

The effects observed when the zinc-insulin complex is titrated are complex. The depression of the ionization has both a kinetic and an equilibrium aspect. For example, at pH 11, a total of one less tyrosine ionizes *initially* in the zinc-insulin sample as compared to the zinc-free sample. In all likelihood this is a reflection that more than one particular tyrosine residue is affected. Based on our knowledge of the X-ray crystal structure of the zinc-insulin complex, residues A-14, B-16, and B-26 could all be affected to varying degrees. After the time-dependent ionization has occurred, a decrease in the total amount of tyrosine ionized in the zinc vs. zinc-free system is found (see Figure 6). The data from the centrifuge studies indicate that some complex still remains in zinc-insulin solutions at basic pH. This decrease in the number of tyrosines ionized at equilibrium would, therefore, be expected. The tyrosine affected is probably tyrosine B-26, although some effect on B-16 and/or A-14 cannot be excluded.

Other investigators (Morris *et al.*, 1970) have attempted to gain information on the state of the tyrosines in insulin by chemically modifying specific tyrosines and subsequently determining the effect on the titration curve of the modified insulin. The present study, however, demonstrates the need to determine first the effect of chemical modification on insulin self-association and interaction with metal ion before attempting to gain information on the state of the tyrosine residues in the molecule.

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A Novel Procedure for the Synthesis of 2'-O-Alkyl Nucleotides[†]

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ABSTRACT: A novel method is presented for the synthesis of 2'-O-alkyl 3'- (or 5'-) nucleotides with a 3':5'-cyclic nucleotide as starting material. This material is methylated or ethylated at the 2'-OH position using an alkyl iodide at alkaline pH. The 2'-O-alkylated cyclic phosphate is cleaved with a 3':5'-cyclic phosphodiesterase or by chemical procedures to yield the 2'-O-alkyl 3'(5')-nucleotide. The 2'-O-

alkyl 5'-nucleotide is then chemically phosphorylated to become the 2'-O-alkyl nucleoside 5'-diphosphate. These compounds are substrates for polynucleotide phosphorylase in the synthesis of polynucleotides. Substantial quantities of poly(2'-O-methyladenylic acid), poly(2'-O-ethyladenylic acid), poly(2'-O-methylinosinic acid), and poly(2'-O-methylcytidylic acid) were prepared by this procedure.

Various 2'-O-methyl nucleotides have been found in tRNAs (Smith and Dunn, 1959; Hall, 1964, 1971; Zachau, 1969) and rRNAs (Hall, 1964, 1971; Brown and Attardi, 1965; Nichols and Lane, 1966) from many sources. Since these com-

pounds can be regarded as analogs of both ribonucleo- and deoxyribonucleotides, various homopolymers (Rottman and Heinlein, 1968; Janion *et al.*, 1970; Zmudzka and Shugar, 1970; Tazawa *et al.*, 1971) and copolymers (Rottman and Johnson, 1969; Simuth *et al.*, 1971) containing 2'-O-methyl nucleotides have been prepared and their physicochemical (Zmudzka and Shugar, 1970; Bobst *et al.*, 1969b; Zmudzka *et al.*, 1969; Alderfer *et al.*, 1971, 1972) and biochemical properties (Dunlap *et al.*, 1971; Gerard *et al.*, 1972) have been studied. In these cases, the 2'-O methyl nucleosides,

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TABLE I: Ultraviolet Maxima and Minima of Nucleosides and Nucleotides.

Compound	pH	λ_{\max} (nm)	λ_{\min} (nm)	Ref
pA	7	259	227	<i>a</i>
pAm	7 (H ₂ O)	259	228	<i>b</i>
ppAm	7 (H ₂ O)	259	228	<i>b</i>
(p<Am spectra are essentially identical with above)				
ppAe	7 (H ₂ O)	259	228	<i>b</i>
(p<Ae and pAe are essentially identical with above)				
N ⁶ -Methyladenosine	7 (H ₂ O)	265	229	<i>c</i>
pI	6	248.5	223	<i>a</i>
pIm	7 (H ₂ O)	248.5	223	<i>b</i>
ppIm	7 (H ₂ O)	248.5	222.5	<i>b</i>
(p<Im is essentially identical with above)				
Im	1	248	<i>g</i>	<i>d</i>
C	7	229.5	226.0	<i>e</i>
		271.0	250.5	
pC	7	272	249	<i>a</i>
pCm	7	228	225	<i>b</i>
		272	250	
ppCm	9	271	250	<i>b</i>
(p<Cm is essentially identical with above)				
N ⁴ -Methylcytidine	7	237	227	<i>f</i>
		271	250	

^a Anonymous (1969). ^b This work. ^c Jones and Robins (1963). ^d Broom and Robbins (1965). ^e Fox and Shugar (1952). ^f Szer and Shugar (1966). ^g Not reported.

were prepared either by specific methylation of unprotected nucleosides using diazomethane (Broom and Robins, 1965; Khwaja and Robins, 1966; Gin and Dekker, 1968; Martin *et al.*, 1968; Robins and Naik, 1971), or by methylation of 3':5'-protected nucleosides using methyl iodide in Purdie's method (Furukawa *et al.*, 1965). Another method for alkylation of cytosine ribonucleosides, which yields various sugar alkylated derivatives, employs the use of dialkyl sulfates in alkaline medium (Kusmierik and Shugar, 1971).

In this study, a nucleoside 3':5'-cyclic phosphate was used as the 3':5'-protected "nucleoside" derivative. When an alkyl iodide was added under alkaline conditions, the desired 2'-O-alkyl derivative was obtained together with some dialkyl derivative. The p<Am¹ or p<Ae thus obtained might exhibit interesting biological properties, since they are analogs of p<A, a compound which plays important roles in many biological processes (Robinson *et al.*, 1968; Jost and Rickenberg, 1971). In addition, cleavage of these 2'-O-alkyl nucleoside 3':5'-cyclic phosphates yielded 2'-O-alkyl nucleoside 5'-phosphates which were phosphorylated to the 2'-O-alkyl nucleoside 5'-diphosphates. These compounds are substrates for polynucleotide phosphorylase in the synthesis of polynucleotides. Substantial quantities of poly(2'-O-methyladenylic acid), poly(2'-O-ethyladenylic

¹ Abbreviations used are: A, adenosine; pA, adenosine 5'-phosphate; Ap, adenosine 3'-phosphate; p<A, adenosine 3':5'-cyclic phosphate; p<Am, 2'-O-methyladenosine 3':5'-cyclic phosphate; p<Ae, 2'-O-ethyladenosine 3':5'-cyclic phosphate; p<eAe, 2'-O-ethyl-N⁶-ethyladenosine 3':5'-cyclic phosphate; ppA, adenosine 5'-diphosphate; I, inosine; C, cytidine.

acid), poly(2'-O-methylinosinic acid), and poly(2'-O-methylcytidylic acid) were prepared by this enzymic procedure.

Experimental Section

Materials and Methods. The following compounds were purchased from Sigma Chemical Co., St. Louis, Mo.: adenosine 3':5'-cyclic phosphate, cytidine 3':5'-cyclic phosphate, and 3':5'-cyclic nucleotide phosphodiesterase (from beef heart, Product P0134). *Escherichia coli* alkaline phosphatase (code BAPC) was purchased from Worthington Biochemical Corp., Freehold, N. J. *Micrococcus luteus* polynucleotide phosphorylase (EC 2.6.6.8) (lot 0302) was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. The maximum molar extinction coefficient adopted for p<A, p<Am, and p<Ae is 14,650; for pAm, pAe, ppAm, and ppAe, 15,400; for p<Im, pIm, and ppIm, 12,200; and for p<C, p<Cm, pCm, Cmp, and ppCm, 9150. The phosphorus analysis procedure used for determination of the polymer extinction coefficients was that of Baginski *et al.* (1967).

Instrumentation. Ultraviolet absorption spectra were recorded by a Cary 15 spectrophotometer at room temperature (24°). Varian HA-100 and HR-220² spectrometers were used for the proton magnetic resonance (pmr) spectra. The spectra were obtained from these instruments at 30 and 20°, respectively. A Varian C-1024 computer of average transients was used with the HA-100 to enhance the signal-to-noise ratio.

Paper Chromatography and Electrophoresis. Descending paper chromatography was performed with Whatman No. 1 or 3MM paper in the following solvent systems: (A) 2-propanol-concentrated ammonium hydroxide-H₂O (7:1:2, v/v), (A') 2-propanol-concentrated ammonium hydroxide-H₂O (8:1:1, v/v), (B) 1-propanol-concentrated ammonium hydroxide-H₂O (55:10:35, v/v), and (C) ethanol-1 M ammonium acetate, pH 5, containing 3.3 mM EDTA (7:3, v/v). Ascending thin-layer chromatography (tlc) was carried out on Eastman cellulose sheets No. 6065, using solvent system D: saturated aqueous ammonium sulfate-1 M sodium acetate-2-propanol (80:18:2, v/v). Paper electrophoresis was performed on a Savant flat-plate apparatus with Whatman No. 3MM paper in 0.05 M triethylammonium bicarbonate (pH 7.5) buffer for 1 hr at a potential of 20 V/cm.

Preparation of 2'-O-Methyladenosine 3':5'-Cyclic Phosphate. Adenosine 3':5'-cyclic phosphate (100 mg) was dissolved in 4 ml of 4 N sodium hydroxide. Addition of dimethylformamide (10 ml) yielded a homogeneous solution to which methyl iodide (2 ml) was added with stirring. A white precipitate formed and the pH dropped to ~7 in a few minutes. The reaction mixture was then diluted with H₂O (1-2 l.) and treated with charcoal (5 g). After removing the liquid, the compounds were eluted from the charcoal with 2% ammonium hydroxide in 50% ethanol. The concentrated eluate was chromatographed in solvent A and gave three uv absorbing bands. The middle band (*R_F* 0.43) was p<Am and was obtained in 50% yield. Its electrophoretic mobility relative to p<A (*R_m*^{p<A}) is 0.98. The ultraviolet (uv), pmr, and chromatographic properties of this compound are listed in Tables I-IV.

Preparation of 2'-O-Methyladenosine 5'-Phosphate. HYDROLYSIS OF 2'-O-METHYLADENOSINE 3':5'-CYCLIC PHOSPHATE.

² Experiments with the 220-MHz instrument were performed at the NMR Regional Facilities Center at the University of Pennsylvania established by N.I.H. Research Grant No. 1 PO7 RR-00542-01 from the Division of Research Facilities and Resources.

TABLE II: Proton Magnetic Resonance Data of Nucleosides and Nucleotides.^a

Compound	Concn (M)	pD	H-8	H-2	H-6	H-5	H-1'	$J_{H-1',2'}$	H-5'-5''	CH ₃ -	Ref
5'-AmMP	0.005	7.2	9.06	8.74			6.66	6.1	4.49	3.92	<i>e</i>
	0.02	8-9 ^b	9.11	8.74			6.66	5.5	<i>c</i>	3.92	<i>f</i>
5'-AeDP	0.014	7.1	9.00	8.72			6.66	5.6	4.65	4.13, 1.57 ^d	<i>e</i>
5'-AMP	0.01	5.9	8.95	8.72			6.60	6.1	4.60		<i>g</i>
Adenosine	0	5.9	8.82	8.73			6.53	6.1	4.35		<i>i</i>
2'-O-Methyladenosine	0	5.9	8.76	8.69			6.58	5.3	<i>c</i>	3.86	<i>h</i>
N ⁶ -Methyladenosine	0	5.9	8.73	8.73			6.49	<i>c</i>	<i>c</i>	3.55	<i>h</i>
5'-ImMP	0.02	6.0	8.94	8.67			6.67	5.6	4.57	3.93	<i>e</i>
5'-IMP	0	6.0	8.92	8.68			6.60	5.6	4.60		<i>g</i>
Inosine	0.09	5.9	8.76	8.65			6.54	5.6	4.37		<i>h</i>
5'-CmMP	0.017	6.0			8.50	6.60	6.51	3.9	4.53	4.00	<i>e</i>
5'-CMP	0.02	5.8			8.47	6.59	6.45	3.7	4.57		<i>g</i>
Cytidine	0.09	5.9			8.29	6.51	6.37	3.7	4.33		<i>h</i>

^a Chemical shifts are in ppm from Me₃Si capillary and coupling constants (*J*) are in Hz; measured at 30°. ^b Temperature 37°. ^c This value not reported. ^d -CH₂- and -CH₃ resonances of the ethyl group. ^e This work. ^f Bobst *et al.* (1969a). ^g J. L. Alderfer and P. O. P. Ts'o, unpublished results. ^h Ts'o *et al.* (1969).

Enzymatic Hydrolysis. p<Am (0.7 mmol) in 100 ml of 0.04 M Tris-Cl (pH 7.5) and 0.002 M MgCl₂ was incubated with 3':5'-cyclic nucleotide phosphodiesterase (5 units) at 37° for 15 hr. This reaction produced pAm in 88% yield following paper chromatography in solvent A. This compound had an R_{m}^{pA} of 1.0, and the uv, pmr, and chromatographic properties are listed for pAm in Tables I-IV.

Chemical Hydrolysis. p<Am (0.2 μmol) was dissolved in 0.1 ml of 0.2 M Ba(OH)₂ in a sealed tube and was heated to 100° for 1 hr. The hydrolysate was chromatographed in solvent B, a procedure which separated unreacted cyclic phosphate from a mixture of pAm and Amp. The mixture was rechromatographed (tlc) with solvent D. This produced two well-resolved spots (see Table III), one spot having the properties listed for pAm in Tables I, II, and IV.

PHOSPHORYLATION OF 2'-O-METHYLADENOSINE. Authentic 2'-O-methyladenosine (1.7 mmol) was phosphorylated with phosphorus oxychloride according to published procedures (Yoshikawa *et al.*, 1967; Yamazaki *et al.*, 1968). The reaction mixture was added to ice-cold 0.1 N sodium hydroxide (200 ml) with stirring and then acidified with 0.1 N HCl to pH 4.³ After charcoal treatment, as described in the preparation of p<Am, the eluate was chromatographed in a DEAE-cellulose column (3.5 × 57 cm, HCO₃⁻) by eluting with triethylammonium bicarbonate (linear gradient, 0-0.2 M, 4-l. total). This produced pAm (R_{m}^{pA} 1.07) and p<Am (R_{m}^{pA} 0.47) in yields of 26 and 45%, respectively, with the chromatographic properties listed in Table III. The p<Am presumably originates from the alkaline conditions used in the work-up procedure.

Preparation of 2'-O-Methylinosine 5'-Phosphate. FROM DEAMINATION OF pAm. pAm (0.134 mmol) was added to 2 N acetic acid (3.5 ml) and 4 N sodium nitrite (2 ml). After 8 hr at room temperature the reaction mixture was evaporated to

dryness, redissolved in 0.4 l. of H₂O, and applied to a DEAE-cellulose column (2.5 × 39 cm, HCO₃⁻). Elution with a linear gradient of ammonium bicarbonate (0-0.3 M, 4-l. total) gave pIm in 92% yield with the properties listed in Tables I, II, and IV.

FROM 2'-O-METHYLINOSINE 3':5'-CYCLIC PHOSPHATE. The starting compound, p<Im, was obtained by deamination of p<Am in a procedure similar to that described in the above paragraph. p<Im (0.17 mmol) was added to 3':5'-cyclic nucleotide phosphodiesterase (5 units) in 100 ml of 0.04 M Tris-Cl and 2 mM MgCl₂ (pH 7.5). After incubation at 37° for 20 hr the hydrolysate was charcoal treated as described for the preparation of p<Am. The eluate from charcoal was

TABLE III: R_F Values of 2'-O-Methyladenosine Phosphates and 2'-O-Methylinosine Phosphates from Various Sources on Thin-Layer Chromatography (Solvent D).

Source of Compound	Adenosine Derivative R_F	Inosine Derivative R_F
Monophosphate obtained by enzymic hydrolysis of methylated cyclic phosphate	0.33	0.61
Monophosphate obtained by phosphorylation of methylated nucleoside	0.33	0.60
Monophosphate mixture obtained from Ba(OH) ₂ hydrolysis of methylated cyclic nucleotide	0.32 and 0.20 ^a	0.61 and 0.54 ^a
5'-Ribonucleotide (authentic sample)	0.35	0.64
3'-Ribonucleotide (authentic sample)	0.15	0.54

^a Compound with the lower R_F value was the major product.

³ A recent personal communication from Dr. B. C. Pal, Oak Ridge National Laboratory, indicated a yield of at least 80% could be achieved in carrying out this phosphorylation reaction (using the same ratio of reactants) simply by pouring the reaction mixture (at 4°) on the triethylammonium bicarbonate solution (6 mol at 4°) instead of 0.1 N NaOH.

TABLE IV: R_F Values of Nucleosides and Nucleotides in Paper Chromatography.

Compound	Solvents		
	A	B	C
A	0.51	0.64	
Am		0.73	
Ae		0.79	
p<A	0.35	0.59	
p<Am	0.43	0.66	
p<Ae	0.57	0.70	
pA	0.06	0.35	0.20
pAm	0.10	0.49	0.30
pAe	0.18	0.57	0.43
ppA			0.09
ppAm			0.14
ppAe			0.20
I		0.56	0.66
Im		0.68	
p<I		0.50	
p<Im		0.55	
pI		0.28	0.41
pIm		0.36	0.56
ppI			0.30
ppIm		0.34	0.34
C	0.45		0.55
Cm	0.56		
p<C	0.31		
p<Cm	0.41		
pC	0.04		0.19
pCm	0.08		0.35
Cmp	0.10		
ppC			0.05
ppCm			0.13

purified by preparative paper chromatography using solvent B. The properties of this compound, pIm, are listed in Tables I, II, and IV.

Preparation of 2'-O-Methylcytidine 3':5'-Cyclic Phosphate. This compound was prepared from cytidine 3':5'-cyclic phosphate by the same general procedure used for preparation of p<Am from p<A. In this case, the overall scale was reduced one-half (50 mg of p<C) and the relative amount of methyl iodide was reduced one-half (0.5 ml of CH₃I). The purified compound was obtained by preparative paper chromatography using solvent A' in 40% yield. The properties of p<Cm are listed in Tables I, II, and IV.

Preparation of 2'-O-Methylcytidine 5'-Phosphate and 2'-O-Methylcytidine 3'-Phosphate. p<Cm (0.69 mmol) was dissolved in 1 N HCl (35 ml) in a sealed tube, and was heated at 100° for 2 hr. The hydrolysate was diluted with H₂O (3 l.), adjusted to pH 8 with ammonium hydroxide, and applied to a DEAE-cellulose column (3 × 43 cm, HCO₃⁻). Linear gradient elution with ammonium bicarbonate (0–0.15 M, 4-l. total) gave a mixture of pCm and Cmp which was desalted by repeated evaporation of water. These isomers were separated on a Dowex 1-X8 column (2.3 × 34 cm, HCO₃⁻, 200–400 mesh) eluted with 0.01 M formic acid. After removing the formic acid by the charcoal procedure as described for p<Am these compounds were obtained in an 8.3% yield for

pCm and 60% yield for Cmp. Their properties are listed in Tables I, II, and IV. The pCm (0.082 mmol) fraction in 2 ml of H₂O was further purified to eliminate any possible contamination from non-2'-O-methylated compounds by treatment with 0.1 M sodium periodate (1 ml) at 25° (3 hr). Lysine (1 M; 1 ml) was added to the mixture and incubated at 37° (27 hr), then 1 M ethylene glycol (0.2 ml) was added; after 1 hr (25°) the mixture was diluted with eight volumes of H₂O and applied to a DEAE-cellulose column (2 × 54 cm, HCO₃⁻). pCm was recovered by linear gradient elution with ammonium bicarbonate (0–0.3 M, 2.4-l. total). The amount of any possible contaminants of N-methyl compounds was determined to be less than 0.5% by careful pmr analysis.

Preparation of 2'-O-Ethyladenosine 5'-Phosphate. pAe was prepared in identical fashion as p<Am except that ethyl iodide was used instead of methyl iodide. Also, after 1.5-hr reaction time, 8 ml of reaction mixture solvent (4 N sodium hydroxide–dimethylformamide–ethyl iodide, 2:5:1, v/v) was added six times at 1-hr intervals. The reaction mixture was then diluted with H₂O (2 l.) and treated with charcoal as previously described for p<Am. The eluate from several similar preparations (~0.8 mmol) was incubated with 3':5'-cyclic nucleotide phosphodiesterase (5 units) at 37° for 15 hr in 0.04 M Tris-Cl (pH 7.5) and 2 mM MgCl₂ (100 ml). After charcoal treatment the mixture was reacted with sodium periodate, lysine, and ethylene glycol as previously described for preparation of pCm. The nucleotide material was reabsorbed to charcoal. Following removal from charcoal, the material was applied to a DEAE-cellulose column (2.5 × 70 cm, HCO₃⁻) and eluted with ammonium bicarbonate (linear gradient, 0–0.2 M, 6-l. total). This yielded a mixture of pAe and pe⁶Ae which was desalted, then applied to a cellulose column (2.5 × 88 cm, solvent A), and eluted with solvent A. This gave pure pAe in 62% yield (starting from p<A) with the properties listed in Tables I, II, and IV.

Preparation of 5'-Diphosphates of 2'-O-Alkyl Nucleotides. Diphosphates of the 2'-O-alkyl 5'-nucleotides were synthesized by the morpholidate procedure of Mollatt and Khorana (1961).

Preparation of 2'-O-Alkyl Nucleosides. The alkyl nucleosides were obtained by treating alkyl nucleotides (1 μmol) with alkaline phosphatase (0.05 mg) in 0.1 M ammonium bicarbonate (0.5 ml) at 37° (0.5 hr). The reaction mixture was then applied to paper and chromatographed in an appropriate solvent for the determination of R_F values.

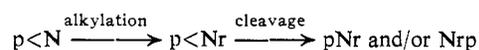
Preparation of 2'-O-Alkyl Polynucleotides. POLY(2'-O-METHYLADENYLIC ACID) [r(Am)_n]. The unprimed polymerization of ppAm with polynucleotide phosphorylase was conducted as described by Rottman and Heinlein (1968). The reaction mixture (5 ml) contained: ppAm (25 mM), Tris-Cl (pH 9.0, 0.1 M), MgCl₂ (5 mM), EDTA (0.4 mM), NaN₃ (1 mM), bovine serum albumin (0.1 mg/ml), and polynucleotide phosphorylase (2.4 mg/ml). After 3 days (37°) the reaction mixture was heated for 2 min (100°), then deproteinized by shaking with a solution of chloroform–isoamyl alcohol (1:1, v/v). The aqueous layer was applied to a Sephadex G-50 column which yielded polymer and substrate fractions. An attempt was made to further deproteinize the early eluted polymer fractions with phenol, and it was found that the polymer was more soluble in the phenol layer. The phenol phase containing the polymer was then successively dialyzed against the following solutions (2 l. each): (1) 0.2 M NaCl–0.02 M sodium phosphate (pH 7.4)–0.001 M EDTA, (2) 0.1 M NaCl–0.01 M sodium phosphate (pH 7.0)–0.001 M EDTA, (3) 0.01 M NaCl–0.01 M sodium phosphate (pH 7.0), and (4 and 5) H₂O. The dialyzed

solution was lyophilized, redissolved in 0.1% sodium dodecyl sulfate (5 ml), and extracted twice with 10 ml of chloroform-isoamyl alcohol mixture (3:1, v/v). The polymer was precipitated by addition of two volumes of cold isopropyl alcohol to afford a yield of about 10% (130 ODU) relative to starting substrate. The uv properties of the polymer in H₂O are λ_{\max} 256 nm, λ_{\min} 230 nm, and A_{\max}/A_{\min} 3.76. This polymer has an average $s_{20,w}$ value of 4.7 S.

POLY(2'-O-ETHYLADENYLIC ACID) [r(Ae)_n]. The reaction mixture (20 ml) for the synthesis of r(Ae)_n contained: ppAe (7 mM), Tris-Cl (pH 8.5, 0.15 M), MnSO₄ (0.01 M), EDTA (0.4 mM), NaN₃ (1 mM), and polynucleotide phosphorylase (2 mg/ml). After incubation (37°) for 120 hr, 5% sodium dodecyl sulfate solution (2 ml), 10% aqueous phenol (2 ml), and then crystalline phenol (4 g) was added with thorough mixing of the reaction mixture. Following centrifugation, the aqueous phase of this mixture was treated three times by this procedure; the polymer was found to be extracted completely from the aqueous layer as determined by paper chromatography. The phenol layer was then dialyzed at 4° extensively against solutions of NaCl, EDTA, and against H₂O. A precipitate formed during dialysis, which presumably was denatured protein, and was removed by filtration after dialysis. The dialyzed solution was extracted with neutralized phenol (pH 7.5) to assure adequate deproteinization. Under this condition, the polymer was more soluble in the aqueous phase. The aqueous phase was again extensively dialyzed as described previously. The polymer in solution after dialysis was obtained in 29% yield (about 400 ODU) relative to starting substrate and had the following properties in H₂O (pH 7.5): λ_{\max} 256 nm, λ_{\min} 232, A_{\max}/A_{\min} 2.48. The polymer has an average $s_{20,w}$ value of 9.0 S.

POLY(2'-O-METHYLINOSINIC ACID) [r(Im)_n]. The reaction mixture (15 ml) for the synthesis of r(Im)_n contained: ppIm (7 mM), Tris-Cl (pH 8.5, 0.15 M), MnSO₄ (0.01 M), Na₂EDTA (0.4 mM), NaN₃ (1 mM), and polynucleotide phosphorylase (2 mg/ml). This solution was incubated (37°) for 48 hr. Water (30 ml) was added to the reaction mixture because of the high viscosity followed by the addition of 5% sodium dodecyl sulfate (4.5 ml) and 1 M sodium citrate (2.5 ml, pH 5.4). This mixture was extracted thrice with 90% phenol (52 ml). After each phenol addition the phases were separated by centrifugation. This resulted in the collection of a white precipitate, which was found to be the desired polynucleotide. Neither the aqueous nor phenol phase contained a substantial amount of polymer as shown by paper chromatography. The precipitate was redissolved in H₂O (40 ml) and extensively dialyzed against solutions of NaCl, EDTA, and H₂O. The yield (400 ODU) of r(Im)_n from this precipitate was 38% of the starting substrate. The uv spectral features in 0.005 M NaOAc (pH 6.0) are λ_{\max} 248 nm, λ_{\min} 223 nm, and A_{\max}/A_{\min} 2.71, and the maximum molar extinction coefficient was determined to be 9910, based on phosphate analysis. The polymer has an average $s_{20,w}$ value of 8 S in 0.005 M NaOAc (pH 6.0).

POLY(2'-O-METHYLCYTIDYLIC ACID) [r(Cm)_n]. This polymer was prepared according to the method of Janion *et al.* (1970). The reaction mixture (8.2 ml) contained: ppCm (7 mM), Tris-Cl (pH 8.5, 0.15 M), MnSO₄ (10 mM), EDTA (0.4 mM), NaN₃ (1 mM), and polynucleotide phosphorylase (2 mg/ml). After incubation (37°) for 50 hr, 5% sodium dodecyl sulfate (0.8 ml) and 10% aqueous phenol (0.8 ml) were added to the reaction mixture. This mixture was extracted three times with 90% phenol (9.8 ml, adjusted to pH 7.5). The aqueous solution was then exhaustively dialyzed against solutions of NaCl, EDTA, and finally water. A polymer solution (210 ODU)

SCHEME I^a

^a N = ribonucleoside; r = alkyl group.

was obtained in about 60% yield relative to starting substrate. The uv properties of r(Cm)_n in 0.01 M Tris-Cl (pH 7.5) are λ_{\max} 268 nm and λ_{\min} 248 nm, and the maximum molar extinction coefficient was determined by phosphorus analysis to be 6200. This polymer has an average $s_{20,w}$ value of 11 S in minimal Eagle's salt solution (0.15 M NaCl-0.01 M sodium phosphate (pH 7.4)-0.001 M MgCl₂).

Results and Discussion

General Methods and Procedures. A generalized two-step procedure describing a new method for synthesizing 2'-O-alkyl nucleotides is illustrated in Scheme I. The applicability of this procedure depends on three factors: (1) the availability of 3':5'-cyclic nucleotides as starting material; (2) the preferential alkylation of the sugar rather than the base moiety; and (3) the ability to cleave the 2'-O-alkyl 3':5'-cyclic nucleotides to give the desired 3'- or 5'-nucleotide (5', in the present case).

Cyclic nucleotides are available commercially or can be synthesized conveniently by activation of 5'-nucleotides (Smith *et al.*, 1961). The selection of alkylating agent and alkylation conditions is dependent in part on one's own preference. Alkylation using an alkyl iodide in sodium hydroxide-dimethylformamide solution was selected because of the ready availability of materials and simplicity in handling reagents. The alkylation proceeded quite rapidly, and generally gave a reaction mixture containing the desired product at about 45-60% of starting material. Another reaction product obtained at about 20-30% of the starting material is the 2'-O-N⁶-dialkyl cyclic nucleotide which was identified by its ultraviolet and chromatographic properties. Cleavage of the 2'-O-alkyl 3':5'-cyclic nucleotides to give 5'-nucleotides is easily accomplished with purine nucleotides using a 3':5'-cyclic nucleotide phosphodiesterase from beef heart. This step occurs in 85-90% yield with the 5'-nucleotide as the exclusive product. In the case of the one alkylated pyrimidine derivative, cytidine, the cyclic nucleotide was completely resistant to the phosphodiesterase at normal reaction conditions. Substituting Mn²⁺ for Mg²⁺ affected hydrolysis of the cyclic phosphate to the 5'-nucleotide only at an extremely slow rate; therefore, acid hydrolysis was used to obtain the 5'-nucleotide. This method is not very satisfactory in obtaining the 5'-nucleotide because the 3' isomer is produced 7- to 8-fold in excess of the 5' isomer. However, the 3'-nucleotide has other utility, for example, as starting material in the synthesis of oligonucleotides (Cramer, 1966; Smith and Khorana, 1959). A cyclic 3':5'-nucleotide phosphodiesterase from heart with specificity for uridine 3':5'-cyclic phosphate has been reported (Hardman and Sutherland, 1965).

Structure Determination. The structural proof of the alkylated nucleotides, starting from known nucleosides or nucleotides, basically requires knowledge of (1) the position of the alkyl group and (2) the location of the phosphate moiety. These structural features were determined by uv and pmr spectroscopy, by comparison of paper chromatographic mobilities to authentic compounds, and by their biochemical properties as substrates for polynucleotide phosphorylase. Tables I and II list the uv and pmr data, respectively, of the

alkylated compounds along with other parent compounds for comparison. For reasons delineated below, the uv and pmr data both indicate that the alkylated nucleotides prepared are in fact 2'-O-alkyl 5'-nucleotides. Alkylation of the sugar moiety does not change the λ_{\max} of the uv spectrum (in fact, the entire spectrum is hardly perturbed), while alkylation of the base unit causes a significant shift in λ_{\max} . The chemical shift (δ) of alkyl (R) protons, as in R-X, is sensitive to the X atom (nitrogen or oxygen) and has a characteristic resonance position (Ts'o *et al.*, 1969). Additional evidence for 2'-O-alkylation comes from the chemical shift of H-1', which has been shown to be sensitive to the substituent cis to it at the C-2' position (Broom *et al.*, 1967). In all the alkylated compounds reported here, the δ (H-1') is displaced downfield relative to nonalkylated compounds. The phosphate position can be determined by pmr from the chemical shift of the protons on the carbon bearing the phosphorylated hydroxyl group. In addition, a phosphate in the 5' position can be specifically detected by its deshielding effect on H-8 of purines and H-6 of pyrimidines. A careful comparison of the pmr data for the alkylated, the nonalkylated and the authentic alkylated compounds listed in Tables I and II completely supports these compounds as 2'-O-alkyl 5'-nucleotides.

Additional structural evidence is obtained by a comparison of the paper chromatographic mobilities of the alkylated compounds to those of authentic samples. Authentic 2'-O-methyladenosine was both deaminated to give Im and phosphorylated by procedures known to give 5'-nucleotides (Yoshikawa *et al.*, 1967; Yamazaki *et al.*, 1968). The similar mobilities of these authentic compounds with the alkylated compounds (Tables II and IV) again indicates they are 2'-O-alkyl 5'-nucleotides.

The biochemical evidence for the 2'-O-alkyl 5'-nucleotides comes from the following facts. (1) When all the alkylated nucleotides were converted to nucleoside diphosphates, these nucleoside diphosphates were found to be substrates of polynucleotide phosphorylase for the synthesis of polynucleotides. (2) Attempts to degrade these polymers by normal conditions of alkaline hydrolysis were completely unsuccessful—a fact anticipated from the known mechanism of alkaline hydrolysis and a 2'-O-alkylated polymer structure. It is quite clear from the combined evidence of uv, pmr, paper chromatography and biochemical data that these alkylated nucleotides are in fact 2'-O-alkyl 5'-nucleotides.

The procedure described in this paper allows synthesis of 2'-O-alkyl 5'-nucleotides from cyclic phosphonucleotides using easily manageable reagents, and provides the alkylated nucleotides in large quantities. The availability of these compounds permits synthesis of the alkylated nucleoside diphosphates, which in turn are polymerized by polynucleotide phosphorylase to give poly(2'-O-alkyl nucleotides). Preparation of four polymers, poly(2'-O-methyladenylic acid), poly(2'-O-methylinosinic acid), poly(2'-O-methylcytidylic acid), and poly(2'-O-ethyladenylic acid) (which has never been prepared before), has been reported in the Experimental Section. These polymers are useful analogs for comparison to the ribose and deoxyribose nucleic acids. Comparative studies of the physicochemical properties of ribosyl, 2'-O-methylribosyl, and deoxyribosyl homopolynucleotides are the subjects of forthcoming papers in this series.

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Subunit Interactions and Ligand Binding in Supernatant Malic Dehydrogenase. Cooperative Binding of Reduced Nicotinamide Adenine Dinucleotide Associated with a Monomer-Dimer Equilibrium of the Protein†

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ABSTRACT: Fluorescence binding studies of reduced nicotinamide adenine dinucleotide (NADH) to S-MDH (supernatant malic dehydrogenase), isolated from beef heart, indicated that the nature of the binding varied with protein concentration. Scatchard plots of NADH titrations showed a curvature convex to the abscissa, characteristic of cooperative binding, at high protein concentrations. With decreasing protein concentration, the Scatchard plots became progressively more linear, approaching the form expected for identical, noninteracting binding sites. The experimental data are consistent with a model assuming a monomer-dimer equilibrium, in which the dimer binds 2 mol of NADH cooperatively. The experimental points were fit by a computer simulation, equating

the dimer to the 8×10^4 molecular weight "native" enzyme, and the monomer to the 4×10^4 molecular weight subunit. Ligand binding constants and the monomer-dimer association constant were evaluated from the theoretical model and the best fit to the experimental points. The molar dissociation constant for the monomer-dimer equilibrium was 2.5×10^{-7} , in reasonably good agreement with a directly measured value of 1×10^{-7} obtained from sedimentation equilibrium studies. The results are discussed in relation to previous studies on this enzyme, and in terms of general allosteric control mechanisms. An appendix is included which presents a theoretical evaluation of Scatchard plots obtained from systems demonstrating cooperative binding.

Previous studies on binding of NADH to the supernatant malic dehydrogenase (S-MDH)¹ had provided evidence for a pH- and NADH-dependent transition of the protein (Cassman, 1970). This transition was of particular interest since it occurred over a pH range where an alteration in the kinetic properties of the enzymes had been previously observed (Cassman and England, 1966).

In order to clarify the molecular events underlying the NADH-dependent effects, a further investigation was initiated into the nature of the NADH binding. The studies reported here indicate that (a) the protein exists in a monomer-dimer equilibrium, (b) NADH binds to both monomer and dimer, and (c) NADH binds cooperatively to the dimer.

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¹ Abbreviations used are S-MDH, supernatant malic dehydrogenase; *F*, fluorescence; $\bar{\nu}$, average mol of NADH bound per mol of enzyme (8×10^4 molecular weight); *n*, total number of NADH binding sites per mol of enzyme; *j*, Hill coefficient; M_w , weight-average molecular weight. All bracketed symbols denote concentrations.

These results are related to previous studies on the enzyme and are also considered in terms of general allosteric control mechanisms.

Materials and Methods

Materials. NADH, oxaloacetate, and D-malate, all of A grade, were obtained from Calbiochem. All other chemicals were standard reagent grade.

Enzyme Purification and Assay. Beef heart supernatant malic dehydrogenase was prepared according to the method of Guha *et al.* (1968). Standard assays were performed in 0.1 M triethanolamine (pH 7.4), using 1×10^{-4} M oxaloacetate and 1×10^{-4} M NADH. Measurements were made at 360 nm, with one unit of enzyme activity defined as a decrease of 0.010 ODU/min. The specific activities were in the range 6.0 – 8.0×10^4 units/min per mg. Protein concentrations were determined by the method of Lowry *et al.* (1951) or from the absorbance at 280 and 260 nm (Layne, 1957). Spectrophotometric measurements were made using a Zeiss PMQ-II spectrophotometer.

Fluorescence Measurements and Calculations. All studies were performed at pH 6.9, in 0.02 M sodium potassium phos-