2′-*O*-Methyladenosine 5′-Triphosphate. A Substrate for Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase of *Pseudomonas putida**

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ABSTRACT: 2′-*O*-Methyladenosine 5′-triphosphate (AmTP) was a substrate for deoxyribonucleic acid dependent ribonucleic acid polymerase of *Pseudomonas putida*. In the DNA-directed synthesis of RNA with AmTP or an equimolar mixture of AmTP and adenosine 5′-triphosphate as the adenyl substrates, a small amount of 2′-*O*-methyladenosine 5′-monophosphate (AmMP) was incorporated into RNA after 60-min incubation. This amounted to 1–2 pmoles of AmMP for each pmole of RNA polymerase added to the reaction mixture. AmMP-containing RNA synthesized in reaction mixtures which contained AmTP as the only added adenyl substrate had a sedimentation coefficient of less than 4 S as determined by sucrose density gradient analysis. Abortive release of the AmMP-containing RNA from the DNA–RNA polymerase–nascent RNA ternary complex did not occur. AmMP-containing RNA synthesized in reaction mixtures which contained both AmTP and ATP as the adenyl substrates was heterogeneous in size with most of the RNA having a sedimentation coefficient of about 30 S. Degradation of the RNA product by alkaline hydrolysis followed by alkaline phosphatase digestion showed that 90% of the AmMP residues were located at the 3′ end of the RNA chain with the remaining 10% at the 5′ end or in the interior of the chain. The studies with AmTP lead to the following conclusions. (1) A free 2′-hydroxyl group is not required for binding of an adenyl substrate by *P. putida* RNA polymerase or for subsequent incorporation into RNA. (2) Following the incorporation of AmMP into the 3′ end of the nascent RNA chain, the rate of RNA chain growth is greatly reduced. The reduction in the rate of RNA chain growth results in the accumulation of nascent RNA chains with AmMP at the 3′ end. The rate-limiting step in RNA synthesis becomes the addition of the next nucleotide to the nascent RNA chain.

A contribution to the understanding of the mechanism of the DNA-directed synthesis of RNA by RNA polymerase (ribonucleoside triphosphate:RNA nucleotidyl transferase, EC 2.7.7.6) has been made through studies of base-altered analogs of the four common ribonucleoside triphosphates. Knowledge of the extent and specificity of incorporation of base-altered analogs by *Escherichia coli* RNA polymerase has added to the understanding of the structural requirements for hydrogen bonding between complementary bases in the RNA synthetic process (Kahan and Hurwitz, 1962) and has led to a definition of the role of base-stacking interactions with nearest neighbors in RNA synthesis (Goldberg and Rabinowitz, 1961; Nishimura *et al.*, 1966; Slapikoff and Berg, 1967; Ikehara *et al.*, 1968).

The mechanism of RNA synthesis has also been studied through the use of substrate analogs altered in the ribose moiety. Studies of 3′-deoxyadenosine 5′-triphosphate (3′-dATP) have indicated that a 3′-hydroxyl group is not required for binding of a nucleoside to *E. coli* or *Micrococcus luteus* RNA polymerase or for incorporation of a nucleotide into the 3′ end of RNA by these enzymes (Shigeura and Boxer, 1964; Shigeura and Gordon, 1965; Sentenac *et al.*, 1968a,b). Once 3′-dAMP is incorporated into the 3′ end of a RNA chain, however, RNA chain growth is terminated. Another substrate analog of ATP, 3′-amino-3′-deoxyadenosine 5′-triphosphate, appears to function in a similar manner as a RNA chain growth terminator (Shigeura *et al.*, 1966).

Studies of analogs of nucleoside triphosphates altered at the 2′ position of the ribose moiety have not clearly defined the requirement for a 2′-hydroxyl group in reactions catalyzed by RNA polymerase. Experiments using 2′-deoxyribonucleoside 5′-triphosphates have indicated that these analogs probably do not bind appreciably to *E. coli* or *M. luteus* RNA polymerase nor are they incorporated into RNA in a native DNA-directed reaction (Weiss and Nakamoto, 1961; Chamberlin and Berg, 1962; Furth *et al.*, 1962; Anthony *et al.*, 1969). Other experiments using denatured calf thymus DNA (Chamberlin and Berg, 1964) and poly[d(A-T)] (Krakow and Ochoa, 1963) as templates indicated that the 2′-deoxyribonucleoside 5′-triphosphates may have substrate activity. Studies with 1-(β-D-arabinofuranosyl)cystosine 5′-triphosphate (ara-CTP)[1] have shown that no appreciable DNA-directed RNA synthesis by *E. coli* RNA polymerase occurred when ara-CTP was used in place of CTP (Cardeilhac and Cohen, 1964). These studies were not designed, however, to detect the direct incorporation of a small amount of labeled ara-CMP into RNA. Chu and Fischer (1968) reported that ara-C is incorporated into both terminal and internal positions of RNA of murine leukemic cells *in vivo* indicating that for this system an unaltered 2′ position on the ribose moiety of a nucleoside triphosphate is not required for incorporation into RNA.

This report describes the effects of 2′-*O*-methyladenosine 5′-triphosphate (AmTP) on the reactions catalyzed by RNA

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[1] The abbreviation used that is not listed in *Biochemistry* 9, 4022 (1970), is: ara-C, 1-(β-D-arabinofuranosyl)cytosine.

polymerase of *Pseudomonas putida*. The purpose of this investigation was twofold. First, we wished to establish whether or not AmTP was a substrate for RNA polymerase, and if so, what effect the incorporation of AmMP into the 3' end of nascent RNA would have on further RNA chain growth. Second, we hoped to prepare 2'-*O*-methyl-containing RNA which was complementary to a DNA template. 2'-*O*-Methylribonucleoside diphosphates have been used to prepare various synthetic polymers using polynucleotide phosphorylase (Rottman and Heinlein, 1968; Rottman and Johnson, 1969). The results of the present studies demonstrated that AmTP is a substrate for *P. putida* RNA polymerase. Following the incorporation of AmMP into RNA, the rate of RNA chain growth was greatly reduced. Consequently, the synthesis of RNA chains containing more than a few AmMP residues was infeasible.

Materials and Methods

Materials. NADP, UDPG, glucose 6-phosphate dehydrogenase, and all the unlabeled 5'-phosphate derivatives of the ribonucleosides were purchased from P-L Biochemicals, Inc. Calf thymus DNA, herring sperm DNA, poly(A), poly(U), poly(C), and phosphoglucomutase were obtained from Sigma Chemical Co. Poly[d(A-T)] was from Miles Laboratories, Inc. *E. coli* alkaline phosphatase, electrophoretically purified, was from Worthington Biochemical Corp. [^{3}H]ATP and [^{3}H]adenosine were obtained from Schwarz BioResearch, Inc. Whatman DEAE-cellulose (DE-1) was from Reeve Angel. Nitrocellulose membrane filters (type B-6) were obtained from Schleicher & Schuell. Wheat germ RNA (Singh and Lane, 1964), *E. coli* B tRNA (Holley *et al.*, 1961), and *Pseudomonas putida* rRNA (Payne and Boezi, 1970) were prepared by published procedures. *P. putida* bacteriophage gh-1 DNA (Lee and Boezi, 1966, 1967) was prepared by the method of Thomas and Abelson (1966). *P. putida* RNA polymerase was purified by the procedure of Johnson *et al.* (1971). The preparations of RNA polymerase used in these experiments were at least 90% pure. UDPG pyrophosphorylase was isolated from calf liver (Albrecht *et al.*, 1966) and recrystallized twice.

Descending paper chromatography employed one of the following solvent systems: (a) isobutyric acid–concentrated NH_4OH–H_2O (66:1:33, v/v), (b) isopropyl alcohol–concentrated NH_4OH–H_2O (7:1:2, v/v), and (c) isopropyl alcohol–concentrated NH_4OH–0.1 M boric acid (7:1:2, v/v).

AmTP was prepared by the procedure of Rottman and Heinlein (1968). AmTP was further purified by descending chromatography on acid-washed Whatman No. 3MM paper. Successive development of AmTP was carried out in solvent systems a and b, and in each case, development was followed by elution from the paper and lyophilization to concentrate the eluant. The purified AmTP contained a small amount of AmDP (about 5%) as determined by chromatography in solvent a.

The synthesis of [^{3}H]AmTP from [^{3}H]adenosine was carried out by methods similar to those described by Rottman and Heinlein (1968). The following modifications in procedure were made. (1) [^{3}H]AmMP prepared from [^{3}H]Am and UMP with wheat seedling phosphotransferase was purified by descending chromatography on Whatman No. 3MM paper in solvent a, and (2) [^{3}H]AmTP prepared from [^{3}H]-AmMP and ATP by treatment with rabbit muscle myokinase was purified in two steps. First, [^{3}H]AmTP and [^{3}H]AmDP were separated from [^{3}H]AmMP and the 5'-phosphate derivatives of adenosine by descending chromatography in solvent c. Second, [^{3}H]AmTP was separated from [^{3}H]-AmDP by electrophoresis in 0.05 M ammonium formate (pH 3.5) on acid-washed Whatman No. 3MM paper using a Hormuth pherograph (Brinkman Instruments, Inc.) (1800 V, 2 hr). The purified [^{3}H]AmTP contained a small amount of [^{3}H]AmDP (about 10%) as determined by chromatography in solvent a.

Analytical Methods. Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. The concentrations of native gh-1 and calf thymus DNA were determined spectrophotometrically based on the extinction coefficient $E_{260}^{1\%}$ 200. The molar extinctions, ϵ(P), used to determine the homopolymer concentrations 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, in 0.1 M NaCl–0.05 M Tris-acetate (pH 7.5) (Ts'o *et al.*, 1962). For poly[d(A-T)], ϵ(P) = 6.7×10^3 at 260 nm and pH 7.5 was used (Radding and Kornberg, 1962).

Assay of RNA Polymerase. The reaction mixture for monitoring the gh-1 DNA-directed synthesis of RNA by radioisotope incorporation contained 20 mM Tris-acetate (pH 8.0), 5 mM 2-mercaptoethanol, 2 mM magnesium acetate, 0.5 mM manganese acetate, 60 mM ammonium acetate, 0.2 mM ATP, and/or 0.2 mM AmTP, 0.2 mM each of UTP, GTP, and CTP, 100 μg/ml of gh-1 DNA, and *P. putida* RNA polymerase. The concentrations of divalent metal ions (Mg^{2+} and Mn^{2+}), monovalent cation (NH_4^+), nucleoside triphosphates, and DNA used in the reaction mixture gave the optimal rate of RNA synthesis. ATP or AmTP was labeled with ^{3}H. RNA polymerase ($\alpha_2\beta\beta'\sigma$) contained a full compliment of σ factor. After incubation at 30°, the total reaction mixture or a sample from it was mixed with 100 μl of 0.1% sodium dodecyl sulfate. Cold 10% trichloroacetic acid–1% sodium pyrophosphate solution (5 ml) and 250 μg of herring sperm DNA were then added. After 15 min at 0–4°, the acid-insoluble product was collected on a membrane filter. Each filter was washed with four 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/l. of toluene.

Reactions using the templates poly[d(A-T)], poly(U), poly(A), poly(C), and denatured calf thymus DNA were monitored using a spectrophotometric assay (Johnson *et al.*, 1968). The assay couples the formation of inorganic pyrophosphate from ribonucleoside triphosphate polymerization to NADP reduction. The reaction mixture with poly[d(A-T)] contained 20 mM Tris-acetate (pH 8.0), 1 mM manganese acetate, 0.4 mM each of ATP and UTP, 45 μM poly[d(A-T)], and 27 μg/ml of RNA polymerase. The reaction mixture with poly(U) contained 100 mM Tris-acetate (pH 8.0), 1 mM manganese acetate, 0.4 mM ATP, 23 μM poly(U), and 20 μg/ml of RNA polymerase. The reaction mixture with poly(A) contained 20 mM Tris-acetate (pH 8.0), 2 mM manganese acetate, 2.8 mM UTP, 36 μM poly(A), and 20 μg/ml of RNA polymerase. The reaction mixture with poly(C) contained 100 mM Tris-acetate (pH 8.0), 1.5 mM manganese acetate, 1.2 mM GTP, 290 μM poly(C), and 240 μg/ml of RNA polymerase. The reaction mixture with denatured calf thymus DNA contained 20 mM Tris-acetate (pH 8.0), 2 mM magnesium acetate, 0.5 mM manganese acetate,

0.4 mM ATP, 94 μg/ml of denatured calf thymus DNA, and 93 μg/ml of RNA polymerase. In addition, all of the above reaction mixtures contained 0.4 mM NADP, 0.4 mM UDPG, and excess phosphoglucomutase, glucose 6-phosphate dehydrogenase, and UDPG pyrophosphorylase. For the concentration of divalent metal ion used in each type of reaction, the optimal concentrations of nucleoside triphosphate(s) and template were used. The amount of RNA polymerase used in each case was rate limiting.

Analysis of the Positions of the [^{3}H]AmMP Residues in RNA. Reaction mixtures (0.5 ml) containing [^{3}H]AmTP (97,000 cpm/nmole), or [^{3}H]AmTP and ATP, 95 μg/ml of RNA polymerase, and the other components of the reaction mixture for the gh-1 DNA-directed synthesis of RNA listed above were incubated for 60 min. After incubation, a sample of each reaction mixture was removed and assayed for ^{3}H-labeled trichloroacetic acid insoluble product. The remainder of each reaction mixture was then treated in the following manner. Trichloroacetic acid to 5% and 2 mg of wheat germ RNA were added. The resultant precipitate was collected by centrifugation and washed by resuspension in five 5-ml portions of cold 5% trichloroacetic acid, once with 5 ml of 95% ethanol, and once with 5 ml of ethanol–ether (3:1, v/v). The washed precipitate together with an additional 8 mg of wheat germ RNA was dissolved in 1 ml of 1.0 N KOH and incubated at room temperature for 90 hr. Cold 4 N $HClO_4$ (0.25 ml) was added and the mixture was centrifuged at 0°. The pellet was washed twice with 1 ml of cold 0.2 N $HClO_4$. The supernatant from the first centrifugation and the washings were combined, neutralized with KOH, and centrifuged to remove $KClO_4$. The neutralized solution which contained 200 A_{260} units was diluted to 6.0 ml and made 0.05 M in $(NH_4)_2CO_3$ (pH 8.5). Following the addition of 0.3 mg of *E. coli* alkaline phosphatase, the solution was incubated at 37° for 24 hr. After lyophilization, the phosphatase digest was dissolved in 1 ml of H_2O. Samples were removed to assay radioactivity by liquid scintillation spectrometry (Bray, 1960). The rest of the phosphatase digest was diluted to 100 ml and applied to a DEAE-cellulose column (1.2 × 15 cm) in the formate form at a flow rate of 1 ml/min. The nucleosides were eluted from the column with 0.005 M ammonium formate followed by the dinucleoside monophosphates with 0.5 M ammonium formate (Price and Rottman, 1970). After repeated lyophilization, samples from each fraction were monitored for radioactivity and for absorbancy at 260 nm. Based on the absorbancy at 260 nm of the nucleoside and dinucleoside monophosphate fractions, the 2′-*O*-methyl content of the carrier wheat germ RNA was 2.5%. The value reported by Singh and Lane (1964) for wheat germ RNA is 1.9%. The total recovery of A_{260} units from the DEAE-cellulose column for each type of reaction mixture was 97% of the A_{260} units present in the neutralized KOH hydrolysate.

Identification of the ^{3}H-labeled material in each fraction was carried out in the following manner. The nucleoside fraction was chromatographed on acid-washed Whatman No. 3MM paper in solvent a. This procedure separated A and Am from G, U, and C. A and Am were then separated by chromatography in solvent c. The dinucleoside monophosphate fraction was subjected to electrophoresis on Whatman No. 1 paper in 0.1 M ammonium bicarbonate (pH 7.8) using a Spinco Model R instrument (400 V, 4 hr). Separation of dinucleoside monophosphates (AmpN) from any contaminating nucleosides is achieved by this procedure.

Sucrose Density Gradient Analysis of [^{3}H]AmMP-Containing RNA and the gh-1 DNA·(RNA Polymerase)·(Nascent RNA) Complex. Reaction mixtures (0.25 ml) containing [^{3}H]AmTP (70,000 cpm/nmole), or [^{3}H]AmTP and ATP, 95 μg/ml of RNA polymerase, and the other components of the reaction mixture for the gh-1 DNA-directed synthesis of RNA as listed above were incubated for 60 min. After cooling to 4°, a sample (0.1 ml) was removed from each reaction mixture and mixed with 10 μl of 5% sodium dodecyl sulfate. The mixture was incubated at 37° for 5 min. After cooling to 4°, the mixture was centrifuged at 5000 rpm for 5 min to remove precipitated sodium dodecyl sulfate. Samples (0.1 ml) of the original reaction mixtures and the sodium dodecyl sulfate treated samples were layered on 4.8-ml 5–20% linear sucrose gradients prepared in 50 mM Tris-acetate (pH 8.0), 100 mM NaCl, and 1 mM dithiothrietol. After centrifugation for 260 min at 39,000 rpm in the Spinco SW39 rotor at 4°, fractions were collected from the bottom of the centrifuge tubes. To determine the position of [^{3}H]AmMP-containing RNA in the gradients, each fraction or a sample of each was precipitated with cold 10% trichloroacetic acid–1% sodium pyrophosphate, collected on a membrane filter, and monitored for radioactivity.

The position of DNA in the gradients from the original reaction mixtures was determined by assaying samples (50 μl) of each fraction for template activity, measured as the incorporation of [^{14}C]AMP into RNA in the presence of excess RNA polymerase. At 32 μg/ml of RNA polymerase, the rate of [^{14}C]AMP incorporation into RNA was proportional to the DNA concentration between 0 and 3 μg per ml. In the sucrose density gradient analyses, *E. coli* B tRNA, *P. putida* rRNA, and gh-1 DNA were used as markers of known sedimentation coefficients.

Results

Incorporation of [^{3}H]AmMP into RNA. [^{3}H]AmMP was incorporated into RNA in a gh-1 DNA-directed reaction catalyzed by *Pseudomonas putida* RNA polymerase (Table I). In the two reaction mixtures which contained RNA polymerase, gh-1 DNA, GTP, CTP, UTP, and [^{3}H]AmTP, 55 and 66 pmoles of [^{3}H]AmMP per ml were incorporated into RNA. If ATP at a concentration equal to that of AmTP was included in the reaction mixture, approximately the same amount of [^{3}H]AmMP was incorporated.

Relative to the amount of AMP incorporated into RNA, the amount of AmMP incorporated was small (Table I). In comparable reaction mixtures, one containing [^{3}H]ATP, the other [^{3}H]AmTP, approximately 200 times as much [^{3}H]AMP (11,400 pmoles/ml) was incorporated into RNA as [^{3}H]AmMP (55–66 pmoles/ml). In the reaction mixtures containing an equimolar mixture of ATP and AmTP, 77 times as much [^{3}H]AMP (5800 pmoles/ml) as [^{3}H]AmMP (75 pmoles/ml) was incorporated into RNA.

Sucrose Density Gradient Analysis of [^{3}H]AmMP-Containing RNA. The sedimentation patterns of [^{3}H]AmMP-containing RNA synthesized in gh-1 DNA-directed reactions with [^{3}H]AmTP or an equimolar mixture of [^{3}H]AmTP and ATP as the adenyl substrates were examined by sucrose gradient centrifugation. The results are presented in Figure 1. Most of the [^{3}H]AmMP-containing RNA synthesized in the reaction mixture which contained [^{3}H]AmTP as the adenyl substrate was small in size, as judged from a sedimentation coefficient of less than 4 S (Figure 1, lower). [^{3}H]AmMP-containing RNA synthesized in the reaction mixture which contained both [^{3}H]AmTP and ATP was of diverse size,

TABLE I: Incorporation of [^{3}H]AmMP or [^{3}H]AMP into RNA.[a]

Expt	Adenyl Substrate(s) Added to the Reaction Mix.	Reaction Mix. Component Omitted	Incubn Time (min)	[^{3}H]AmMP or [^{3}H]AMP Incorpd (pmoles/ml)
1	[^{3}H]AmTP	None	0	0
	[^{3}H]AmTP	None	30	66
	[^{3}H]AmTP	RNA polymerase	30	0
	[^{3}H]AmTP, ATP	None	30	75
	[^{3}H]AmTP, ATP	gh-1 DNA	30	4
	AmTP, ATP	None: [^{3}H]AmTP added immediately before reaction was terminated	30	0
2	[^{3}H]AmTP	None	30	55
	[^{3}H]AmTP, ATP	None	30	72
3	[^{3}H]ATP	None	0	0
	[^{3}H]ATP	None	30	11,400
	[^{3}H]ATP	gh-1 DNA	30	57
	[^{3}H]ATP, AmTP	None	30	5,800

[a] Reaction mixtures (0.05 ml) containing the components described in Materials and Methods for the gh-1 DNA-directed synthesis of RNA were incubated for the times indicated. After incubation, the amount of radioactivity in the trichloroacetic acid insoluble product was determined as described in Materials and Methods. [^{3}H]AmTP and [^{3}H]ATP where indicated were present at 70,000 and 15,000 cpm per nmole, respectively. RNA polymerase was at 63 μg/ml. The values for the incorporation of [^{3}H]-AmMP and [^{3}H]AMP were those obtained after the subtraction of 16 and 35 pmoles per ml, respectively, which were the amounts of ^{3}H-labeled substrates which were trapped on the membrane filters from reaction mixtures terminated at zero time.

TABLE II: Position of the [^{3}H]AmMP Residues in RNA.[a]

Expt	Adenyl Substrate(s) Added to the Reaction	Cl_3CCOOH-Insoluble RNA Product Formed (cpm)	Alkaline Phosphatase Digest (cpm)	Nucleoside Fraction (cpm)	Dinucleoside Monophosphate Fraction (cpm)
1	[^{3}H]AmTP	11,000	500		
2	[^{3}H]AmTP, ATP	10,000	9700	7700	900

[a] Reaction mixtures (0.5 ml) containing the components for the gh-1 DNA-directed synthesis of RNA described in Materials and Methods were incubated for 60 min, and were treated as described in Materials and Methods.

with most of the [^{3}H]AmMP-containing RNA having a sedimentation coefficient of about 30 S (Figure 1, upper).

Position of the [^{3}H]AmMP Residues in RNA. The RNA produced in gh-1 DNA-directed reactions with [^{3}H]AmTP or [^{3}H]AmTP and ATP as the adenyl substrates was washed with cold trichloroacetic acid to remove unreacted substrates and was then degraded with KOH followed by alkaline phosphatase. As a result of this procedure, AmMP residues at the 3' end of the RNA chain were converted into Am, and those at the 5' end or in the interior of the RNA chain were converted into dinucleoside monophosphates (AmpN). Separation of Am and AmpN was achieved by chromatography on DEAE-cellulose. The results are shown in Table II.

For the analysis of the RNA synthesized in the reaction mixture which contained [^{3}H]AmTP as the adenyl substrate, only 5% of the radioactivity in the [^{3}H]AmMP-containing RNA product was recovered after the washing procedure with trichloroacetic acid prior to KOH hydrolysis. As shown by the sucrose density gradient analysis (Figure 1, lower), [^{3}H]AmMP-containing RNA produced in the reaction mixture with [^{3}H]AmTP as the adenyl substrate was small in size. Consequently, it was lost during the repeated washing with cold trichloroacetic acid. Insufficient radioactivity was present in the alkaline phosphatase digest (Table II, expt 1) to permit analysis of the nucleoside and dinucleoside monophosphate content by DEAE-cellulose chromatography.

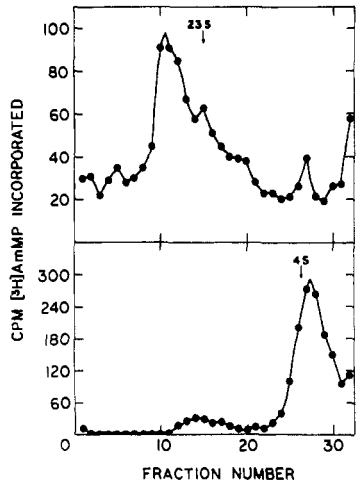

FIGURE 1: Sucrose density gradient analysis of the [³H]AmMP-containing RNA product. Reaction mixtures containing the components of the gh-1 DNA-directed synthesis of RNA with [³H]-AmTP (lower) or [³H]AmTP and ATP (upper) were treated with sodium dodecyl sulfate and analyzed by sucrose gradient centrifugation as described in Materials and Methods.

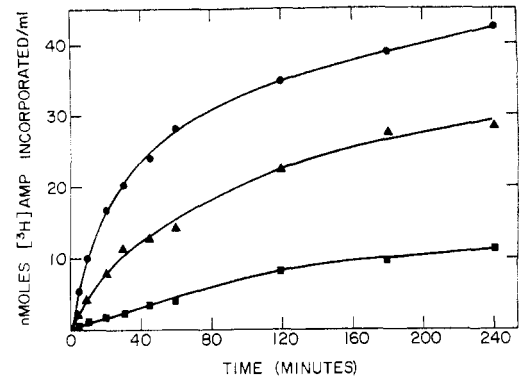

FIGURE 3: Effect of AmTP on the incorporation of [³H]AMP into RNA. The reaction mixtures contained, in a volume of 0.5 ml, those components for the gh-1 DNA-directed synthesis of RNA listed in Materials and Methods. RNA polymerase was present at 33 μg/ml and [³H]ATP at 15,000 cpm/nmole. At various times during the incubation, 50-μl aliquots were removed and assayed for radioactivity in the trichloroacetic acid insoluble product as described in Materials and Methods. Incorporation of [³H]AMP is shown in the absence of AmTP (●), and in the presence of 0.2 mM (▲) and 0.8 mM (■) AmTP.

For the analysis of the RNA synthesized in the reaction mixture which contained [³H]AmTP and ATP as the adenyl substrates (Table II, expt 2), 97% of the radioactivity in the [³H]AmMP-containing RNA product was recovered in the alkaline phosphatase digest. The alkaline phosphatase digest was separated into nucleoside and dinucleoside monophosphate fractions by chromatography on DEAE-cellulose. The nucleoside fraction contained 90% of the radioactivity recovered from the DEAE-cellulose column and the dinucleoside monophosphate contained 10%. Analysis of the nucleoside fraction from the DEAE-cellulose column by paper chromatography confirmed that the radioactive material was [³H]Am. Analysis of the dinucleoside monophosphate fraction by paper electrophoresis showed that the fraction was not contaminated with labeled Am and that the radioactive material present had the same electrophoretic mobility as authentic dinucleoside monophosphates.

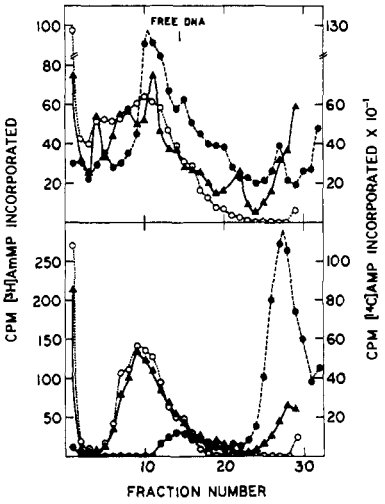

FIGURE 2: Sucrose density gradient analysis of the DNA·(RNA polymerase)·([³H]AmMP-containing RNA) ternary complex. Samples of reaction mixtures containing the components of the gh-1 DNA-directed synthesis of RNA with [³H]AmTP (lower) or [³H]-AmTP and ATP (upper) were analyzed by sucrose gradient centrifugation or treated with sodium dodecyl sulfate and then analyzed by sucrose gradient centrifugation. (●) [³H]AmMP-containing RNA of the samples that had been treated with sodium dodecyl sulfate. (▲) [³H]AmMP-containing RNA of samples that had not been treated with the detergent. (○) DNA, as monitored by template activity measured as the incorporation of [¹⁴C]AMP into RNA in the presence of an excess of RNA polymerase.

Nonrelease of [³H]AmMP-Containing RNA from the gh-1 DNA·(RNA Polymerase)·(Nascent RNA) Complex. The data presented in Table II led to the conclusion that most of the [³H]AmMP incorporated into RNA was at the 3′ end of RNA chains. A possible explanation for this conclusion is that the addition of an AmMP residue to the 3′ end of a nascent RNA chain induced the release of that chain from the DNA·(RNA polymerase)·(nascent RNA) ternary complex before RNA chain propagation could continue. To look for release of [³H]AmMP-containing RNA, sucrose density gradient analysis was used. After incubation, samples of reaction mixtures containing the components of gh-1 DNA-directed RNA synthesis with [³H]AmTP or [³H]AmTP and ATP as the adenyl substrates were treated with sodium dodecyl sulfate, and centrifuged through sucrose gradients. The position in the gradients of [³H]AmMP-containing RNA, that is [³H]AmMP-containing RNA released from the ternary complex, was determined. Other samples of the reaction mixtures, not treated with sodium dodecyl sulfate, were also centrifuged through sucrose gradients. The position in the gradients of the DNA·(RNA polymerase)·(nascent RNA) ternary complex was determined to be that position where [³H]AmMP-containing RNA and DNA cosedimented at speeds faster than that of free [³H]AmMP-containing RNA or free DNA. The results are presented in Figure 2. In the case of the analysis of the reaction mixture containing [³H]-AmTP as the adenyl substrate, the data show that approximately 85% of the [³H]AmMP-containing RNA cosedimented with DNA in the ternary complex. This result leads to the conclusion that release of [³H]AmMP-containing RNA from the ternary complex had not occurred to any significant degree. In the case of the analysis of the reaction mixture containing both [³H]AmTP and ATP as the adenyl substrates, it is not clear how much, if any, release of [³H]AmMP-

TABLE III: The Effect of AmTP on Several Reactions Catalyzed by RNA Polymerase.[a]

Expt	NTP Substrates Added	Polymer Added	AmTP (mM)	Initial Velocity: nmoles of NADPH Produced/min per ml	Inhibn (%)
1	ATP, UTP	Poly[d(A-T)]		2.6	
	ATP, UTP	Poly[d(A-T)]	0.4	1.5	42
	UTP	Poly[d(A-T)]		0.1	
	UTP	Poly[d(A-T)]	0.4	0.1	
	ATP, UTP			<0.1	
2	ATP	Poly(U)		1.0	
	ATP	Poly(U)	0.4	0.5	50
		Poly(U)	0.4	0	
	ATP			0	
3	ATP	dDNA[b]		1.4	
	ATP	dDNA[b]	0.4	0.2	86
		dDNA[b]	0.4	0	
	ATP			0	
4	UTP	Poly(A)		3.1	
	UTP	Poly(A)	0.2	3.1	0
	UTP	Poly(A)	1.0	3.3	0
		Poly(A)	2.8	0	
	UTP			0	
5	GTP	Poly(C)		4.5	
	GTP	Poly(C)	0.4	4.2	7
		Poly(C)	0.4	0	
	GTP			0	

[a] The reaction mixtures, in a volume of 0.25 ml, contained the components described in Materials and Methods. The production of NADPH was measured as described in Materials and Methods. [b] dDNA-denatured calf thymus DNA.

containing RNA from the ternary complex had occurred because of the difficulty in distinguishing between free RNA and RNA in the ternary complex.

Absence of Synthesis of Trichloroacetic Acid Soluble Oligonucleotides. In all of the experiments described above in which AmTP was the adenyl substrate, only the formation of trichloroacetic acid insoluble RNA was monitored. In order to determine if trichloroacetic acid soluble oligonucleotides were being synthesized, the reaction was monitored by measuring inorganic pyrophosphate formation using a spectrophotometric assay (Materials and Methods). In a reaction mixture containing 50 μg/ml of RNA polymerase, 100 μg/ml of gh-1 DNA, 0.2 mM each of GTP, CTP, UTP, and AmTP, and the components of the coupled assay system, no inorganic pyrophosphate formation was observed. Thus, within the limits of sensitivity of the assay (a rate of formation of 0.02 nmole of inorganic pyrophosphate/min per ml of reaction mixture or greater), no DNA-directed reaction was detected, indicating that RNA polymerase was not repeatedly initiating the synthesis and release of trichloroacetic acid soluble oligonucleotides.

AmTP as an Inhibitor of RNA Polymerase Catalyzed Reactions. Although the presence of ATP at an equimolar concentration to that of [^{3}H]AmTP did not decrease the amount of [^{3}H]AmMP incorporated into RNA (see Table I), the presence of AmTP in reaction mixtures containing [^{3}H]ATP decreased the amount of [^{3}H]AMP incorporated. In reaction mixtures containing RNA polymerase, gh-1 DNA, and 0.2 mM each of GTP, CTP, UTP, and [^{3}H]ATP, the initial rate of [^{3}H]AMP incorporation into RNA was inhibited by 63 and 90% in the presence of 0.2 and 0.8 mM AmTP, respectively (Figure 3). The extent of [^{3}H]AMP incorporation at 240 min was reduced by 30 and 72%, respectively, by the two concentrations of AmTP.

The polymerization of ATP and UTP directed by poly[d(A-T)], and the polymerization of ATP directed by poly(U) or denatured calf thymus DNA were inhibited by AmTP (Table III). AmTP had little or no effect on the poly(A)-directed polymerization of UTP or the poly(C)-directed polymerization of GTP. These results indicate that AmTP was an inhibitor of only those reactions which used ATP as a substrate.

Discussion

AmTP was a substrate for the gh-1 DNA-directed synthesis of RNA by *Pseudomonas putida* RNA polymerase. By virtue of its structure and the fact that it inhibited only those RNA polymerase catalyzed reactions which used ATP as a substrate, AmTP may be regarded as a substrate analog of ATP. Thus, a free 2′-hydroxyl group is not required for binding of an adenyl substrate by *P. putida* RNA polymerase or for subsequent incorporation into RNA. However, in both gh-1 DNA-directed reactions studied, the amount of AmMP incorporated was small. With either AmTP or an equimolar mixture of AmTP and ATP as the adenyl substrates, approximately 1–2 pmoles of AmMP was incorporated in 60 min/pmole of enzyme added to the reaction mixtures. The pmoles of RNA polymerase was calculated using mol wt 5×10^5 (Johnson *et al.*, 1971).

AmMP-containing RNA synthesized in the reaction mixture which contained AmTP as the only added adenyl substrate was small in size, as judged from a sedimentation coefficient of less than 4 S. AmMP-containing RNA synthesized in the reaction mixture which contained AmTP and ATP was heterogeneous in size with most of the RNA having a sedimentation coefficient of 30 S. Under similar assay conditions, RNA synthesized in gh-1 DNA-directed reactions with the four common nucleoside triphosphates (GTP, CTP, UTP, and ATP) can achieve an even larger size, as judged from a sedimentation coefficient of 45 S (G. F. Gerard and J. A. Boezi, unpublished results).

AmMP incorporated into RNA in the reaction mixture which contained an equimolar mixture of AmTP and ATP was found both at the 3′ end of the RNA chain, and at the 5′ end or in the interior of the chain. Most of the AmMP was at the 3′ end of the chain. The presence of some AmMP at the 5′ end or in the interior of the RNA chain shows that nascent RNA chain growth can continue following the incorporation of AmMP. Thus, AmTP does not function as a chain terminator as is the case with 3′-dATP (Shigeura and Boxer, 1964).

The location of AmMP incorporated into RNA in the reaction mixture which contained AmTP as the only added adenyl substrate was not determined. Since there was no significant RNA chain release from the ternary complex, each enzyme molecule could have initiated the synthesis of only one RNA chain. Because there was only 1–2 pmoles of AmMP incorporated per pmole of enzyme, there was therefore probably only 1–2 AmMP residues/RNA chain. Most of the AmMP-containing RNA synthesized in this reaction mixture was estimated to have a sedimentation coefficient of somewhat less than 4 S. As estimated from the sucrose gradient profile, the sedimentation coefficient was approximately 3 S which corresponds to a chain length of 50–75 nucleotides (Madison, 1968). In order to explain the synthesis of RNA of this chain length containing only 1–2 AmMP residues, two alternatives are suggested. First, the RNA had an unusual base composition being very low in adenyl content. Second, the RNA had a base composition which reflected the overall base composition of the gh-1 DNA template (Lee and Boezi, 1966) with a mole per cent adenyl groups equal to 22%, but the additional adenyl groups were from ATP present as a contaminant in the reaction mixtures. Consistent with the second alternative is the observation that a slow rate of RNA synthesis (approximately 0.5% of the control rate) was detected in reaction mixtures which contained CTP, GTP, and UTP, but no added ATP (G. F. Gerard and J. A. Boezi, unpublished results). This observation suggests that one of the stock nucleoside triphosphates was contaminated with a small amount of ATP.

The results presented in this paper lead us to the following understanding of the mechanism of incorporation of AmMP into RNA by *P. putida* RNA polymerase. The substrate, AmTP, binds on the enzyme surface in place of ATP, and is incorporated into the 3′ end of the nascent RNA chain. The rate of incorporation of AmMP into the 3′ end of RNA is probably slower than that of AMP. This reduced rate is due to a lower affinity of the enzyme for AmTP than for ATP and/or a slower rate of phosphodiester-bond formation with AmTP as substrate than with ATP. The rate of initiation of synthesis of RNA chains is probably also slower with AmTP than with ATP. If ATP is present in the reaction mixture, competition between AmTP and ATP for binding on the enzyme results. Following the incorporation of AmMP into the 3′ end of the nascent RNA chain, the rate of RNA chain growth is greatly reduced. The reduction in rate of RNA chain growth results in the accumulation of nascent RNA chains with the AmMP at the 3′ end. The bulky 2′-*O*-methyl group at the 3′ end of the nascent RNA chain could impede translocation of RNA polymerase relative to the DNA template and nascent RNA chain and/or could hinder phosphodiester-bond formation with the incoming nucleoside triphosphate. Whatever the reason, the addition of the next nucleotide to the chain becomes the rate-limiting step in RNA synthesis. Abortive release of the RNA from the ternary complex does not occur. Following the addition of the next nucleotide, the rate of RNA chain growth may return to the normal *in vitro* rate, but perhaps not until the AmMP residue in the nascent RNA chain leaves the enzyme surface.

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Deoxyribonucleic Acid Polymerase with Rat Liver Ribosomes and Smooth Membranes. Purification and Properties of the Enzymes*

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ABSTRACT: Two enzymes that incorporate deoxyribonucleotides into DNA have been found in association with ribosomes and with a smooth membrane fraction isolated from rat liver. These enzymes have been partially purified by fractionation with ammonium sulfate and chromatography on DEAE-cellulose and phosphocellulose. The membrane and ribosome associated polymerases behave differently during the course of purification. Purified membrane polymerase is free of exo- and endonuclease activities but the purified ribosome polymerase contains a small amount of endonuclease activity. The purified enzymes have different pH optima but similar Mg^{2+} requirements. The ribosome-associated polymerase and the membrane-associated enzyme exhibit maximal activity at 9.0 and 8.0 pH units and at 15 and 10 mM Mg^{2+}, respectively. After purification both enzymes prefer "activated" DNA as primer, although the ribosome-associated enzyme retains some activity with native DNA primer. All four deoxyribonucleoside triphosphates are required by both enzymes for maximal activity, but the purified DNA polymerase from ribosomes has about 25% of maximal activity in the presence of only a single deoxyribonucleoside triphosphate. This activity does not appear to be a terminal addition enzyme since experiments with poly(dA) and poly[d(A-T)] demonstrate a requirement for base pairing of the deoxyribonucleoside triphosphate with the template. The properties of the ribosome- and smooth membrane-associated DNA polymerases suggest that they are not mitochondrial in origin. However, the DNA-synthesizing activity associated with ribosomes is similar to the activity extracted from highly purified rat liver nuclei. The DNA-synthesizing activity associated with the membrane fraction is not detectable in the KCl-extractable or nonextractable portion of rat liver nuclei.

Deoxyribonucleic acid polymerase activity has invariably been found in the postmicrosomal supernatant solution when mammalian tissue homogenates are prepared in aqueous media (Bollum and Potter, 1958; Davidson *et al.*, 1958). In fact, with the exception of a few specific studies of nuclear (Howk and Wang, 1969; Gold and Helleiner, 1964) and mtDNA polymerases (Kalf and Ch'ih, 1968; Meyer and Simpson, 1968), most of the investigations of the enzyme in mammalian tissues have used the postmicrosomal supernatant solutions as the starting material for isolation of the enzyme. Bollum and Potter (1958) and Keir and Smellie (1962) originally suggested that a portion of the DNA polymerase in mammalian cells may reside in the cytoplasm, as well as in the nucleus. However, these investigators acknowledged the possibility of artifact production during tissue homogenization. The recent demonstration of a unique mtDNA polymerase (Kalf and Ch'ih, 1968; Meyer and Simpson, 1968) clearly indicates that some of the DNA polymerase found in the cytoplasm is not representative of the nuclear enzyme. The question of whether the remaining DNA polymerase activity found in the cytoplasm is of nuclear origin must be examined critically and with an open mind. In particular, the demonstration of DNA associated with microsomes and polysomes (Bond *et al.*, 1969; Schneider and Kuff, 1969; Bell, 1969) raises the question of whether other unique ctDNA[1] polymerases might not exist. The demonstration of distinct membrane-associated and soluble DNA-synthesizing activities in bacteria (Okazaki *et al.*, 1970) clearly indicates the need for a critical examination of the distribution of DNA-synthesizing activity in higher organisms.

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[1] The abbreviations used are: ctDNA, cytoplasmic deoxyribonucleic acid; poly(dA), the homopolymer of deoxyriboadenylate; poly[d(A-T)], the alternating copolymer of deoxyriboadenylate and deoxyribothymidylate; poly(U), the homopolymer of uridylic acid; PMB, *p*-hydroxymercuribenzoate.