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Polymers Containing 2′-*O*-Methylnucleotides. II. Synthesis of Heteropolymers*

Fritz Rottman and Karol Lynn Johnson

ABSTRACT: Heteropolymers containing 2′-*O*-methyladenylic acid, 2′-*O*-methylcytidylic acid, and nonmethylated nucleotides have been synthesized with polynucleotide phosphorylase from their corresponding nucleoside 5′-diphosphates. The distribution of 2′-*O*-methylnucleotides in these polymers was not completely random but occurred in pairs. The frequency of this paired incorporation indicates some form of cooperative polymerization of 2′-*O*-methylnucleotides in the presence of nonmethylated nucleotides. The addition of dimethyl sulfoxide to the polymerization reaction enhanced the random incorporation of 2′-*O*-methyladenosine 5′-diphosphate in the presence of cytidine 5′-diphosphate but had little effect on the incorporation of 2′-*O*-methylcytidine 5′-diphosphate in the presence of uridine 5′-diphosphate. Certain restrictions on the formation of heteropolymers by *Micrococcus lysodeikticus* polynucleotide phosphorylase were noted. 2′-*O*-Methyladenosine 5′-diphosphate formed heteropolymers with cytidine 5′diphosphate and uridine 5′-diphosphate but not guanosine 5′-diphosphate and adenosine 5′-diphosphate. 2′-*O*-Methylcytidine 5′-diphosphate formed heteropolymers with uridine 5′-diphosphate but would not form a homopolymer nor would it form heteropolymers with cytidine 5′-diphosphate, adenosine 5′-diphosphate, or guanosine 5′-diphosphate. A heteropolymer containing 2′-*O*-methyladenosine, 2′-*O*-methylcytidine, and uridine was synthesized from the corresponding nucleoside 5′-diphosphates.

Polynucleotide phosphorylase is known to catalyze the polymerization of 2′-*O*-methyladenosine 5′-diphosphate forming a large molecular weight homopolymer, poly-2′-*O*-methyladenylic acid (Rottman and Heinlein, 1968). Evidence for the formation of a heteropolymer containing 2′-*O*-methyladenylic acid and uridylic acid was also presented. However, the possibility of forming RNA molecules more closely approximating those isolated from natural sources in both 2′-*O*-methyl content and distribution was not examined.

The widespread distribution of 2′-*O*-methylnucleotides in RNA from many organisms has been amply demonstrated (Smith and Dunn, 1959; Biswas and Meyers, 1960; Hall, 1964; Tamaoki and Lane, 1968). Information concerning the possible function of this modification is minimal (Starr and Sells, 1969). Methylation of preribosomal 45S RNA in HeLa cells has been reported to be a prerequisite for its maturation and processing into 18S and 28S RNA (Vaughn *et al.*, 1967). This processing may involve the 2′-*O*-methyl group since the major site of methylation in

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this RNA molecule is the 2'-hydroxyl group of ribose rather than purine or pyrimidine bases. The 2-'*O*-methyl groups, which are incorporated at the 45S precursor stage, are believed to be retained in the 28S and 18S products, even though approximately 50% of the nucleotides initially present in 45S RNA is eliminated in the maturation process (Wagner *et al.*, 1967; Weinberg *et al.*, 1967).

Some studies on the function of 2'-*O*-methylnucleotides in RNA may profit by the availability of homopolymers in which all of the residues contain 2'-*O*-methyl groups. The effect of the modification would thus be amplified. Physical measurements on poly-2'-*O*-methyladenylic acid indicate that this polymer has a greater tendency for ordered structure than polyadenylic acid (Bobst *et al.*, 1969) and may permit measurements of the contribution of 2'-hydroxyl groups in RNA to intramolecular structure. However, most experiments designed to study the biological function of 2'-*O*-methylnucleotides can better employ polynucleotides containing these modifications in trace amounts and in random sequence as they occur in RNA isolated from natural sources.

Materials and Methods

Chromatographic Systems. Descending paper chromatography was carried out using the following solvent systems: (A) *n*-propyl alcohol–concentrated NH_4OH–H_2O (55:10:35, v/v), (B) isopropyl alcohol–concentrated NH_4OH–0.1 M boric acid (7:1:2, v/v), (C) ethyl acetate–*n*-propyl alcohol–H_2O (4:1:2, v/v, upper phase), (D) 95% ethyl alcohol–1 M ammonium acetate (pH 7.5) (7:3, v/v), (E) saturated ammonium sulfate–H_2O–isopropyl alcohol (79:19:2, v/v), and (F) isobutyric acid–concentrated NH_4OH–H_2O (66:1:33, v/v). Whatman No. 1 paper was employed for routine qualitative separations. Whatman No. 40 paper was used when ultraviolet-absorbing materials were eluted for quantitative estimation.

Paper electrophoresis was also performed on Whatman No. 40 paper in the cold with a Spinco Model R instrument (450 V, 2 hr). The solvents used in electrophoresis were: (A) 0.05 M Tris-acetate (pH 7.5) and (B) 0.075 M triethylammonium bicarbonate (pH 7.8). Solvent B, which employs a volatile buffer, was used when the products were to be isolated for further enzymatic treatment.

Materials. ADP, CDP, UDP, and GDP, from P-L Biochemicals, Inc., were adjusted to pH 9 with NaOH before use. *Micrococcus lysodeikticus* polynucleotide phosphorylase (type I) was obtained from P-L Biochemicals, Inc., and dialyzed 18 hr against 0.01 M Tris-Cl (pH 8.1), 0.001 M EDTA, 0.01 M NaCl, and 0.001 M β-mercaptoethanol. This dialysis was necessary to retard contaminating nucleases which otherwise degraded the polymers formed over the long incubation periods required for polymerization. *Escherichia coli* alkaline phosphatase (Worthington Biochemicals Corp.) was dialyzed 18 hr in 0.01 M Tris-Cl (pH 8.1) to remove ammonium sulfate. The phosphodiesterase from crude Russell's viper snake venom (Sigma Chemical Co.) was purified by the method described by Gray and Lane (1967) to eliminate phosphatase contamination. Incubation for 18 hr in the presence of a large excess of enzyme failed to demonstrate detectable phosphatase activity on AMP. Pancreatic ribonuclease (type IIIA) was purchased from Sigma Chemical Co. Dimethyl sulfoxide was distilled under vacuum before use in both enzyme and chemical reactions.

AmDP[1] was synthesized as reported earlier (Rottman and Heinlein, 1968), with the exception that the morpholidate displacement reaction, as described in the synthesis of CmDP, was substituted for the myokinase step when large amounts of AmDP were required. The synthesis of [^{3}H]AmDP, [^{3}H]-ADP, and their use in a filter assay with polynucleotide phosphorylase have also been described.

2'-O-Methylcytidine 5'-Diphosphate. Chemical methylation of cytidine by treatment with diazomethane in 1,2-dimethoxyethane was carried out by the method of Martin *et al.* (1968). The products of a reaction obtained from 2.5 g of cytidine were separated on a 4 × 40 cm Dowex 1(OH^-) column (Bio-Rad AG1-X2, 200–400 mesh) by elution with methanol (Dekker, 1965). 2'-*O*-Methylcytidine appeared after elution with 850 ml of 30% methanol. The product was rechromatographed on an identical column to eliminate traces of contamination which chromatographed faster than Cm on paper chromatography in solvent systems B and C. Isolated Cm migrated with naturally occurring Cm obtained from wheat germ RNA in solvent systems A, B, and C. The yield of purified Cm, based on the amount of cytidine reacted, was 17–20%.

2'-*O*-Methylcytidine 5'-monophosphate was prepared from Cm by phosphorylation with 2-cyanoethyl phosphate using a modification of a procedure described by Tener (1962) and employed earlier in the synthesis of AmMP. The reaction with unprotected Cm results in a number of products, the major one being 2'-*O*-methylcytidine 5'-phosphate. CmMP was isolated from this mixture by chromatography on a Dowex 1 (formate) column (Bio-Rad AG1-X2, 200–400 mesh). Unreacted Cm was eluted with water. Elution with 0.1 M formic acid resulted in the appearance of three ultraviolet-absorbing fractions. The middle fraction (CmMP) was well resolved from two smaller unidentified fractions and was recovered after repeated lyophilization to remove formic acid. Chromatography of CmMP showed it to be homogeneous in solvent systems B and E. The yield of CmMP based on the amount of Cm used was 19%. Treatment with *E. coli* alkaline phosphatase converted the isolated product into Cm and resulted in the concomitant release of 1 mole of inorganic phosphate/mole of CmMP.

2'-*O*-Methylcytidine 5'-diphosphate was synthesized by displacement of the morpholidate derivative (Moffatt, 1967) with the tri-*n*-butylamine salt of orthophosphate. The monotriethylammonium salt of CmMP (0.9 mmole) was dissolved in 50 ml of 50% aqueous *t*-butyl alcohol. Morpholine (0.3 ml) was added and the solution was gently refluxed. While refluxing, a second solution composed of 720 mg of dicyclohexylcarbodiimide in 25 ml of *t*-butyl alcohol was slowly added from a separatory funnel over a 45-min period. Examination of the reaction mixture by paper electrophoresis in 0.03 M phosphate buffer (pH 7.5) usually indicated incomplete conversion into the morpholidate derivative in which case an equivalent amount of morpholine and dicyclohexylcarbodiimide was added in a similar manner. Following confirmation of complete conversion by

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Am, 2'-*O*-methyladenosine; AmMP, 2'-*O*-methyladenosine 5'-phosphate; AmDP, 2'-*O*-methyladenosine 5'-diphosphate; Cm, 2'-*O*-methylcytidine; CmMP, 2'-*O*-methylcytidine 5'-phosphate; CmDP, 2'-*O*-methylcytidine 5'-diphosphate; poly (Am,U), copolymer of Am and U.

TABLE I: Paper Chromatography of Ribose- and 2′-*O*-Methylribose-Containing Cytosine Derivatives

Compound	R_F Solvent A	R_F Solvent B	R_F Solvent C	R_F Solvent D
C	0.64	0.54	0.10	
Cm	0.74	0.66	0.16	
CMP	0.38	0.04	0.01	0.22
CmMP	0.46	0.15	0.03	0.36
CDP	0.38	0.05	0.03	0.11
CmDP	0.46	0.19	0.04	0.24

paper electrophoresis the material was evaporated to one-third the volume, extracted three times with ether, and further evaporated (aqueous fraction) to dryness. The residue was rendered anhydrous by three evaporations with anhydrous pyridine under vacuum followed by two evaporations with anhydrous benzene.

The tri-*n*-butylamine salt of orthophosphate (2 mmoles) was prepared separately by repeated evaporations of phosphoric acid (0.11 ml) and tri-*n*-butylamine (1.0 ml) from anhydrous pyridine and anhydrous benzene. The product was dissolved in 4 ml of freshly distilled dimethyl sulfoxide, which had been stored over molecular seive, and added to the dry morpholidate derivative of CmMP. Two additional rinses with dimethyl sulfoxide resulted in a total volume of 12 ml. The flask was tightly sealed and incubated 2 days at 37°. Following the addition of 80 ml of water, the material was placed on a 2.5 × 40 cm DEAE column in the carbonate form. After elution of free morpholine with water, a linear gradient of triethylammonium bicarbonate (0.005–0.35 M) was used to develop the column. CmDP eluted in a large peak approximately midway in the gradient following CmMP and small amounts of unidentified material. Triethylammonium bicarbonate was removed by repeated lyophilization and evaporation from methanol. The product was dissolved in methanol and precipitated as the sodium salt by the addition of NaI and acetone, washed with acetone, and dried.

Chromatography in solvent systems A, B, and D showed the product to be homogeneous with the exception of approximately 5% CmMP contamination. Treatment with *E. coli* alkaline phosphatase caused the liberation of 1.8 μmoles of P_i/μmole of CmDP and also produced Cm, as confirmed by paper chromatography. The yield of purified CmDP based on the amount of CmMP used in this reaction was 61%. The mobilities of Cm, CmMP, and CmDP in various solvent systems are recorded in Table I.

Synthesis of Heteropolymers. Polymerization of nucleoside 5′-diphosphates by polynucleotide phosphorylase was followed by phosphate release and chromatography in solvent A, in which polymers remain at the origin. Reaction mixtures contained 0.1 M Tris-Cl^- (pH 9.0), 5 mM $MgCl_2$, 0.4 mM EDTA, 1 mM NaN_3, 2.4 mg/ml of polynucleotide phosphorylase, 35 mM nucleoside 5′-diphosphates, and the optional addition of dimethyl sulfoxide. The reaction mixtures were incubated at 37° for various lengths of time and deproteinized by repeated (three times) shaking with $CHCl_3$ and isoamyl alcohol. Mononucleotides were removed by four precipitations from 0.2 M potassium acetate and two volumes of ethanol and the resulting polymers were shown to be free of small molecular weight material by chromatography in solvent A.

Degradation of Polymers. Complete degradation of heteropolymers to mononucleotides was accomplished by incubation with purified Russell's viper snake venom phosphodiesterase; 3.5 A_{260} units of polymer in a 1-ml solution containing 0.1 M $(NH_4)_2CO_3$ (pH 8.8), 0.01 M $MgCl_2$, and 0.7 mg of phosphodiesterase was incubated for 18 hr at 37° and lyophilized.

Partial degradation to detect the presence and distribution of 2′-*O*-methyl groups was carried out by either NaOH or pancreatic ribonuclease treatment which will not degrade phosphodiester bonds adjacent to 2′-*O*-methyl groups. Subsequent incubation with *E. coli* alkaline phosphatase removed terminal phosphate from mononucleotides and oligonucleotides.

Digestion of 10 A_{260} units of polymer in 0.2 ml of 0.5 N NaOH for 18 hr at 37° was terminated by dilution to 2 ml and neutralization with Dowex 50 (H^+) to pH 8.0. The resulting solution of mononucleotides and oligonucleotides was made 0.1 M with respect to $(NH_4)_2CO_3$ (pH 8.8) and incubated 6 hr at 37° with 0.05 mg of *E. coli* alkaline phosphatase.

Alternatively, 10 A_{260} units of polymer in 0.2 ml was treated with pancreatic RNase (1 mg/ml) and incubated at 37° for 18 hr. The reaction mixture was diluted to 1 ml and incubated in the presence of 0.1 M $(NH_4)_2CO_3$ (pH 8.8) and 0.025 mg/ml of *E. coli* alkaline phosphatase for 6 hr at 37° to produce free nucleoside and oligonucleotides without a terminal phosphate.

Results

Effect of 2′-O-Methylnucleotides on the Rate of Polymerization. Earlier studies on the kinetics of AmDP polymerization into the homopolymer, poly Am, showed an extremely slow rate of polymer formation relative to poly A synthesis from ADP even though the apparent K_m for AmDP and ADP were similar. Thus, it was not surprising to find an inhibition by 2′-*O*-methylnucleotides on the over-all rate of heteropolymer formation. Increasing proportions of AmDP to CDP (Figure 1A) and CmDP to UDP (Figure 1B) caused a decreased rate of polymerization as determined by phosphate release. By extending the incubation time for those reactions containing higher proportions of the 2′-O-methylated substrate it was possible to obtain approximately the same degree of polymerization in all reactions.

The incorporation of AmDP in the presence of CDP was studied in more detail to ascertain that AmDP was in fact polymerized in the presence of CDP and to determine if its rate of incorporation was similar to the rate of total polymerization assayed by phosphate release. [^{3}H]AmDP incorporation in the presence of CDP was detected by precipitating the polymer produced with trichloroacetic acid and filtration on Millipore filters. The rate of [^{3}H]AmDP incorporation, as observed in the 1:3 and 1:10 reaction mixtures (Figure 2), and the time at which equilibrium is reached is dependent upon the proportion of 2′-O methylated to normal nucleotide. Total polymerization of [^{3}H]AmDP is less in the 1:20 reaction mixture since it represents a smaller percentage of the total nucleoside 5′-diphosphates present than in the 1:10 and 1:3 reactions.

Restrictions on Heteropolymer Formation. The availability of CmDP theoretically permits the synthesis of various polymers containing CmMP with polynucleotide phosphorylase.

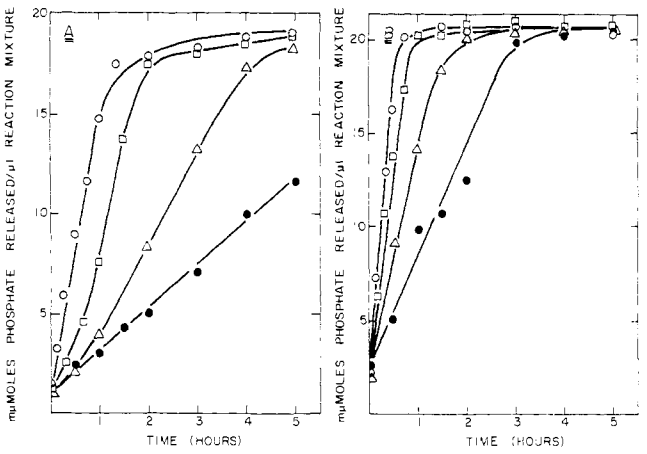

FIGURE 1: The effect of 2′-*O*-methylnucleotide concentration on the rate of polymer formation. Reaction mixtures contained the components described in Methods in which the total concentration of nucleoside 5′-diphosphates of 35 mM was proportioned as indicated. At various time intervals, 5-μl aliquots were removed and assayed for P_i. A comparable aliquot was chromatographed in solvent A to verify polymer formation. The rates of formation of (Am,C) polymers are shown in Figure 1A and (Cm,U) polymers in 1B. The ratio of methylated to nonmethylated nucleotides present in each reaction mixture was: (—○—○—○—), 1:20; (—□—□—□—), 1:10; (—△—△—△—), 1:6; and (—●—●—●—), 1:3.

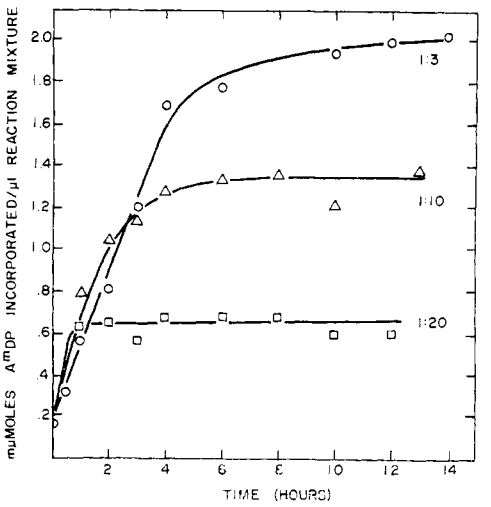

FIGURE 2: [^{3}H]AmDP incorporation in the presence of CDP. Each reaction mixture contained the components described in Methods with the exception of nucleoside 5′-diphosphate concentrations. The concentrations of [^{3}H]AmDP and CDP in the 1:3, 1:10, and 1:20 reactions were 10:30, 5:50, and 2.5:50 mM, respectively. Aliquots (5 μl) were removed at various time intervals, diluted into 200 μl of cold H_2O and mixed with 2 ml of cold 5% trichloroacetic acid. The acid-treated aliquot was poured over a Millipore filter which was dried and counted in toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene.

However, there appears to be some restriction on the capacity of this substrate to be polymerized by *Micrococcus lysodeikticus* polynucleotide phosphorylase. CmDP alone is not incorporated into poly Cm under conditions where CDP gives poly C and AmDP gives poly Am. Neither is CmDP copolymerized in the presence of CDP, ADP, and GDP to form heteropolymers. Various ratios of 2′-*O*-methylnucleotide to nonmethylated (from 1:1 to 1:12) in the presence or absence of dimethyl sulfoxide seem to have little effect on the ability of these mixtures to copolymerize. However, polynucleotides are formed in reaction mixtures containing CmDP and either CDP or ADP. The polymers appear to be homopolymers of the nonmethylated substrates since alkaline hydrolysis and subsequent electrophoresis failed to demonstrate the presence of any 2′-*O*-methyl-containing oligonucleotides. CmDP is incorporated in the presence of UDP in various ratios to form heteropolymers containing CmpU and CmpCmpU sequences (Table III). The efficient incorporation of CmDP into poly (Cm,U) is rather striking in light of its lack of incorporation into other heteropolymers.

In a similar manner, AmDP does not appear to be copolymerized with certain nucleoside 5′-diphosphates. Using conditions which produce poly (Am,C) and poly (Am,U) in good yield, AmDP is not polymerized in the presence of GDP. AmDP is polymerized in the presence of ADP and ADP is polymerized in the presence of AmDP, but the product is not the heteropolymer, poly (Am,A). Incorporation of either substrate is strongly dependent upon the relative concentration of the other. As indicated in Figure 3A, AmDP but not ADP is polymerized when the ADP to AmDP ratio is 1:1. The results shown were obtained when both substrates were present at 25 mM concentrations. Lowering the concentration of each substrate to 15 mM gave identical results. When the ratio of ADP to AmDP is raised to 4:1 (Figure 3B), ADP is slowly incorporated but AmDP is not. The lack of AmDP incorporation into these polymers was verified by alkaline digestion of the polymers and subsequent electrophoresis. No alkaline-stable oligonucleotides were observed. The addition of dimethylformamide or dimethyl sulfoxide had no effect on AmDP incorporation. Therefore, it does not seem possible to synthesize poly (Am,A) heteropolymers containing small amounts of AmMP using these conditions.

A heteropolymer containing three nucleotides (AmMP, CmMP, and UMP) was synthesized from the corresponding diphosphates with polynucleotide phosphorylase. The product was characterized by two methods. Following removal of protein and repeated precipitation from ethanol to eliminate mononucleotide contamination, the polymer was degraded with purified snake venom phosphodiesterase as described above. Separation of the 5′-mononucleotides produced by this treatment was accomplished in solvent F. The ratio of AmDP:CmDP:UDP in the reaction mixture was 1:1:10. The ratio of AmMP:CmMP:UMP present in the product and produced by phosphodiesterase digestion was determined by elution of the corresponding ultraviolet-absorbing areas of the chromatogram as described below and was determined to be 0.6:3.6:10, respectively. The R_F of these nucleotides in solvent F were: AmMP, 0.78; CmMP, 0.54; and UMP, 0.21. Chemically synthesized nucleotides were used as standards for chromatography. Alternatively, poly (Am,Cm,U) was degraded with snake venom phosphodiesterase plus alkaline phosphatase and the resultant nucleosides separated in solvent C. The nucleosides produced migrated with authentic Am, Cm, and U standards.

Characterization of Polymers. Nucleotide composition of poly (Am,C) and poly (Cm,U) was determined by exhaustive digestion with snake venom phosphodiesterase, as described in Methods, and subsequent chromatography in solvents B and D. The ultraviolet-absorbing spots, corresponding to

TABLE II: Distribution of AmMP in Poly (Am,C) and AMP in Poly (A,C).[a]

Input Ratio AmDP:CDP	Product Ratio[b] AmMP:CMP	% Me_2SO Present	Polymerization Time (hr)	% of Total Nucleosides in			AmpC/ AmpAmpC Found	AmpC/ AmpAmpC Theor
				C	AmpC	AmpAmpC		
1:6	1:13.6	0	1.5	88	7.6	4.6	1.6	14.6
1:6	1:11.8	10	2.5	85	11.9	3.7	3.2	12.8
1:6	1:14.3	20	3	88	9.5	2.5	3.8	15.3
1:6	1:12.1	30	7	86	12.2	1.4	8.7	13.1
Input Ratio ADP:CDP	Product Ratio AMP:CMP	% Me_2SO Present	Polymerization Time (hr)	% of Total Nucleosides in			ApC/ ApApC Found	ApC/ ApApC Theor
				C	ApC	ApApC		
1:6	1:15.1	0	1.5	88	10.8	1.2	9.0	16.1

[a] Conditions for the synthesis of poly (Am,C) and poly (A,C) are described in Methods. The concentrations of AmDP and ADP were 5 mM, and that of CDP was 30 mM. Me_2SO was added to the final concentration indicated as per cent of the total volume. The polymers were degraded with RNase and treated with alkaline phosphatase, and the products were characterized by paper electrophoresis in 0.05 M Tris-acetate (pH 7.8) as described in Methods. The per cent of total nucleosides present in oligonucleotides was calculated using molar extinction values which reflect the contribution of each nucleoside at the λ_{max} of the other. [b] The product ratios of AmMP to CMP were calculated from these results and were similar to those obtained by total degradation with snake venom phosphodiesterase as described in Methods.

TABLE III: Distribution of 2′-*O*-Methylnucleotides in Poly (Cm,U).[a]

Input Ratio CmDP:UDP	Product Ratio CmMP:UMP	% Me_2SO Present	Polymerization Time (hr)	% of Total Nucleosides in			CmpU/ CmpCmpU Found	CmpU/ CmpCmpU Theor
				U	CmpU	CmpCmpU		
1:6	1:8.6	0	6.5	81.6	10.9	7.5	1.4	9.6
1:6	1:7.9	10	6.5	80.3	11.2	8.6	1.3	8.9
1:6	1:7.0	20	7.5	77.8	13.8	8.4	1.6	8.9
1:6	1:7.8	30	23.5	79.1	15.1	6.0	2.5	8.8
1:12	1:12.5	0	3	86.5	9.6	3.9	2.5	13.5
1:12	1:13.3	10	3	87.2	9.2	3.7	2.5	14.3
1:12	1:11.6	20	3	85.5	10.9	3.6	3.0	12.6
1:12	1:12.3	30	6	85.9	11.3	3.0	3.7	13.3
1:12	1:8.8	40	24	81.8	12.8	4.6	2.8	9.8

[a] The synthesis of poly (Cm,U) and subsequent degradation with NaOH and *E. coli* alkaline phosphatase is described in Methods. Paper electrophoresis and calculations of the molar percentage of nucleosides in each fraction were performed as described in the legend to Table II. The concentrations of CmDP and UDP were 5 and 30 mM, respectively, for the 1:6 ratio and 2.5 and 30 mM for the 1:12 ratio. The ratios of isolated products, CmMP:UMP, were determined by snake venom phosphodiesterase treatment as described in Methods.

known standards, were eluted by cutting the paper into small pieces and suspending in 0.1 N HCl for 4 hr at room temperature. Equivalent areas, devoid of visible ultraviolet-absorbing material, were used as controls.

Heteropolymers containing 2′-*O*-methylnucleotides were degraded with NaOH or pancreatic RNase followed by *E. coli* alkaline phosphatase as described in Methods. The mixture of nucleosides and oligonucleotides obtained were separated by paper electrophoresis in a 0.075 M triethylammonium bicarbonate (pH 7.8) system. Nucleosides migrated slightly toward the cathode (−1.0 cm) while dinucleoside monophosphates (+2.5 cm) and trinucleoside diphosphates (+4.0 cm) migrated toward the anode. Ultraviolet areas corresponding to each component were eluted with water and lyophilized to remove the bicarbonate buffer. It was necessary at this point to further purify the oligonucleotides to eliminate traces of alkaline phosphatase which was carried through the electrophoretic separation and interfered with the interpretation of subsequent phosphodiesterase digestion. The oligonucleotides were chromatographed in solvent D, eluted, and repeat-

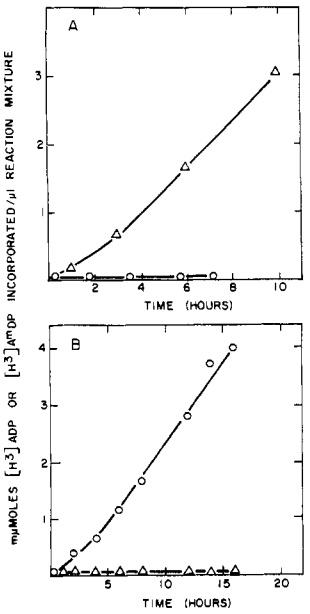

FIGURE 3: Incorporation of [³H]AmDP in the presence of ADP and the incorporation of [³H]ADP in the presence of AmDP. Polymerization conditions are those described in Methods. Figure 3A shows the incorporation obtained when the AmDP:ADP ratio is 1:1. The concentration of each substrate is 25 mM. The upper curve (△—△—△) represents the incorporation of [³H]AmDP in the presence of nonradioactive ADP. The lower curve (O—O—O) shows the lack of [³H]ADP incorporation in the presence of nonradioactive AmDP under identical conditions. The incorporation of each substrate when present at a 1:4 AmDP:ADP ratio is given in part B. O—O—O was obtained with 12.5 mM AmDP and 50 mM [³H]ADP while the △—△—△ was obtained with 12.5 mM [³H]AmDP and 50 mM ADP.

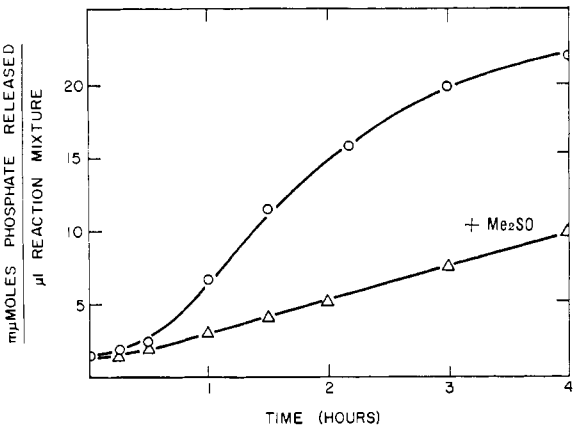

FIGURE 4: Effect of Me_2SO on the rate of polymer formation. Conditions for polymer synthesis are given in Methods. The concentrations of AmDP and CDP were 5 and 30 mM, respectively; 30% Me_2SO by volume was added to one reaction mixture. The reaction was followed as described in the legend to Figure 1.

edly lyophilized. Exhaustive treatment of the isolated oligonucleotides with snake venom phosphodiesterase produced nucleosides and mononucleotides in the expected ratio. AmpC and AmpAmpC produced Am plus pC and Am, pAm, plus pC, respectively, in the ratio of 1.1:1.0 and 1.1:1.0:1.1. Likewise, CmpU and CmpCmpU produced Cm plus pU and Cm, pCm, plus pU in a ratio of 1.0:1.1 and 1.1:1.0:1.2.

However, the amount of trinucleoside diphosphate relative to dinucleoside monophosphate was in all cases much greater than predicted by random polymerization. It appeared as if the incorporation of 2′-*O*-methylnucleotide was not random but partially cooperative (Tables II and III). Since this does not correspond to the natural distribution of these modified nucleotides, attempts were made to synthesize polymers in which the 2′-*O*-methylnucleotides were not paired.

Effect of Dimethyl Sulfoxide on 2′-O-Methylnucleotide Incorporation. Incubation conditions with polynucleotide phosphorylase were modified in a number of ways in an attempt to inhibit the paired incorporation of 2′-*O*-methylnucleotides. Various concentrations of dimethyl sulfoxide ranging from 10 to 40% were found to influence the incorporation of AmDP in the presence of CDP, increasing the ratio of AmpC to AmpAmpC (Table II). The final concentration of dimethyl sulfoxide present in routine reaction mixtures was 30% and represents a compromise between increased randomization and overall inhibition of polymerization in high concentrations of dimethyl sulfoxide. The effect of 30% dimethyl sulfoxide on the rate of heteropolymer formation is presented in Figure 4. Concentrations of dimethyl sulfoxide exceeding 40% permitted only limited synthesis of poly (Am,C) (1:6) and completely inhibited the synthesis of poly (Cm,U) (1:6).

Other organic solvents were tested to stimulate randomization of polymerization. Various concentrations of methanol, ethanol, and dimethylformamide were added to the reaction mixture to determine concentrations of perturbants which would still permit reasonable poly (Am,C) and poly (Cm,U) formation in 24 hr. It was found that polynucleotide phosphorylase would catalyze the formation of poly (Am,C) (1:6) and poly (Cm,U) (1:6) in 20% methanol, 10% ethanol, or 15% dimethylformamide. However, none of these solvents appeared to be as effective as dimethyl sulfoxide in stimulating random polymerization and were not further examined.

The size of poly (Am,C) (1:6) molecules formed with polynucleotide phosphorylase in the presence of 30% dimethyl sulfoxide varies with the time of incubation, as might be expected. Analysis of purified polymers on 5–20% sucrose gradients with tRNA markers indicated the product was 2–3 S after 4 hr, 4 S at 5 hr, and 5 S at 7 hr.

Since dimethyl sulfoxide did not seem to effect the paired incorporation of CmMP in poly (Cm,U) formed from an input ratio of 1:6, the same studies were carried out in reaction mixtures in which the input ratio of CmDP to UDP was 1:12 (Table III). These later reactions gave polymers with similar 2′-*O*-methylnucleotide content as the poly (Am,C) reactions in Table II. However, the distribution of CmMP was not altered by various concentrations of dimethyl sulfoxide at either input ratio.

The ratio of nucleotides present in the product does not directly reflect the ratio in the reaction mixture. This is also observed with normal (A,C) polymerization reactions (Grunberg-Manago, 1963). A (1:6) (Am,C) input results in a (1:12) (Am,C) polymer (Table II), as determined by both snake venom digestion and calculations based on the amount of alkaline-stable oligonucleotides remaining. In comparison, the products of (1:6) and (1:12) (Cm,U) reactions, as determined by either method, more closely approximate the input ratio (Table III).

Discussion

The formation of heteropolymers containing 2′-*O*-methylnucleotides with *M. lysodeikticus* polynucleotide phosphorylase takes place with certain restrictions. AmDP is copolymerized with UDP and CDP but not GDP or with its unmethylated analog, ADP. CmDP is not polymerized to form homopolymer, poly Cm, yet it copolymerizes with UDP to form poly (Cm,U). A similar observation has been reported by Ikehara *et al.* (1969) using 8-substituted purine nucleotides and polynucleotide phosphorylase; heteropolymers containing these modified nucleotides were synthesized but homopolymer synthesis was not observed. CmDP is not incorporated in the presence of ADP, GDP, or CDP. These restrictions are not understood. This is especially true with reactions involving AmDP in which poly Am is synthesized when AmDP is present either by itself or in 1:1 ratio with ADP (Figure 3A) and poly A is synthesized at high ratios of A:Am without concomitant AmMP incorporation (Figure 3B). In the later case the rate of polymerization of ADP is greatly reduced by the presence of AmDP which is consistent with apparent K_m's for each substrate which are similar. In this rather peculiar situation, AmDP can act as both substrate and/or inhibitor, depending upon its relative concentration to ADP.

It would appear that 2′-*O*-methylnucleotides can react in the presence of polynucleotide phosphorylase in at least three possible ways: (1) they can form 2′-O-methylated homopolymers, even in the presence of normal nucleoside diphosphates (Figure 3A), (2) they can be incorporated into heteropolymers containing normal nucleoside diphosphates, and (3) they can partially inhibit the polymerization of normal nucleoside diphosphates without undergoing self-polymerization (Figure 3B).

The linear incorporation of [^{3}H]AmDP in the presence of CDP at high AmDP:CDP ratios (Figure 2) and rather abrupt fall off at later incubation times indicate that [^{3}H]AmDP was being incorporated progressively as the polymer lengthened rather than by a discontinuous incorporation of [^{3}H]AmDP at any one period of incubation. Other experiments not shown here assayed the AmDP and CDP remaining after various times of polymerization. The results showed a constant ratio of AmDP to CDP in each reaction at all stages of polymerization and are consistent with the data obtained with [^{3}H]AmDP.

Heteropolymers formed from a mixture of 2′-O-methylated and unmethylated nucleotides reflected a tendency for cooperative incorporation of the 2′-O-methylated species. To a much smaller degree, adjacent pairs of 2′-*O*-methylnucleotides occur in natural RNA, obviously arising from a different mechanism involving methylation of polynucleotide chains. Thus Singh and Lane (1964) have shown that 10% of the 2′-*O*-methylnucleotides found in a commercial ribonucleate preparation is present as adjacent 2′-*O*-methylnucleotides, a value considerably higher than one would expect by a statistical prediction of the random placement of a trace nucleotide.

It should be noted that the tendency for cooperative incorporation apparently does not extend beyond two adjacent 2′-*O*-methylnucleotides since long runs of such nucleotides were not observed. Oligonucleotides containing more than two 2′-*O*-methylnucleotides could have been detected by these methods if the increased frequency of adjacent 2′-*O*-methylnucleotide incorporation was maintained beyond two nucleotides. This implies a mechanism by which the initial incorporation of one 2′-*O*-methylnucleotide facilitates the subsequent incorporation of a second, but not a third like molecule.

In some unknown manner dimethyl sulfoxide interferes with this tendency for cooperative incorporation with 2′-*O*-methyladenosine, thereby enhancing random incorporation. Poly (Am,C) formed in the presence of 30% dimethyl sulfoxide resembles its nonmethylated analog, poly (A,C), in the distribution of A and Am within the respective heteropolymers (Table II). Thus it appears dimethyl sulfoxide induces a randomization of AmMP approximating its unmethylated analog AMP when copolymerized with CMP. The effect of dimethyl sulfoxide may be on the substrate, the enzyme, or the manner in which the enzyme recognizes a more hydrophobic substrate than unmethylated nucleotides in a more hydrophobic media than water. Dimethyl sulfoxide was effective in randomizing AmMP incorporation yet had no apparent effect on CmMP incorporation under identical conditions.

We have noted some variability between different *M. lysodeikticus* polynucleotide phosphorylase preparations in their efficiency in catalyzing heteropolymer formation with 2′-*O*-methylnucleotides. These differences were observed with enzyme preparations purified in our laboratory by the method of Singer (1966) to stage 6 and also within different lots of enzyme purchased from P-L Biochemicals, Inc. This may reflect various degrees of contamination with primer oligonucleotides. However the involvement of primer could not be demonstrated by further purification to remove primer or the separate addition of ApApA primer.

The availability of heteropolymers which are methylated only in the 2′-hydroxyl position permits a number of studies related to the possible function of methylation in RNA. Experiments designed to measure the contribution of 2′-*O*-methyl groups to the conformation of RNA molecules, the effect of 2′-*O*-methylnucleotides on digestion by specific nucleases, and the template properties of these heteropolymers in cell-free amino acid incorporating systems are in progress.

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N^6-(Δ^2-Isopentenyl)adenosine. Biosynthesis in Transfer Ribonucleic Acid *in Vitro**

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ABSTRACT: Several molecular species of transfer ribonucleic acid contain an N^6-(Δ^2-isopentenyl)adenosine residue adjacent to the 3′ end of the anticodon. The biosynthesis of this component has been investigated. Yeast and rat liver contain an enzyme that catalyzes *in vitro* the transfer of the Δ^2-isopentenyl group from Δ^2-isopentenyl pyrophosphate to a receptor adenosine residue in homologous suitably treated transfer ribonucleic acid resulting in the synthesis of the N^6-(Δ^2-isopentenyl)adenosine component of transfer ribonucleic acid. The enzyme has been purified 50–100-fold from yeast. Δ^3-Isopentenyl pyrophosphate is not a substrate. The enzyme does not catalyze the attachment of the isopentenyl group to the native transfer ribonucleic acid but does catalyze the reaction with permanganate-treated transfer ribonucleic acid. The permanganate treatment is specific for cleavage of the Δ^2-isopentenyl groups of transfer ribonucleic acid leaving adenosine residues. Therefore, a specific adenosine residue in the transfer ribonucleic acid serves as the receptor site of the Δ^2-isopentenyl group. Support for this conclusion comes from an experiment in which the N^6-(Δ^2-isopentenyl)adenosine residues are rendered insensitive to permanganate oxidation.

The transfer ribonucleic acid is treated with iodine which reacts specifically with the N^6-(Δ^2-isopentenyl)adenosine residues and prevents further reaction with permanganate. Transfer ribonucleic acid treated first with iodine and then with permanganate does not serve as a substrate. The enzyme systems from both yeast and rat liver catalyze a significant incorporation of Δ^2-isopentenyl groups into untreated transfer ribonucleic acid from *Escherichia coli* B.

The Δ^2-isopentenyl group has been detected in the tRNA of all organisms investigated. This group occurs as the parent nucleoside, N^6-(Δ^2-isopentenyl)adenosine, in the tRNA of yeast, animal, plant, and *Lactobacillus acidophilus* (Biemann *et al.*, 1966; Fittler *et al.*, 1968a; Hall *et al.*, 1966, 1967; Peterkofsky, 1968; Robins *et al.*, 1967; Staehelin *et al.*, 1968). A hydroxylated derivative, N^6-(*cis*-4-hydroxy-3-methylbut-2-enyl)adenosine, occurs in the tRNA of plant tissue (Hall *et al.*, 1967) and a methylthio derivative, N^6-(Δ^2-isopentenyl)-2-methylthioadenosine, occurs in the tRNA of *Escherichia coli* B (Burrows *et al.*, 1968; Harada *et al.*, 1968). Moreover, it always occurs adjacent to the 3′ end of the presumed anticodon in several tRNAs whose sequence is known (Goodman *et al.*, 1968; Madison and Kung, 1967; Staehelin *et al.*, 1968; Zachau *et al.*, 1966).

N^6-(Δ^2-Isopentenyl)adenosine possesses cytokinin activity; it promotes cell division and cell differentiation in plant tissue (Hall and Srivastava, 1968; Leonard *et al.*, 1966). This nucleoside and its derivatives are the only known naturally occurring purine derivatives that possess cytokinin activity, but there is no evidence that their presence in tRNA is related to their physiological activity.

A knowledge of the mechanism of the biosynthesis of N^6-(Δ^2-isopentenyl)adenosine may shed light on the significance of this compound to tRNA structure, and further, might give some indication of the reason for its biological activity. Accordingly, a study of the biosynthetic pathway has been undertaken.

Studies carried out on the mevalonate-utilizing bacteria, *L. acidophilus*, *in vivo*, have shown that the Δ^2-isopentenyl group of N^6-(Δ^2-isopentenyl)adenosine in the tRNA of this organism is derived from mevalonic acid (Fittler *et al.*, 1968a; Peterkofsky, 1968). Fittler *et al.* (1968b) demonstrated *in vitro* that the Δ^2-isopentenyl group derived from mevalonic acid is incorporated into the preformed RNA molecule. This reaction was catalyzed by a crude extract obtained from yeast or rat liver.

The enzyme catalyzing the formation of N^6-(Δ^2-isopen-

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