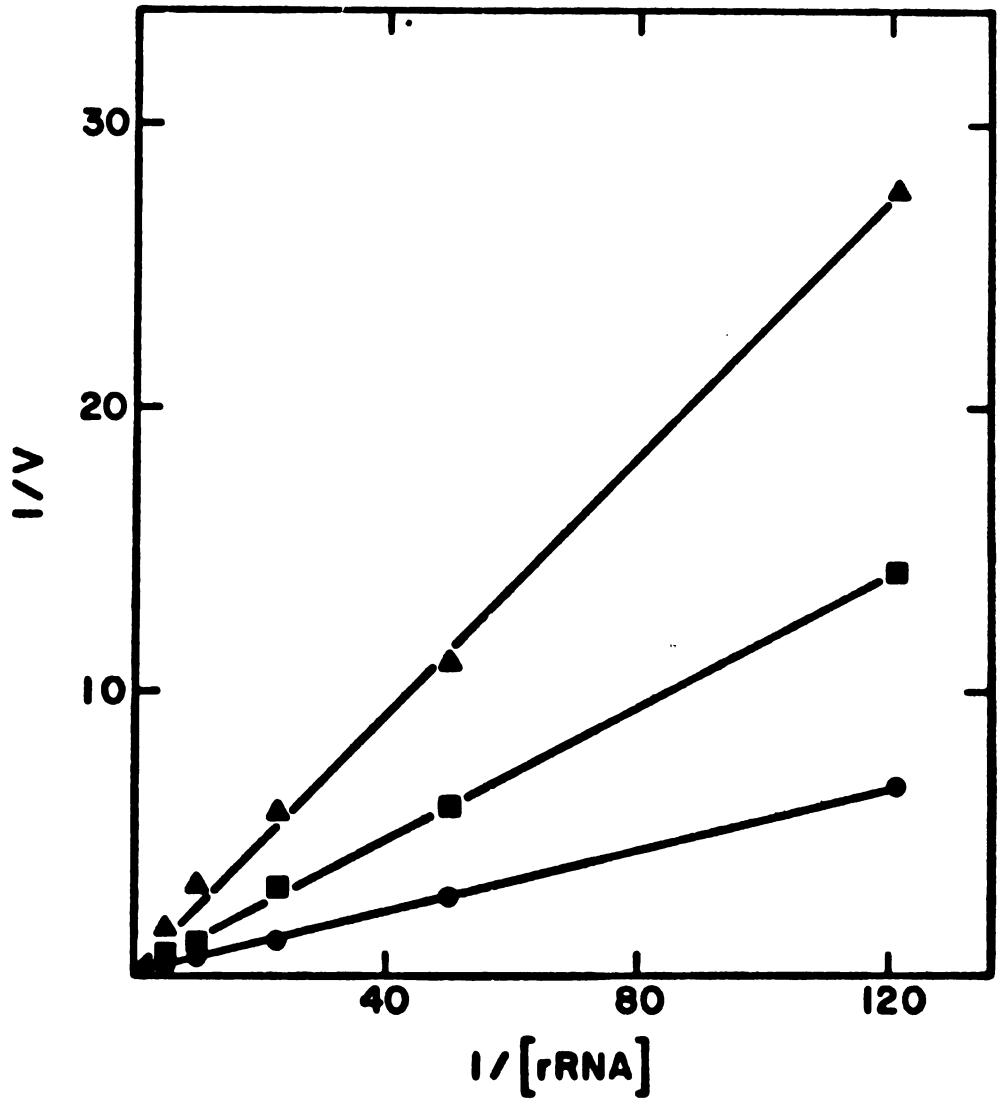
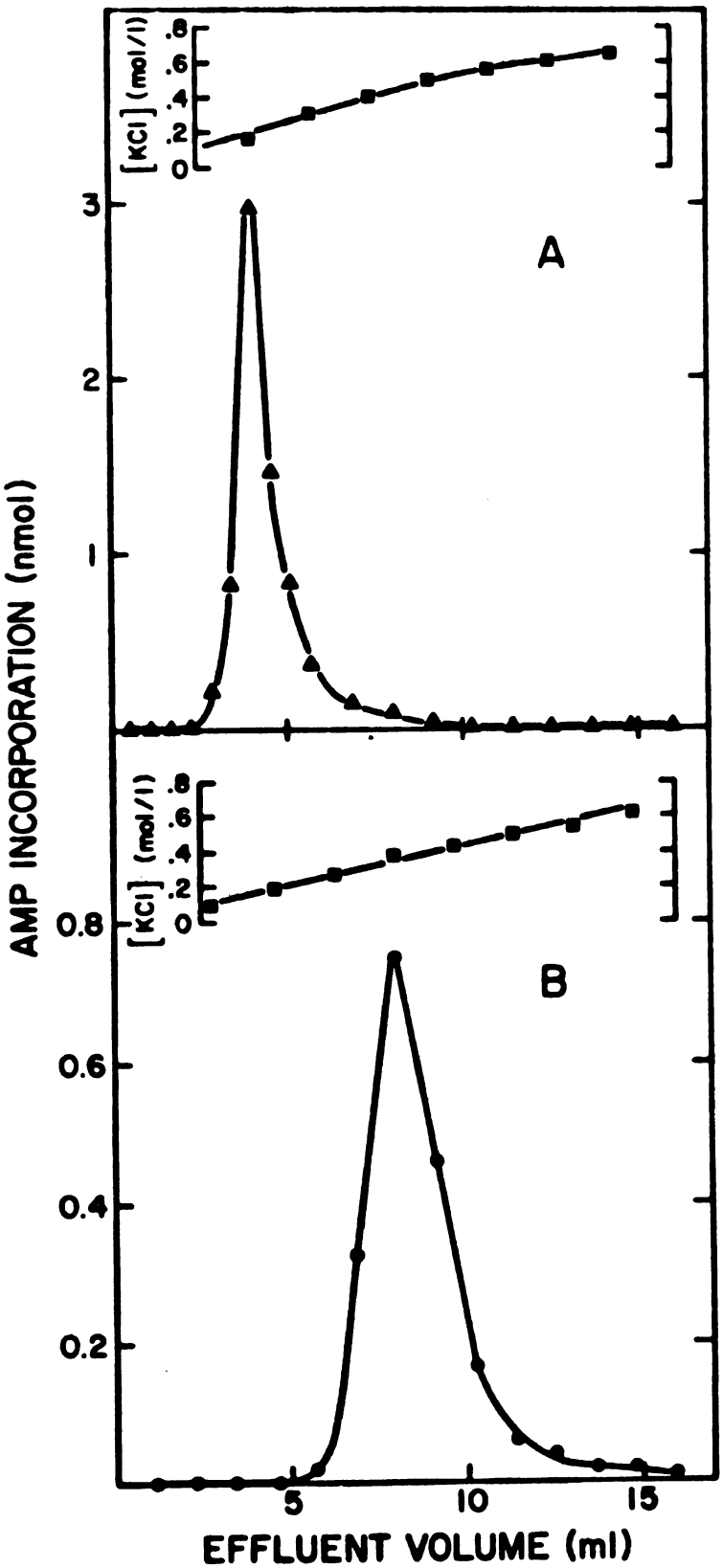

30
20
10
1/V
40
80
120
1/[rRNA]

Figure 6.--Affinity chromatography of fractions concentrated phosphocellulose A and concentrated phosphocellulose B on poly(U)-Sepharose. A 100 µl sample of the concentrated phosphocellulose A enzyme fraction was applied to a column (0.5 x 3 cm) of poly(U)-Sepharose, prepared as described under EXPERIMENTAL PROCEDURE, equilibrated with 10 m*M* potassium phosphate (pH 7.1), 100 m*M* KCl, 5 m*M* 2-mercaptoethanol and 100 µg per ml bovine serum albumin (panel A). Following application of the enzyme fraction, the column was washed with 2.5 ml of the same buffer. A 20 ml linear gradient from 0.1 to 1.0 *M* KCl in 10 m*M* potassium phosphate (pH 7.1), 5 m*M* 2-mercaptoethanol and 100 µg per ml bovine serum albumin was used to elute the enzyme. The column was developed at 0.2 ml per min and 28 fractions of 0.6 ml each were collected. For the assay of ATP-incorporating activity (▲), 25 µl samples of the fractions were assayed in the reaction mixture described under EXPERIMENTAL PROCEDURE. Similarly, a 25 µl sample of the concentrated phosphocellulose B enzyme fraction was applied to the same poly(U)-Sepharose column and eluted as described above (panel B). For the assay of ATP-incorporating activity (●), 25 µl samples of the fractions were assayed in the reaction mixture described under EXPERIMENTAL PROCEDURE. The KCl concentration was determined from conductivity measurements (■).


[KCl] (mol/l)
0
.2
.4
.6
.8
A
B
AMP INCORPORATION (nmol)
0.2
0.4
0.6
0.8
1
2
3
5
10
15
EFFLUENT VOLUME (ml)

Structural Properties of Adenylyltransferases A and B

Determination of the Sedimentation Coefficient of Adenylyltransferases A and B.--Glycerol gradient centrifugation was used to determine the sedimentation coefficients of adenylyltransferases A and B. The results are presented in Figure 7. With horse blood hemoglobin ($s^{\circ}_{20,w}$ = 4.2 S) as the reference protein, the sedimentation coefficient[5] of adenylyltransferase A was calculated to be 9.3 S and that for adenylyltransferase B 4.3 S. For typical globular proteins, sedimentation coefficients ($s^{\circ}_{20,w}$) of 9.3 S and 4.3 S correspond to molecular weights of 185,000 and 58,000, respectively (36).

Determination of the Stoke's Radius for Adenylyltransferase B Using Gel Filtration on Bio-Gel P-100.--Kd values for adenylyltransferase B and for the reference proteins alkaline phosphatase, hemoglobin, and cytochrome c were determined for the Bio-Gel P-100 column that was used as the last step in the purification procedure (Figure 3). The inverse error function complements of the Kd values of the reference proteins were plotted against their Stokes' radii (37). Using this plot as the standard curve, a Stoke's radius of 2.9 nm for adenylyltransferase B was determined from its inverse error function complement of Kd.

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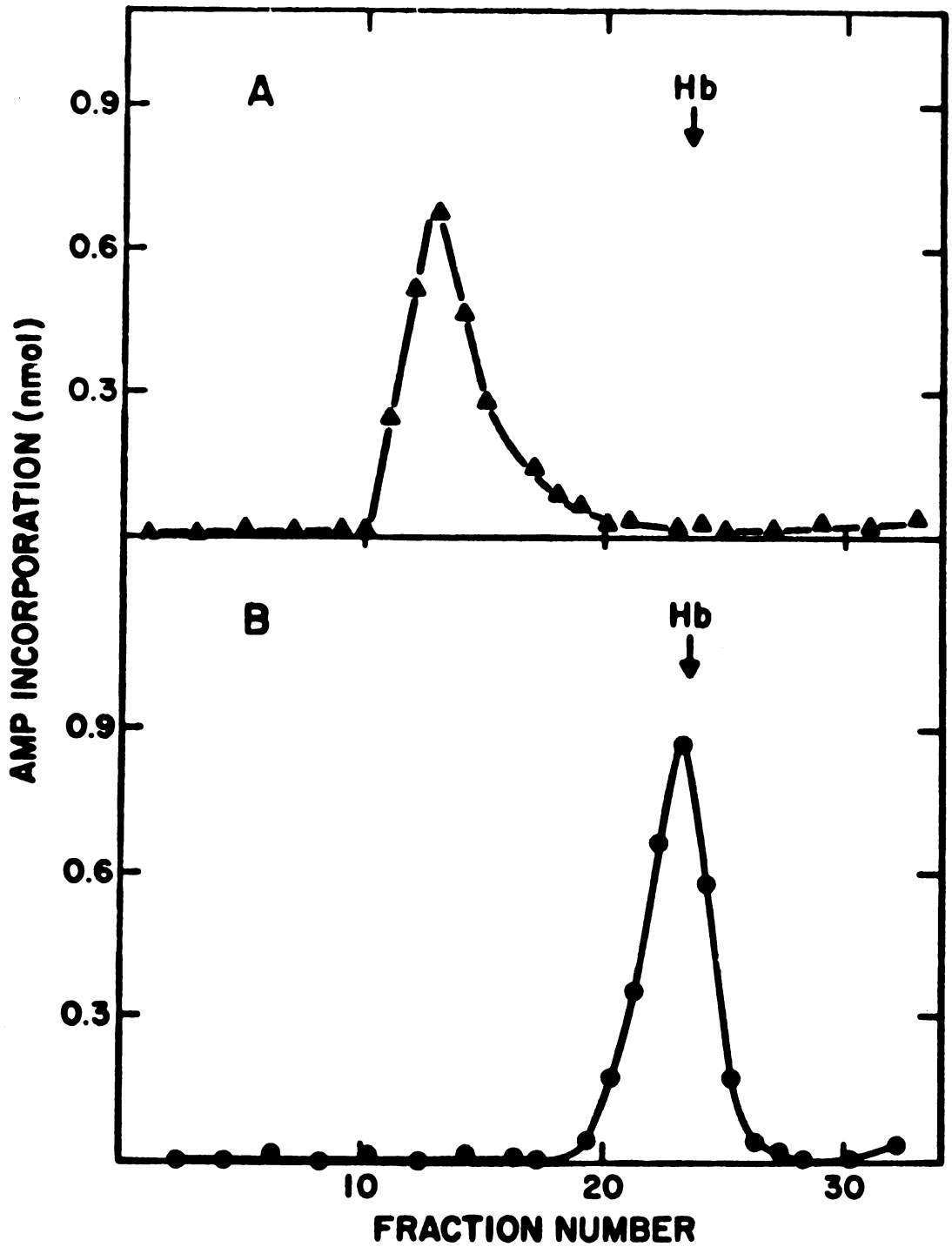
[5]The enzyme concentration was much less than 0.1 mg per ml, so this is essentially a $s^{\circ}_{20,w}$ value; however, 0.01% bovine serum albumin was present.

Figure 7.--Glycerol gradient centrifugation of adenylyltransferases A and B. The protein solutions, 200 µl of either adenylyltransferase A (panel A) or adenylyltransferase B (panel B), were layered on separate 10 to 30% linear glycerol gradients (4.8 ml) prepared in 10 m*M* potassium phosphate (pH 7.1), 1*M* KCl, 0.5 m*M* dithiothreitol and 100 µg per ml bovine serum albumin. After centrifugation in a Spinco SW 50L rotor at 46,000 rpm for 21 hours at 4°, fractions (0.15 ml) were collected from the bottom of the centrifuge tube. The ATP-incorporating activity of adenylyltransferase A (▲) and adenylyltransferase B (●) were assayed on 25 µl samples in the reaction mixture described under EXPERIMENTAL PROCEDURE, except that the specific radioactivity for [^{3}H]ATP was 6.1 x 10^3 cpm per nmol. Hemoglobin (Hb) was used as a marker of known sedimentation coefficient.


A
Hb
0.9
0.6
0.3
B
Hb
0.9
0.6
0.3
AMP INCORPORATION (nmol)
10
20
30
FRACTION NUMBER

From a Stoke's radius (r) of 2.9 nm and a sedimentation coefficient (s) of 4.3 S, a molecular weight (M.W.) for adenylyltransferase B of 52,000 can be calculated from the equation[6] (38).

$$M.W. = \frac{6\pi\eta Nrs}{1 - v\rho}$$

Determination of the Polypeptide Composition of Adenylyltransferase B Using SDS Polyacrylamide Gel Electrophoresis.--A sample of the phosphocellulose B enzyme fraction was subjected to SDS polyacrylamide gel electrophoresis (Figure 8) essentially as described by Johnson *et al.* (39), except that the concentration of acrylamide was 10% (w/v) and that of bis-acrylamide was 0.368% (w/v). In parallel experiments, *P. putida* DNA-dependent RNA polymerase, bovine serum albumin, pepsin and pancreatic ribonuclease A were analyzed electrophoretically. When compared to the relative migration of these polypeptides of known molecular weight, the four Commassie blue stained[7] protein bands of fraction phosphocellulose B had molecular weights of 53,000, 47,000, 14,000 and 12,000 in the weight ratio of 1.2 : 1.1 : 1.0 :

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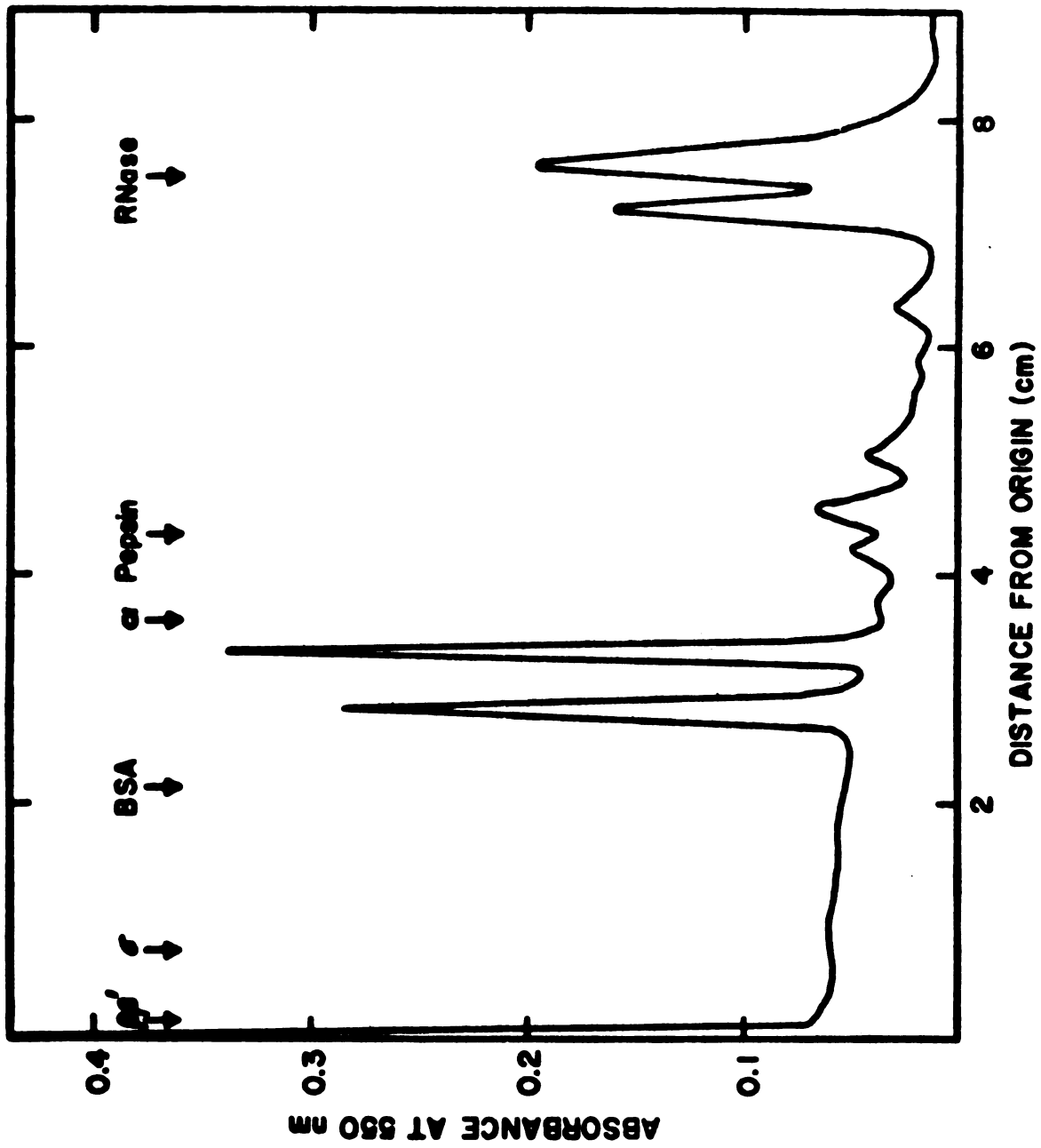
[6]Also, where N is the Avogadro number, the viscosity of the solvent (η) is assumed to be 0.01 poise, the partial specific volume of the protein (v) is assumed as 0.725 cm^3 per g, and the value of the density of the solvent (ρ) is taken as 1.0 g per cm^3.

[7]For an alternative staining procedure to that described (39), see Appendix.

Figure 8.--SDS-polyacrylamide gel electrophoresis of fraction phosphocellulose B. SDS-polyacrylamide gel electrophoresis was performed essentially as described by Johnson *et al.* (39). The phosphocellulose B enzyme fraction (4 µg) was incubated at 80° for 30 min in a 100 µl solution containing 0.1 *M* sodium phosphate (pH 7.1), 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 5% (v/v) glycerol. The mixture (60 µl) was layered upon a SDS-polyacrylamide gel (11 cm) prepared from an acrylamide-SDS mixture containing 10% (w/v) acrylamide and 0.368% (w/v) bis-acrylamide. Electrophoresis was performed at 25° for 6.5 hours at 8 ma per gel in 0.1 *M* sodium phosphate (pH 7.1), and 0.1% (w/v) SDS. The protein was stained with Coomassie brillant blue. The densimetric tracing at 550 nm was made with a Gilford linear transport. In parallel experiments, *P. putida* DNA-dependent RNA polymerase, bovine serum albumin, pepsin and ribonuclease I were electrophoresed as described (39). The molecular weights of the polypeptides used as standards were 165,000, 155,000, 98,000, and 44,000 for β', β, σ, and α subunits of *P. putida* RNA polymerase, 68,000 for bovine serum albumin (BSA), 37,000 for pepsin, and 13,700 for pancreatic ribonuclease, type I-A (RNase) (39).


RNase
α
Pepsin
BSA
0.4
0.3
0.2
0.1
2
4
6
8
DISTANCE FROM ORIGIN (cm)
ABSORBANCE AT 550 nm

2.0. A sample of the enzymatic activity peak from Bio-Gel P-100 gel filtration of fraction phosphocellulose B in the absence of bovine serum albumin was subjected to the same SDS polyacrylamide gel electrophoresis system. Only two polypeptides of molecular weights 53,000 and 47,000 were present. Electrophoresis was also performed on a sample of fraction phosphocellulose A in the same SDS polyacrylamide gel system (data not shown). Of the two possible polypeptides representing adenylyltransferase activity, only the 53,000 molecular weight species was common to both fractions phosphocellulose A and B.

DISCUSSION

ATP:RNA adenylyltransferase was purified from *P. putida* by a procedure similar to that described earlier (16). However, the scale of the purification was increased over ten-fold, from 40 to 500 grams of cells, and another step, Bio-Gel P-100 chromatography was added. In addition, improvements to the procedure were introduced to increase the recovery of enzymatic activity. PMSF, a protease inhibitor, at a concentration of 0.1 m*M* and 2-mercaptoethanol at a concentration of 5 m*M* were added to all buffers used in the purification. The addition of at least 0.25 *M* KCl to buffers subsequent to DEAE-Sephadex chromatography, and 50% glycerol in the final storage buffer stabilized enzyme activity. Through phosphocellulose chromatography the improved scheme resulted in an increased specific activity of nearly four times over that previously reported. With the inclusion of Bio-Gel P-100 chromatography the enzyme was estimated to be purified another 2.5-fold.

As a result of the revised purification scheme, a previously undetected ATP-incorporating activity was discovered. It was evident in phosphocellulose chromatography as a distinct peak of activity eluting separately from the previously reported adenylyltransferase (Figure 2). The

activity was indistinguishable by all kinetic criteria from the adenylyltransferase originally described by Payne and Boezi (16). Both of these ATP-specific, primer-dependent activities were optimally active at pH 9.5, were inhibited by inorganic pyrophosphate but not by orthophosphate, and demonstrated the same specificity for primer. The divalent metal ion requirement for both activities was preferentially satisfied by Mg^{2+}, rather than Mn^{2+}, with an optimum at 20 m*M*. Within experimental error, both enzymes had identical values for the Km of ATP, the Ki for 3'-dATP and the Km for ribosomal RNA. When synthetic polynucleotides were added to ribosomal RNA-primed reactions, the effect on the AMP incorporation by either enzyme was the same. The Ki values determined for poly(U) and poly(dT) were equivalent within experimental error. The amount of rifamycin AF/013 or AF/DNFI required to inhibit 50% the rate of AMP incorporation by either enzyme was identical. Neither activity was inhibited by rifampicin. This newly detected ATP-incorporating activity was designated adenylyltransferase A, while the previously reported activity was designated adenylyltransferase B.

It was not possible to distinguish between these two adenylyltransferases catalytically. However, an examination of their chromatographic and sedimentation properties indicates that these enzymes have different structures. A difference in elution from anionic-exchange chromatography

provided the basis for the initial detection of the new adenylyltransferase. During chromatography on phosphocellulose (Figure 2), adenylyltransferase A eluted at 0.3 *M* KCl, while adenylyltransferase B eluted at 0.6 *M* KCl. When subjected to poly(U)-Sepharose chromatography, adenylyltransferase A eluted at 0.2 *M* KCl and adenylyltransferase B eluted at 0.4 *M* KCl (Figure 6). Another difference observed for these enzymes was in their molecular weights. When chromatographed on Bio-Gel P-100, adenylyltransferase A was excluded while adenylyltransferase B was included in the gel (Figure 3). From its elution on Bio-Gel P-100 relative to protein standards, adenylyltransferase B was estimated to have a molecular weight of 52,000. By sedimentation through glycerol gradients, adenylyltransferase A was determined to have a sedimentation coefficient ($s^{\circ}_{20,w}$) of 9.3 S and adenylyltransferase B of 4.3 S (Figure 7). From these determinations the molecular weight of adenylyltransferase A was estimated to be approximately 185,000 and that of adenylyltransferase B to be 50,000 to 60,000.

During the purification of adenylyltransferase, a peak of enzymatic activity eluted at approximately 4 liters in Bio-Gel P-200 gel filtration (Figure 1). When compared to the elution volumes of protein standards, the elution of adenylyltransferase activity corresponded to a molecular weight of approximately 50,000 to 60,000. Pooling of this activity in the effluent from 3.6 to 4.3 liters during the

purification selected against material of molecular weight greater than about 65,000. If a sample of this pooled activity was subjected to glycerol gradient centrifugation under the conditions of Figure 7, there was only a single 4.3 S peak of adenylyltransferase activity. Thus, only the activity corresponding to adenylyltransferase B was present at this point in the purification. Adenylyltransferase A must then have arisen during the purification procedure, subsequent to Bio-Gel P-200 gel filtration but prior to phosphocellulose chromatography.

The generation of adenylyltransferase A during the purification was not the result of a random aggregation. Several facts indicate that this enzyme has some specific structure. Adenylyltransferase A eluted as a sharp peak of activity in both phosphocellulose and poly(U)-Sepharose chromatographies. Glycerol gradient centrifugation of adenylyltransferase A revealed a single, well-defined peak of AMP incorporation. If adenylyltransferase A was first preincubated with ribonuclease, then subjected to the glycerol gradient centrifugation, there was no alteration in the sedimentation profile. This indicated that the specific structure of adenylyltransferase A did not include a bound RNA molecule.

Since adenylyltransferases A and B are identical catalytically, it is probable that adenylyltransferase A contains as part of its structure adenylyltransferase B.

Although there is no direct evidence, the interpretation of the data available supports this conclusion. The specific structure of adenylyltransferase A could be generated from the 50,000 to 60,000 molecular weight material of Bio-Gel P-200 chromatography, adenylyltransferase B, by several mechanisms. Adenylyltransferase A could be the result of a simple self-association of adenylyltransferase B. It is not likely that adenylyltransferase A would be a dimer of adenylyltransferase B, since a typical globular protein of 100,000 to 120,000 has a sedimentation coefficient of 6-7 S. However, a trimer or a tetramer of adenylyltransferase B is possible since these would have $s^{\circ}_{20,w}$ values close to that of adenylyltransferase A (9.3 S).

Adenylyltransferase A could alternatively be the result of the specific association of a unique protein or proteins with adenylyltransferase B. When SDS polyacrylamide gel electrophoresis patterns of enzyme fractions phosphocellulose A and B were compared, the 53,000 molecular weight polypeptide corresponding to adenylyltransferase B was present in both patterns. In addition, several polypeptides of the molecular weight range 50,000 to 80,000 were found in A but not in B. These additional polypeptides are likely candidates for the specific binding to the catalytic polypeptide, adenylyltransferase B, to form adenylyltransferase A.

From the limited amount of structural data on these adenylyltransferases, it is not possible to decide which of these mechanisms is applicable. However, it is clear that the governing process is not readily reversible. Since a simple, readily reversible association is an equilibrium process, once the species have been separated the equilibrium should re-establish. Once adenylyltransferases A and B were separated, however, no further appearance of adenylyltransferase A was detected in fractions containing adenylyltransferase B or vice versa.

Poly A polymerase of *E. coli* has been implicated by some (35,42) to be a subunit of DNA-dependent RNA polymerase. Sippel (14) compared purified "ATP-RNA adenyltransferase" of *E. coli* to RNA polymerase by electrophoresis in SDS polyacrylamide gels. None of the polypeptides of RNA polymerase had the same mobility as that for the "adenyltransferase." Schäfer *et al.* (43) demonstrated that even purified *E. coli* RNA-primed polynucleotidepyrophosphorylase, which can synthesize poly(A), was not a subunit of RNA polymerase. When adenylyltransferase B is compared to *P. putida* DNA-dependent RNA polymerase by SDS polyacrylamide gel electrophoresis (Figure 8), it is clear that these two enzymes also do not have any polypeptides in common.

P. putida adenylyltransferase B is one of the most highly purified poly A polymerases from the particulate fraction of a prokaryote. Recently, Sippel (14) has

characterized a highly purified "adenyltransferase" from the particulate fraction of *E. coli*. These two particulate enzymes have some similar catalytic, chromatographic and structural properties. Both of the ATP-specific, primer-dependent adenylyltransferases have an unusually strong affinity for phosphocellulose. Elution of activity required the presence of greater than 0.5 *M* KCl. To maintain activity buffers used in the later steps of purification were high in ionic strength. Molecular weight analysis indicated that either activity was a single polypeptide of 50,000 to 60,000. Several catalytic differences, however, demonstrate that these enzymes are distinct from one another. The *E. coli* enzyme had a pH optimum of 8, had a preference for Mn^{2+} over Mg^{2+}, was inhibited by inorganic orthophosphate, and was primed as well by Qβ RNA as it was by transfer RNA or ribosomal RNA. In addition, optimal enzymatic activity occurred in the presence of 0.35 *M* KCl. Although the data was not presented, the *P. putida* enzyme was optimally active at 20 m*M* KCl and completely inhibited by 200 m*M* KCl.

The rifamycin family of antibiotics have been used as potent inhibitors of many nucleic acid enzymes. These semisynthetic derivatives of the natural fermentation product of *Streptomyces mediterranei* have been postulated to interact with the nucleoside triphosphate binding site of the template-requiring DNA and RNA polymerases (for a review of rifamycins, see Riva and Silvestri [34]). Only

a few of the derivatives have been examined for their effect on poly A polymerases. One derivative, rifampicin, was ineffective in inhibiting the ATP-incorporating activity of the prokaryotic (14,15,35) and eukaryotic enzymes (2,3, 5,6). On the other hand, AF/AP and AF/ABDMP were effective inhibitors of the HeLa enzyme (3); and AF/013, at 100 µg per ml, inhibited the rat liver nuclear enzyme (2). The effects of fourteen derivatives of rifamycin, all effective inhibitors of *P. putida* DNA-dependent RNA polymerase, on the ATP-incorporating activity of adenylyltransferase was presented in this report. Of these derivatives only two, AF/013 and AF/DNFI, inhibited the AMP incorporation of *P. putida* ATP:RNA adenylyltransferase. AF/013 is a strong inhibitor, as 50% inhibition is obtained at only 5 µg per ml. ATP:RNA adenylyltransferase, a non-template-requiring polymerase, can be added to the list of nucleic acid polymerizing enzymes that are inhibited by AF/013 (32,33,44).

The characteristics of the nucleoside triphosphate binding site of ATP:RNA adenylyltransferase were examined. It was demonstrated, as shown in Table II (and [16]), that this enzyme preferentially utilized ATP as substrate. The base specificity for substrate was also demonstrated by the minimal amount of inhibition of AMP incorporation observed when GTP, CTP or UTP was present (16). The amount of inhibition is an indication of the binding efficiency of the three ribonucleoside 5'-triphosphates relative to

ATP. Additional characteristics of substrate binding were obtained from studies on the inhibition of AMP incorporation by analogs of ATP. Three analogs, 2'-dATP, AmTP and ε-ATP did not affect the rate of AMP incorporation. On the other hand, a fourth analog, 3'-dATP, was an effective inhibitor of ATP for the enzyme. The failure of the analogs altered at the 2'-position of the ribose moeity to compete with ATP is interpreted as a failure to bind to the enzyme. Thus, the hydroxyl at the 2'-position seems to be required of a potential substrate for binding. However, the inhibition of AMP incorporation and, therefore, competitive binding demonstrated by 3'-dATP indicates that for the 3'-position either a hydrogen or a hydroxyl substituent is satisfactory. The large variability in selectivity of substituents between the 2'- and the 3'-positions may be an expression of the evolutionary pressure on the enzyme to distinguish between the only two naturally-encountered isomers, ATP and 2'-dATP.

The ribosomal RNA-primed reaction catalyzed by ATP:RNA adenylyltransferase was inhibited upon the addition of synthetic non-priming homopolynucleotides. A study of this inhibition has lead to a characterization of the primer binding site of the enzyme. The reaction was inhibited to varying extents depending upon the polynucleotide added. Poly(U), poly(dT) and poly(G) were the strongest, poly(C) and poly(I) were intermediate and poly(A), poly(Am)

poly(dA) and poly(Um) were ineffective as inhibitors. Since the kinetics of inhibition by poly(U) and poly(dT) were competitive with ribosomal RNA (Figure 5), these non-priming polynucleotides did not inhibit by irreversibly binding to the enzyme. One explanation for the inhibition is to presume that poly(U) or poly(dT) hydrogen-bonded to the adenylate residues of the partially synthesized product. The resulting complex then was not a primer for the reaction. This would not explain, however, why poly(G), poly(C) or poly(I) inhibited. In addition, this contradicts the fact that poly(Um), which would be expected to bind to the adenylate residues at least as efficiently as poly(U), did not inhibit the reaction.

Another explanation for the inhibition involves the competition of the synthetic polynucleotides with ribosomal RNA directly for the primer binding site on the enzyme. The variation in effectiveness of the synthetic homopolynucleotides for inhibition could be related to their differences in secondary structure. The primer binding site would then seem to prefer single-stranded polynucleotides with little secondary structure. However, binding alone is not sufficient criteria for a polynucleotide to prime AMP incorporation. Assuming the Km represents the dissociation constant, the fact that the Km for ribosomal RNA is ten times the Ki for poly(U) indicates that adenylyltransferase binds poly(U) much more efficiently. However,

poly(U) does not prime the reaction. Thus, in addition to the general property of binding nucleic acids, the enzyme also requires some 3'-terminal base specificity of the polynucleotide for primer.

The strong affinity demonstrated by adenylyltransferase for the non-priming poly(U) led to the possibility that the enzyme would bind to a column of immobilized poly(U). When adenylyltransferase was subjected to poly(U)-Sepharose chromatography (Figure 6), the enzyme was bound. Upon application of a salt gradient, adenylyltransferase activity eluted with nearly 100% recovery. Affinity chromatography of this enzyme on poly(U)-Sepharose could provide an alternative method for the purification of adenylyltransferase to greater specific activities and higher yields. When enzyme fraction pH 5 supernatant was applied to the column, however, no appreciable amount of activity was retained. The failure of this fraction to bind to the column was probably due to the enzyme's strong association in impure fractions, and at low salt concentrations, with macromolecular components of the cell (16). Thus, for the *P. putida* enzyme this column would only be successful in later stages of the purification, but poly(U)-Sepharose chromatography might prove valuable in the purification of poly A polymerase from other sources.

The ATP:RNA adenylyltransferase of *P. putida* has been studied in some detail. Several kinetic and structural

characterizations of the enzyme were made. Further kinetic studies on the primer-binding site could determine whether the enzyme also requires a specific 3'-terminal sequence, or if only the 3'-terminal residue is important. Additional investigation on the structure of adenylyltransferase A could lead to deducing the proper mechanism for its formation. This may also provide for an understanding of whether the formation of such a protein complex with the catalytic polypeptide, adenylyltransferase B, is of biological significance or merely an artifact of the purification procedure.

REFERENCES

1. Winters, M. A., and Edmonds, M. (1973) *J. Biol. Chem. 248,* 4756-4762.

2. Niessing, J., and Sekeris, C. E. (1973) *Nature New Biol. 243,* 9-12.

3. Mans, R. J. (1973) *Fed. Eur. Biochem. Soc. Lett. 33,* 245-246.

4. Tsiapalis, C. M.; Dorson, J. W.; De Sante, D. M.; and Bollum, F. J. (1973) *Biochem. Biophys. Res. Commun. 50,* 737-743.

5. Coleman, M. S.; Hutton, J. J.; and Bollum, F. J. (1974) *Nature 248,* 407-409.

6. Jacob, S. T., and Schindler, D. G. (1972) *Biochem. Biophys. Res. Commun. 48,* 126-134.

7. Burkard, G., and Keller, E. B. (1974) *Proc. Nat. Acad. Sci. U.S.A. 71,* 389-393.

8. Venkataraman, P. R., and Mahler, H. R. (1963) *J. Biol. Chem. 238,* 1058-1067.

9. Haff, L. A., and Keller, E. B. (1973) *Biochem. Biophys. Res. Commun. 51,* 704-710.

10. Twu, J. S., and Bretthauer, R. K. (1971) *Biochemistry 10,* 1576-1582.

11. Hyatt, E. A. (1967) *Biochim. Biophys. Acta 142,* 246-253.

12. Hyatt, E. A. (1967) *Biochim. Biophys. Acta 142,* 254-262.

13. August, J. T.; Ortiz, P. J.; and Hurwitz, J. (1962) *J. Biol. Chem. 237,* 3786-3793.

14. Sippel, A. E. (1973) *Eur. J. Biochem. 37,* 31-40.

15. Modak, M. J., and Srinivasan, P. R. (1973) *J. Biol. Chem. 248,* 6904-6910.

16. Payne, K. J., and Boezi, J. A. (1970) *J. Biol. Chem. 245,* 1378-1387.

17. Moss, B.; Rosenblum, E. N.; and Paoletti, E. (1973) *Nature New Biol. 245,* 59-63.

18. Stoltzfus, C. M.; Morgan, M.; Banerjee, A. K.; and Shatkin, A. J. (1974) *J. Virol. 13,* 1338-1345.

19. Darnell, J. E.; Jelinek, W. R.; and Molloy, G. R. (1973) *Science 181,* 1215-1221.

20. Brawerman, G. (1974) *Annu. Rev. Biochem. 43,* 621-642.

21. Hirsch, M., and Penman, S. (1973) *J. Mol. Biol. 80,* 379-391.

22. Hirsch, M., and Penman, S. (1974) *J. Mol. Biol. 83,* 131-142.

23. Edmonds, M., and Kopp, D. W. (1970) *Biochem. Biophys. Res. Commun. 41,* 1531-1537.

24. Perry, R. P.; Kelley, D. E.; and LaTorre, J. (1972) *Biochem. Biophys. Res. Commun. 48,* 1593-1600.

25. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; and Randall, R. J. (1951) *J. Biol. Chem. 193,* 265-275.

26. Bollum, F. J. (1966) in *Procedures in Nucleic Acid Research.* Edited by S. L. Cantoni and D. R. Davies. New York: Harper and Row, p. 579.

27. Ayad, S. R., and Blamire, J. (1972) *Techniques of Nucleic Acid Fractionation.* New York: John Wiley and Sons, p. 42.

28. Ts'o, P. P.; Helmkamp, G. K.; and Sander, C. (1962) *Biochim. Biophys. Acta 55,* 584-600.

29. Rottman, F.; Friderici, K.; Comstock, P.; and Khan, M. K. (1974) *Biochemistry 13,* 2762-2771.

30. Poonian, M. S.; Schlabach, A. J.; and Weissbach, A. (1971) *Biochemistry 10,* 424-427.

31. Failla, D., and Santi, D. V. (1973) *Anal. Biochem. 52,* 363-368.

32. Chamberlin, M. J., and Ring, J. (1972) *Biochem. Biophys. Res. Commun. 49,* 1129-1136.

33. Küpper, H.; McAllister, W.; and Bautz, E. K. F. (1973) *Eur. J. Biochem. 38,* 581-586.

34. Riva, S., and Silvestri, L. G. (1972) *Annu. Rev. Microbiol. 26,* 199-224.

35. Ohasa, S., and Tsugita, A. (1972) *Nature New Biol. 240,* 35-38.

36. Holleman, W. (1966) Ph.D. thesis, Michigan State University, E. Lansing, Michigan.

37. Ackers, G. K. (1967) *J. Biol. Chem. 242,* 3237-3238.

38. McGuinness, E. T. (1973) *J. Chem. Educ. 50,* 826-830.

39. Johnson, J. C.; DeBacker, M.; and Boezi, J. A. (1971) *J. Biol. Chem. 246,* 1222-1232.

40. Jaenicke, R., and Knof, S. (1968) *Eur. J. Biochem. 4,* 157-163.

41. Garen, A., and Levinthal, C. (1960) *Biochim. Biophys. Acta 38,* 470-483.

42. Terzi, M.; Cascino, A.; and Urbani, C. (1970) *Nature 226,* 1052-1054.

43. Schäfer, R.; Zillig, W.; and Priess, H. (1972) *Fed. Eur. Biochem. Soc. Lett. 25,* 87-90.

44. Gerard, G. F.; Gurgo, C.; Grandgenett, D. F.; and Green, M. (1973) *Biochem. Biophys. Res. Commun. 53,* 194-201.

SECTION III

THE EFFECT OF 3'-DEOXYADENOSINE 5'-TRIPHOSPHATE ON THE REACTION CATALYZED BY ATP:RNA ADENYLYLTRANSFERASE FROM NOVIKOFF HEPATOMA

ABSTRACT

A Mn^{2+}-dependent ATP:RNA adenylyltransferase was purified from the cytoplasm of Novikoff hepatoma (strain N1-S1). The activity was primer-dependent, had a pH optimum of 8, and was sensitive to the sulfhydryl reagent, *p*-hydroxymercuribenzoate. This enzyme had a sedimentation coefficient ($s^{\circ}_{20,w}$) of 4.1 S. The preferred primers were polyadenylic acid and yeast transfer RNA. Rifamycin SV AF/013 inhibited the enzyme; 50% of the activity remained at 20 µg per ml. The drug cordycepin (3'-deoxyadenosine) did not affect adenylyltransferase activity. However, the triphosphate derivative of the drug, 3'-deoxyadenosine 5'-triphosphate, was a competitive inhibitor with ATP for the reaction. The Km for ATP and the Ki for 3'-deoxyadenosine 5'-triphosphate were 2×10^{-5} *M*.

INTRODUCTION

ATP:RNA adenylyltransferase (EC 2.7.7.19), or as it is more commonly known by the trivial name, poly A (Adenylate) polymerase, was first isolated nearly fifteen years ago from calf thymus nuclei (1). Since then there have been numerous reports of the activity in prokaryotes (2-4), eukaryotes (5-10), and viral cores (11,12). Recently, the discovery in eukaryotes of 3'-terminal polyadenylation of messenger RNA (mRNA) and of its precursor, heterogeneous DNA-like nuclear RNA (HnRNA), has lead to the postulation that enzymes of this type catalyze the posttranscriptional synthesis of those polyadenylate (poly[A])segments (for a review, see Darnell *et al.* [13] and Brawerman [14]). Polyadenylation of these DNA transcripts is believed to be important for the effective processing and the functionality of mRNA. The *in vivo* polyadenylation of these RNAs is sensitive to the drug cordycepin. If the eukaryotic adenylyltransferase is responsible for this polyadenylation, then it should also be sensitive to the drug. The AMP incorporation by the adenylyltransferase purified from calf thymus nuclei (5) or from rat liver nuclei (6), however, was not affected by the presence of cordycepin. The triphosphate derivative of cordycepin (3'-dATP) is probably

the form in which cordycepin would inhibit adenylyltransferase activity. The effect that 3'-dATP has on adenylyltransferase activity has not been studied previously.

This report describes some of the basic properties of the ATP:RNA adenylyltransferase from Novikoff hepatoma cells. In addition, there is presented the first evidence that a eukaryotic adenylyltransferase is effectively inhibited by 3'-dATP. In agreement with previous results, cordycepin itself was found ineffective in inhibiting the *in vitro* synthesis of poly(A).

EXPERIMENTAL PROCEDURE

Materials

The homopolyribonucleotides, *p*-hydroxymercuribenzoate and calf thymus DNA were from Sigma Chemical Co. Poly(dT)[1] and Qβ RNA were purchased from Miles Laboratories, Inc. Dithiothreitol, ADP, 2'-dATP and all unlabeled 5'-triphosphate derivatives of the ribonucleotides were obtained from P-L Biochemicals, Inc. ^{3}H-labeled nucleotides were purchased from Schwarz/Mann. Whatman DEAE-cellulose (DE52) and cellulose phosphate (P-1) were from H. Reeve Angel, Inc. Nitrocellulose membrane filters (type B-6) were obtained from Schleicher and Schuell, Inc. Nogalomycin, actinomycin, and α-amanitin were from Upjohn, Merck, and Henley and Co., respectively. Rifamycin derivatives were the gifts of Dr. Luigi Silvestri, Gruppo Lepetit, Inc., Milan, Italy. Yeast tRNA, 3'-dA, and 3'-*O*-methylATP were the gifts of Dr. Fritz Rottman of this department. Chromatographically pure 3'-dATP was a gift of Ron Desrosiers also of this department. *Pseudomonas putida* ribosomal RNA was prepared according to a procedure previously described (3). Qβ RNA was purified by sedimentation through a sucrose gradient before use. Calf thymus DNA was activated by a previously described method (15).

[1]The abbreviations used are listed in the *Journal of Biological Chemistry 249*, 1 (1974).

Analytical Methods

Protein concentration was determined by the method of Lowry *et al.* (16) with bovine serum albumin as the standard. The concentrations of calf thymus DNA and Qβ RNA were determined spectrophotometrically based on the extinction coefficient $E_{260}^{1\%} = 200$. The molar extinctions, ε(P), used to determine the concentration of ribosomal and transfer RNA at 260 nm were 7.4×10^3 (17) and 7.2×10^3 (18), respectively. The molar extinctions, ε(P), for the homopolynucleotides were: 10.5×10^3 at 257 nm, 9.2×10^3 at 260 nm and 6.5×10^3 at 267 nm for poly(A), poly(U), and poly(C), respectively (19) and 8.1×10^3 at 260 nm for poly(dT) (17). The molar extinction used to determine spectrophotometrically the concentrations of 3'-dA, 3'-dATP and 3'-*O*-methylATP at 259 nm was 1.5×10^4.

Assay of ATP:RNA Adenylyltransferase

The standard assay measured the conversion of [^{3}H]ATP into acid-insoluble form. Unless otherwise indicated, the complete reaction mixture (0.2 ml) contained 100 m*M* Tris-HCl (pH 8), 1 m*M* $MnCl_2$, 1 m*M* dithiothreitol, 100 μg per ml bovine serum albumin, 0.1 m*M* [^{3}H]ATP (100 cpm per pmol), a saturating amount of poly(A) and adenylyltransferase. After incubation for 30 min at 37°, the reaction was terminated by addition of 2 ml cold 10% trichloroacetic acid -1% sodium pyrophosphate. Bovine serum albumin, 50 μl of a 5 mg per ml solution, was then added as carrier. After 5 min at 0°, the mixture was centrifuged at 10,000 x *g* for 2 min. The supernatant solution was removed and discarded.

The pellet was dissolved in 0.35 ml of 0.5 *N* NaOH. Following the addition of cold 10% trichloroacetic acid -1% sodium pyrophosphate and incubation for 5 min at 0°, the acid-insoluble material was collected on a membrane filter. The filter was then washed and dried. The radioactivity in the filter was determined by counting in 5 ml of a scintillation fluid containing 4 g of 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)] thiophene per liter of toluene.

One unit of enzymatic activity is defined as one pmol of AMP incorporated into acid-insoluble form per 30 min. Specific activity is expressed as units per mg protein. The amounts of enzyme activity which were added to the reaction mixtures were within the activity range which gave direct proportionality with the rate of AMP incorporation for 30 min.

Partial Purification of ATP:RNA Adenylyltransferase

The N1-S1 strain of Novikoff hepatoma cells were grown in Swimm's S-77 media supplemented with 4 m*M* glutamine and 10% calf serum (20). The cells were grown in an atmosphere of 5% CO_2 in air in sealed screw cap Erlenmeyer flasks or spinner flasks with a doubling time of approximately 12 hours at 37°. Cells were harvested by centrifugation when growth had reached 8 x 10^5 cells per ml (mid-logrithmic phase). All subsequent procedures were performed at 0-4°. For a typical purification about 1 x 10^9 cells were used. The cells were washed by suspension in Basal Salt Solution

(21), followed by low speed centrifugation (1000 x *g* for 2 min). The cells were swollen by suspension in 15 ml of 10 m*M* Tris-HCl (pH 7.6), 6 m*M* KCl, and 5 m*M* $MgCl_2$ for 5 min, then lysed by bringing the suspension to 0.5% (v/v) NP-40. The supernatant fraction of a low speed centrifugation of the lysed cells was centrifuged at 80,000 x *g* for 60 min. The resulting supernatant fraction was dialyzed against 10 m*M* Tris-HCl (pH 8) and 5 m*M* 2-mercaptoethanol (Cytoplasmic Extract).

The cytoplasmic extract enzyme fraction was passed through a 4 x 14 cm column of DEAE-cellulose equilibrated with 10 m*M* Tris-HCl (pH 8) and 5 m*M* 2-mercaptoethanol. The enzymatic activity absorbed to the column was eluted by the application of column buffer containing 150 m*M* KCl. The peak activity fractions were pooled, then dialyzed against 10 m*M* potassium phosphate (pH 7.1) and 5 m*M* 2-mercaptoethanol (DEAE-cellulose).

The DEAE-cellulose enzyme fraction was absorbed onto a 0.9 x 7 cm column of phosphocellulose equilibrated with 10 m*M* potassium phosphate (pH 7.1) and 5 m*M* 2-mercaptoethanol. A linear gradient of 0 to 1 *M* KCl in column buffer applied to the phosphocellulose column produced a single peak of enzymatic activity, eluting at approximately 0.4 *M* KCl. Fractions containing peak enzymatic activity were pooled (Adenylyltransferase), and used as a source of enzyme for all assays reported here. Adenylyltransferase had a

specific activity of 1×10^5 units per mg protein, representing a 15-fold purification and a 60% recovery from the cytoplasmic extract fraction. Enzymatic activity was stable for at least one month when stored at 4°.

RESULTS AND DISCUSSION

Properties

The characteristics of the reaction catalyzed by the soluble enzyme from Novikoff hepatoma purified through phosphocellulose chromatography are presented in Table I. The incorporation of AMP into acid-insoluble form was dependent upon added enzyme, an exogenous RNA primer and Mn^{2+}. Mg^{2+} could not substitute for Mn^{2+} at either 2.5 m*M* or 10 m*M*. The activity was sensitive to the sulfhydryl reagent, *p*-hydroxymercuribenzoate. Even though dithiothreitol or bovine serum albumin were not required, their presence stabilized optimal activity. In separate experiments it was determined that optimal activity was achieved at pH 8 and at 0.5 m*M* $MnCl_2$.

The specificity of the enzyme for ATP as substrate is displayed in Table II. The rate of reaction was greatest when ATP was present. UTP, CTP, or GTP were not utilized as substrates by adenylyltransferase. The small amount of incorporation when 2'-dATP or ADP was present might possibly be a reflection of the impurity of this preparation.

The primer specificity of adenylyltransferase was investigated (Table III). Of the synthetic homopolyribonucleotides tested at saturating concentrations, poly(A) was the most effective primer of AMP incorporation. Yeast

TABLE I.--Characteristics of the Reaction Catalyzed by Adenyltransferase.*

Components of the Reaction Mixture	Relative Incorporation of AMP
Complete	100 (230 ± 1)
minus enzyme	0
minus poly(A)	0
minus Mn^{2+}	0
minus Mn^{2+}, plus 2.5 m*M* or 10 m*M* Mg^{2+}	0
minus bovine serum albumin	82
minus dithiothreitol	84
minus dithiothreitol, plus 1 m*M* *p*-hydroxymercuribenzoate	4

*The complete reaction mixture (0.2 ml) contained 100 m*M* Tris-HCl (pH 8), 1 m*M* $MnCl_2$, 1 m*M* dithiothreitol, 100 μg per ml bovine serum albumin, 0.1 m*M* [^{3}H]ATP (100 cpm per pmol), a saturating amount of poly(A) and an appropriate amount of enzyme. The pmol of [^{3}H]AMP incorporated into acid-insoluble product after 30 min of incubation at 37° is given in parentheses.

TABLE II.--Nucleotide Specificity for Adenylyltransferase.*

Nucleotide	Relative Incorporation of Mononucleotides
ATP	100 (129 ± 1)
2'-dATP	19
ADP	12
CTP	4
GTP	0
UTP	0

*The complete reaction mixture (0.2 ml) contained 100 m*M* Tris-HCl (pH 8), 1 m*M* $MnCl_2$, 1 m*M* dithiothreitol, 100 μg per ml bovine serum albumin, a saturating amount of poly(A), an appropriate amount of enzyme and 0.1 m*M* labelled nucleoside di- or triphosphate (100 cpm per pmol) as indicated. The pmol of [^{3}H]nucleotide monophosphate incorporated into acid-insoluble product after 30 min of incubation at 37° is given in parentheses.

TABLE III.--Primer Specificity for Adenylyltransferase.*

Primer	Relative Incorporation of AMP
Poly(A)	100 (237 ± 1)
Poly(C)	21
Poly(U)	3
Poly(dT)	2
S. cerevisiae transfer RNA	79
P. putida ribosomal RNA	27
Qβ RNA	12
Activated calf thymus DNA	0
None	0

*Reaction mixtures (0.2 ml) contained 100 m*M* Tris-HCl (pH 8), 1 m*M* $MnCl_2$, 1 m*M* dithiothreitol, 100 μg per ml bovine serum albumin, 0.1 m*M* [^{3}H]ATP (100 cpm per pmol), an appropriate amount of enzyme and a saturating amount of primer. The pmol of [^{3}H]AMP incorporated into acid-insoluble product after 30 min of incubation at 37° is given in parentheses.

transfer RNA provided the greatest priming activity of the natural RNAs. Neither polydeoxynucleotide, poly(dT) nor activated calf thymus DNA, functioned as a primer for the reaction.

Adenylyltransferase was subjected to centrifugation in 5-20% sucrose gradients containing 50 m*M* Tris-HCl (pH 7.5), 250 m*M* KCl and 5 m*M* 2-mercaptoethanol (data not shown). When compared to the relative migration of *E. coli* alkaline phosphatase ($s^{\circ}_{20,w}$ = 6.3 S), the single, sharp peak of adenylyltransferase activity was determined to have a sedimentation coefficient $(s_{20,w})^2$ of 4.1 S.

In summary, the properties of this adenylyltransferase are similar to those reported for the Mn^{2+}-dependent enzyme from other eukaryotic sources (8-10). For example, the adenylyltransferase from the cytoplasm of calf thymus had a pH optimum of 8.3, was effectively primed by poly(A) and had a sedimentation coefficient ($s^{\circ}_{20,w}$) of 3.5 S (8).

Inhibitors

Several rifamycin derivatives, actinomycin, nogalomycin and α-amanitin were tested for their effect on the AMP incorporation by adenylyltransferase. As shown in Table IV, the presence of nogalomycin, actinomycin or α-amanitin had little or no effect on the rate of AMP incorporation. Of

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[2]The enzyme concentration was much less than 0.1 mg per ml, so this is essentially a $s^{\circ}_{20,w}$ value.

TABLE IV.--The Effect of Inhibitor Compounds on Adenylyltransferase.*

Inhibitor Added to the Reaction Mixture	Relative Incorporation of AMP
Experiment I	
None	100 (93 ± 1)
Rifamycin AF/AP	122
Rifampicin	122
Rifamycin PR/19	100
Rifamycin AF/BO	67
Rifamycin AF/DNFI	22
Rifamycin AF/013	11
Experiment II	
None	100 (119 ± 1)
Nogalomycin	92
Actinomycin	125
α-amanitin	117

*Reaction mixtures (0.2 ml) contained 100 m*M* Tris-HCl (pH 8), 1 m*M* $MnCl_2$, 1 m*M* dithiothreitol, 100 μg per ml bovine serum albumin, 0.1 m*M* [^{3}H]ATP (100 cpm per pmol), a saturating amount of poly(A) and an appropriate amount of enzyme. In addition, in Experiment I the rifamycin derivatives were added to reaction mixtures to a final concentration of 50 μg per ml. In Experiment II nogalomycin, actinomycin or α-amanitin were added to reaction mixtures to a final concentration of 50, 50, or 20 μg per ml. The pmol of [^{3}H]AMP incorporated into acid-insoluble product after 30 min of incubation at 37° is given in parentheses.

the rifamycin derivatives tested, only AF/DNFI and AF/013 were effective inhibitors of adenylyltransferase. In a separate experiment, the concentration of AF/013 that gave 50% inhibition was determined to be 20 µg per ml. The adenylyltransferase from Novikoff hepatoma, a non-template-requiring polymerase, can be added to the list of nucleic acid polymerizing enzymes that are inhibited by AF/013 (22).

Substrate Analogs

Several structural analogs of ATP were examined for their effect on the ATP-incorporating activity of adenylyltransferase. The rate of AMP incorporation was measured at various concentrations of ATP in the absence and in the presence of each analog. The data was analyzed using Lineweaver-Burk double reciprocal plots (Figure 1). At a concentration of 1 m*M*, 3'-dA (cordycepin) had no effect on the rate of AMP incorporation. However, 3'-dATP and 3'-*O*-methylATP were equally effective as competitive inhibitors of ATP. The Km[3] for ATP as well as the Ki for 3'-dATP or 3'-*O*-methylATP was $2 \pm 1 \times 10^{-5}$ *M*.

Practically all eukaryotic mRNA (except histone mRNA) isolated contains a 3'-terminal poly(A) sequence. As a part of the current model for eukaryotic mRNA biogenesis, the poly(A) sequences are proposed to be posttranscriptionally

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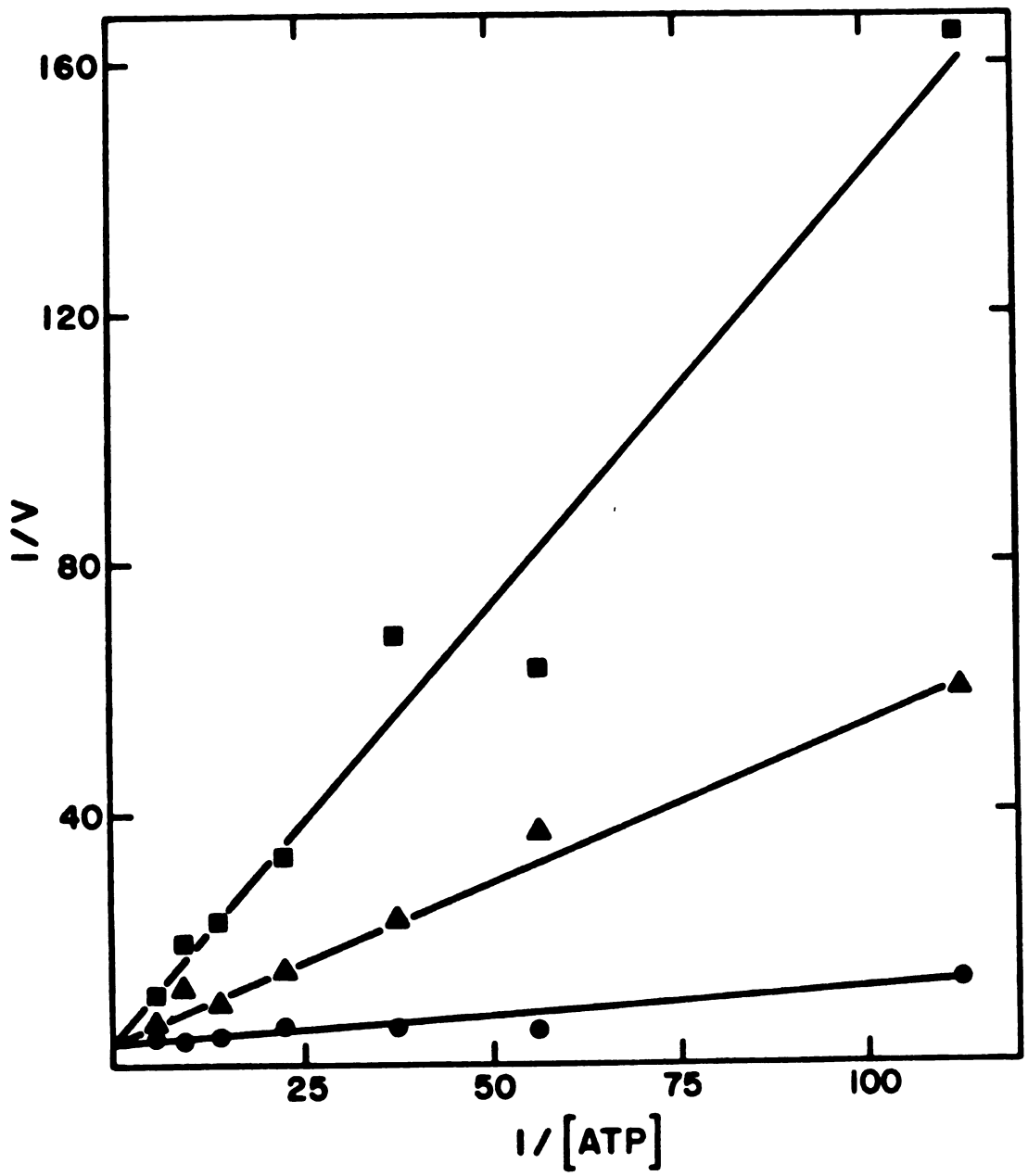
[3]A linear least squares analysis of the data provided the source for the reported values of Km or Ki. The variances following the values represent multiple determinations.

Figure 1.--The effect of ATP structural analogs on AMP incorporation by adenylyltransferase. The complete reaction mixture was that described in the EXPERIMENTAL PROCEDURE, except that varying amounts of [^{3}H]ATP (100 cpm per pmol) were present. AMP incorporated in the complete reaction mixture ( ), in the complete reaction mixture plus 8.8 x 10^{-5} *M* 3'-dATP ( ), and in the complete reaction mixture plus 5 x 10^{-4} *M* 3'-*O*-methyl ATP ( ). The addition of 1 x 10^{-3} *M* 3'-dA to the complete reaction mixture did not alter the rate of AMP incorporation. Velocity is in pmol AMP incorporated per 30 min; and substrate is in m*M* ATP.


160
120
80
40
1/V
25
50
75
100
1/[ATP]

synthesized on the 3'-hydroxyl end of the heterogeneous nuclear RNA (HnRNA) (13). The presence of these sequences on the DNA transcripts is associated with the effective processing of mRNA from HnRNA and for the appearance of mRNA in the cytoplasm. These functions for the poly(A) sequence were derived from observations of the *in vivo* effects of the drug cordycepin (3'-dA). In cells treated with 3'-dA, HnRNA synthesis continued, but little mRNA appeared with polyribosomes. Also, the poly(A) sequences of HnRNA and mRNA isolated from these cells were significantly shorter. Similar results have been observed for Novikoff hepatoma cells.[4]

Since poly A polymerase appeared as a likely candidate for catalyzing the posttranscriptional synthesis of poly(A), 3'-dA was examined for its effect on this enzyme. When *in vitro* reactions catalyzed by the enzyme from the nuclei of calf thymus (5) or of rat liver (6) included 3'-dA, no effect on the rate of AMP incorporation was detected. This is reasonable since the triphosphate derivative (3'-dATP) is probably the form in which cordycepin would be active against adenylyltransferase. This report provides the first *in vitro* evidence that 3'-dATP does inhibit the poly(A) synthesis catalyzed by a eukaryotic poly A polymerase. It also confirms the previous observations

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[4]R. Desrosiers, personal communication.

that 3'-dA does not effectively inhibit the *in vitro* synthesis of poly(A).

REFERENCES

1. Edmonds, M., and Abrams, R. (1960) *J. Biol. Chem. 235,* 1142-1149.

2. August, J. T.; Ortiz, P. J.; and Hurwitz, J. (1962) *J. Biol. Chem. 237,* 3786-3793.

3. Payne, K. J., and Boezi, J. A. (1970) *J. Biol. Chem. 245,* 1378-1387.

4. Sippel, A. E. (1973) *Eur. J. Biochem. 37,* 31-40.

5. Winters, M. A., and Edmonds, M. (1973) *J. Biol. Chem. 248,* 4756-4762.

6. Niessing, J., and Sekeris, C. E. (1973) *Nature New Biol. 243,* 9-12.

7. Burkard, G., and Keller, E. B. (1974) *Proc. Nat. Acad. Sci.* U.S.A. *71,* 389-393.

8. Tsiapalis, C. M.; Dorson, J. W.; DeSanta, D. M.; and Bollum, F. J. (1973) *Biochem. Biophys. Res. Commun. 50,* 737-743.

9. Giron, M. L., and Huppert, J. (1972) *Biochim. Biophys. Acta 287,* 438-447.

10. Walter, T. J., and Mans, R. J. (1970) *Biochim. Biophys. Acta 217,* 72-82.

11. Moss, B.; Rosenblum, E. N.; and Paoletti, E. (1973) *Nature New Biol. 245,* 59-62.

12. Stoltzfus, C. M.; Morgan, M.; Banerjee, A. K.; and Shatkin, A. J. (1974) *J. Virol. 13,* 1338-1345.

13. Darnell, J. E.; Jelinek, W. R.; and Molloy, G. R. (1973) *Science 181,* 1215-1221.

14. Brawerman, G. (1974) *Annu. Rev. Biochem. 43,* 621-642.

15. Schlabach, A.; Fridlender, B.; Bolden, A.; and Weissbach, A. (1971) *Biochem. Biophys. Res. Commun. 44,* 879-885.

16. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; and Randall, R. J. (1951) *J. Biol. Chem. 193,* 1593-1600.

17. Bollum, F. J. (1966) in*Procedures in Nucleic Acid Research.* Edited by S. L. Cantoni and D. R. Davies. New York: Harper and Row, p. 579.

18. Ayad, S. R., and Blamire, J. (1972) *Techniques of Nucleic Acid Fractionation.* New York: John Wiley and Sons, p. 42.

19. Ts'o, P. P.; Helmkamp, G. K.; and Sander, C. (1962) *Biochim. Biophys. Acta 55,* 584-600.

20. Morse, P. A., Jr., and Potter, V. R. (1965) *Cancer Res. 25,* 499-508.

21. Plagemann, P. G. W., and Roth, M. F. (1969) *Biochem. 8,* 4782-4789.

22. Gerard, G. F.; Gurgo, C.; Grandgenett, D. P.; and Green, M. (1973) *Biochem. Biophys. Res. Commun. 53,* 194-201.

APPENDIX

APPENDIX

A RAPID, SIMPLE STAINING TECHNIQUE FOR PROTEINS SEPARATED IN POLYACRYLAMIDE GEL ELECTROPHORESIS

Introduction

The electrophoresis of proteins in polyacrylamide gels is a quick, simple procedure for the separation of proteins. Sophisticated analytical measurements, for example, the determination of molecular weight (1), can be obtained relatively easily. One drawback to this technique has been the tedious routine to locate separated proteins. By a conventional staining technique employing Coomassie Brilliant Blue R250 in trichloroacetic acid followed by various destaining solutions and procedures (2), a minimum of 20 to 24 hours was required for protein identification. For experiments requiring optimal sensitivity (about 0.1 μg protein) with low, uniform backgrounds such a lengthy procedure has been acceptable. Decreased time for protein band visualization has been obtained by electrophoretically destaining, but some band mobility has also occurred. However, for many situations such delays, extreme sensitivity or involved techniques were undesirable. This is the case, for example, with the use of preparative gel

electrophoresis as part of a purification procedure, or in the analysis of large numbers of samples.

A new staining procedure has been developed to achieve the goals of simplicity and quickness without significantly sacrificing sensitivity or background uniformity. The staining procedure originated from ideas presented in articles from the laboratories of Diezel *et al.* (3) and Malik and Berrie (4). The first report (3) introduced the methyl substituted triphenylmethane dye, Coomassie Brilliant Blue G250. This dye was less soluble in 12.5% trichloroacetic acid than the conventionally used parent dye, Coomassie Brilliant Blue R250. This fact meant the reduction of dye penetration into non-protein areas of the polyacrylamide gels, requiring less destaining and thus providing shorter times for protein band visualization. As a consequence, interior portions of the gel containing protein were not stained, lowering the protein-stained band intensity and, thereby, stain sensitivity. The second paper (4) described a method using a stain containing sulfuric acid, trichloroacetic acid and Coomassie Brilliant Blue R250. The protein-stained color produced was usually not of sufficient contrast compared to the background for satisfactory protein detection or sensitivity. To reduce backgrounds destaining was required, but destaining also caused the concomitant reduction in intensity of protein-stained areas. The integration of these two procedures has

produced a staining technique which is rapid (as quick as 30 min), requires little or no destaining, has a sensitivity of at least 1 μg of protein per band, and is simple to perform.

Stain Preparation

Coomassie Brilliant Blue G250 (xylene brilliant cyanin G) was purchased from K and K Laboratories, Inc. To a 0.2% (w/v) aqueous solution of the dye was added an equal volume of 2 *N* (5.6% [v/v]) H_2SO_4. After the solution was mixed well, it was allowed to stand for at least three hours. The precipitated solution was filtered through Whatman No. 1 by gravity flow. To the clear brown filtrate was added one-nineth volume of 10 *N* KOH. Trichloroacetic acid was added to the resulting dark purple solution to a final concentration of 12% (w/v). The resulting clear, light blue solution was ready for use.

The stain can be stored for several months without loss of effectiveness. Upon storage the solution color will change towards grey, but no change in the resulting protein-stained color or intensity is observable. The stain may be reused several times, but the pH of the solution must be maintained below 1.0.

Comments

After electrophoresis, the polyacrylamide gel can be added directly to the staining solution; for example,

15 ml of stain is used in a test tube for a 0.7 x 10 cm cylindrical gel. Within 30 minutes protein bands of 5 to 10 µg are evident, while maximum development is achieved in 5 to 8 hours. Gels can be stored in the staining solution without overstaining or increasing the background level. When stored in water, there is a marked color intensification and background reduction producing optimal sensitivity. A rapid loss of all stain occurs if the gels are placed in solutions containing sulfuric acid, trichloroacetic acid, or acetic acid. The stained gels can be scanned densimetrically at 640 nm. A linear relationship was observed between absorbance and protein concentration in the concentration range of 1 µg to at least 10 µg per band. When a tracking dye, for example, pyronin B, is used, the dye does not become obscured by the stain, permitting direct measurements of relative mobilities.

This staining procedure can be used with SDS as well as non-dissociating polyacrylamide gels. The stain does contain a sufficient concentration of potassium ions to precipitate SDS in the gel, causing a clouded background. However, the resulting background will not obscure the visual detection of most protein-stained bands. When clear backgrounds are desired, especially for densimetric scanning, gels containing SDS should be rinsed with 10% (w/v) trichloroacetic acid, 33% (v/v) methanol, or water for up to 48 hours prior to stain application. If electrophoresis

is performed in the presence of less than 0.1% (w/v) SDS, then significant gel clouding is not encountered. Alternatively, NaOH can be substituted for KOH in preparing the stain, reducing the amount of interference by SDS precipitation (5). However, this modification has not been met with complete success.

This staining procedure has also been effectively used in other laboratories in this department. A part of the purification procedure of rat pancreas zymogen granule membrane proteins employed preparative slab gel electrophoresis (5). The rapid identification of the proteins using this staining technique enabled the subsequent purification steps to follow without long delays. Similarly, the purification of rat brain hexokinase utilized gel electrophoresis of antibody-enzyme complex (6). This stain provided the quick identification of the protein for subsequent elution, reducing the duration of the purification by one to two days. In another case, the lipoproteins of insect hemolymph were subjected to polyacrylamide gel electrophoresis as part of a study of the effect of filipin on insects (7). This protein stain was chosen for its fast development and clear, uniform backgrounds, which were well-suited for densimetric scanning. The quick visualization of the results also allowed for many more samples to be analyzed than by the conventional staining procedures. Finally, the simplicity and almost

infallibility of this staining technique made it practical for use in the teaching laboratory. The experiment in BCH 805 on gel electrophoresis of proteins was greatly simplified by use of this stain. The problems encountered by the students, that is, proper staining and destaining intervals and procedures, were eliminated by the substitution of the conventional technique with the procedure described here.

REFERENCES

1. Weber, K., and Osborn, M. (1969) *J. Biol. Chem. 244,* 4406-4412.

2. Johnson, J. C.; DeBacker, M.; and Boezi, J. A. (1971) *J. Biol. Chem. 246,* 1222-1232.

3. Diezel, W.; Kopperschläger, G.; and Hofmann, E. (1972) *Anal. Biochem. 48,* 617-620.

4. Malik, N., and Berrie, A. (1972) *Anal. Biochem. 49,* 173-176.

5. MacDonald, R. J. (1974) Ph.D. thesis, Michigan State University, E. Lansing, Michigan.

6. Felgner, P., personal communication.

7. Schroeder, F. (1973) Ph.D. thesis, Michigan State University, E. Lansing, Michigan.