The Preparation and Properties of O-Methylated Adenosine Derivatives*

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ABSTRACT: By reacting diazomethane with adenosine in a partially aqueous medium, 2′-*O*-methyladenosine, 3′-*O*-methyladenosine, 5′-*O*-methyladenosine, and 2′,3′-di-*O*-methyladenosine have been prepared in 38, 11, 1.5, and 3.5% yields, respectively. These yields are in general accord with the known mechanism of action of diazomethane and with the acidities of the individual hydroxyl groups of adenosine as determined by ion-exchange chromatography of the O-methylated derivatives on Dowex 1 (OH^-) columns, *i.e.*, the more acidic the hydroxyl groups, the greater the extent of methylation. Replacement of the 2′- and/or 3′-hydroxyl group of adenosine by a methoxyl group slightly stabilized the glycosyl bond to acid-catalyzed hydrolysis. For 0.1 N HCl, 97.5°, the following first-order rate constants were determined: adenosine, 10×10^{-4} sec^{-1}; 5′-*O*-methyladenosine, 10×10^{-4} sec^{-1}; 3′-*O*-methyladenosine, 7.5×10^{-4} sec^{-1}; 2′-*O*-methyladenosine 6.0×10^{-4} sec^{-1}; and 2′,3′-di-*O*-methyladenosine, 3.5×10^{-4} sec^{-1}.

Methylation also affects the rate of color development in the orcinol reaction. Relative to adenosine, the following color yields were obtained using standard conditions (6 N HCl, 20 min at 100°): 2′-*O*-methyladenosine, 23%; 3′-*O*-methyladenosine, 100%; 5′-*O*-methyladenosine, 125%; and 2′,3′-di-*O*-methyladenosine, 15%. These values provide further evidence regarding the chemical basis of the orcinol test. Adenosine deaminase from calf intestinal mucosa was found to be inactive toward 5′-*O*-methyladenosine but active toward the derivatives bearing methyl groups on the 2′- and/or 3′-oxygens.

While it is now clearly established that both t- and rRNAs are frequently methylated at the 2′-oxygen of some of their ribose residues (Smith and Dunn, 1959; Singh and Lane, 1964a,b; Hall, 1964), our knowledge of the chemical behavior and biological significance of 2′-*O*-methyl nucleoside residues is, at best, fragmentary. Recent progress in methylating procedures (Broom and Robins, 1965; Khwaja and Robins, 1966) has made possible selective O methylation of adenosine and cytidine. Using the Broom and Robins (1965) procedure which employs diazomethane in aqueous dimethoxyethane, we have obtained on reaction of adenosine: 3′-*O*-methyladenosine, 5′-*O*-methyladenosine, and 2′,3′-di-*O*-methyladenosine in addition to the 2′-*O*-methyladenosine initially reported (Broom and Robins, 1965). These methylated adenosines can be readily separated by a combination of cellulose and anion-exchange chromatography making use, in the case of the latter technique, of the variation in acidity of the remaining hydroxyl groups of the derivatized adenosines (Dekker, 1965). In this paper we describe the isolation and properties of several methylated adenosines and their behavior toward (1) acid hydrolysis, (2) enzymatic deamination, and (3) the orcinol reaction.

The preparation of 3′-*O*-methyladenosine has also been accomplished by the coupling of the appropriate methylated sugar derivative with adenine (Tong *et al.*, 1967). Our results are in general agreement with those of Martin *et al.* (1968) who have prepared 2′- and 3′-*O*-methyladenosines and 2′- and 3′-*O*-methylcytidines as a part of their continuing study of methods for differentiating between 2′- and 3′-substituted nucleosides and the selectivity of various reagents for the 2′- and 3′-hydroxyl groups of nucleosides.

Materials and Methods

Reagents and Substrates. *N*-Nitrosomethylurea was either purchased from Rare and Fine Chemicals or synthesized by the procedure of Arndt (1943). $^{14}CH_3$-labeled *N*-nitrosomethylurea was prepared from [^{14}C]-methylamine hydrochloride supplied by New England Nuclear Corp. AxpAp[1] was provided by Dr. Byron Lane and 3′-*O*-methyladenosine, prepared from suitably protected 3-*O*-methylribose (Tong *et al.*, 1967), was a gift of Dr. Leon Goodman. Calf intestinal adenosine deaminase was purchased from Calbiochem (lot 54850, B grade, 230 units/mg).[2]

Microanalysis and Spectral Measurements. All analyses were performed by the Microchemical Laboratory, Department of Chemistry, University of California,

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[1] The symbol Ax has been used to represent 2′-*O*-methyladenosine (see Smith and Dunn, 1959). AxpAp would therefore be a dinucleotide in which the adenylyl moiety bears the 2′-*O*-methyl group.

[2] A unit is defined as that amount of enzyme which catalyzes the deamination of 1 μmole of adenosine/min at 37°.

Berkeley. Nuclear magnetic resonance spectra were measured with a Varian Model A-60 spectrometer using tetramethylsilane as external standard and D_2O as solvent. Ultraviolet absorption spectra were measured on a Cary, Model 14, or Beckman spectrophotometer.

Electrophoresis and Chromatography. Paper electrophoresis was conducted with a modification of the instrument described by Crestfield and Allen (1955). Electrophoretic mobilities were measured in 0.1 M sodium tetraborate on Whatman No. 3MM paper at a potential gradient of 8 V/cm. Mobilities were expressed as migration relative to ribose (M_R). Paper chromatography was performed in descending fashion using all-glass apparatus. The following solvent systems were employed: for nucleosides and nucleoside derivatives: (A) *n*-butyl alcohol–water–concentrated ammonia (86:14:5), (B) isopropyl alcohol–water–concentrated ammonia (7:2:1), (C) ethyl acetate–*n*-propyl alcohol–water (4:1:2), and (D) isopropyl alcohol–water–concentrated ammonia (50:35:10); for ribose and ribose derivatives: (E) *n*-butyl alcohol saturated with water and boric acid, using paper dipped in saturated boric acid and dried, (F) *n*-butyl alcohol saturated with water, (G) *n*-butyl alcohol–ethyl alcohol–water–concentrated ammonia (40:10:49:1), (H) pyridine–ethyl acetate–water (1:2:2); and for adenine and methylated adenines: (I) isopropyl alcohol–5% aqueous ammonium sulfate (1:19), (J) dimethylformamide–concentrated ammonia–isopropyl alcohol (25:10:65). Solvents A–C were previously employed by Broom and Robins (1965), E and F by Barker *et al.* (1955), F–H by Brown *et al.* (1954), and I and J by Townsend *et al.* (1964). For ion-exchange fractionation of nucleosides, an analytical grade of Dowex 1-2X (Bio-Rad AG 1-2X, 200–400 mesh) was used in the hydroxide form. Columns were packed, converted into the hydroxide form, and washed free of alkali as described by Austin *et al.* (1963). Nucleosides were eluted with water or aqueous methanol (Dekker, 1965). The elution patterns were monitored and fractions were collected with a G. M. E. Model TL fractionator equipped with a Texas Instrument recorder. For quantitative estimation of the material in peak fractions, A_{260} values were read at the spectrophotometer.

O-Methylation of Adenosine. The procedure of Khwaja and Robins (1966) for the methylation of adenosine was followed with some modifications. The diazomethane is generated by carefully adding 20 g (192 mmoles) of *N*-nitrosomethylurea, over a period of 5 min, with continuous mixing by magnetic stirrer, to 105 ml of 1,2-dimethoxyethane and 45 ml of 50% (w/w) KOH in a salt–water–ice bath. After stirring for 15 min, the aqueous layer is frozen in an acetone–Dry Ice bath and the ethereal diazomethane solution is decanted into a flask containing KOH pellets and allowed to dry for 20 min at 0°. The resulting solution containing 5.5 g (131 mmoles) of diazomethane/100 ml (as determined by standardization against benzoic acid) is poured into a solution of 1 g (3.74 mmoles) of adenosine in 40 ml of water at 80°. The reaction mixture is allowed to cool to room temperature and then stirred overnight. The thick syrup remaining after evaporation at reduced pressure is taken up twice in 20 ml of 95% ethanol, reevaporating after each wash.

Acid Hydrolysis of Nucleosides for Paper Chromatographic Analysis. Equal volumes (10 μl) of nucleoside in water (1 A_{260} unit) and 0.2 N HCl were combined, sealed in a capillary, and treated for 3.5 hr at 100° before applying to paper.

Demethylation with 40% hydrobromic acid was carried out as described by Hough and Theobald (1963).

Periodate Oxidation. The susceptibility of 2′-*O*-methyladenosine, 3′-*O*-methyladenosine, and 2′,3′-di-*O*-methyladenosine to periodate oxidation was tested by the spectrophotometric method of Dixon and Lipkin (1954). An alternate method was utilized for 5′-*O*-methyladenosine to provide additional information. Periodate oxidation, in this case, was carried out in a capillary tube with 0.3 M periodate, using 1 A_{260} unit of nucleoside. After 15 min the excess periodate was reduced with bisulfite and the products were chromatographed in solvent D. The chromatogram was then sprayed with 0.4% 2,4-dinitrophenylhydrazine in 2 N HCl giving a yellow spot where dialdehyde had been produced by the oxidation. The spot turned purple when sprayed with 10% NaOH (see test for carbonyl compounds described by Bland, 1949). Because of the high periodate concentration used in the latter procedure, the method can be applied to nucleosides with a *trans*-glycol structure, *e.g.*, arabinofuranosyl or xylofuranosyl derivatives, if a 48-hr reaction time is used.

Orcinol Reaction for Pentose. This was carried out according to Mejbaum (1939) using a 20-min reaction period. Absorbance at 670 mμ was read on a Beckman DU-2 and compared with a standard curve obtained by measuring the A_{670} of the products resulting when varying concentrations of adenosine were subjected to the orcinol reaction under identical conditions. Longer heating periods were examined to determine the maximum color yield possible.

Formaldehyde Determination. The adenosine derivative (0.5 μmole in 0.2 ml of 0.1 N HCl) was hydrolyzed at 100° for 3.5 hr. Sodium metaperiodate (0.3 ml of 0.2 M) was added and the pH was brought to *ca.* 1.5 by addition of 10 μl of 12 N HCl. Oxidation of the free sugar was allowed to proceed for 6 hr. A 0.1-ml aliquot was withdrawn and excess periodate was reduced with 0.1 ml of 20% sodium bisulfite. After bringing the volume of the sample to 1 ml by addition of water, 5 ml of chromotropic acid reagent was added. The sample was heated at 100° for 30 min and the A_{570} was determined with a Beckman DU-2 spectrophotometer. Adenosine was used to prepare a standard curve. Formaldehyde determination with chromotropic acid is discussed by MacFadyen (1945) and Frisell *et al.* (1954).

Adenosine deaminase reactivity was assayed by the general procedure of Kaplan (1955). The reaction of O-methylated adenosine derivatives was determined by incubating 4×10^{-5} M solutions with 2.3 units of adenosine deaminase for 24 hr at 25° and measuring any decrease in A_{265}.

Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer using Bray's (1960) solution.

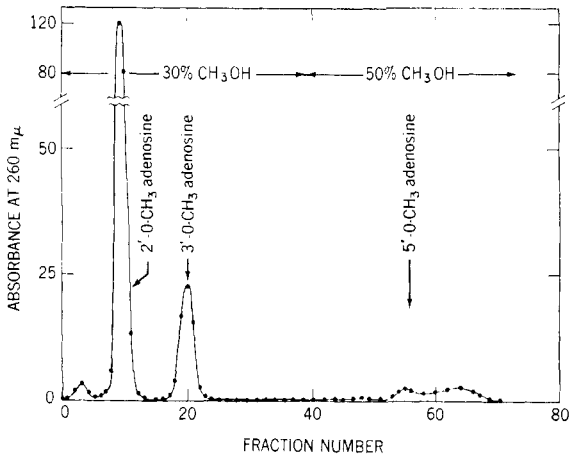

FIGURE 1: Fractionation of monomethylated adenosine derivatives on a Bio-Rad AG 1-2X (OH^-) column, 200–400 mesh, 1.6 × 21 cm; 16 ml/tube at 1 ml/min; aqueous methanol as eluent.

Measurement of Rates of Acid Hydrolysis. Aliquots (100 μl) of a solution of methylated adenosine (0.18 μmole) in 0.1 N HCl were pipetted into micro test tubes and the latter were sealed with parafilm and placed in a water bath maintained at 97.5 + 0.5°. At intervals tubes were removed and plunged into ice–water, and the contents were brought to neutrality by adding 2.5 ml of 0.05 M sodium phosphate buffer (pH 7.0). The remaining nucleoside was converted into the corresponding inosine derivative by the action of adenosine deaminase and the A_{265} was measured at the completion of reaction (Kalckar, 1947). The concentration of unhydrolyzed nucleoside can be calculated from the equation

$$C_{\text{inosine}} = \frac{A_{265}^{\text{obsd}} - \epsilon_{265}^{\text{adenine}} C_{\text{total}}}{\epsilon_{265}^{\text{inosine}} - \epsilon_{265}^{\text{adenine}}}$$

where C represents the concentration in millimoles and ϵ represents the millimolar extinction coefficient. The values utilized were $\epsilon_{265}^{\text{adenine}}$ 11.4 and $\epsilon_{265}^{\text{inosine}}$ 5.16. In the case of 5′-*O*-methyladenosine, which is not a substrate of adenosine deaminase, aliquots of the hydrolysate were removed at intervals and applied to Whatman No. 1 paper. Chromatography and estimation of both starting material and adenine were performed as described by Martin *et al.* (1968).

Results

Separation of Individual O-Methylated Adenosines and Characterization. The crude reaction mixture from the treatment of adenosine with diazomethane was separated into unmethylated, monomethylated, and dimethylated adenosine by preparative paper chromatography using solvent A. A total of 75 mg could be conveniently fractionated on a 46.5 × 57 cm sheet of Whatman No. 3MM paper; R_F's were: adenosine, 0.27; monomethylated adenosine, 0.47; and dimethylated adenosine, 0.65. The monomethylated material (up to 600 mg), after elution and evaporation, was dissolved in 3 ml of 30% methanol and applied to a 1.6 × 21 cm column of

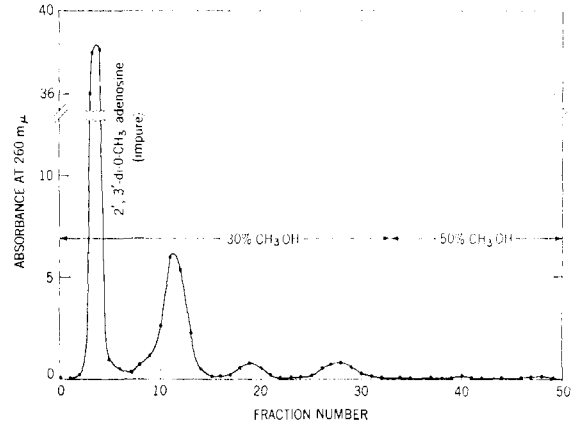

FIGURE 2: Fractionation of dimethylated adenosine derivatives on a Bio-Rad AG 1-2X (OH^-) column, 200–400 mesh, 1.6 × 21 cm; 16 ml/tube at 1 ml/min; aqueous methanol as eluent.

Bio-Rad AG 1-2X (OH^-) (200–400 mesh) previously equilibrated with the eluent, 30% methanol. Fractions of 16 ml were collected at 1 ml/min and the elution pattern was followed at 260 mμ (Figure 1). The first major peak after the elution of a minor unidentified component was 2′-*O*-methyladenosine; the following peak was 3′-*O*-methyladenosine. After the eluent was changed to 50% methanol, 5′-*O*-methyladenosine was eluted together with other minor methylation products. The fractions containing 2′-*O*-methyladenosine and 3′-*O*-methyladenosine were independently combined and concentrated to dryness; yields based on adenosine were 38 and 11%, respectively. Both substances were crystallized from methanol for characterization. For 2′-*O*-methyladenosine the results were: mp 201.5°, $[\alpha]_{589}^{22}$ −57.5° (lit. (Khwaja and Robins, 1966), mp 201–202°, $[\alpha]_D^{23}$ −57.9°); *Anal.* Calcd for $C_{11}H_{15}N_5O_4$: C, 46.96; H, 5.38; N, 24.90. Found: C, 46.76; H, 5.20; N, 24.76. For 3′-*O*-methyladenosine the results were: mp 178–180°, $[\alpha]_{589}^{22}$ −59° (lit. (Tong *et al.*, 1967), mp 182–183°, $[\alpha]_{589}^{25}$ −57°); *Anal.* Found: C, 46.77; H, 5.18; N, 25.18. 5′-*O*-Methyladenosine was also prepared by methylating 2′,3′-*O*-isopropylideneadenosine under the same conditions used for adenosine. After removal of the isopropylidene group by the method of Hampton *et al.* (1965), the monomethylated nucleoside was isolated by paper and anion-exchange chromatography as before. Although the yield was only 3%, which is twice that obtained directly from adenosine, isolation was simplified by the absence of appreciable amounts of other monomethylated adenosines. The ultraviolet spectrum of the purified material was similar to that of adenosine; A_{280}/A_{260} was 0.18 at pH 7.0. The amount of material available did not permit crystallization and physical characterization.

To purify 2′,3′-di-*O*-methyladenosine, the crude dimethylated band from the paper chromatographic separation was fractionated on a 1.6 × 21 cm Bio-Rad AG 1-2X (OH^-) column (Figure 2) as in the case of the monomethylated material. The first peak is composed of 2′,3′-di-*O*-methyladenosine plus small amounts of base- and sugar-methylated components and an unidentified impurity (about 50% of the A_{260} units) which is alkali

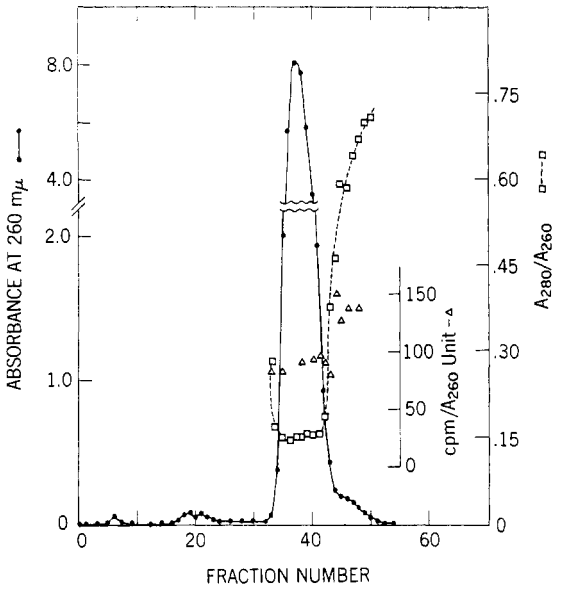

FIGURE 3: Purification of $^{14}CH_3$-labeled 2′,3′-di-*O*-methyladenosine on a Bio-Rad AG 1-2X (OH^-) column, 200–400 mesh, 1.1 × 100 cm; 9.6 ml/tube at 0.8 ml/min; water as eluent.

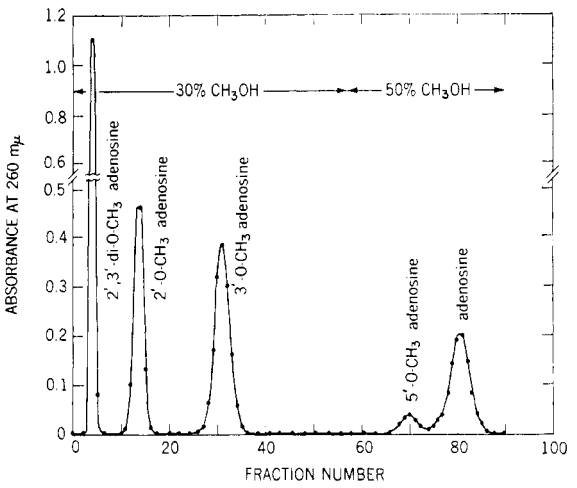

FIGURE 4: Separation of a synthetic mixture of adenosine plus purified O-methylated adenosine derivatives on a Bio-Rad AG 1-2X (OH^-) column, 200–400 mesh, 1.6 × 22 cm; 30% methanol for fractions 1–56 at 12 ml/tube per 12 min; 50% methanol for fractions 57–100 at 18 ml/tube per 12 min.

labile. From the A_{280}/A_{260} ratios measured throughout the peak and from sugar analysis after acid degradation of the total material, the second peak appears to be a mixture of di- and trimethylated adenosine derivatives with methyl groups on both the 2′-oxygen and on the heterocyclic base. No attempt was made to identify this material or the other minor components. To further purify the 2′,3′-di-*O*-methyladenosine, the first peak was evaporated to dryness, taken up in 0.5 ml of 0.2 N NaOH, and heated in a boiling-water bath for 40 min. The solution was then neutralized with 0.5 ml of 0.2 N HCl and added directly to a 1.1 × 100 cm Bio-Rad AG 1-2X (OH^-) column previously equilibrated with water. The 2′,3′-di-*O*-methyladenosine which is stable to the alkaline treatment was eluted with water, incompletely resolved from some unidentified material of higher A_{280}/A_{260} ratio. The fractions comprising the leading edge of the peak, with A_{280}/A_{260} ratio of *ca.* 0.15, were combined and recycled through a Bio-Rad AG 1-2X (OH^-) column of 1.1 × 100 cm. Elution with water at 9.6 ml/tube per 12 min gave the pattern seen in Figure 3. The methylated adenosine in this case had been prepared using diazomethane derived from $^{14}CH_3$-labeled *N*-nitrosomethylurea, thus providing an additional parameter for gauging purity. From Figure 3 it can be seen that those fractions (34–42) with a constant A_{280}/A_{260} ratio had approximately 90 cpm/A_{260} unit. Since pure CH_3-labeled 3′-*O*-methyladenosine isolated from the same reaction mixture had 45 cpm/A_{260} unit, fractions 34–42 were known to contain a dimethylated adenosine. After combining these fractions and evaporating to dryness, 2′,3′-di-*O*-methyladenosine was obtained in 3.5% yield (based on adenosine); mp 177°, $[\alpha]_{589}^{22}$ −49°.

Identification of each of the four methylated adenosines shown in Figure 4 was achieved in the following manner. (1) Acid hydrolysis gave adenine, identified by paper chromatography using the solvents of Townsend *et al.* (1964), and a methylated ribose which could be identified by its electrophoretic migration relative to ribose in 0.1 M sodium tetraborate (Brown *et al.*, 1954). When taken in the order in which they were eluted in Figure 4, the methylated nucleosides gave the following sugars, respectively: 2,3-di-*O*-methylribose, M_R 0.00 (lit. M_R 0.00); 3-*O*-methylribose, M_R 0.92 (lit. M_R 0.90); 2-*O*-methylribose, M_R 0.50 (lit. M_R 0.50); 5-*O*-methylribose M_R 0.96 (lit. M_R 0.99). The sugars were further identified by paper chromatography in solvents ystems E–H (Brown *et al.*, 1954; Barker *et al.*, 1955), confirming the above assignment. (2) Demethylation and hydrolysis with hydrobromic acid gave adenine and a sugar which ran coincidently with authentic ribose on paper chromatography in ethyl acetate–acetic acid–formic acid–water (18:3:1:4) (Hough and Jones, 1962). (3) Nuclear magnetic resonance gave the following results: (a) for 2′-*O*-methyladenosine: τ 1.75 (s) and 1.90 (s) for H(8) and H(2), τ 3.89 (d; J = 6 cps) for H(1′), and τ 6.56 (s) for methoxy protons; (b) for 3′-*O*-methyladenosine: τ 1.75 (s) and 1.92 (s) for H(8) and H(2), τ 3.98 (d; J = 6 cps) for H(1′), and τ 6.42 (s) for methoxy protons; (c) for 2′,3′-di-*O*-methyladenosine: τ 1.69 (s) and 1.80 (s) for H(8) and H(2), τ 3.82 (d; J = 6.5 cps) for H(1′), and τ 6.44 and 6.54 for the protons of the two methoxy groups; and (d) for 5′-*O*-methyladenosine: τ 1.76 (s) and 1.89 (s) for H(8) and H(2), τ 3.94 (d; J = 5.0 cps) for H(1′), and τ 6.62 (s) for methoxy protons. Integration showed the proper number of protons. In accordance with Reese's rule for 2′- and 3′-isomeric derivatives of ribonucleosides, the proton magnetic resonance of H(1) was at higher field for 3′-*O*-methyladenosine than for 2′-*O*-methyladenosine (Fromageot *et al.*, 1966). (4) On treatment with periodate, 5′-*O*-methyladenosine was oxidized while 2′-*O*-methyladenosine and 3′-*O*-methyladenosine were inert. (5) When $^{14}CH_3$-labeled adenosine derivatives were prepared, 2′,3′-di-*O*-methyladenosine was found to have

TABLE I: Kinetics of Acid Hydrolysis (0.1 N HCl, 97.5°).

Compound	k^1 ($\times 10^4$)[a] (sec^{-1})	$t_{1/2}$[a] (sec)
Adenosine	10.0	690
5′-*O*-Methyladenosine	10.0	690
3′-*O*-Methyladenosine	7.5	920
2′-*O*-Methyladenosine	6.0	1150
2′,3′-Di-*O*-methyladenosine	3.5	1970
2′-Deoxyadenosine		<30[b]

[a] Values have an accuracy of ±15% or better. [b] Accurate value not measurable under conditions used.

twice as many counts per minute per mole as either 2′-*O*-methyladenosine or 3′-*O*-methyladenosine. (6) When the purified 2′,3′-di-*O*-methyladenosine was subjected sequentially to acid hydrolysis and periodate oxidation, the yield of formaldehyde was quantitative when compared with that produced by an equivalent amount of adenosine under identical conditions. Similarly, 2′-*O*-methyladenosine and 3′-*O*-methyladenosine gave the expected amounts of formaldehyde. 5′-*O*-Methyladenosine gave no formaldehyde. (7) 5′-*O*-Methyladenosine failed to react with adenosine deaminase whereas the adenosine derivatives methylated at oxygens 2′ and/or 3′ were deaminated. The results are in accord with those of Chassy and Suhadolnik (1967) and Bloch *et al.* (1967) who have demonstrated that the 2′- and 3′-hydroxyl groups of adenosine are not essential for substrate activity whereas the 5′-hydroxyl group is essential.

The Order of Elution of O-Methylated Adenosines from Dowex 1 (OH^-) Columns. When a synthetic mixture was made of adenosine plus the four purified O-methylated adenosines and subjected to anion-exchange chromatography, the compounds were eluted in the order 2′,3′-di-*O*-methyladenosine, 2′-*O*-methyladenosine, 3′-*O*-methyladenosine, 5′-*O*-methyladenosine, and adenosine (Figure 4).

Kinetics of Acid Hydrolysis. At 97.5°, the acid-catalyzed hydrolysis of each of the four methylated derivatives appears to be a first-order reaction. A comparison of the enzymatic assay and the chromatographic assay, using 2′-*O*-methyladenosine as test substance, gave first-order rate constants of 6.0 and 5.9 $\times 10^{-4}$ sec^{-1}, respectively. Rate constants and half-times of hydrolysis are shown in Table I.

Orcinol Reaction. Under standard conditions (6 N HCl, 20 min, 100°), 2′-*O*-methyladenosine gave a color yield that was 23% that of adenosine; 3′-*O*-methyladenosine, 100%; 5′-*O*-methyladenosine, 125%; and 2′,3′-di-*O*-methyladenosine, 15%. 5′-Adenylic acid gave 138% of the color produced by adenosine in agreement with earlier results (Kerr and Seraidarian, 1945; Albaum and Umbreit, 1947), and AxpAp, an O-methyl-

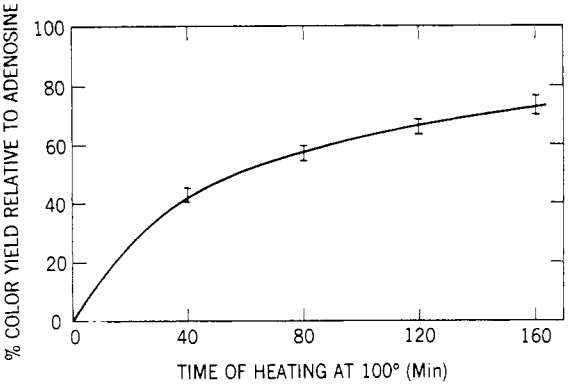

FIGURE 5: Color yield of 2′-*O*-methyladenosine as a function of time of heating in the orcinol reaction. Values are expressed as a percentage of the color yield of an equivalent amount of adenosine under identical conditions.

ated dinucleotide derived from wheat germ RNA (Singh and Lane, 1964a,b), gave 56% of the color of 2 M equiv of adenosine as earlier observed by Singh and Lane (1964b). To determine whether the variation in color yield was related to the rate of color development, the relative color yields were determined as a function of time of heating. The results with 2′-*O*-methyladenosine are shown in Figure 5 from which it can be seen that color development is slow and begins to plateau at about 180 min. In agreement with Albaum and Umbreit (1947), adenosine was found to give maximum color at 40 min at which time the A_{670} value was 115% of that at 20 min. With longer periods of heating the color decayed. Back-extrapolation of the decay curves to the zero-time intercept permitted calculation of the maximum theoretical color yield which was 125% of the color obtained at 20 min. The color yield *vs.* time profile for 5′-*O*-methyladenosine showed that maximum color was obtained in 20 min. All substances gave the same spectral envelope for the orcinol color suggesting a common chromophoric product.

Discussion

The nature of the solvent employed in the reaction of diazomethane and adenosine has a profound effect on the site(s) of methylation. For example, when adenosine in aqueous solution is treated with ethereal diazomethane (Haines *et al.*, 1964), 1-methyladenosine is produced in low yield. In contrast, Friedman *et al.* (1963), using methanol–ether as solvent, found 2′-deoxyadenosine to be inert. Bredereck (1947) had earlier made a similar observation using the more soluble 2′,3′,5′-tri-*O*-acetyladenosine. More recently, Broom and Robins (1965) made the very practical discovery that treatment of adenosine with diazomethane in the homogeneous system, dimethoxyethane–water, led to preferential O methylation, *i.e.*, sugar hydroxyl methylation. While there is no simple explanation for this last result, it is probably related to the considerably higher concentration of both reactants in a single phase and to the increased acidity of the hydroxyl groups of the sugar relative to the amidine group of the heterocyclic base in the homogeneous polar solvent. From the

known mechanism of action of diazomethane, the most strongly acidic group of adenosine might be expected to be methylated most readily. Information regarding the acidities of the hydroxyl groups of adenosine comes from several sources. (1) Titration, both potentiometric (Levene *et al.*, 1926a,b) and thermal (Izatt *et al.*, 1965), demonstrates that adenosine has an acidic group of $pK_a \simeq 12.5$ and that this is most likely associated with the 2′,3′-*cis*-glycol structure (Izatt *et al.*, 1965). (2) Rapid equilibration of acyl or aminoacyl groups between the 2′- and 3′-oxygens (Wolfenden *et al.*, 1964; Reese and Trentham, 1965) suggests similarity in the acidities of the 2′- and 3′-hydroxyl groups. (3) Indirectly, the distribution of products resulting from the alkaline hydrolysis of adenosine 2′,3′-cyclic phosphate gives the same information. Since the ratio of 3′-phosphate to 2′-phosphate is 60:40 (Dekker and Khorana, 1954), it suggests that the conjugate bases of the 2′- and 3′-hydroxyl groups are of roughly equivalent strength with the former being slightly the weaker. (4) Loss of either the 2′- or 3′-hydroxyl group causes a dramatic decrease in acidity of adenosine (Izatt *et al.*, 1965). More direct evidence is obtained from ion-exchange chromatography of adenosine and its methylated derivatives on Dowex 1-2X (OH^-) columns where elution volumes can be correlated with the acidity of the nucleoside (Dekker, 1965; Gin and Dekker, 1967). From Figure 4 it can be seen that methylation of the 2′- and/or 3′-oxygen of adenosine leads to a dramatic decrease in elution volume while methylation of the 5′-oxygen has a rather minor effect. This indicates that the 2′,3′-*cis*-glycol group is responsible for the pK_a of 12.5. Furthermore the elution of 2′-*O*-methyladenosine just prior to 3′-*O*-methyladenosine supports the earlier evidence that the 2′-hydroxyl group is slightly more acidic than the 3′-hydroxyl group. This is not unexpected since the additional inductive effect of the aglycone is operative at the 2′ position.

Because of the high and roughly similar acidity of the 2′- and 3′-hydroxyls it was not surprising to find that both groups reacted with diazomethane under the conditions of Broom and Robins (1965). The somewhat higher yield of the 2′- than of the 3′-*O*-methyladenosine (ratio $\sim$ 3:1) again supports the assignment of a lower pK_a to the 2′-hydroxyl group. The very low yield of 5′-*O*-methyladenosine (1.5%) indicates that the 5′-hydroxyl is a considerably weaker acid than either the 2′- or 3′-hydroxyl group. However, since the molar ratio of water to adenosine in the diazomethane reaction mixture was 600:1, a 1.5% yield is significant and suggests that the 5′-hydroxyl of adenosine is a slightly stronger acid than water (probable pK_a = 15.0–15.5). By the same reasoning one can conclude that the remaining secondary hydroxyl group of adenosine, after methylation of either the 2′- or 3′-oxygen, is a stronger acid than the primary, *i.e.*, the 5′-hydroxyl group. This follows from the observed yield of 3.5% 2′,3′-di-*O*-methyladenosine compared with 1.5% 5′-*O*-methyladenosine.

Some N-methylated products were formed under the conditions described. However, since 41% unreacted adenosine was recovered, these were minor components and could not represent more than 5% of the total products. No single N-methylated component was present in greater than 1% yield with the possible exception of $O^{2'}$,N^6-dimethyladenosine which appeared to be a major component of the second large peak in the dimethyladenosine fraction (Figure 2). Because of the small quantity available, absolute identification of this substance was not attempted. The presence of numerous minor, unidentified components complicated the purification of 2′,3′-di-*O*-methyladenosine which is, itself, formed in very low yield.

It is frequently assumed that 2′-*O*-methyl nucleosides are chemically similar to the corresponding 2′-deoxynucleosides. Determination of the rates of hydrolysis in mild acid showed that for this reaction the assumption is false. In Table I it can be seen that the glycosyl bond of 2′-*O*-methyladenosine is more stable than that of adenosine, whereas the glycosyl bond of 2′-deoxyadenosine is markedly less stable. The latter observation can be explained in part by the strong inductive effect of the 2′-hydroxyl group (relative to a 2′-hydrogen) which hinders protonation of the ring oxygen, an obligatory step for hydrolysis. The rather small changes in rate associated with the O methylation of adenosine are consistent with the rough similarity in electron-withdrawing power of methoxyl and hydroxyl groups. Whether the decrease in rate observed on methylation is due to conformational changes in the sugar ring resulting from the more bulky methoxyl group, to solvation effects, or to other factors will only be established when more extensive kinetic studies have been made on a broader range of O-methylated nucleosides. It can only be noted at this time that there appears to be a correlation between the acidity of a hydroxyl group and the decrease in rate of hydrolysis resulting from its methylation, *i.e.*, the more acidic the hydroxyl, the greater the decrease in rate of glycosyl-bond hydrolysis when it is replaced by methoxyl. The greater stabilizing effect of a 2′ substituent than of a 3′ substituent has also been noted by Furukawa *et al.* (1965) and Martin *et al.* (1968).

The results of the orcinol test on 2′-*O*-methyladenosine emphasize the need for extended heating times if maximum color is to be realized from 2′-O-methylated nucleosides or nucleotides. The failure of 2′-*O*-methyl nucleosides to react appreciably in 20 min has already been noted by Singh and Lane (1964a). The conflicting report by Correll (1964) that 2(3)-*O*-methylribose and 2,3-di-*O*-methylribose gave complete reaction with orcinol in 40 min is not supported by the present data. Even after heating for 160 min the color yield of 2′-*O*-methyladenosine is only 75% that of adenosine. No further increase is possible because of concurrent degradative reaction. In contrast to the slow rate of color development observed for 2′-*O*-methyladenosine, 3′-*O*-methyladenosine reacts at the same rate as adenosine and gives an identical color yield at both 20- and 40-min heating time. Furthermore, 5′-*O*-methyladenosine reacts faster than adenosine and at 20 min gives 125% the color of adenosine. With longer heating periods, the difference in color yield diminishes. These results are reminiscent of those obtained with adenosine phosphates (Albaum and Umbreit, 1947), for which it was

found that orcinol color development is more rapid with adenosine 5′-phosphate than with adenosine 3′-phosphate or adenosine. The significance of the results in the latter case is clouded by the possibility of phosphate migration. Nevertheless, the results with the *O*-methyl derivatives and with the *O*-phosphate derivatives support a mechanism which involves: (1) initial cleavage of the glycosyl bond, (2) subsequent attack of the 2′-oxygen at C-5′ with displacement of the protonated phosphate or protonated methoxyl group to give a five-membered ring, and (3) dehydration in the strong acid milieu to give furfural which in turn reacts with orcinol to give the green color. The ease of conversion into furfural of compounds with 2,5-anhydro rings is well known (Cifonelli *et al.*, 1955; Ekenstein and Blanksma, 1910). Such a mechanism would explain the slow reaction of 2′-O-methylated derivatives which have to be demethylated to acquire the essential free 2′-hydroxyl group. Moreover, since it will be easier to protonate the 5′-methyl ether of 5′-*O*-methyladenosine than the 5′-hydroxyl group of adenosine, the more rapid reaction of the former compound is explained. Singh and Lane (1964a) have likewise proposed that the 2′-*O*-methyl group interferes with furfural formation in the case of 2′-*O*-methyladenosine. They have also presented similar arguments to the above to explain the color yields of a series of dinucleoside phosphates including AxpA (Singh and Lane, 1964b). In view of the ready loss of a 3′-phosphate group under the conditions of the orcinol test (Albaum and Umbreit, 1947), it was to be expected that AxpAp would give a similar color yield to that observed for AxpA.[3] The comparable reaction of 3′-*O*-methyladenosine and adenosine is probably due to the facile loss in strong acid of the 3′-*O*-methyl group of the former compound by a β-elimination process either before or after 2,5-anhydro ring closure. The very slow rate of color development of 2′,3′-di-*O*-methyladenosine may be related to the decreased probability of either methoxyl being protonated due to the presence of a neighboring group of similar basicity.

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[3] In comparing our result on AxpAp to that of Singh and Lane (1946b) on AxpA, it should be noted that the color value in the latter reference is expressed as moles of ribose per mole of nucleoside residue rather than moles of ribose per mole of nucleoside diphosphate, as mistakenly stated (private communication from Dr. Lane).

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Purification and Properties of Inosine Monophosphate: Pyrophosphate Phosphoribosyltransferase (EC 2.4.2.8) from Brewers Yeast*

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ABSTRACT: Inosine monophosphate (IMP):pyrophosphate phosphoribosyltransferase from brewers yeast has been purified 234-fold over the original high-speed supernatant by heat denaturation, ammonium sulfate fractionation, DEAE-cellulose chromatography, and hydroxylapatite chromatography. IMP synthesis parallels guanosine monophosphate (GMP) synthesis over the entire purification range. Considerable denaturation of the enzyme occurs below pH 5.2 and above pH 10.

A broad pH optimum for activity with guanine between pH 7.0 and 8.0 and for hypoxanthine activity between pH 8.0 and 9.0 was observed. Both activities require the presence of a divalent metal ion. Optimal Mg^{2+} concentration for both activities is 1×10^{-3} M. The K_m values at pH 7.4 and 25° are 7.7×10^{-6} M for guanine, 1.8×10^{-5} M for hypoxanthine, 2.4×10^{-5} M for 5-phosphorylribose 1-pyrophosphate (PRPP) in the presence of guanine, and 4.2×10^{-5} M for PRPP in the presence of hypoxanthine. The apparent activation energies, determined by an Arrhenius plot, are 11,600 cal/mole for hypoxanthine as substrate and 5700 cal/mole below 19° and 11,300 cal/mole above 19° for guanine as substrate. Evidence is presented which supports the concept of a single enzyme catalyzing both IMP and GMP synthesis.

The direct conversion of hypoxanthine into IMP[1] was first recognized by Williams and Buchanan (1953). Pigeon liver extracts were demonstrated to be capable of forming IMP in a reaction involving hypoxanthine, ribose 5-phosphate, and ATP without the participation of inosine as an intermediate. It was later shown (Kornberg *et al.*, 1954) that ribose 5-phosphate was phosphorylated by ATP to form PRPP which reacted with hypoxanthine to form IMP. This general class of enzymes, the purine nucleotide pyrophosphorylases, was first described by Kornberg *et al.* (1955) who showed that an extract of dried brewers yeast contained purine nucleotide pyrophosphorylase activity with adenine, hypoxanthine, and guanine as substrates.

An enzyme has recently been extensively purified from Ehrlich ascites tumor cells (Hori and Henderson, 1966) which catalyzed the condensation of adenine and PRPP to form AMP. Another pyrophosphorylase system has been partially purified from beef liver (Korn *et al.*, 1955), red blood cells (Preiss and Handler, 1957), Ehrlich ascites tumor cells (Atkinson and Murray, 1965), and brewers yeast (Kornberg *et al.*, 1955). This enzyme system catalyzes a condensation reaction of hypoxanthine or guanine with PRPP to form IMP or GMP. A partial separation of the hypoxanthine activity from the guanine activity in the above-mentioned tumor system has been achieved by the use of electrophoresis. Littlefield (1964) has reported different cell lines derived from a single strain in which the ratio of IMP to GMP pyrophosphorylase activity is altered. Adye and Gots (1966) using *Salmonella typhimurium* obtained a variety of altered forms of the enzyme from mutant strains of this organism. In some cases partial loss of the hypoxanthine activity with retention of all the guanine activity was observed while in other cases partial loss of the guanine activity and retention of all of the hypoxanthine activity was reported.

The still-unresolved problem of the number of specific purine nucleotide pyrophosphorylases which exist and the related question of the specificity of purine nucleotide pyrophosphorylases with respect to purine sub-

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[1] The abbreviation used in this paper that is not defined in *Biochemistry 5*, 1445 (1966), is: PRPP, 5-phosphorylribose 1-pyrophosphate.