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# 2′-*O*-($\alpha$-Methoxyethyl)nucleoside 5′-Diphosphates as "Single-Addition" Substrates in the Synthesis of Specific Oligoribonucleotides with Polynucleotide Phosphorylase†

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ABSTRACT: In the presence of adenosine trinucleotide and 2′(3′)-*O*-($\alpha$-methoxyethyl)adenosine 5′-diphosphate, polynucleotide phosphorylase catalyzes the addition of only one nucleotide residue to the oligonucleotide to form adenosine tetranucleotide containing an $\alpha$-methoxyethyl substituent at its terminus. The terminal nucleoside of this product has been removed by alkaline hydrolysis and shown to be 2′-*O*-($\alpha$-methoxyethyl)adenosine. In addition, the mixed 2′- and 3′-*O*-($\alpha$-methoxyethyl)adenosine 5′-diphosphates have been separated and characterized, and the conclusion that the 2′ isomer is the enzymatically active species has been confirmed. The structures of these two substituted nucleoside diphosphates were assigned by comparing the chromatographic and mass spectral properties of the products obtained from them by enzymatic dephosphorylation with the corresponding properties of 2′- and 3′-*O*-($\alpha$-methoxyethyl)adenosine synthesized by separate routes. 2′-*O*-($\alpha$-Methoxyethyl)adenosine was obtained by the reaction of 3′,5′-di-*O*-acetyladenosine with methyl vinyl ether followed by alkaline hydrolysis of the acetyl groups. The 3′ isomer was prepared by the limited reaction of 5′-*O*-acetyladenosine with methyl vinyl ether and also by the partial acid hydrolysis of 2′,3′-di-*O*-($\alpha$-methoxyethyl)adenosine. The mono($\alpha$-methoxyethyl) derivatives of each of the other three common ribonucleoside 5′-diphosphates can also be separated into two species and it is presumed that the enzymatically active species in each case is the corresponding 2′ isomer.

A new approach to the synthesis of polynucleotides of defined sequence has recently been developed in this laboratory (Mackey and Gilham, 1971). The basis of the method lies in the use of a nucleotide polymerizing enzyme with chemically modified substrates where the chemical modification is such that it permits the addition of only one nucleotide at a time to a growing polynucleotide chain. The particular case under study is that of polynucleotide phosphorylase with nucleoside 5′-diphosphates containing an *O*-($\alpha$-methoxyethyl) substituent group. This modification fulfills all the requirements for the realization of such a synthetic method: (i) the group is stable under the conditions of the enzyme reaction, (ii) it permits the addition of one nucleotide to an acceptor oligonucleotide and prevents the addition of a second, (iii) the group is readily removed under chemical conditions that do not affect the structural integrity of the oligonucleotide product, and (iv) after the removal of the blocking group the product is then available for a second single addition, and so on. This paper contains both a detailed description of the study reported in the above preliminary communication and a discussion of the evidence supporting the contention that, in the substrate, 2′(3′)-*O*-($\alpha$-methoxyethyl)nucleoside 5′-diphosphate, it is the 2′ isomer that is the enzymatically active species.

*Reaction of $\alpha$-Methoxyethyladenosine Diphosphates with Adenosine Trinucleotide.* The limited reaction of adenosine 5′-diphosphate with methyl vinyl ether yields a mixture of the disubstituted and monosubstituted derivatives together with unreacted nucleotide and these can be separated readily by paper chromatography. The monosubstituted product, on further chromatography, separates into a faster moving and a slower moving component and these are shown below to be the 2′ isomer (II) and the 3′ isomer (I), respectively (Figure 1). These derivatives have been tested both separately and in combination in addition reactions using adenosine trinucleotide as the acceptor oligonucleotide. The 2′ isomer was found to permit the addition of a single nucleotide to the acceptor molecule to the same extent as a mixture of the 2′ and 3′ isomers, whereas the 3′ isomer alone gave essentially no addition. These reaction mixtures were analyzed on columns of a polystyrene anion exchanger using a solvent system containing 20% ethanol and a linear gradient of chloride ion (Ho and Gilham, 1973). In this system the starting materials

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and the product, $\alpha$-methoxyethyladenosine tetranucleotide (pApApApA-ME, III), are all well resolved.

The tetranucleotide, III, has been isolated and its structure has been determined by alkaline hydrolysis. For this analysis the tetranucleotide was prepared by the reaction of the trinucleotide with a mixture of the $\alpha$-methoxyethyladenosine 5′-diphosphates containing about equal quantities of the 2′ and 3′ isomers. Alkaline hydrolysis of the tetranucleotide followed by ion-exchange chromatography gave the products: $\alpha$-methoxyethyladenosine, adenosine 2′(3′)-phosphate, and adenosine 2′(3′),5′-diphosphate in the ratio of 1.0:2.0:1.0 (Figure 2). In order to determine the structure of the terminal nucleoside of III a larger scale synthesis was performed in which the trinucleotide was converted to III in essentially quantitative yield. The tetranucleotide was hydrolyzed with alkali and the products were separated by paper chromatography. The $\alpha$-methoxyethyladenosine obtained in this way was shown, by the comparison of chromatographic properties, to be identical with the two diastereomers of the 2′ isomer (IVa and IVb). No 3′-*O*-($\alpha$-methoxyethyl)adenosine (VI) could be detected in the hydrolysate.

*Dephosphorylation of $\alpha$-Methoxyethyladenosine Diphosphates.* The structures of the two isomeric nucleoside diphosphates used in the above reactions were determined by dephosphorylating them with alkaline phosphatase and comparing the products with 2′- and 3′-*O*-($\alpha$-methoxyethyl)-adenosine synthesized as described below. The comparisons of the substituted nucleosides were made on the basis of their chromatographic properties and the mass spectra of their trimethylsilyl derivatives. Thus, on dephosphorylation, the diphosphate (II) that had the higher $R_F$ value gave a product identical with 2′-*O*-($\alpha$-methoxyethyl)adenosine (IV), while the isomer with the lower $R_F$ value (I) gave a product identical with 3′-*O*-($\alpha$-methoxyethyl)adenosine (VI).

*Synthesis of 2′- and 3′-O-($\alpha$-Methoxyethyl)adenosine.* The 2′-substituted adenosine (IV) was prepared by the reaction of methyl vinyl ether with 3′,5′-di-*O*-acetyladenosine. The synthesis and the characterization of this diacetate have been reported previously (Brown *et al.*, 1954; Fromageot *et al.*, 1966) and it has been used before for the synthesis of 2′-substituted adenosines (*e.g.*, 2′-*O*-tetrahydropyranyladenosine, Griffin *et al.*, 1968). The acid-catalyzed reaction with methyl vinyl ether was complete within 5 min at 10° and, after removal of the acetyl groups with ammonia, the product could be fractionated, by adsorption chromatography, into two derivatives, which were obtained in equal quantities. The two derivatives have similar uv spectra, proton magnetic resonance (pmr) spectra, mass spectra, and elemental compositions but possess different melting points and molar rotations. On the basis of these observations they are assigned the structures of the two diastereomers of 2′-*O*-($\alpha$-methoxyethyl)adenosine (IVa and IVb).

It was considered desirable to also synthesize the 3′ isomer in order to confirm the structures of the diphosphates, I and II. This isomer was obtained by the limited reaction of 5′-*O*-acetyladenosine with methyl vinyl ether. The reaction conditions were similar to those used for the partial methoxyethylation of adenosine 5′-diphosphate and, after the subsequent removal of the acetyl group with ammonia, the products were separated by paper chromatography. Two bands of products were obtained together with some unchanged adenosine. The band with the higher $R_F$ value contained the diastereomers of 2′,3′-di-*O*-($\alpha$-methoxyethyl)adenosine (V) since this was the major product obtained when 5′-*O*-acetyladenosine was treated with methyl vinyl ether for an extended period of time.

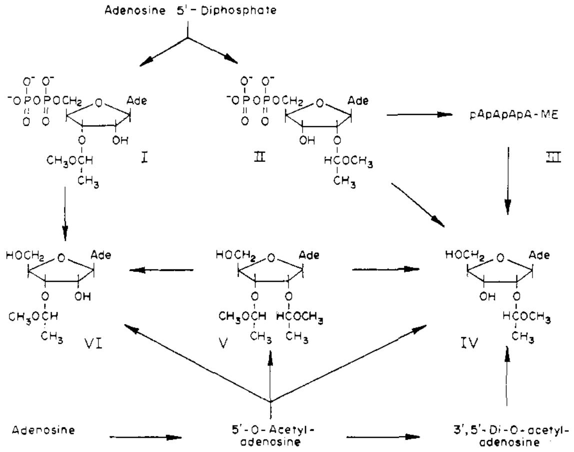

FIGURE 1: Reaction scheme for adenosine derivatives.

The band of products with the lower $R_F$ value was separated into two components by a second paper chromatographic system. The component with the higher $R_F$ in this system was identical with 2′-*O*-($\alpha$-methoxyethyl)adenosine and the second component was assigned the structure 3′-*O*-($\alpha$-methoxyethyl)-adenosine (VI). The latter derivative can also be shown, by thin-layer chromatography, to consist of two diastereomeric forms. Further confirmation of these structural assignments was obtained by the observation that the limited acid hydrolysis of 2′,3′-di-*O*-($\alpha$-methoxyethyl)adenosine yielded two monosubstituted adenosines which were chromatographically indistinguishable from the 2′- and 3′-*O*-($\alpha$-methoxyethyl)-adenosine synthesized as described above.

*Proton Magnetic Resonance and Mass Spectra.* Further support for the structural assignments of the derivatives, IV–VI, has come from a study of their pmr and mass spectra. In a study of the structure of nucleic acid components by pmr (Ts'o *et al.*, 1969) it has been pointed out that, in aqueous solution, purine nucleosides undergo stacking interactions, causing a concentration dependence of their chemical shifts, and that this concentration dependence can be greatly reduced by carrying out pmr studies in solvents such as dimethyl sulfoxide or dimethylformamide. In order to avoid this problem in the present study, the solvent used was DMSO–$D_2O$ (9:1, v/v) in which the concentration dependence should also be relatively small. The assignment of the resonance positions of the purine base protons (Table I) are made according to the values compiled by Ts'o *et al.* (1969) for other adenosine derivatives, and the positions of the ribose protons are identified on the basis of the assignments derived by Jardetzky (1963) and Sonnenbichler *et al.* (1963).

Fromageot *et al.* (1966) have pointed out that, for a pair of 2′ and 3′ derivatives of a nucleoside, the H-1′ resonance of the 2′ isomer is located further downfield than that of the 3′ isomer, and that the coupling constant, $J_{\text{H-1}'-\text{H-2}'}$, of the 3′ isomer is larger than that of the 2′ isomer. In the present work the H-1′ doublet of the 3′-*O*-($\alpha$-methoxyethyl)adenosine appears near the location of the corresponding resonance in adenosine whereas the H-1′ resonances of the 2′ isomers, IVa and IVb, are shifted 0.10–0.12 ppm downfield from the adenosine resonance.

In the case of the protons of the $\alpha$-methoxyethyl group it was expected that their resonance positions would be found near those listed for dimethyl acetal: $\delta$ 1.20, 4.5, 3.21 for the $CH_3$ group, acetal CH, and $OCH_3$ group protons, respectively (Sadtler Standard Spectra, 1967). Accordingly, the peaks at

TABLE I: Pmr Spectral Data of Adenosine Derivatives.

| | Chemical Shifts[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Acetal Protons | | |
| Compound | H-8 | H-2 | H-1′ | H-2′ | H-3′ | H-4′ | CH | OCH$_3$ | CCH$_3$ |
| Adenosine | 8.32 (s) | 8.13 (s) | 5.88 (d) | 4.58 (m) | 4.17 (m) | 4.05 (m) | | | |
| 2′-*O*-(α-Methoxyethyl)adenosine (IVa) | 8.37 | 8.13 | 6.00 | 4.72 | 4.27 | 4.05 | 4.63 | 2.87 | 1.07 (d, $J$ = 5 Hz) |
| 2′-*O*-(α-Methoxyethyl)adenosine (IVb) | 8.37 | 8.13 | 5.98 | 4.73 | 4.22 | 4.03 | 4.55 | 2.82 | 1.15 (d, $J$ = 5 Hz) |
| 3′-*O*-(α-Methoxyethyl)adenosine (VI) | 8.37 | 8.17 | 5.90 | 4.68 | 4.27 | 4.12 | 4.82 | 3.27 | 1.30 (d, $J$ = 5 Hz) |
| 2′,3′-Di-*O*-(α-Methoxyethyl)adenosine (V) | 8.42 | 8.20 | 6.05 | 4.87 | 4.35 | 4.17 | 4.82 | 3.30 | 1.32 (d, $J$= 5 Hz) |
| | | | | | | | 4.68 | 2.83 | 1.17 (d, $J$ = 5 Hz) |

[a] Chemical shifts are given in ppm downfield from Me$_4$Si as internal standard. The spectra were recorded on a Varian A-60 spectrometer with a sample concentration of 25–50 mg/ml of solvent, Me$_2$SO-$d_6$–D$_2$O (9:1, v/v).

2.82–3.30 are assigned to the OCH$_3$ protons in the α-methoxyethyl derivatives. The value, 3.27, obtained for the 3′ isomer is close to that listed for dimethyl acetal and this resonance also appears in the spectrum of the di(α-methoxyethyl) derivative. However, the disubstituted adenosine also produces a peak at 2.83 and this is about the value found in the spectra of the two diastereomers of the 2′ derivative. This shift to higher field of the resonances of the substituent group at the 2′ position relative to the 3′ position can be explained in terms of the shielding effect of the purine ring system. Cushley *et al.* (1967) have studied the shielding and deshielding effects of the bases, uracil and thymine, in various acetylated nucleosides. They have shown that the changes in the 2′-acetoxy resonance caused by the hydrogenation of the 5,6 double bond can be understood in terms of the conformation of the acetoxy group relative to the negative shielding zone of that double bond. In the present case, however, a molecular model of 2′-*O*-(α-methoxyethyl)adenosine shows that the methoxy group can be readily located within the presumptive positive shielding cone of the purine ring system whereas, in the 3′ isomer, this conformation is not possible. It will be seen in Table I that the resonances of the acetal CH and CCH$_3$ protons in the 2′ isomer are also shifted to higher field positions relative to those of the 3′ isomer.

The first indication that the diphosphate, II, was the active substrate came from an examination of the mass spectra of the two blocked nucleosides obtained by the dephosphorylation of I and II (Mackey and Gilham, 1971). The spectra were carried out on the trimethylsilyl derivatives of these compounds in order to prevent any possible migration of the methoxyethyl groups. These spectra have now been compared with those obtained from the trimethylsilyl derivatives of IVa and IVb synthesized from 3′,5′-di-*O*-acetyladenosine. In the use of mass spectra for the determination of structures of O-substituted nucleosides the most useful fragment ion is that which contains the base, C-1′, and C-2′ together with any substituent located at C-2′ (Biemann and McCloskey, 1962; Eggers *et al.*, 1966; Shaw *et al.*, 1970). Thus, the spectrum of the trimethylsilyl derivative of the nucleoside prepared by dephosphorylation of II indicated the ion, *m/e* 235, that corresponds to this fragment, containing, in this case, a methoxyethyl group at C-2′. The corresponding nucleoside derivative prepared from the diphosphate, I, did not produce this ion. Instead, its spectrum showed a new ion, *m/e* 249, and this corresponds to a similar fragment containing, in this case, a trimethylsilyl group at C-2′. In addition, the mass spectrum of the derivatized nucleoside obtained from II was found to be indistinguishable from those obtained from the trimethylsilyl derivatives of IVa and IVb.

## Conclusion

The details of the synthesis of the mono(α-methoxyethyl) derivatives prepared from the diphosphates of the other three common nucleosides, guanosine, uridine, and cytidine, will be discussed in a forthcoming publication. It will suffice here to mention that, in each case, the monosubstituted derivative may be separated into two components by paper chromatography. It can be shown that, in each case, it is the component with the higher $R_F$ value that is enzymatically active, and it is presumed that these derivatives are also the 2′-substituted nucleoside diphosphates.

Apart from its obvious value in the field of specific polynucleotide synthesis, the finding that single addition results from the 2′ substitution of nucleoside 5′-diphosphates with the α-methoxyethyl group adds another interesting aspect to the study of the catalytic activity of polynucleotide phosphorylase. A number of 2′-substituted nucleoside diphosphates have already been shown to undergo polymerization in the presence of this enzyme. For example, the 5′-diphosphates of 2′-*O*-methyladenosine (Rottman and Heinlein, 1968), 2′-*O*-methylcytidine (Zmudzka *et al.*, 1969), 2′-*O*-methyluridine (Zmudzka and Shugar, 1970), 2′-azido-2′-deoxyuridine (Torrence *et al.*, 1972), 2′-fluoro-2′-deoxyuridine (Janik *et al.*, 1972), 2′-chloro-2′-deoxyuridine, and 2′-chloro-2′-deoxycytidine (Hobbs *et al.*, 1972) are all capable of polymerizing to high molecular weight polymers with polynucleotide phosphorylase. Deoxynucleoside diphosphates where the 2′-hydroxyl is replaced by hydrogen show only limited addition to the 3′ terminus of an oligonucleotide (Kaufmann and Littauer, 1969; Bon *et al.*, 1970), although, in the presence of Mn$^{2+}$ ions, deoxyadenosine 5′-diphosphate is capable of copolymerization with adenosine 5′-diphosphate (Chou and Singer, 1971).

There have only been two cases reported where the modified diphosphate allows only single addition to an oligonucleotide chain: 2′-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates (Mackey and Gilham, 1971) and 2′(3′)-*O*-isovalerylnucleoside

5′-diphosphates (Kaufmann *et al.*, 1971), and, in the light of the present work, it seems likely that the enzymatically active component in the latter case is also the 2′ isomer. The simplest explanation to account for the differences in activity (polymerization *vs.* single addition) of the various 2′-substituted diphosphates would involve a consideration of the steric hindrance of the reactivity of the 3′ hydroxyl group of the oligonucleotide by the substituent group at the 2′ position after the first addition to the primer molecule has been made. It has recently been shown that 2′-*O*-ethyladenosine 5′-diphosphate can be polymerized by polynucleotide phosphorylase (Tazawa *et al.*, 1972) so that the α-methoxyethyl group may well be the smallest substituent that is capable of restricting the reaction to single addition. In this regard it would be of some interest to synthesize and test the activity of a 2′-*O*-isopropylnucleoside 5′-diphosphate. However, it should be noted that, in each of the cases listed above, where a 2′-substituted nucleoside diphosphate has been shown to undergo polymerization, it is possible that the type of reaction observed is dependent on the experimental conditions used, and that, in the presence of polynucleotide phosphorylase from a different source or in the presence of a different divalent cation, some of these diphosphates may also be restricted to single addition reactions.

## Experimental Section

*Materials.* Methyl vinyl ether was purchased from K and K Laboratories, Plainview, N. Y. *E. coli* alkaline phosphatase (Grade BAPF, Worthington Biochemical Corp., Freehold, N. J.) was assayed by the method of Garen and Levinthal (1960), and the unit of activity is that quantity of enzyme that liberates 1 μmol of *p*-nitrophenol from *p*-nitrophenyl phosphate per minute at 25°. Adenosine, poly(A), and *M. luteus* polynucleotide phosphorylase (Type 15) were purchased from P-L Biochemicals Milwaukee, Wis.; a unit of activity of this enzyme is defined as the amount that catalyzes the incorporation of 1 μmol of $^{32}$P into adenosine diphosphate in the presence of poly(A) per 15 min at 37° (Singer, 1966). Pig liver nuclei ribonuclease was prepared by the method of Heppel (1966) and a unit of activity is defined as that amount which liberates 1 μmol of acid-soluble adenylic acid residues from poly(A) in 1 hr.

*Preparation of Adenosine Trinucleotide.* Poly(adenylic acid) (0.3 g) was dissolved in 0.05 M potassium phosphate–0.01 M magnesium chloride buffer (pH 7.0 (20 ml)) and treated with pig liver nuclei ribonuclease (25 units). A few crystals of thymol were added and the mixture was incubated at 37° for 36 hr. The products were separated on a column (1.5 × 55 cm) of DEAE-cellulose (Whatman DE-23) using 6 l. of 7 M urea–0.05 M Tris-acetate buffer (pH meter reading, 8.0) containing a linear gradient of 0–0.5 M sodium acetate, at a flow rate of 50 ml/hr. The products present in the series of peaks obtained were identified as pA, pApA, pApApA, pApApApA, etc., by the comparison of their mobilities in paper chromatographic and electrophoretic analysis. The combined fractions corresponding to the trinucleotide peak contained 1160 $\mathrm{ODU_{260\ nm}}$, and the product was recovered in a salt-free condition by a modification of the method described by Mandeles and Kammen (1966). The trinucleotide fraction was adjusted to pH 3 with hydrochloric acid and then stirred with acid-washed activated charcoal (Norit A, 1 g) for 15 min. The suspension was then filtered through a membrane filter (0.45 μ pore size) and the charcoal was washed with several 10-ml portions of water. The adsorbed trinucleotide was then recovered by washing the charcoal with several 10-ml portions of the mixture: ethanol–water–concentrated ammonia (600:400:6.5, v/v). Evaporation of the solvent from the combined washings gave the pure adenosine trinucleotide (990 $\mathrm{ODU_{260\ nm}}$).

*Enzyme-Catalyzed Addition of 2′- and 3′-O-(α-Methoxyethyl)adenosine 5′-Diphosphates to Adenosine Trinucleotide.* The mixed 2′ and 3′ isomers of α-methoxyethyladenosine diphosphate were prepared as described by Mackey and Gilham (1971). On extended chromatography on Whatman 3 MM paper with solvent A the two isomers separated with the 2′ isomer (II) and the 3′ isomer (I) displaying $R_F$ values of 0.30 and 0.26 (relative to that of adenosine), respectively. Each product was eluted with dilute ammonia and concentrated by evaporation *in vacuo*. During the evaporation the pH of the solution was kept between 9 and 10 by the periodic addition of small amounts of sodium hydroxide in order to avoid any hydrolysis of the methoxyethyl group. These diphosphates were used separately or combined in three enzyme reactions. Each reaction (total volume 0.1 ml) contained adenosine trinucleotide (64 nmol), Tris-chloride (pH 8.0) (10 μmol), manganese chloride (1 μmol), polynucleotide phosphorylase (0.4 unit), and the diphosphate I (70 nmol), or the diphosphate II (84 nmol), or a mixture of I (70 nmol) and II (84 nmol). The mixtures were kept at 37° for 5 hr and then separated by chromatography on a column (30 × 0.5 cm) of Dowex 1-X2 (−400 mesh) ion-exchange resin using 200 ml of 20% ethanol containing a linear gradient of 0.1–1.0 M ammonium chloride (pH 8.5) at a flow rate of 20 ml/hr. In this system the nucleoside diphosphates, adenosine trinucleotide, and the product, 2′-*O*-(α-methoxyethyl)adenosine tetranucleotide (III), have retention volumes of 46, 90, and 104 ml, respectively. The yields obtained for this tetranucleotide using the different substituted diphosphates, I, II, and a mixture of I and II, were 6, 37, and 42 nmol, respectively.

*Characterization of α-Methoxyethyladenosine Tetranucleotide (III).* The tetranucleotide, III, was prepared from adenosine trinucleotide and 2′(3′)-*O*-(α-methoxyethyl)adenosine 5′-diphosphate and isolated as described above. The appropriate fractions from the ion-exchange chromatography were dialyzed for 6 hr against water containing a few drops of concentrated ammonia. The solution containing about 7 $\mathrm{ODU_{260}}$ of the tetranucleotide was then treated with 1 M sodium hydroxide (0.1 ml) and the solution was concentrated *in vacuo* to about 0.5 ml and then kept at 37° for 20 hr. The products of the alkaline hydrolysis were separated by ion-exchange chromatography using a solvent containing an ammonium chloride gradient in 20% ethanol (Ho and Gilham, 1973). The column (100 × 0.2 cm) was packed with a slurry of AG1-X4 (−400 mesh) ion-exchange resin in 20% ethanol solution containing 0.5 M ammonium chloride whose pH had been adjusted to 10 with ammonia. The column was then washed with 20% ethanol and the following additions were made: 1 M sodium hydroxide (0.3 ml), water (2 × 1 ml), alkaline hydrolysate (0.5 ml), and water (2 × 0.5 ml). Fractionation was then carried out, under pressure, with 200 ml of 20% ethanol containing a linear gradient of 0–0.5 M ammonium chloride (pH 10) at a flow rate of 6 ml/hr, and the elution pattern obtained is shown in Figure 2. Spectrophotometric analysis at 260 nm of the products showed that the adenosine 2′(3′),5′-diphosphate, adenosine 2′(3′)-phosphate, and α-methoxyethyladenosine were formed in the mole ratio of 1.0:2.0:1.0.

For the identification of the terminal nucleoside of III a larger scale synthesis of the tetranucleotide was necessary.

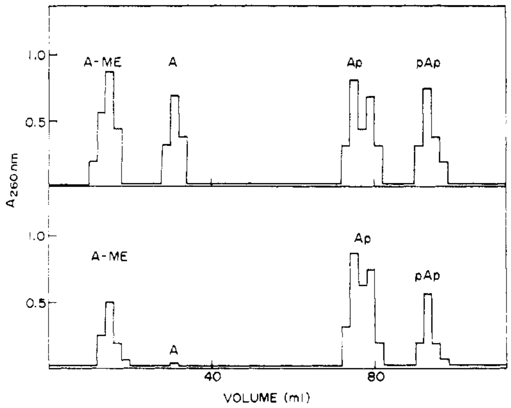

FIGURE 2: Elution patterns from the analysis of the tetranucleotide, III. The column (100 × 0.2 cm) of AG 1-X4 (−400 mesh) ion-exchange resin (Bio-Rad Laboratories, Richmond, Calif.) was prewashed with 20% ethanol under pressure before the following additions were made by means of a sample injection loop: 1 M sodium hydroxide (0.3 ml), water (2 × 1 ml), alkaline hydrolysate (0.5 ml), and water (2 × 0.5 ml). Separation was effected by elution at 6–10 ml/hr, under pressure, with 200 ml of 20% ethanol containing a linear gradient of 0–0.5 M ammonium chloride (pH 10). The upper elution pattern was obtained from the separation of a mixture of 2′(3′)-*O*-(α-methoxyethyl)adenosine (A-ME), adenosine (A), adenosine 2′(3′)-phosphate (Ap), and adenosine 2(3′),5′-diphosphate (pAp). The lower pattern was obtained from the alkaline hydrolysate of the tetranucleotide, III.

The reaction mixture (2.5 ml) contained 2.5 mM 2′(3′)-*O*-(α-methoxyethyl)adenosine 5′-diphosphate, adenosine trinucleotide (1.1 mM), polynucleotide phosphorylase (10 units), 0.01 M manganese chloride, and 0.1 M sodium tris(hydroxymethyl)methylaminopropanesulfonate (pH 9.0 at 20°). After 11 hr at 37° the mixture was applied to Whatman 3 MM paper and the substituted adenosine diphosphate was separated from the product by chromatography with the solvent system: *n*-propyl alcohol–concentrated ammonia–water (50:10:35, v/v). The tetranucleotide, which was obtained in essentially quantitative yield, was eluted with dilute ammonia and concentrated to dryness *in vacuo*. The residue was dissolved in 0.25 M sodium hydroxide and kept at 37° for 20 hr. The mixture was then brought to about pH 9 with Dowex 50W-X8 ($H^+$) ion-exchange resin and the products were separated by paper chromatography with solvent A. The α-methoxyethyladenosine obtained in this way was shown to be chromatographically indistinguishable from the two diastereomers of the 2′ isomer, IVa and IVb (Table II).

*Dephosphorylation of α-Methoxyethyladenosine Diphosphates.* Each isomeric methoxyethyladenosine diphosphate (30 $ODU_{260}$) in water (0.5 ml) was treated with 0.5 ml of 0.04 M Tris-chloride–0.02 M magnesium chloride buffer (pH 8) at 20° and a solution of alkaline phosphatase (0.3 unit in 0.1 ml of $H_2O$). After incubation for 5 hr at 37° portions of the reaction mixtures were chromatographed in various systems (Table II). The product obtained from the diphosphate, II (the isomer with the higher $R_F$ value), gave $R_F$ values identical with those of the synthetic 2′-*O*-(α-methoxyethyl)adenosine while the product from the other isomer, I, gave $R_F$ values equivalent to those of 3′-*O*-(α-methoxyethyl)adenosine. Each of the products was converted to its trimethylsilyl derivative for mass spectral analysis. The dried nucleoside (*ca.* 1 mg) was added to dry acetonitrile (15 μl) and 15 μl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, Ill.) and the mixture was heated at 100° for 2 hr in a sealed vial. Spectra were taken in a Hitachi-Perkin-Elmer RMU-6D spectrometer at 70 eV using the direct inlet probe. The spectrum of the nucleoside obtained from diphosphate, II, contained: $M^+$, 469, and fragment ions $M^+ - 15(CH_3)$, 454; $M^+ - 59(CH_3OCHCH_3)$, 410; $M^+ - 31(CH_3O)$, 438; $M^+ - 103(CH_2OSiMe_3)$, 366; and an ion, *m/e* 235, corresponding to the base + C-1′ and C-2′ together with the α-methoxyethyl moiety. The spectrum of the nucleoside from the diphosphate, I, gave a similar fragmentation pattern except for the absence of the *m/e* 235 ion and the presence of a peak at *m/e* 249, corresponding to an ion consisting of the base, C-1′, C-2′, and a trimethylsilyl group.

TABLE II: $R_F$ Values of Compounds.

| Compound | Solvent A[a] | Solvent B[b] | Solvent C[c] |
|---|---|---|---|
| Adenosine | 1.00 | 1.00 | 0.03 |
| 2′-*O*-(α-Methoxyethyl)adenosine 5′-diphosphate (II) | 0.30 | 2.20 | |
| 3′-*O*-(α-Methoxyethyl)adenosine 5′-diphosphate (I) | 0.26 | 1.70 | |
| 2′-*O*-(α-Methoxyethyl)adenosine (IVa) | 1.30 | 0.90 | 0.29 |
| 2′-*O*-(α-Methoxyethyl)adenosine (IVb) | 1.30 | 0.90 | 0.34 |
| 3′-*O*-(α-Methoxyethyl)adenosine (VI) | 1.30 | 0.70 | 0.21, 0.26 |
| 2′,3′-Di-*O*-(α-methoxyethyl)-adenosine (V) | 1.40 | 0.60 | 0.41 |
| 2′-*O*-(α-Methoxyethyl)adenosine from adenosine tetranucleotide (III) | 1.30 | 0.90 | 0.29, 0.34 |
| 5′-*O*-Acetyladenosine | | | 0.13 |
| 3′,5′-Di-*O*-acetyladenosine | | | 0.28 |

[a] Descending chromatography on Whatman No. 1 paper with isopropyl alcohol–concentrated ammonia–water (70:10:20, v/v). Values given are relative to the $R_F$ value of adenosine in this solvent. [b] Descending chromatography on Whatman No. 1 paper with a mixture of water (100 ml) and ammonium sulfate (40 g) adjusted to pH 8.5 with concentrated ammonia. Values listed are relative to the $R_F$ value of adenosine in this solvent. [c] Thin-layer chromatographic $R_F$ values determined on Eastman 6060 silica gel chromagram sheets with chloroform–methanol (96:4, v/v).

*3′,5′-Di-O-acetyladenosine.* 5′-*O*-Acetyladenosine was converted to 3′,5′-di-*O*-acetyladenosine by the method of Fromageot *et al.* (1967) and, after recrystallization from ethanol, the product had mp 147–148° (Brown *et al.*, 1954). Its pmr spectrum contained peaks in the positions described (Fromageot *et al.*, 1966) for the 3′,5′-diacetate.

*2′-O-(α-Methoxyethyl)adenosine (IV).* 3′,5′-Di-*O*-acetyladenosine (203 mg) and *p*-toluenesulfonic acid monohydrate (134 mg) were stirred in dioxane (1.9 ml) for 2 hr at 10°. Methyl vinyl ether (0.6 ml) was added and the reaction mixture was kept at 10° for 5 min. Ammonia (7 M, 10 ml) was then added and the mixture was allowed to stand at room temperature for 20 hr. The solution was evaporated to dryness *in vacuo*, and the residue was dissolved in 10 ml of acetone–methanol (1:1, v/v) containing a few drops of ammonia, and was then fractionated by dry column chromatography (Loev and Goodman, 1967). Silica gel Woelm (300 g) was shaken with 30 ml of the elution solvent chloroform–methanol (96:4, v/v) and packed into a dry column (2.5 × 100 cm). A 5-g portion of the equilibrated gel was mixed with the solution of the product and the solvent was removed *in vacuo*. This silica gel was then added to the top of the dry column and the total column was then eluted with chloroform–methanol (96:4, v/v) at 150 ml/hr. Fractions of 20 ml each were collected and their contents analyzed by thin-layer chromatography (Table II). The diastereomer with the faster mobility (IVb) was located in fractions 56–60 while that of slower mobility (IVa) appeared in fractions 62–72. The diastereomer IVa was concentrated by evaporation of the solvent and recrystallized from chloroform, 36 mg, 19%, mp 125–126°. *Anal.* Calcd for $C_{13}H_{19}N_5O_5$: C, 48.00; H, 5.89; N, 21.53. Found: C, 48.11; H, 5.85; N, 21.46. The product had $\lambda_{max}$ 259 nm ($\epsilon$ 14,600), $\lambda_{min}$ 227 nm in water at pH 7; and gave $[m]_{300} = -2300°$ in dioxane–water (4:1, v/v). The pmr data and $R_F$ values for this derivative are listed in Tables I and II.

The diastereomer IVb, which eluted first from the silica gel column, was concentrated and purified further by chromatography in chloroform–methanol (96:4, v/v) on a thin-layer plate (1000 $\mu$, Kontes Glass Co., Vineland, N. J.) which had been coated with silica gel (GF-254, E. Merck, Darmstadt, Germany) and dried at 80° for 3 hr before use. The product was extracted with chloroform–methanol (4:1, v/v, containing a few drops of ammonia) and, after the removal of the solvent *in vacuo*, it was recrystallized from chloroform, 31 mg, 17%, mp 159–161°. *Anal.* Calcd for $C_{13}H_{19}O_5N_5$: C, 48.00; H, 5.89; N, 21.53. Found: C, 47.59; H, 5.80; N, 21.24. This had $\lambda_{max}$ 259 nm ($\epsilon$ 14,500), $\lambda_{min}$ 227 nm in water at pH 7 and gave $[m]_{300} = -1800°$ in dioxane–water (4:1, v/v). Under these conditions adenosine gave a value for $[m]_{300}$ of $-2000°$. The pmr data and $R_F$ values for this product are listed in Tables I and II.

The trimethylsilyl derivatives of both IVa and IVb were prepared as described above and the mass spectra of these derivatives were found to be identical with the mass spectrum of the trimethylsilyl derivative of the nucleoside obtained by the dephosphorylation of diphosphate, II.

*Reaction of 5′-O-Acetyladenosine with Methyl Vinyl Ether.* *p*-Toluenesulfonic acid monohydrate (1 g) and 308 mg of 5′-*O*-acetyladenosine (Fromageot *et al.*, 1967) were dissolved in dry dimethyl sulfoxide (8 ml) and the solution was frozen in an ice bath. Methyl vinyl ether (15 ml) at 0° was added and the mixture was stirred until it was homogeneous. The reaction was allowed to proceed at 0° for 15 min and then 7 M ammonia (25 ml) was added. The mixture was allowed to come to room temperature and stirred for about 10 hr. The product was concentrated *in vacuo* to an oil and fractionated by dry column chromatography and subsequently by thin-layer chromatography as described above for the preparation of 2′-*O*-($\alpha$-methoxyethyl)adenosine. The mixture of 2′,3′-di-*O*-($\alpha$-methoxyethyl)adenosine (V) diastereomers (9400 $ODU_{260\ nm}$, 72%) had a uv spectrum similar to that of adenosine, and its pmr and chromatographic data are listed in Tables I and II.

The reaction of 5′-*O*-acetyladenosine with methyl vinyl ether under the same conditions but for shorter times (3–9 min), followed by hydrolysis of the acetyl group, gave smaller amounts of V and more of a product which had an $R_F$ value (paper chromatography with solvent system A) corresponding to that of 2′-*O*-($\alpha$-methoxyethyl)adenosine. This product split into two bands on paper chromatography in solvent system B and the component with the higher $R_F$ value corresponded to that of the mixture of 2′-*O*-($\alpha$-methoxyethyl)adenosine diastereomers (IVa and IVb). The band with the lower $R_F$ value was chromatographically identical with the 3′-*O*-($\alpha$-methoxyethyl)adenosine (VI) obtained from the partial hydrolysis of the diderivative, V, described below.

*Hydrolysis of 2′,3′-Di-O-($\alpha$-methoxyethyl)adenosine.* A solution of the di($\alpha$-methoxyethyl) derivative in water was brought to pH 2.5 with dilute hydrochloric acid. After 20 min at room temperature the reaction mixture was neutralized with dilute ammonia and the products were separated by chromatography on Whatman No. 3 MM paper with solvent system A. Together with some unchanged material, two bands with lower $R_F$ values (1.3 and 1.0 relative to that of adenosine) were obtained. The band (relative $R_F$ value, 1.3) containing the monosubstituted adenosines was eluted with dilute ammonia and concentrated. The mixture was then separated, by paper chromatography with solvent system B, into 2′-*O*-($\alpha$-methoxyethyl)adenosine and 3′-*O*-($\alpha$-methoxyethyl)adenosine with $R_F$ values of 0.9 and 0.7 (relative to that of adenosine), respectively. Each band was eluted with dilute ammonia and the extracts were mixed with excess ethanol to precipitate the ammonium sulfate. After removal of the salt by filtration the solutions were concentrated *in vacuo* and the products were further purified by thin-layer chromatography as described above. The pmr and chromatographic data for these products are listed in Tables I and II and it will be noted that, in solvent system C, the 3′ isomer can also be shown to consist of two diastereomers.

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# Synthesis and Characterization of Potential Interferon Inducers. Poly(2′-azido-2′-deoxyuridylic acid)[†]

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ABSTRACT: 2′-Azido-2′-deoxyuridine (I) was converted in a series of steps to 2′-azido-3′-acetyl-2′-deoxyuridine (IV) which was phosphorylated with 2-cyanoethyl phosphate to give 2′-azido-2′-deoxyuridine 5′-monophosphate (V) from which the 5′-diphosphate (VI) was prepared by the morpholidate procedure. Polynucleotide phosphorylase polymerized VI in the presence of $Mg^{2+}$ or $Mn^{2+}$ to poly(2′-azido-2′-deoxyuridylic acid) [poly(dUz)] which was completely resistant to degradation by 0.3 N KOH and pancreatic ribonuclease A, as well as DNase I. Circular dichroism studies indicate that at 2.5°, poly(dUz) assumes a secondary structure similar to that of poly(U) (most likely a single hairpin). Ultraviolet and circular dichroism–temperature profiles in a number of buffer systems indicate that this structure has a thermal stability which is somewhat greater than poly(U). This finding indicates that neither a 2′-OH group nor a 2′-oxygen atom is necessary for the formation of ordered structure in poly(U). The construction of mixing curves at the isochromic wavelengths for this system revealed that poly(dUz) can form both double- and triple-stranded complexes with poly(A), *i.e.*, poly(dUz)·poly(A) and 2poly(dUz)·poly(A). A study of the course of the thermal dissociation of these complexes appeared to indicate that both the doubly and triply stranded complexes undergo monophasic transitions directly to constituent homopolymers (3 → 1 and 2 → 1, respectively). If the assignment of the above transitions (3 → 1 and 2 → 1) is correct, then the introduction of the azido group into the C-2′ position of poly(U) does not have a significant effect on the stabilities of both the triple- and double-stranded complexes when compared with the corresponding complexes in the poly(A)–poly(U) system.

Perhaps the most rewarding approach to the elucidation of the role of specific functional groups in the physical and biological properties of nucleic acids has been the synthesis of modified nucleosides and their conversion to 5′-diphosphates, followed by polymerization by the enzyme polynucleotide phosphorylase (Grunberg-Manago, 1963). In contrast to the chemical modification of a preformed polynucleotide, such an approach has the advantages of widening the possibilities for structural changes and leads to chemically well-defined high molecular weight homopolymers. In most instances, the substrate specificity of polynucleotide phosphorylase is the only limitation to this approach, but, fortunately, the nonspecific enzyme accepts a relatively wide variety of substrate modifications which can be extended even further by the use of manganous ion as cofactor (Babinet *et al.*, 1965; Thang *et al.*, 1965; Zmudzka *et al.*, 1969a,b; Rottman and Heinlein, 1968; Chou and Singer, 1971; Torrence and Witkop, 1972; Mackey and Gilham, 1971) or possibly through the use of matrix-bound enzyme (Brentnall and Hutchinson, 1972). By such techniques, a number of polynucleotides containing 2′-fluoro (Janik *et al.*, 1972; Hendler *et al.*, 1971), 2′-amino (Hobbs *et al.*, 1972a), 2′-chloro (Hobbs *et al.*, 1971, 1972b), 2′-*O*-methyl (Zmudzka *et al.*, 1969a,b; Rottman and Heinlein, 1968; Zmudzka and Shugar, 1970; Tazawa *et al.*, 1972), and 2′-*O*-ethyl (Khurshid *et al.*, 1972; Tazawa *et al.*, 1972) substituents have been introduced and studied recently. Such 2′-modified polynucleotides can be powerful tools in the elucidation of the role of the 2′-hydroxyl group in RNA and the factors responsible for the clinically promising phenomenon of the induction of interferon by synthetic polynucleotides (Colby, 1971; Kleinschmidt, 1972). In this paper[1] we describe the synthesis and characterization of poly(2′-

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[1] A preliminary account of a portion of this work has appeared (Torrence *et al.*, 1972).