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Polynucleotides Containing 2′-*O*-Methyladenosine. I. Synthesis by Polynucleotide Phosphorylase*

Fritz Rottman and Karen Heinlein

ABSTRACT: 2′-*O*-Methyladenosine 5′-phosphate, which was chemically synthesized from adenosine, was converted into 2′-*O*-methyladenosine 5′-diphosphate with rabbit muscle myokinase. This nucleoside diphosphate was slowly polymerized by polynucleotide phosphorylase to form poly-2′-*O*-methyladenylic acid. The rate of incorporation of this modified substrate was only $^1/_{100}$ that obtained with adenosine 5′-diphosphate. Addition of oligonucleotide primer stimulated the incorporation of 2′-*O*-methyladenosine 5′-diphosphate in both crude and primer-dependent polynucleotide phosphorylase preparations. The apparent K_m for 2′-*O*-methyladenosine 5′-diphosphate in the presence of oligonucleotide primer was determined to be 1.8×10^{-2} M. Studies on the size of the product revealed that poly-2′-*O*-methyladenylic acid synthesized in the absence of primer sedimented at approximately 13–15 S while polymer formed in the presence of primer sedimented at 5–6 S. 2′-*O*-Methyladenosine 5′-diphosphate was polymerized by polynucleotide phosphorylase in the presence of uridine 5′-diphosphate. The product of this reaction was shown to be a heteropolymer resulting from mixed incorporation of both substrates by its susceptibility to both alkali and ribonuclease.

The occurrence of 2′-*O*-methylribose in RNA was first reported by Smith and Dunn (1959). It has since been found in RNA obtained from many sources (Biswas and Myers, 1960; Hall, 1964; Singh and Lane, 1964; Correll, 1965) including purified phenylalanyl-tRNA (RajBhandary *et al.*, 1967). Recent studies on rRNA isolated from HeLa cells indicate that the major site of methylation in this RNA molecule is the 2′-hydroxyl group of ribose (Wagner *et al.*, 1967).

In addition to conferring resistance to nucleases which hydrolyze polynucleotides through the formation of 2′,3′ cyclic intermediates, 2′-*O*-methyl groups have been shown to influence the susceptibility of RNA components toward other enzymes. Snake venom phosphodiesterase attacks oligonucleotides containing 2′-*O*-methyl groups with difficulty (Gray and Lane, 1967), and 2′-*O*-methylribonucleoside 5′-phosphates are resistant to snake venom 5′-nucleotidase (Honjo *et al.*, 1964). Recently, Norton and Roth (1967) have reported the isolation of a ribonuclease from *Anacystis nidulans* which is specific for phosphodiester linkages containing a 2′-*O*-methyl group.

The participation of 2′-hydroxyl groups in internal hydrogen bonding between adjacent nucleotides in RNA has been postulated to be a significant component of the forces favoring ordered structure within polynucleotide chains (Riley *et al.*, 1966; Sato *et al.*, 1966; Ts'o *et al.*, 1966). Therefore, substitution at the 2′ position with an *O*-methyl group may influence both the physical and biological properties of an RNA molecule.

In considering possible approaches to the synthesis of RNA molecules containing large amounts of 2′-*O*-methyl groups, polynucleotide phosphorylase-catalyzed polymerization seemed rather unlikely in light of previous work on the substrate specificity of this enzyme. Although the enzyme has been shown to utilize nucleoside diphosphates which are extensively modified in the base moiety, alterations of the ribose diphosphate moiety were believed to be unacceptable (Grunberg-Manago, 1963). Thus, inversion of the 2′-hydroxyl of ribose to form the arabinose derivative produced an inactive substrate (Michelson *et al.*, 1962; Lucas-Lenard and Cohen, 1966). Conversion of either the 2′- or 5′-hydroxyl of the nucleoside diphosphate into the corresponding deoxy analog likewise resulted in molecules lacking substrate activity for polynucleotide phosphorylase (Grunberg-Manago, 1963; Yengoyan and Rammler, 1966).

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This report describes the synthesis of A^mDP[1] using rabbit muscle myokinase and its subsequent incorporation into large molecular weight polymers by polynucleotide phosphorylase.

Materials and Methods

Chromatographic Methods. Analyses were carried out by descending chromatography on Whatman No. 1 paper in the following solvent systems: (A) *n*-propyl alcohol–concentrated NH_4OH–H_2O (55:10:35), (B) isopropyl alcohol–concentrated NH_4OH–H_2O (7:1:2), (C) isopropyl alcohol–concentrated NH_4OH–0.1 M boric acid (7:1:2), (D) 95% ethyl alcohol–1 M ammonium acetate (pH 7.5) (7:3), (E) *n*-butyl alcohol–concentrated NH_4OH–H_2O (86:5:14), (F) ethyl acetate–*n*-propyl alcohol–H_2O (4:1:2, upper phase), and (G) saturated $(NH_4)_2SO_4$–H_2O–isopropyl alcohol (78:18:2).

Thin-layer chromatography was performed on Eastman Chromagram cellulose sheets (no. 6065) and developed in solvent A. Polynucleotides and long oligonucleotides remain near the origin in this solvent system while mononucleotides and short oligonucleotides migrate further, depending upon their chain length.

Enzymes. The polynucleotide phosphorylase from *Micrococcus lysodeikticus* used in these studies was of three different types: (1) lyophilized polynucleotide phosphorylase was purchased from P-L Laboratories, Inc.; (2) the enzyme was purified from a commercial preparation of dried *M. lysodeikticus* cells (Miles Chemical Co.) by the method of Singer (1966) to stage VI. This preparation did not require oligonucleotide primer for activity with ADP as a substrate, and in these studies behaved identically with the commercial (P-L Laboratories, Inc.) preparation and (3) primer-dependent polynucleotide phosphorylase was prepared by trypsin treatment of the commercial P-L preparation according to the method of Klee and Singer (1967). Initial incorporation of ADP into polymer catalyzed by this enzyme was stimulated 40-fold by the addition of ApApApA primer.

Rabbit muscle myokinase was obtained from Calbiochem as an ammonium sulfate suspension. The enzyme preparation was dialyzed against 0.1 M Tris-acetate (pH 7.2) before use to remove ammonium sulfate. *Escherichia coli* alkaline phosphatase (Worthington Biochemical Corp., chromatographically purified) was passed over Sephadex G-25 to remove ammonium sulfate. Snake venom phosphodiesterase and micrococcal nuclease were obtained from Worthington Biochemical Corp.

2′-O-Methyladenosine. An aqueous solution of adenosine was treated with diazomethane in 1,2-dimethoxyethane as described by Broom and Robins (1965). Although the primary product of this reaction is A^m, other compounds were present in the reaction mixture, presumably resulting from methylation at the 3′- and 5′-hydroxyl positions as well as the purine ring itself. The mixture of products can be conveniently separated on Dowex 1 (OH^-) (Bio-Rad AG 1-X2, 200–400 mesh) using a separation procedure described by Dekker (1965) in which nucleosides are eluted with aqueous methyl alcohol. In a typical experiment, the reaction mixture obtained with 5 g of adenosine was taken to dryness, dissolved in 40 ml of 30% methyl alcohol, and applied to a Dowex column (5 × 60 cm) which had previously been equilibrated with 30% methyl alcohol. Elution with 30% methyl alcohol resulted in the separation of 2′-*O*-methyladenosine from small amounts of contaminants which preceded it (di-*O*-methyl and ring-substituted derivatives) and a larger contaminant (3′-*O*-methyladenosine) which was eluted later. Isolated 2′-*O*-methyladenosine was estimated to be greater than 97% pure by paper chromatography in solvent systems A–C. Conditions employed would have detected an ultraviolet-absorbing contaminant exceeding 2% of the total. The product migrated with naturally occurring 2′-*O*-methyladenosine obtained from wheat germ RNA in solvent systems A, C, E, and F. The yield of purified 2′-*O*-methyladenosine varied from 15 to 20%.

Heating a solution of synthetic 2′-*O*-methyladenosine with Dowex 50 (H^+) produced the free sugar which was characterized by electrophoresis in 0.1 M sodium borate (pH 9.45). Relative to ribose (1.0), 2′-*O*-methylribose (0.56) migrated similarly to 2′-deoxyribose (0.53), while 3′-*O*-methylribose (0.91), obtained from material presumed to be 3′-*O*-methyladenosine, migrated with 3′-deoxyribose (0.95) which was obtained from cordycepin (Rottman *et al.*, 1963).

2′-O-Methyladenosine 5′-Phosphate. The chemical phosphorylation of A^m was carried out using a modification of the method described by Tener (1962) for the synthesis of deoxycytidine 5′-phosphate. Unprotected A^m (6 mmoles) was allowed to react with the pyridinium form of 2-cyanoethyl phosphate (3 mmoles) in the presence of dicyclohexylcarbodiimide (10 g), dry pyridine (40 ml), dry dimethylformamide (20 ml), and a few grains of Dowex 50 in the pyridinium form for 65 hr at room temperature. Following the addition of 60 ml of 50% aqueous pyridine, the mixture was extracted three times with cyclohexane, allowed to stand another 24 hr at room temperature, and evaporated under vacuum to dryness. Hydrolysis of the cyanoethyl group was accomplished by treatment with 250 ml of 7 N NH_4OH at 70° for 6 hr. The reaction mixture was evaporated to a small volume, cooled in ice, and filtered to remove dicyclohexylurea.

A^mMP was isolated by chromatography on Dowex 1 (formate) (Bio-Rad AG 1-X2, 200–400 mesh). Unreacted A^m was eluted with water, followed by several uncharacterized products which were eluted with 0.1 M formic acid. A^mMP appeared immediately after changing to 1.0 M formic acid and was concentrated to dryness by lyophilization. A small contaminant representing approximately 1% of the material in the A^mMP

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: A^m, 2′-*O*-methyladenosine; A^mMP, 2′-*O*-methyladenosine 5′-phosphate; A^mDP, 2′-*O*-methyladenosine 5′-diphosphate; A^mTP, 2′-*O*-methyladenosine 5′-triphosphate; poly A^m, poly 2′-*O*-methyladenylic acid; poly (U, A^m), random copolymer of U and A^m; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

peak was removed by chromatography on a DEAE-cellulose column in the carbonate form using a linear gradient of triethylammonium bicarbonate (0.01–0.4 M, pH 7.8). The purified product was homogenous in solvent systems A, C, D, and G. The yield of pure A^mMP based on the amount of A^m used in the initial reaction was 21%.

Treatment of A^mMP with *E. coli* alkaline phosphatase resulted in complete conversion into A^m and the quantitative release of 1.1 moles of inorganic phosphate/mole of A^mMP. A^mMP was refractory to the action of snake venom 5′-nucleotidase (Sigma) under conditions which gave complete hydrolysis of adenosine 5′-phosphate and deoxyadenosine 5′-phosphate. This lack of activity is consistent with previous studies using this enzyme and 2′-*O*-methylnucleoside 5′-phosphates (Honjo *et al.*, 1964).

A^mMP was also synthesized enzymatically by the transfer of phosphate from uridine 5′-phosphate to A^m in the presence of a phosphotransferase preparation obtained from wheat seedlings (Rottman *et al.*, 1963). The product formed in this reaction was identical with that synthesized chemically. Although the yield obtained with the enzymatic reaction, which ranged from 20 to 25%, was slightly higher than chemical synthesis, the large amount of uridine 5′-phosphate required in this reaction made its use impractical. Chemical synthesis was generally employed for large-scale preparations.

2′-O-Methyladenosine 5′-Diphosphate. A^mMP was allowed to react with ATP in the presence of rabbit muscle myokinase and resulted in the production of AMP, ADP, A^mDP, and A^mTP. A typical reaction mixture contained 0.005 M A^mMP, 0.005 M ATP, 0.01 M magnesium acetate, 0.05 M Tris-Cl^- (pH 7.8), 3.2 mg of dialyzed rabbit muscle myokinase, and water to a total volume of 100 ml. Following incubation for 1.5 hr at 37°, the reaction mixture was diluted to 150 ml with water and 1.03 g of $NaIO_4$ (4.8 mmoles) and 5.5 ml of cyclohexylamine (48 mmoles) were added (Neu and Heppel, 1964). The reaction mixture was incubated an additional 1.5 hr at 45° to destroy ribose-containing nucleotides. Ethylene glycol (8.9 mmoles) was added to consume excess $NaIO_4$, and a precipitate which had formed at the time of cyclohexylamine addition was removed by filtration. The filtrate was applied directly to a Dowex 1 (Cl^-) column (Bio-Rad AG 1-X2, 200–400 mesh, 4 × 38 cm) and the column was developed at 4°. Adenine, a by-product resulting from $NaIO_4$ treatment of the ribose-containing nucleotides, was eluted with water. A^mDP, A^mTP, and the starting material, A^mMP, were separated by elution with a linear gradient of 0–0.2 M LiCl in a total volume of 4 l. of 0.01 N HCl. Fractions were adjusted to an alkaline pH upon collection by the addition of NH_4OH. A^mMP appeared after approximately 15% of the gradient had passed through the column, A^mDP at 30% immediately following a large amount of IO_3^-, and A^mTP at 90%. Pooled fractions obtained from the Dowex column were diluted to 0.07 M Cl^- concentration, applied to a DEAE column (2 × 40 cm, carbonate form), and washed with water until free of Cl^-. Elution with a linear gradient of triethylammonium bicarbonate (pH 7.8) from 0.01 to 0.4 M, resulted in a homogeneous product which was recovered by lyophilization. Over-all recoveries based on the amount of A^mMP used in the original myokinase reaction were: A^mMP, 33%; A^mDP, 40%; and A^mTP, 9%.

A^mDP and A^mTP were characterized by paper chromatography of sufficient amounts of material to detect contaminants present in excess of 2%. Both compounds were homogeneous in solvent systems A, C, and D, with the exception of small amounts of A^mMP (<5%) in A^mDP and A^mDP (<5%) in A^mTP. R_F values for these compounds are given in Table I. Treatment of each compound with *E. coli* alkaline phosphatase resulted in the release of 1.7 moles of phosphate/mole of A^m in A^mDP and 3.0 moles of phosphate/mole of A^m in A^mTP. In both cases, A^m was the only ultraviolet-absorbing compound present after phosphatase treatment.

TABLE I: Paper Chromatography of Ribose- and 2′-*O*-Methylribose-Containing Nucleosides and Nucleotides.

Compound	R_F Solvent A	R_F Solvent C	R_F Solvent D
Adenosine	0.66	0.35	0.63
A^m	0.76	0.69	0.71
AMP	0.50	0.04	0.16
A^mMP	0.60	0.23	0.27
ADP	0.46	0.02	0.07
A^mDP	0.52	0.17	0.15
ATP	0.35	0.01	0.05
A^mTP	0.45	0.10	0.09

A^mDP was also synthesized chemically from A^mMP by displacement of the diphenyl phosphate derivative of A^mMP with phosphate. Conditions employed were similar to those described by Michelson (1964) for the synthesis of guanosine 5′-diphosphate. Owing to the low yield of A^mDP and the high loss of A^mMP by conversion into a form which could not be recovered, the myokinase reaction was generally preferred.

[^{3}H]A^mDP was synthesized from [^{3}H]adenosine (Nuclear-Chicago) using the combination of reactions described above. [^{3}H]Adenosine was diluted with cold adenosine before treatment with diazomethane. The specific activity of purified [^{3}H]A^mDP was approximately 57 μCi/mmole.

Polynucleotide Phosphorylase Assay. The polymerization of [^{3}H]A^mDP was followed by trapping the acid-insoluble product on Millipore filters. A typical reaction mixture contained the following components: 0.1 M Tris-Cl^- (pH 9.0), 5 mM $MgCl_2$, 0.4 mM EDTA, 1 mM NaN_3, 25 mM A^mDP, 0.21 mM ApApApA (as mononucleotide), 0.1 mg/ml of bovine serum albumin, and 2.4 mg/ml of polynucleotide phosphorylase. Following incubation at 37° for various time intervals, a 4-μl aliquot was removed, diluted into 200 μl of cold H_2O, and

mixed with approximately 2 ml of cold 5% trichloroacetic acid. The trichloroacetic acid treated aliquot was poured over a Millipore filter which was then dried and assayed in a liquid scintillation spectrometer in a fluor containing toluene PPO–POPOP. The validity of the assay was established by chromatography of aliquots on Whatman No. 1 paper in solvent A, cutting the developed chromatogram into small strips, and counting directly. The per cent of total radioactivity remaining with polymer controls at the origin was identical with that trapped on Millipore filters.

The unmodified radioactive substrate, [^{3}H]ADP, which was used for purposes of comparison in polymerization studies, was synthesized from [^{3}H]AMP and ATP with myokinase using the conditions described above for the synthesis of A^{m}DP. The specific activity of purified [^{3}H]ADP was approximately 28 μCi/mmole. The oligonucleotides ApApApA and UpUpUpU were prepared from poly A and poly U, respectively, using methods described previously (Rottman and Nirenberg, 1966).

Results

Time Course and Enzyme Concentration. The incorporation of A^{m}DP into polymer was catalyzed by polynucleotide phosphorylase as shown in Figure 1A. However, the reaction proceeds very slowly relative to the natural substrate ADP (Figure 1B). Under identical conditions, the reaction with ADP is essentially over after 1.5 hr while A^{m}DP is still being incorporated after 30 hr. Calculation of the rates of substrate incorporation from the linear region of the curves presented in Figure 1 indicate that 77 mμmoles of A^{m}DP is incorporated into polymer/hr per mg of enzyme, while 8350 mμmoles of ADP/hr per mg of enzyme are polymerized under identical reaction conditions. The total amount of A^{m}DP polymerized after 30-hr incubation varied between 20 and 30%, depending upon the conditions employed.

The dependence of the reaction upon polynucleotide phosphorylase concentration is shown in Figure 2. Relatively high concentrations of enzyme were used to obtain a significant reaction with A^{m}DP. In view of the long incubation times, NaN_3 was included in each reaction mixture to prevent microbial contamination. To further verify that the polymerization observed was catalyzed by polynucleotide phosphorylase, a time course was run in the presence of 0.25 M orthophosphate, a product of the reaction. The reaction was completely inhibited, as expected, when followed for 22 hr (data not shown).

A slight lag in A^{m}DP incorporation was noted in the absence of ApApApA primer with the commercial (P-L) polynucleotide phosphorylase (Figure 1A). Addition of oligonucleotide primer effectively eliminated the lag observed with this enzyme and also increased the total amount of polymer synthesized. Similar results were obtained with polynucleotide phosphorylase which was purified to stage VI in our laboratory (see Materials and Methods). However, when trypsin-treated primer-dependent polynucleotide phosphorylase was used, the

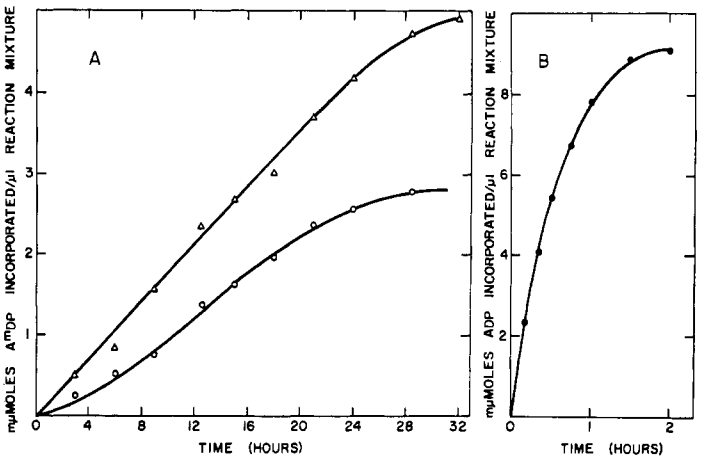

FIGURE 1: Polymerization of A^{m}DP and ADP by polynucleotide phosphorylase. Reaction mixtures contained, in a volume of 75 μl, 0.1 M Tris-Cl$^-$ (pH 9.0), 5 mM $MgCl_2$, 0.4 mM EDTA, 1 mM NaN_3, 0.1 mg/ml of bovine serum albumin, 25 mM A^{m}DP or ADP, 0.21 mM ApApApA (as mononucleotide), where indicated, and 2.4 mg/ml of polynucleotide phosphorylase (P-L). Following incubation at 37°, 4-μl aliquots were removed and assayed as described in Materials and Methods. Polymerization of A^{m}DP is shown in A in the presence (△) and absence (○) of ApApApA. For comparison, the polymerization of ADP (●) is shown in B in the presence of ApApApA.

polymerization of A^{m}DP was completely dependent upon primer addition (Figure 3A), while ADP showed a small amount of incorporation in the absence of primer (Figure 3B).

The effect of $MgCl_2$ and KCl concentration on the incorporation of A^{m}DP was studied. The optimal molar ratio of A^{m}DP to Mg^{2+} appeared to range from 2:1 to 5:1. Only under conditions of low Mg^{2+} (10:1) was the reaction inhibited. The presence of 0.2 M KCl in the reaction mixture had little effect on A^{m}DP incorporation as determined by the Millipore filter assay.

Determination of Apparent K_m for A^{m}DP. The large difference in the rate of incorporation of A^{m}DP and

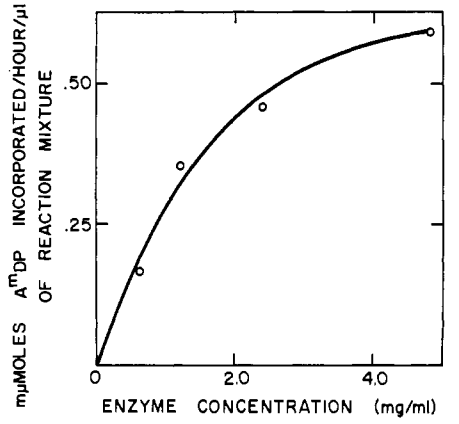

FIGURE 2: Dependence of A^{m}DP incorporation upon polynucleotide phosphorylase concentration. Reaction mixtures contained the components described in the legend to Figure 1 with the exception of ApApApA which was omitted and the concentration of polynucleotide phosphorylase (P-L) which was varied as indicated. Incubation and assay of 4-μl aliquots was carried out as described in the legend to Figure 1.

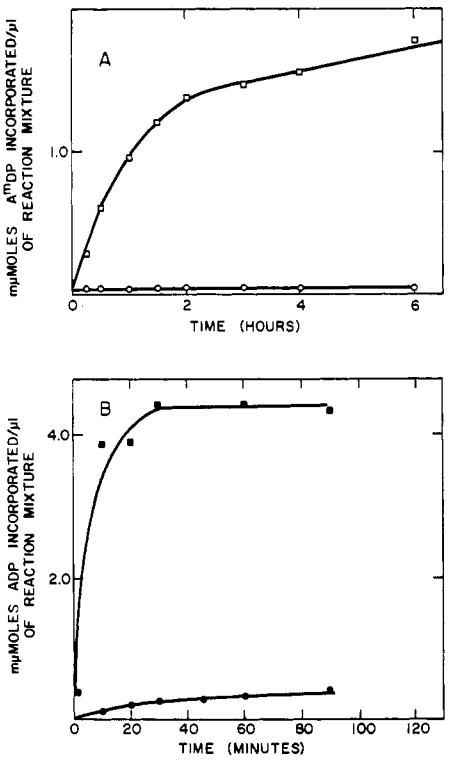

FIGURE 3: Stimulation of A^mDP and ADP incorporation by oligonucleotide with primer-dependent polynucleotide phosphorylase. The components of each reaction mixture were identical with those described in the legend to Figure 1 except for polynucleotide phosphorylase which was prepared by trypsin treatment as described in Materials and Methods. Assays were performed as described in the legend to Figure 1. Polymerization of A^mDP in the presence (□) and absence (○) of ApApApA is shown in A, while similar results obtained with ADP in the presence (■) and absence (●) of ApApApA are shown in B.

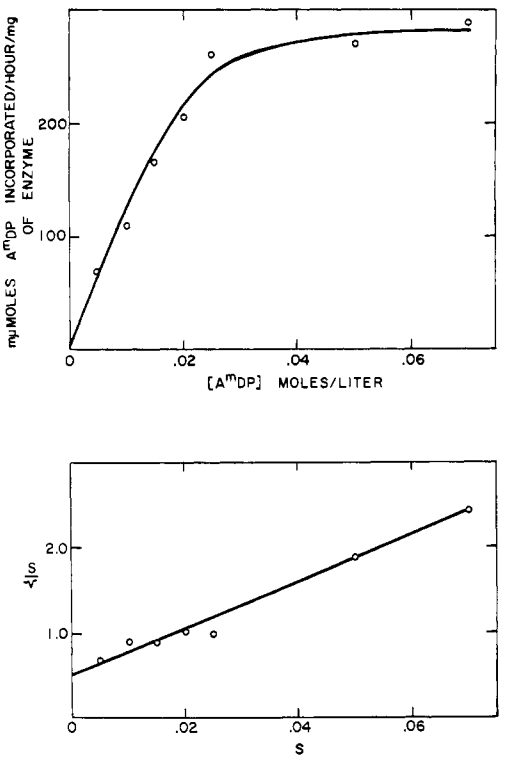

FIGURE 4: Determination of apparent K_m for A^mDP. Each reaction mixture contained, in a volume of 30 μl, the components described in the legend to Figure 1 including ApApApA primer. The concentration of A^mDP was varied as indicated. Initial velocities were determined as described in the text.

ADP prompted us to determine the apparent K_m for A^mDP and compare it with that of ADP. These experiments were conducted in the presence of ApApApA primer to eliminate the initial lag and thus permit a more accurate estimation of initial velocities. Initial velocities were determined by assay of 4-μl aliquots removed at five different time intervals during the first 8-hr incubation. Rates were linear throughout the assay except with reaction mixtures containing substrate concentrations below 0.01 M in which synthesis fell off after 3–4 hr. The apparent K_m of A^mDP calculated from the data shown in Figure 4 was 1.8×10^{-2} M. For purposes of comparison, the apparent K_m for ADP was determined using [^{3}H]ADP. Reaction conditions, including the concentration of ApApApA primer and the method of assay, were identical. The apparent K_m for ADP determined in this manner ranged in values from 1 to 3×10^{-2} M.

Poly-2′-O-methyladenylic Acid Characterization. In the absence of ApApApA primer, reaction mixtures containing A^mDP became very viscous after 24–30-hr incubation, indicating the formation of large molecular weight polymers. Chromatography of aliquots spotted on Whatman No. 1 paper or thin-layer chromagram sheets and developed in solvent A showed a large amount of ultraviolet-absorbing material remaining at the origin with polymer standards. To further characterize this polymer, a 1.8-ml reaction mixture containing the components described in Figure 1 (with the exception of primer which was omitted) was incubated for 31 hr. Protein was removed by shaking with $CHCl_3$ and isoamyl alcohol. Attempts to separate A^mDP from poly A^m by alcohol precipitation were not successful as the precipitate contained large amounts of A^mDP. A^mDP was removed by extensive dialysis and recovered from the dialysate. The yield of purified poly A^m was 22%.

Treatment of poly A^m with 0.1 N NaOH for 18 hr at 37° was without effect, while poly A treated in the same manner was completely degraded to adenylic acid as determined by paper chromatography in solvent A. Hydrolysis of poly A^m was accomplished by the concerted action of snake venom phosphodiesterase, micrococcal nuclease, and *E. coli* alkaline phosphatase. The reaction mixture contained the following: 0.075 M Tris-Cl^- (pH 9.0); 0.015 M $CaCl_2$; poly A^m, 2 μmoles/ml (as mononucleotide); snake venom phosphodiesterase, 0.15 mg/ml; micrococcal nuclease, 0.015 mg/ml; and *E. coli* alkaline phosphatase, 0.1 mg/ml. After incubation for 18 hr at 37°, a portion of the reaction mixture was spotted on Whatman No. 1 paper and developed in solvent C. The only ultraviolet-absorbing product was the free nucleoside, A^m. Treatment of poly A^m with snake venom phosphodiesterase and *E. coli* alkaline

TABLE II: Formation and Characterization of Poly (U, A^m).[a]

	UpUpUpU Added (μmole/ml) (as mononucleotide)	Poly (U, A^m) Isolated (μmole/ml) (as mononucleotide)
Control	None	1.18
	0.15	1.28
	0.37	0.92
Alkaline hydrolysis	None	0.08
	0.15	0.05
	0.37	0.04

[a] Reaction mixtures contained the components described in Figure 1 with the exception of 0.01 M [^{3}H]A^mDP, 0.03 M UDP, and the optional addition of UpUpUpU instead of ApApApA. Following incubation for 25 hr at 37°, a 10-μl aliquot was removed from each reaction mixture, precipitated with 5% trichloroacetic acid, and filtered as described previously. A second 10-μl aliquot was treated with 0.1 N NaOH for 18 hr at 37°, precipitated with trichloroacetic acid, and filtered.

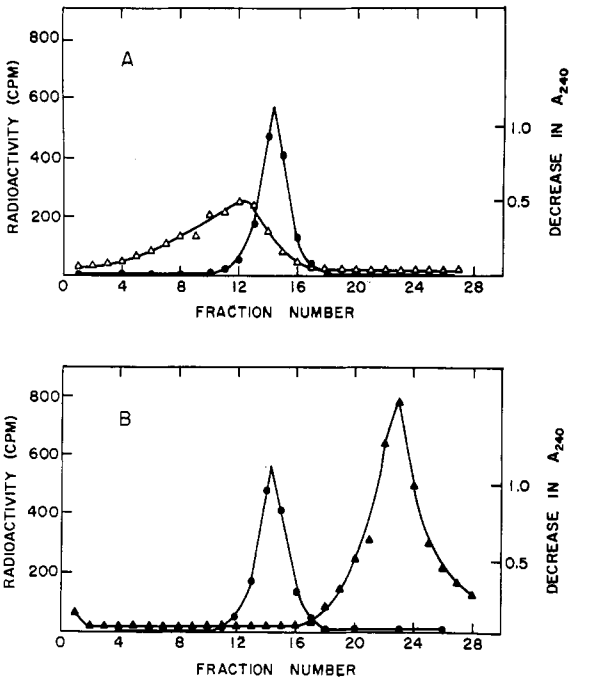

FIGURE 5: Sucrose gradient pattern of poly A^m synthesized in the presence and absence of ApApApA primer. A reaction mixture containing the components described in Figure 1 including [^{3}H]A^mDP was incubated at 37° for 34 hr. A 17-μl aliquot was removed, mixed with catalase, and layered on a 4.8-ml sucrose gradient of 5–20% sucrose in 0.1 M Tris-Cl^- (pH 9). Following centrifugation in a SW 39 rotor at 39,000 rpm for 9 hr, the tube was punctured and fractions were collected. Each fraction was assayed for catalase activity by removing a 10-μl aliquot, incubating with H_2O_2, and measuring the decrease in absorption at 240 mμ. The location of poly A^m was determined by treating the remainder of each fraction with 5% trichloroacetic acid and collecting the labeled precipitate on Millipore filters. The sedimentation profile of poly A^m formed in the absence of ApApApA primer (△) is shown in A. Catalase activity (●) peaks near tube 14. Poly A^m formed in the presence of ApApApA primer (▲) is shown in B.

phosphatase (omitting micrococcal nuclease) released only small amounts of A^m which is consistent with the low activity of this diesterase on bonds adjacent to 2′-*O*-methyl groups (Gray and Lane, 1967).

The size distribution of poly A^m formed in the presence and absence of oligonucleotide primer was determined by sucrose density gradient centrifugation. The observation that the reaction mixture developed noticeable viscosity only in the absence of primer molecules suggested a relation between the addition of primer and chain length of the product. As shown in Figure 5, poly A^m synthesized without primer (Figure 5A) sedimented faster than the catalase marker (11 S) with a broad range of sizes at approximately 13–15 S, while poly A^m formed in the presence of primer (Figure 5B) sedimented at approximately 5–6 S. Separate experiments not shown here in which poly A^m synthesis was carried out for shorter time periods in the presence and absence of primer indicated that the chain length of the polymer was also proportional to incubation time. Thus, the products in reaction mixtures incubated for 24 hr in the presence and absence of primer were 4–5 and 11 S, respectively.

Heteropolymers Containing 2′-O-Methyladenosine. The slower rate of polymerization of A^mDP relative to ADP led us to test the possible incorporation of A^mDP in the presence of an unmodified ribose-containing substrate. UDP was chosen as the second substrate to facilitate characterization of the product. Incorporation of [^{3}H]A^mDP into polymer does occur in the presence of UDP, as shown in Table II. Furthermore, the product is a heteropolymer resulting from the mixed incorporation of A^mDP and UDP rather than simultaneous formation of poly A^m and poly U. This was demonstrated by treatment of the product with 0.1 N NaOH for 18 hr at 37° which converted the [^{3}H]A^mMP present in the polymer into an acid-soluble form. The effect of UpUpUpU addition on the total amount of A^mDP incorporated and the susceptibility of the product to 0.1 N NaOH appears to be negligible (Table II).

The formation of poly (U, A^m) was further established by characterizing the products resulting from RNase treatment of the polymer. Poly (U, A^m) isolated from a reaction mixture containing UDP and nonradioactive A^mDP, as described in Table II, was deproteinized with $CHCl_3$–isoamyl alcohol and precipitated by the addition of two volumes of ethyl alcohol. Poly (U, A^m) (1 μmole, as mononucleotide) was treated with pancreatic RNase (0.2 mg/ml), *E. coli* alkaline phosphatase (0.1 mg/ml), and NH_4HCO_3 (pH 7.8) (0.05 M) in a total volume of 0.5 ml. Following incubation for 18 hr at 37°, the reaction mixture was lyophilized to remove the volatile buffer. The dried material was dissolved in 1 ml of H_2O and applied to a DEAE-carbonate column (0.7 × 6

cm). Nucleosides were eluted with water followed by oligonucleotides which were eluted with 0.3 M triethylammonium bicarbonate. The oligonucleotide-containing fraction was evaporated to dryness to remove the buffer and dissolved in a small volume of water. Chromatography of an aliquot on Whatman DEAE paper developed with 0.2 M ammonium formate confirmed the oligonucleotide nature of this material. The remaining oligonucleotide was treated exhaustively with snake venom phosphodiesterase, as described previously, and the products were chromatographed on Whatman No. 1 paper in solvent C. Three ultraviolet-absorbing compounds were detected on the chromatogram corresponding to A^m, uridine 5′-phosphate, and small quantities of A^mMP.

A separate sample of poly (U, A^m) was treated with a combination of pancreatic RNase and *E. coli* alkaline phosphatase as described above to determine the ratio of U to A^m in the polymer. The input ratio of UDP to A^mDP was 3:1. Separation of uridine from oligonucleotides containing A^m was carried out by electrophoresis at pH 3.5. Calculations based on a spectrophotometric determination of A^m in the oligonucleotides indicated that the ratio of U to A^m in the polymer was 4.2:1.

Discussion

The presence of 2′-*O*-methyl groups in nucleotides and RNA molecules apparently influences the substrate properties of these molecules in a differential manner when tested with various enzymes. Thus, 2′-*O*-methyl nucleoside 5′-phosphates (*e.g.*, A^mMP) are not dephosphorylated by snake venom 5′-nucleotidase, and phosphodiester bonds adjacent to 2′-*O*-methyl groups are only slowly hydrolyzed by snake venom phosphodiesterase. Both ribose- and 2′-deoxyribose-containing molecules are efficiently hydrolyzed by both enzymes. On the other hand, A^mMP is a substrate for rabbit muscle myokinase, and this reaction has been used in these studies for the production of A^mDP. In addition to a high yield of A^mDP, A^mTP can be isolated from the reaction mixture. A^mTP represents an interesting analog of ATP which is being tested for biological activity in several systems.

Polynucleotide phosphorylase can be used to catalyze the polymerization of A^mDP, thereby permitting the formation of polynucleotides which are completely and exclusively methylated in the 2′ position. On the basis of previous studies which indicated a requirement for an unaltered ribose moiety for substrate activity, one might expect A^mDP to be inactive. Apparently, the presence of a 2′-*O*-methyl group results in a substrate which more closely resembles an unsubstituted ribonucleotide than 2′-deoxyribonucleotide, since the latter is not incorporated into polymer by polynucleotide phosphorylase. This was confirmed by an experiment we performed in which an equivalent amount of dADP was substituted for A^mDP in a typical reaction mixture. Under conditions which led to 20–25% conversion of A^mDP into polymer, no detectable dADP (<2%) incorporation was observed. The reactivity of A^mDP suggests that nucleoside diphosphates possessing sugar moieties other than unsubstituted ribose can still function as substrates for polynucleotide phosphorylase. More importantly, it permits formation of polynucleotides containing 2′-*O*-methyl groups in high yields.

The effect of oligonucleotide primer on the elimination of the initial lag in the reaction has been observed previously with ribonucleotide substrates (Singer *et al.*, 1960). Primer stimulation of A^mDP incorporation is apparent in both the commercial P-L polynucleotide phosphorylase and the trypsin-treated, primer-dependent preparation. Only the latter enzyme preparation is primer dependent when the substrate is ADP. This increased dependency upon primer for A^mDP incorporation is without explanation and apparently reflects something more than variance in the rates of incorporation for both substrates.

The lower rate of A^mDP incorporation (approximately 100×) relative to that of ADP under identical conditions might possibly result from either a greatly reduced affinity for A^mDP by polynucleotide phosphorylase in comparison with the usual substrate, ADP, or slower formation of diester bonds adjacent to A^mDP subsequent to its binding. The close agreement of apparent K_m values obtained for A^mDP and ADP under similar conditions argues against a large difference in substrate affinity as the basis for the reduced rate of polymerization. On the other hand, the results are consistent with, but do not prove, inhibited phosphodiester bond formation between 2′-*O*-methyl-containing nucleotides in a step subsequent to substrate binding.

The formation of poly (U, A^m) heteropolymers is consistent with the above explanation. In these experiments, UDP and A^mDP were incorporated together into a mixed copolymer implying some degree of effective competition by both substrates for polynucleotide phosphorylase catalyzed polymerization. These experiments eliminate the exclusive formation of two homopolymers; *i.e.*, poly U and poly A^m, but do not prove that the incorporation of substrates is completely random.

The molecular weight of poly A^m is rather large as determined by its sedimentation in a sucrose gradient and is a function of the presence of oligonucleotide primer in the reaction mixture. Increasing concentrations of primer caused the synthesis of more polynucleotide chains of shorter average chain length, as might be expected if the observed synthesis represents addition of A^mMP residues to primer molecules.

The polymerization of A^mDP by polynucleotide phosphorylase has permitted the synthesis of RNA molecules which are extensively methylated in the 2′-OH position. The physical and biological properties of these molecules are now under investigation.

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