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Chloroacetaldehyde-Modified Dinucleoside Phosphates. Dynamic Fluorescence Quenching and Quenching Due to Intramolecular Complexation†

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ABSTRACT: The 12 possible dinucleoside phosphates combining adenosine and cytidine with adenosine, cytidine, guanosine, and uridine were readily converted to the 1,N^6-ethenoadenosine (εA) and 3,N^4-ethenocytidine (εC) analogs by reaction with chloroacetaldehyde. Those dinucleoside phosphates containing 1,N^6-ethenoadenosine are fluorescent in neutral solution, while those containing 3,N^4-ethenocytidine are not since 3,N^4-ethenocytidine is fluorescent only in the protonated form. Chloroacetaldehyde modification in general renders the dinucleoside phosphates more resistant to nucleolytic cleavage. Dinucleoside phosphates of the form εCpN were completely resistant to the action of pancreatic RNase A, and those of the form εApN and εCpN were highly resistant to the action of RNase T_2. Static and dynamic quenching parameters were determined from the values measured for the fluorescence lifetimes and quantum efficiencies of the seven 1,N^6-ethenoadenosine dinucleoside phosphates. From the fluorescence quenching parameters, it was possible to determine the proportion of internally complexed or folded conformations *vs.* open or unfolded conformations at 25°. Guanosine and 1,N^6-ethenoadenosine participate equally well in stacking interactions in the dinucleoside phosphates, and in general greater intramolecular association was observed in the dinucleoside phosphates containing purines than those containing pyrimidines. The sequence effects on intramolecular association observed in the 1,N^6-ethenoadenosine dinucleoside phosphates are identical with those observed for their unmodified counterparts. The fluorescence quenching parameters of UpεA and εCpεA indicate a lower degree of base–base interaction than in their εApU and εApεC counterparts. The circular dichroic spectra of εCpεA *vs.* εApεC confirm the very weak base–base intramolecular interaction in the former modified dinucleoside phosphate. The results obtained with the chloroacetaldehyde-modified dinucleoside phosphates are directly applicable to the fluorescent modification of tRNAs by reaction with chloroacetaldehyde.

In seeking to develop new methods for the fluorescent modification of nucleic acid bases, related coenzymes, and tRNAs, we have recognized the value of obtaining basic information concerning the fluorescence properties and enzymatic properties of modified dinucleoside phosphates while beginning to explore similar features in specific tRNAs. Applications of fluorescent labeling of tRNAs have already proved useful (Ward *et al.*, 1969; Brdar and Reich, 1972; Maelicke *et al.*, 1974; Churchich, 1963; Millar and Steiner, 1966; Pachmann *et al.*, 1973; Lynch and Schimmel, 1974; Wintermeyer and Zachau, 1974; Friest *et al.*, 1972; Secrist *et al.*, 1971; Yang and Söll, 1973, 1974a,b; Beardsley and Cantor, 1970). The chloroacetaldehyde modification of adenosine to the fluorescent 1,N^6-ethenoadenosine (Secrist *et al.*, 1972; Spencer *et al.*, 1974) and of cytidine to 3,N^4-ethenocytidine, fluorescent in the protonated form (Barrio *et al.*, 1972a), is capable of extension to di- and oligonucleotides and to tRNAs. We wish to report the chloroacetaldehyde modification of 12 possible dinucleoside phosphates that combine adenosine or cytidine with adenosine, cytidine, guanosine, or uridine to give the corresponding 1,N^6-ethenoadenosine (εA)[1] and 3,N^4-ethenocytidine (εC)[1] derivatives (Figure 1).

Modification at the 1 and N^6 positions of adenosine and

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[1] Abbreviations following the IUPAC–IUB Commission on Biochemical Nomenclature recommendations (1971) are used throughout. The abbreviation ε stands for etheno so that ε-adenosine (εAdo) is 3-β-D-ribofuranosylimidazo[2,1-*i*]purine (1,N^6-ethenoadenosine) and ε-cytidine (εCyd) is 5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[2,1-*c*]pyrimidine (3,N^4-ethenocytidine) (Secrist *et al.*, 1972; Barrio *et al.*, 1972a); εAde-C_3-εAde, or εAde(9$(CH_2)_3$9)εAde, stands for 9,9′-trimethylenebis(1,N^6-ethenoadenine).

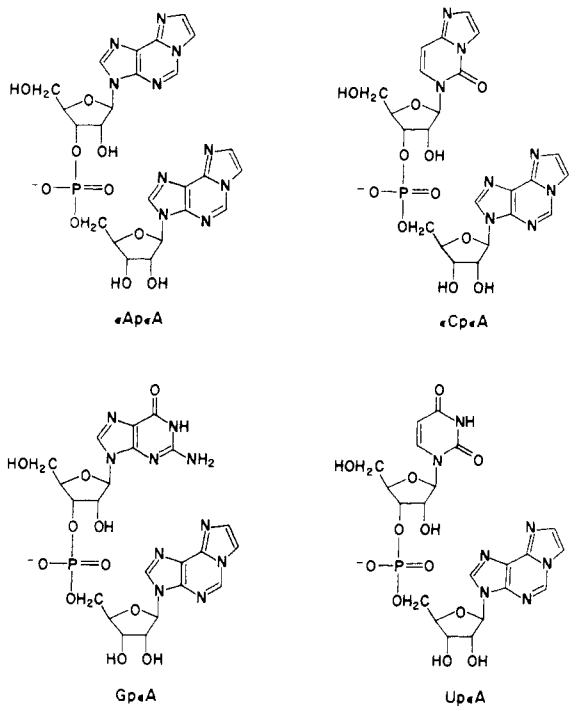

FIGURE 1: Representative structural formulas of four chloroacetaldehyde-treated dinucleoside phosphates.

the 3 and N^4 positions of cytidine by chloroacetaldehyde might be expected to change the specificity of nucleases in the hydrolysis of chloroacetaldehyde-modified tRNA. Kochetkov *et al.* (1972) found that εCpU, under conditions which completely hydrolyzed CpU, was resistant to pancreatic ribonuclease A. Janik and coworkers (1973) reported that poly (εA) and poly (εC) are more resistant to the action of pancreatic ribonuclease A and snake venom phosphodiesterase than unmodified poly(A) and poly(C). To determine any changes in substrate specificity which result from the formation of the new heterocyclic systems, we have studied the behavior of the 12 ε-dinucleoside phosphates with the common nucleases: snake venom phosphodiesterase, bovine spleen phosphodiesterase, pancreatic ribonuclease A, ribonuclease T_1, and ribonuclease T_2.

1,N^6-Ethenoadenosine cannot form a Watson–Crick base pair with uridine since the binding sites at 1 and N^6 on adenosine are covered. This is exemplified experimentally by the fact that poly(εA) synthesized from εADP using *Escherichia coli* polynucleotide phosphorylase does not complex with poly(U) (Janik *et al.*, 1973). However, substitution of 20% or less of the adenosine residues with 1,N^6-ethenoadenosine in chloracetaldehyde-treated poly(A) does not appear to block the formation of the bi- and trihelical species characteristic of the acid form of poly(A) or the complex of poly(A) with poly(U) (Steiner *et al.*, 1973). The fluorescence intensity of poly(εA) is less than one-seventh that of εAMP (Janik *et al.*, 1973; Lehrach and Scheit, 1973), indicating that the fluorescence of 1,N^6-ethenoadenosine is quenched by its neighbors in the polynucleotide chain. Steiner and coworkers (1973) noted greater quenching of fluorescence in poly(A) containing 80% 1,N^6-ethenoadenosine than in poly(A) with lower degrees of substitution and suggest that homologous interactions of 1,N^6-ethenoadenosine are more efficient in quenching fluorescence than are adenosine-1,N^6-ethenoadenosine interactions. 3,N^4-Ethenocytidine also cannot form Watson-Crick base pairs since the 3 and N^4 binding sites are covered. Poly(εC) made from εCDP using polynucleotide phosphorylase does not complex with poly(I) and shows no significant fluorescence in neutral solution (Janik *et al.*, 1973). The lack of fluorescence is actually not surprising since 3,N^4-ethenocytidine itself exhibits significant fluorescence intensity only at pH values lower than 3.5 (Barrio *et al.*, 1972a). For a greater understanding of the interactions in polynucleotides and the resulting changes in the fluorescence properties of 1,N^6-ethenoadenosine units, we have investigated the intramolecular complexing of the base units by detailed studies of fluorescence lifetimes and relative quantum efficiencies of ε-dinucleoside phosphates containing the highly fluorescent 1,N^6-ethenoadenosine. These data can be used to generate the relative proportions of open and closed (intramolecularly complexed) conformations (Spencer and Weber, 1972). Since optical properties of ribonucleic acids, including those having double helical regions, may be satisfactorily accounted for by considering only nearest neighbor interactions (Cantor and Tinoco, 1965), studies of the ε-dinucleoside phosphates are also directly applicable to an understanding of the properties of chloroacetaldehyde-modified tRNAs.

Experimental Procedure

The dinucleoside phosphates used were obtained from commercial sources: adenylyl(3′→5′)adenosine (ApA), adenylyl(3′→5′)guanosine (ApG), guanylyl(3′→5′)adenosine (GpA), adenylyl(3′→5′)uridine (ApU), uridylyl(3′→5′)adenosine (UpA), cytidylyl(3′→5′)adenosine (CpA), cytidylyl(3′→5′)cytidine (CpC), guanylyl(3′→5′)cytidine (GpC), and uridylyl(3′→5′)cytidine (UpC) from Sigma Chemical Co., and adenylyl(3′→5′)cytidine (ApC), cytidylyl(3′→5′)guanosine (CpG), and cytidylyl(3′→5′)uridine (CpU) from Calbiochem. They were used directly and purification was effected after chloroacetaldehyde reaction. Chloroacetaldehyde was purchased from Pfaltz and Bauer as a 40% solution in water, diluted to 20%, and distilled under reduced pressure. For the enzyme studies, venom phosphodiesterase (EC 3.1.4.1, potency 0.361; residual phosphatase activity), bovine (pancreatic) ribonuclease A (EC 2.7.7.16, approximately 2500 units/mg), and ribonuclease T_1 (EC 2.7.7.26), 311,000 units/mg) were purchased from Worthington Biochemicals Corp. Bovine spleen phosphodiesterase (EC 3.1.4.1, 23 units) and crude ribonuclease T_2 (EC 2.7.7.26, 50 units/mg, contains ribonuclease T_1 activity) were purchased from Sigma Chemical Co. Highly purified ribonuclease T_2 (EC 2.7.7.26, 500 units) was a gift from Sankyo Chemical Co., which we gratefully acknowledge. *Escherichia coli* alkaline phosphatase purchased from Worthington Biochemicals Corp. was purified according to the procedure of Kirkegaard *et al.* (1972) to give a stock enzyme solution of 84.5 units/ml. 1,N^6-Ethenoadenosine 5′-phosphate (εAMP) (Secrist *et al.*, 1972), 9,9′-trimethylenebisadenine (Browne *et al.*, 1968), and 1,N^6-etheno-9-propyladenine (3-propylimidazo[2,1-*i*]purine) (Spencer *et al.*, 1974) were prepared according to previously reported procedures. All buffer solutions were prepared from commercial reagents of high purity in glass-distilled water.

General Procedure for the Preparation of ε Dinucleoside Phosphates. Reaction of the dinucleoside phosphate of choice with a 50-fold excess of 1.5–2.0 M chloroacetaldehyde (pH 4.5–3.5) for 3–5 days at 23° produced the etheno-bridged derivative. The optimum pH of reaction for the adenosine-containing dinucleoside phosphates was 4.5,

while 3.5 was the optimum pH for those containing cytidine. The pH of reaction was periodically adjusted to the optimum value by addition of dilute sodium bicarbonate solution. The extent of reaction was monitored using cellulose tlc plates (Eastman No. 6065) developed with isobutyric acid–NH_4OH–H_2O (75:1:24, v/v). The final product was decolorized with charcoal and evaporated to dryness *in vacuo*. Purification was effected by dissolving in a minimum amount of water followed by addition of ethanol to precipitate the modified dinucleoside phosphate. For fluorescence studies, the $1,N^6$-ethenoadenosine-containing dinucleoside phosphates were extensively purified using high-pressure liquid anion-exchange chromatography or gel filtration. The first method involved treatment of 2 mg of the ϵ-dinucleoside phosphate in 200 μl of 0.2 M Tris-HCl buffer (pH 8.1) with 2 units of *E. coli* alkaline phosphatase (20 μl) (Kirkegaard *et al.*, 1972) for 1 hr followed by gel filtration on a 0.7 × 34 cm column of Bio-Gel P-2 (minus 400 mesh) and elution with 0.05 M NH_4OAc at 1 ml/min (200 psi) to remove the phosphatase and the nucleosides generated. Alternatively, the modified dinucleoside phosphates could be purified by anion exchange chromatography on a 0.7 × 18 cm column of Aminex A-28 and elution with 0.05 M KH_2PO_4 (pH 7.0)/0.05 M NH_4Cl in 30% ethanol at 0.6 ml/min (350 psi). All the ϵ-adenosine dinucleoside phosphates can be purified by anion exchange chromatography, while phosphatase treatment followed by gel filtration works well with all of the ϵ-dinucleoside phosphates except those containing guanosine.

Identical values of fluorescent lifetimes determined by phase and modulation measurements using the cross-correlation fluorometer described by Spencer and Weber (Spencer and Weber, 1969; Spencer *et al.*, 1969) were required for proof of fluorescent purity since impure samples may yield differences of 1–3 nsec when the two techniques are used.

9,9′-Trimethylenebis(1,N^6-ethenoadenine) Hydrochloride (3,3′-Trimethylenebis(imidazo[2,1-i]purine) Hydrochloride). 9,9′-Trimethylenebisadenine (190 mg) was stirred in a 50-fold excess of 1.5–2.0 M chloroacetaldehyde (pH 4.5) for 24 hr at 40°. During the course of the reaction, the pH was maintained between 4.0 and 4.5 by addition of dilute sodium bicarbonate solution. Decolorization with charcoal followed by evaporation to dryness *in vacuo* gave a slightly yellow solid. Upon recrystallization with water–isopropyl alcohol and drying, 217 mg of the 9,9′-trimethylenebis(1,N^6-ethenoadenine) monohydrochloride monohydrate was obtained: $\lambda_{max}(H_2O)$ pH 2, 275 (ϵ 20,900); pH 7.0, 260 (sh), 267 (9100), 277 (8300), and 296 (5100); pH 12, 260 (sh), 267 (11,700), 277 (10,600), and 300 nm (brsh).

Anal. Calcd for $C_{17}H_{15}ClN_{10} \cdot H_2O$: C, 49.34; H, 4.15; N, 33.93. Found: C, 49.22; H, 3.88; N, 33.95.

For fluorescence studies, the 9,9′-trimethylenebis(1,N^6-ethenoadenine) hydrochloride was dissolved in absolute ethanol, neutralized with an equivalent of sodium methoxide, and subjected to column chromatography on Sephadex LH-20, elution with absolute ethanol. Fluorescent purity was monitored by homogeneity of fluorescence lifetimes as determined by phase and modulation techniques (Spencer and Weber, 1969; Spencer *et al.*, 1969).

The absorption spectra were recorded on a Cary 15 spectrophotometer in aqueous solution at concentrations on the order of 10^{-5} M. All absorption spectra were determined against the appropriate blank using a matched set of sample and reference cells. All pH values were measured with a Brinkman 101 pH meter. The preparative high-pressure liquid chromatography was carried out using a Chromatronix Cheminert Metering Pump IV equipped with a Chromatronix Model 230 Dual Channel Absorbance Detector and Hewlett-Packard Model 680 recorder.

The circular dichroism spectra were recorded on a Jasco Model J-40 spectropolarimeter, which had been calibrated against camphorsulfonic acid. The solvent used was 0.05 M sodium phosphate buffered to pH 7.0. Concentrations for the dinucleoside phosphates were computed from the known extinction coefficients of the constituent monomers using the ultraviolet absorption spectra before and after complete enzymatic hydrolysis to estimate the hypochromic effect in each compound. The units used for molar ellipticity [θ] are (degrees-liter/mole-centimeter) × 100.

Fluorescence Emission Studies. Fluorescence lifetimes were determined from both phase and modulation measurements on the sub-nanosecond cross-correlation spectrofluorometer of Spencer and Weber (1969) using exciting light modulated at 14.2 MHz. Excitation was at 305 nm, selected by a monochromator and a CS-7-54 Corning filter, and emission was observed through a CS-0-52 Corning filter. Fluorescence emission spectra were recorded on a Hitachi Perkin-Elmer MPF-2A spectrofluorometer. All fluorescence intensities given are normalized for differences in absorptions of the samples and are therefore direct quantitative representations of relative quantum efficiencies. All fluorescence measurements were made at 25°.

Enzyme Studies. The dinucleoside phosphates, both unmodified and modified with chloroacetaldehyde, were prepared for enzyme assays as 1.0 mM aqueous solutions, determined spectrophotometrically; 20-μl aliquots were then used for the assay. Enzymes were dissolved in distilled water, and 10-μl aliquots were used for assays. Appropriate buffers were used in 10-μl portions, bringing the total volume of each assay sample to 40 μl. Controls lacking enzyme were prepared by substituting 10 μl of distilled water for the enzyme solution. All assays were performed at 25°.

Release of nucleoside upon enzymatic cleavage was monitored at selected intervals by high-pressure liquid chromatography using a Varian 4100 liquid chromatogram equipped with a uv monitor and a $\frac{1}{8}$ × 35 in. stainless steel column packed with Vydac pellicular anion exchange resin. We wish to thank Dr. John A. Katzenellenbogen of the University of Illinois for kindly allowing us to use this high-pressure liquid chromatography system. Adequate separation of the remaining nucleoside phosphate from the nucleoside and mononucleotide generated by enzymatic cleavage was accomplished upon elution with KH_2PO_4 buffer, pH 6.8 (0.15–0.30 M), at flow rates ranging from 50 to 200 ml/hr (Figure 2). Some enzyme reactions were also followed using thin-layer chromatography (1:1 ethanol–ether with Eastman silica gel chromatograms), as previously described by Henderson *et al.* (1973).

With those dinucleoside phosphates containing $1,N^6$-ethenoadenosine, enzyme assays were also performed on 3.0-ml aliquots of dinucleoside phosphate solution in the appropriate buffer having an absorbance of 0.2 at 305 nm. Upon the addition of 20 μl of enzyme stock solution, the enzymatic hydrolysis was followed by the increase in fluorescence intensity at 415 nm as the quenching action on the $1,N^6$-ethenoadenosine moiety is lost with cleavage of the dimer (Figure 3).

Ribonuclease T_1 stock solution was prepared in a concentration of 10 μg/ml; 0.2 M Tris-HCl (pH 7.5) was added as

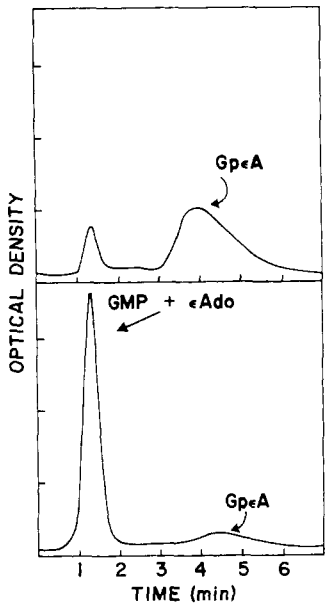

FIGURE 2: (Top) High-pressure liquid chromatogram of the ε-dinucleoside phosphate GpεA (see Experimental Procedure); (bottom) high-pressure liquid chromatogram of the ε-dinucleoside phosphate GpεA following 30-min treatment with ribonuclease T_1 (see Experimental Procedure).

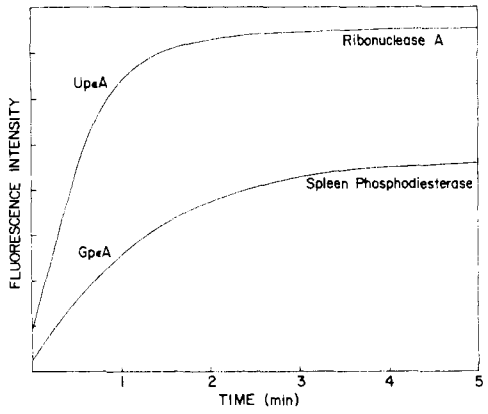

FIGURE 3: Kinetic curves obtained by following the increase in fluorescence intensity at 410 nm (excitation at 305 nm) as the quenching of the ε-adenosine in UpεA and GpεA is lost upon hydrolysis with pancreatic ribonuclease A and with bovine spleen phosphodiesterase.

a buffer to maintain the pH of the reaction. Ribonuclease T_1 may be regarded as a guanylribonuclease, cleaving on the 3′ side of the guanosine residue in the dinucleoside phosphate to give 3′-guanylic acid.

The stock solution of the Sigma brand ribonuclease T_2 (containing RNase T_1 activity) was prepared in a concentration of 10 mg/ml; potassium acetate buffer (0.2 M) was added to maintain pH 4.75, maximizing the RNase T_2 activity and minimizing the RNase T_1 activity. The stock solution of the Sankyo brand ribonuclease T_2 was prepared in a concentration of 500 units/ml using the same potassium acetate buffer (0.2 M, pH 4.75) to maintain the pH during the reaction. The enzyme acts as an endonuclease which releases nucleoside from the 3′ end of the dinucleoside phosphate.

Venom phosphodiesterase stock solution was prepared in a concentration of 5 mg/ml; stock Tris-HCl buffer (0.4 M with 0.04 M $MgCl_2$) was used to maintain pH 8.8. Since the enzyme is an exonuclease, nucleosides at the 5′ end of the dinucleoside phosphate were released.

Spleen phosphodiesterase (23 units) was dissolved in 2 ml of distilled water; stock Tris-HCl buffer (0.2 M, pH 7.5) was added as buffer. This exonuclease operates in a manner complementary to venom phosphodiesterase releasing the nucleoside at the 3′ end of the dinucleoside phosphate.

Pancreatic ribonuclease A stock solution was prepared in a concentration of 10 mg/ml; 0.2 M Tris-HCl (pH 7.5) was used as a buffer. The enzyme is an endonuclease which requires a pyrimidine residue on the 3′ side of the dinucleoside phosphate releasing a 3′-pyrimidine monophosphate.

Results

Chemistry. Using conditions previously described (Secrist *et al.*, 1972), the 12 possible adenosine- and cytidine-containing dinucleoside phosphates reacted readily with chloroacetaldehyde, following a course of reaction similar to that observed for the constituent nucleosides. Although reaction with chloroacetaldehyde proceeds more rapidly at elevated temperature, it was carried out at room temperature to minimize hydrolysis of the phosphodiester linkages. Because the rate of chloroacetaldehyde condensation decreases rapidly as the pH of reaction departs from the optimum values of 4.5 for adenosine and 3.5 for cytidine (Kochetkov *et al.*, 1972), the pH of the reaction was maintained in the optimum range to ensure rapid modification.

Although no cleavage of the dinucleoside phosphates was observed during modification, each reprecipitated ε-dinucleoside phosphate did contain some ribonucleoside and ribonucleotide due to the presence of varying amounts of monomer in the commercial starting material. Such contamination was in no case greater than a few per cent, as illustrated in the chromatogram in Figure 2 of reprecipitated GpεA. For enzyme studies, further purification of the reprecipitated ε-dinucleoside phosphate was not required. However, extensive purification of those ε-dinucleoside phosphates used in fluorescence studies was required to remove all traces of free, unquenched εAMP (or εAdo) since the fluorescence quantum efficiency of εAMP (or εAdo) is in most cases several times greater than that of the ε-diribonucleoside phosphates. In the case of GpεA, for which the quantum efficiency is $\frac{1}{30}$th that of εAMP, an impurity of 1% by weight of unquenched εAMP would result in the measurement of 30% more fluorescence emission over that due to GpεA. Therefore, all of the $1,N^6$-ethenoadenosine dinucleoside phosphates used in the fluorescence studies were extensively purified (see Experimental Procedure) to ensure that the fluorescence emission would be resulting from the phosphodiester alone. Ultimate purification was recognized as being achieved when identical values for fluorescence lifetimes, as measured by phase and modulation using the sub-nanosecond cross-correlation spectrofluorometer of Spencer and Weber (1969), were obtained, since wide differences in the values determined by the two techniques result when a mixture of fluorophores is present.

Enzyme Results. The purpose of the enzyme assays performed was to assess the effect of chloroacetaldehyde modification on substrate specificity in nucleolytic digestion. Accordingly, all assays were designed primarily to compare modified diribonucleoside phosphates with their unmodified counterparts. For this reason, care was taken that each sample of etheno-bridged substrate used in the enzyme assays was free of all unmodified starting material, although no effort was made to remove ribonucleosides or ribonucleotides from the reprecipitated ε-dinucleoside phosphates. No attempts were made to optimize the conditions of the

assays, but rather conditions were chosen that clearly reflected the differences in reactivity of the substrates used.

The high-pressure liquid chromatographic system used to monitor the hydrolysis of the dinucleoside phosphates (see Experimental Procedure) was chosen because of its sensitivity and ease of operation. For each enzyme system, a relative ordering of the dinucleoside phosphates as substrates was obtained by comparing peak areas of eluted ribonucleoside and ribonucleotide with the peak area of the remaining dinucleoside phosphate in several aliquots taken at selected intervals of time. The chromatograms of recrystallized GpεA before and after 30 min of RNase T_1 treatment in Figure 2 demonstrate the capability to estimate about every 8 min the extent of hydrolysis of the substrate in an individual aliquot. The results presented in Table I obtained using this chromatographic system clearly show the difference in reactivity of modified *vs.* unmodified dinucleoside phosphates and also indicate some differences in reactivity among the ε-dinucleoside phosphates.

Exploiting the fluorescence properties of $1,N^6$-ethenoadenosine, more precise rates of hydrolysis could be obtained for the ε-dinucleoside phosphates by following the increase in fluorescence intensity at 415 nm as the quenching in the εAdo-containing substrates is lost upon enzymatic cleavage. The kinetic curves shown in Figure 3 show the type of results which can be obtained using this method. Because $3,N^4$-ethenocytidine shows no fluorescence at the pH values used in the enzyme assays, kinetic curves could be obtained only from the $1,N^6$-ethenoadenosine-containing dinucleoside phosphates.

Exonucleases. In all cases, the exonucleases snake venom phosphodiesterase and bovine spleen phosphodiesterase, which operate in manners complementary to one another and show no preference for any individual nucleotide linkages (Bernardi and Bernardi, 1971; Laskowski, 1971), were found to cleave the modified dinucleoside phosphates more slowly than their unmodified counterparts. Snake venom phosphodiesterase hydrolyzed the substrates more rapidly than bovine spleen phosphodiesterase. While UpεA was completely hydrolyzed by the venom exonuclease in less than 4 min, UpεC required the longest time, more than 30 min, for complete hydrolysis. Although no quantitative conclusion could be drawn, venom phosphodiesterase seemed to hydrolyze more slowly, the ε-Ado-containing substrates having the modified nucleotides in the 3′ position of the dinucleoside phosphate.

Endonucleases. Enzyme assays using pancreatic RNase A were carried out with the modified dinucleoside phosphates UpεA, UpεC, εCpεA, εCpG, εCpU, and εCpεC. Although the RNase A rapidly hydrolyzed UpεA ($t_{1/2} \sim 1$ min) and UpεC, the presence of the modified nucleoside in the 5′ end of the dinucleoside phosphate slowed the rate of hydrolysis in comparison with UpA and UpC. While assays containing εCpU and εCpεA showed no signs of cleavage following 2-hr RNase A treatment, εCpεC and εCpG showed traces of hydrolysis after 2-hr treatment; however, the hydrolysis proceeded at such a slow rate that it was almost negligible.

Assays utilizing RNase T_1 with GpεC and GpεA indicated that the presence of ε-adenosine or ε-cytidine in the 5′ end of the dinucleoside phosphate slowed the action of RNase T_1. While GpA was completely hydrolyzed following 30-min treatment with RNase T_1 using the assay described, the GpεA assay required longer reaction time for completion. Comparison of the two modified substrates showed that GpεC was hydrolyzed more rapidly than GpεA, indicating that the modification of cytidine with chloroacetaldehyde has less effect on substrate specificity than modification of adenosine.

TABLE I: Relative Reactivities of the Twelve ε-Dinucleoside Phosphates and Their Unmodified Counterparts in Nucleolytic Enzyme Reactions.[a]

	Exonucleases		Endonucleases		
Substrate	Venom Phosphodiesterase	Spleen Phosphodiesterase	RNase A	RNase T_1	RNase T_2
εApεA	+++	+++	0	0	−
ApA	+++	+++	0	0	+++
εApεC	+++	+++	0	0	−
ApC	+++	+++	0	0	+++
εCpεA	+++	++	−	0	−
CpA	+++	+++	+++	0	+++
εApU	+++	++	0	0	−
ApU	+++	+++	0	0	+++
UpεA	+++	+++	+++	0	++
UpA	+++	+++	+++	0	+++
εApG	+++	+++	0	0	−
ApG	+++	+++	0	0	+++
GpεA	+++	+++	0	++	+
GpA	+++	+++	0	+++	+++
εCpεC	+++	+++	+	0	+
CpC	+++	+++	+++	0	+++
εCpG	+++	+++	+	0	+
CpG	+++	+++	+++	0	+++
GpεC	+++	+++	0	+++	++
GpC	+++	+++	0	+++	+++
εCpU	+++	+++	−	0	−
CpU	+++	+++	+++	0	+++
UpεC	++	+++	+++	0	++
UpC	+++	+++	+++	0	+++

[a] Symbols used are: +++, hydrolysis complete in less than 30 min; ++, hydrolysis complete in less than 2 hr; +, incomplete hydrolysis following 2-hr incubation; −, no hydrolysis observed; 0, no assay was performed.

In enzyme assays utilizing RNase T_2 with each of the 12 ε-dinucleoside phosphates, again the modified substrates were hydrolyzed more slowly than their unmodified counterparts. The results shown in Table I were obtained using a crude enzyme preparation of RNase T_2 which contained some RNase T_1 activity (see Experimental Procedure). In assays using this enzyme, the modified substrates containing $1,N^6$-ethenoadenosine in the 3′ end of the dinucleoside phosphate showed no signs of hydrolysis following 2-hr incubation. The inability of the RNase T_2 preparation to hydrolyze ε-dinucleoside phosphates with modified nucleosides in the 3′ end was also observed in the ε-cytidine analogs since εCpεA and εCpU showed no signs of hydrolysis and εCpεC and εCpG showed only traces of hydrolysis following 2-hr incubation. However, the observed base specificity of RNase T_2 for $1,N^6$-ethenoadenosine and to a lesser degree for $3,N^4$-ethenocytidine is far from absolute and can be overcome by increasing the amount of enzyme present. When a stock solution of the highly purified Sankyo-brand

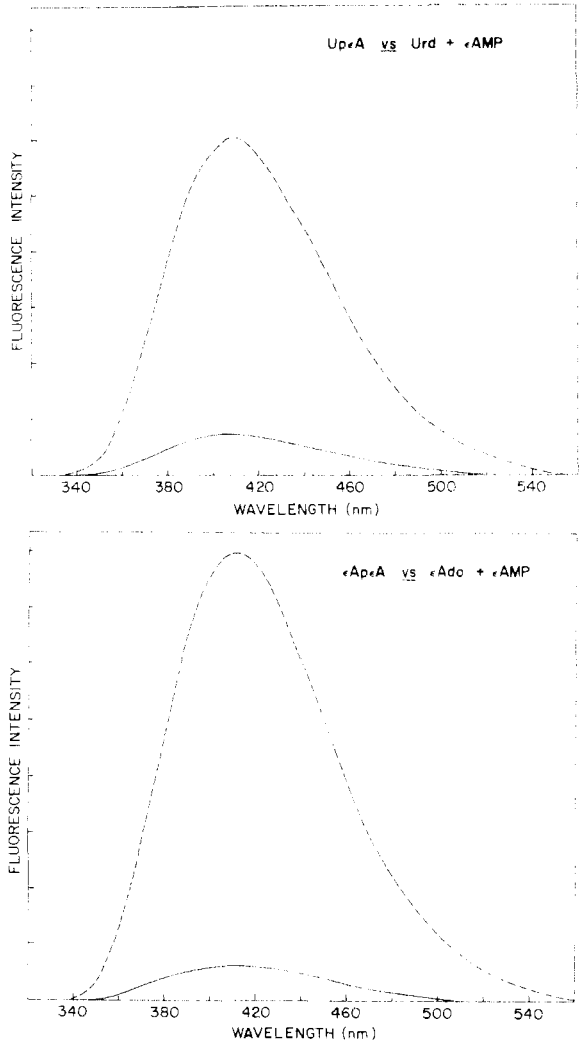

FIGURE 4: The technical fluorescence emission spectra (excitation at 305 nm) of the ε-dinucleoside phosphates UpεA and εApεA before (—) and after (- - -) complete enzymatic hydrolysis with snake venom phosphodiesterase.

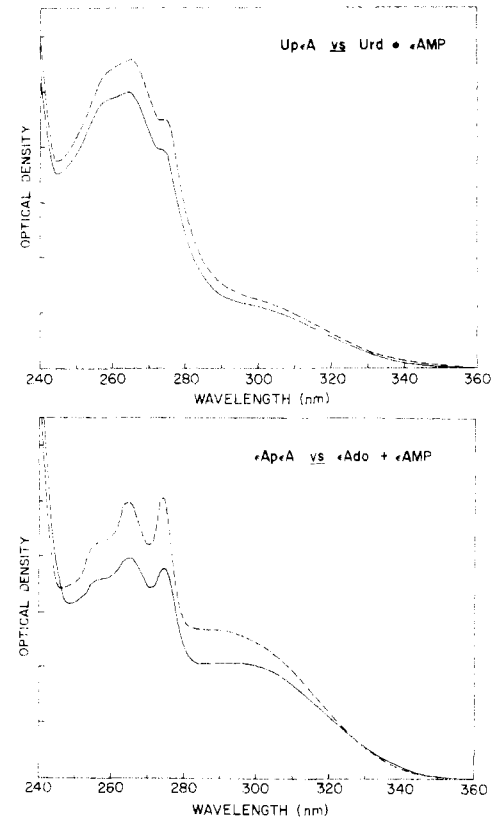

FIGURE 5: The ultraviolet absorption spectra of the ε-dinucleoside phosphates UpεA and εApεA before (—) and after (- - -) complete enzymatic hydrolysis with snake venom phosphodiesterase.

RNase T_2 (see Experimental Procedure) was used in the same assay, the ε-dinucleoside phosphates that were previously resistant to the action of RNase T_2 were fully hydrolyzed following 2-hr incubation.

Fluorescence Results. For the fluorescence studies, each compound was purified until identical values for fluorescence lifetimes were obtained by phase and modulation measurements using the cross-correlation fluorometer described by Spencer and Weber (1969). The close agreement between the phase and modulation values indicated that the emission observed was a single exponential decay and thus confirmed the fluorescent purity of each sample. The relative quantum efficiencies were determined by integration and comparison of the peak areas of the corrected fluorescence emission spectra recorded before and after complete enzymatic hydrolysis, examples of which are shown for εApεA and for UpεA in Figure 4. Corrections for differences in absorption (Figure 5) at the exciting wavelength before and after enzymatic hydrolysis were made so that the number of photons absorbed in both instances was equal. The values measured for the fluorescence lifetimes and quantum efficiencies of the seven $1,N^6$-ethenoadenosine dinucleoside phosphates and of the trimethylene-bridged dinucleotide model compound 9,9′-trimethylenebis-$1,N^6$-ethenoadenine are presented in Table II and reflect the quenching of the $1,N^6$-ethenoadenosine fluorescence present in each dinucleoside phosphate.

The sensitivity to environment in neutral solution of the fluorescence lifetime and quantum efficiency of $1,N^6$-ethenoadenosine has previously been of great value in investigations concerning the extent of intramolecular complexing in the modified coenzymes ϵNAD^+ and εFAD (Barrio *et al.*, 1972b, 1973). Among the dinucleoside phosphate molecules in which intramolecularly complexed forms are in dynamic equilibrium with open or extended forms, the quenching of fluorescence observed can be separated into time-independent and time-dependent processes. Some molecules are quenched immediately upon excitation because of the close proximity of the two nucleosides prior to excitation, producing a time-independent or static component, while the remaining excited molecules are subject to quenching by time-dependent or dynamic processes occurring during the lifetime of the excited state (Spencer, 1970). Assuming that under the experimental conditions used the contribution of the internally complexed molecules to the fluorescence emission was negligible in comparison with that contributed by the open forms, the parameter $\gamma = (F/F_0)(\tau_0/\tau)$, where F_0 and τ_0 are the quantum efficiency and lifetime of εAMP and F and τ are the corresponding quantities of the dinucleoside phosphate, has been used to characterize these processes which diminish the fluorescence of the $1,N^6$-ethenoadenosine in these compounds. When $\gamma = 1$, *i.e.*, $F/F_0 = \tau/\tau_0$, this corresponds to dynamic quenching in which the process that reduces the yield is strictly competitive with emission; when $\gamma = F/F_0$, *i.e.*, $\tau/\tau_0 = 1$, it corresponds to processes in which the quantum yield is exclusively reduced by a dark process, *i.e.*, one which predates excitation; and $1 > \gamma > F/F_0$ indicates the coexistence of both types of

TABLE II: Fluorescence Properties and Intramolecular Complexing of the Seven 1,N^6-Ethenoadenosine Dinucleoside Phosphates and 9,9′-Trimethylenebis(1,N^6-ethenoadenine).

Compd	Fluorescence Lifetime (nsec)	Quantum efficiency (Rel to εAMP)[a]	Deg of Internal Assn (%) $(1-\alpha)$ (±5%)	Dynamic Quenching: Efficiency τ/τ_0	Dynamic Quenching: Rate Constant k_+* ($\times 10^8$ sec^{-1}) $(1/\tau - 1/\tau_0)$	Static Quenching: Efficiency γ[b]	Static Quenching: Equilibrium Constant $[(1/\alpha) - 1]$
εApεA	4.5	0.072	68	0.20	1.79	0.37	2.12
εAde-C_3-εAde[c]	4.5	0.094[c]	65	0.19	1.80	0.49	1.86
εApG	4.6	0.084	62	0.20	1.74	0.42	1.63
GpεA	2.1	0.029	72	0.09	4.33	0.32	2.57
εApεC	8.2	0.179	58	0.36	0.79	0.50	1.38
εCpεA	12.3	0.490	15	0.53	0.38	0.92	0.18
εApU	5.5	0.143	44	0.24	1.39	0.60	0.79
UpεA	3.1	0.107	28	0.13	2.80	0.79	0.39

[a] Relative quantum efficiencies were determined by integration and comparison of corrected fluorescence emission spectra before and after complete enzymatic hydrolysis. All spectra were corrected for differences in absorption so that the number of photons absorbed before and after hydrolysis was equal. [b] $\gamma = F/F_0$, τ_0/τ; where F/F_0 is the relative quantum efficiency *vs.* εAMP and τ_0 is 23.0 nsec for εAMP (Spencer and Weber, 1972). [c] Quantum efficiency measured relative to 9-propyl-1,N^6-ethenoadenine (εAde-C_3); τ_0 = 23.6 nsec for εAde-C_3.

quenching processes. Accordingly, the value of γ for each compound given in Table II corresponds to the fraction of absorption transitions by free, unquenched fluorophore in the ground state relative to the total number of absorptions. Since the molar absorption coefficients of the open form (ϵ_0) and of the complexed form (ϵ_c) are not equal because of hypochromic effects, the degree of static quenching γ does not represent directly the degree of dissociation α of the dinucleoside phosphate but must be corrected for the difference in extinction coefficients of the two forms. In the dilute solutions used, the approximation $\alpha = (\bar{\epsilon}/\epsilon_0)(\gamma)$, where $\bar{\epsilon} = \epsilon_0\alpha + \epsilon_c(1-\alpha)$, can be used to obtain α from γ by substituting the extinction coefficient at the exciting wavelength of the dinucleoside phosphate for $\bar{\epsilon}$ and that of the component monomers following complete enzymatic hydrolysis for ϵ_0 (Spencer, 1970). Accordingly, the calculated values of $1-\alpha$ presented in Table II correspond to the degree of intramolecular association or the per cent of intramolecular complex present in each of the 1,N^6-ethenoadenosine dinucleoside phosphates. Thermodynamic parameters have previously been obtained from temperature dependence studies of the optical properties of several dinucleoside phosphates (Davis and Tinoco, 1968; Brahms *et al.*, 1967).

Because the low degree of intramolecular association (15%) of the two nucleosides in εCpεA indicated by the high relative quantum efficiency and long lifetime (relative to εAMP) determined for the 1,N^6-ethenoadenosine moiety in this dinucleoside phosphate seemed inconsistent, *prima facie*, with the results obtained for the other dinucleoside phosphates, especially those for εApεC, we made certain that we were dealing with the 3′ to 5′-linked dinucleoside phosphate and not with one having a 2′ to 5′ or 5′ to 5′ linkage. A second sample of CpA was obtained from an alternate commercial source and was converted to εCpεA using identical experimental conditions. The fluorescence lifetime and relative quantum efficiency of the second sample of εCpεA were identical with those measured for the original sample. To investigate further the intramolecular interactions of εCpεA in comparison with those in εApεC, the circular dichroic spectra in neutral solution of εApεC, εCpεA, and a mixture of the component nucleosides were recorded and are shown in Figure 6. The circular dichroic (CD) spectrum of εApεC indicates a large change from the summation of CD values for the individual components, indicative of stacking (Warshaw and Tinoco, 1966; Warshaw and

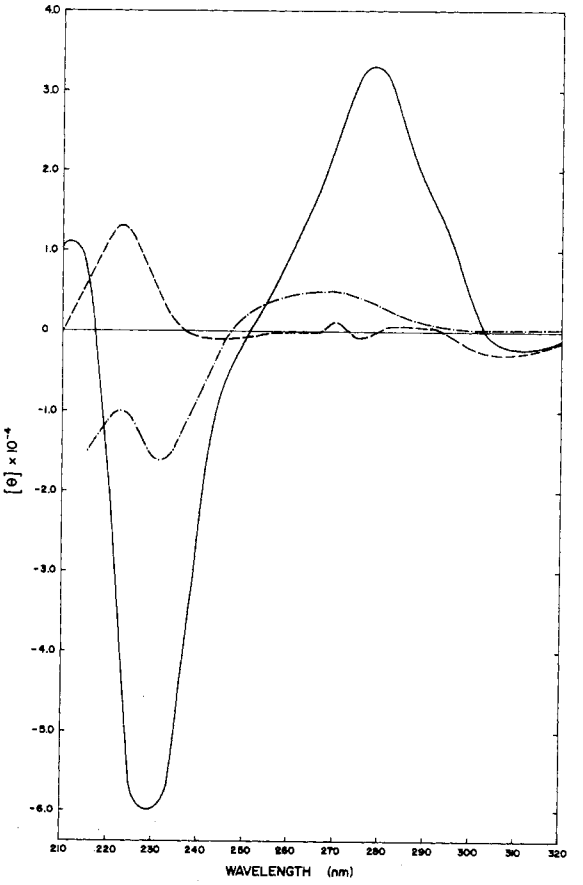

FIGURE 6: The circular dichroic spectra of the ε-dinucleoside phosphates εApεC (—) and εCpεA (- - -) and of their component nucleosides ε-adenosine and ε-cytidine (– • –) in aqueous solution buffered to pH 7.0 (0.05 M phosphate buffer).

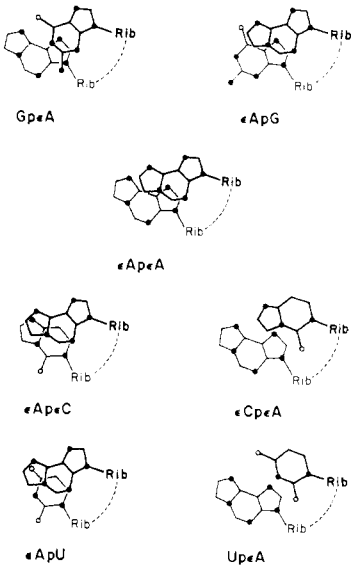

FIGURE 7: Conformations of the seven 1,N^6-ethenoadenosine dinucleoside phosphates as viewed normal to the planes of the bases and drawn as though they were part of an RNA-11 helix (Arnott, 1971; Bugg *et al.*, 1971).

Cantor, 1971) and indicative further of dissymmetrical stacked conformations similar to those previously observed with other dinucleoside phosphates (Brahms *et al.*, 1967). By contrast, the circular dichroism of εCpεA is closer to that of the component parts, showing no induced CD effect and indicating that the contribution of dissymmetrical stacked conformations to εCpεA in buffered solution is very small.

Discussion

The masking of N-1 and N^6 of adenosine and N-3 and N^4 of cytidine in the reaction of these dinucleoside phosphates with chloroacetaldehyde renders them more resistant to the action of the common nucleases, and this fact in itself gives an indication of the limitations on substrate structure. The observed resistance of the etheno-bridged substrates to exonuclease digestion was expected on the basis of previous results which have shown that the modified nucleosides in tRNA are more resistant to the action of snake venom phosphodiesterase than are the normal bases (Laskowski, 1971). Consistent with the previous observation that substitution of a bulky function at N-3 of the pyrimidine moiety invariably renders normal substrates resistant to RNase A (Richards and Wyckoff, 1971), the results of Kochetkov and coworkers (1972) with εCpU and the results presented here show that the dinucleoside phosphates of the general formula 3,N^4-ethenocytidylyl(3′→5′) nucleoside are not hydrolyzed by pancreatic RNase A. In line with the observations made in the cleavage of GpεA and GpεC by RNase T_1, the rate of cleavage of the 3′-guanylyl phosphodiester bond by RNase T_1 is known to be sensitive to the neighboring nucleoside (Uchida and Egami, 1971). With respect to the results with RNase T_2, although substitution of a bulky group at N^6 of adenosine has been shown to have no effect on RNase T_2 action, the masking of N-1 upon modification with chloroacetaldehyde was expected to increase its resistance to RNase T_2 cleavage since substitution at N-1 has previously produced increased resistance (Uchida and Egami, 1971). The increased resistance of etheno-bridged dinucleoside phosphates and oligonucleotides to nucleolytic digestion has implications in further studies of chloroacetaldehyde-treated tRNA, since the conditions of enzymatic hydrolysis must therefore be more strenuous to ensure total hydrolysis of all phosphodiester bonds present. Further, the high degree of resistance of εCpN phosphodiester bonds to RNase A action and of εApN phosphodiester bonds to RNase T_2 action can actually be turned to use in sequencing chloroacetaldehyde-treated oligonucleotides and tRNAs.

Although the quenching of 1,N^6-ethenoadenosine in the dinucleoside phosphate shows a dependence on the identity of the neighboring nucleoside, examination of the fluorescence quantum efficiency and lifetime of the 1,N^6-ethenoadenosine in the compounds studied indicates that the fluorescence emission is greatly quenched in both dynamic and static processes by all of the neighboring nucleosides. In taking the degree of intramolecular association as an indication of the stacking interaction present in each dinucleoside phosphate, guanosine and 1,N^6-ethenoadenosine seem to participate in stacking interactions in the dinucleoside phosphate equally well, since the $1 - \alpha$ values for GpεA and εApεA are in the same range. The lower values observed for the degree of internal association of εApU and UpεA indicate less base–base interaction between uridine and 1,N^6-ethenoadenosine than in the case of guanosine or 1,N^6-ethenoadenosine. According to the degree of intramolecular association calculated for εApεC, the ability of 3,N^4-ethenocytidine to participate in stacking interactions seems to be intermediate between 1,N^6-ethenoadenosine and uridine attached through the 5′-hydroxyl; however, the $1 - \alpha$ value calculated for εCpεA falls below that of UpεA (attachment through the 3′-hydroxyl). The ordering of purine–1,N^6-ethenoadenosine interactions above pyrimidine–1,N^6-ethenoadenosine interactions is consistent with the general ordering of base–base interactions found in dinucleoside phosphates (Warshaw and Tinoco, 1965, 1966) and in dinucleotide analogs (Browne *et al.*, 1968) as judged by optical properties including hypochromism, optical rotary dispersion (ORD), and fluorescence emission (the latter at low temperature).

The dependence of the extent of base–base interactions on the sequence of the nucleosides in the dinucleoside phosphate has previously been observed in hypochromism and ORD studies of the 16 possible dinucleoside phosphates (Warshaw and Tinoco, 1966) and clearly arises from the constraints imposed by the ribose–phosphate–ribose backbone of the dinucleoside phosphate. For greater insight concerning the patterns of base–base interactions allowed by the ribose–phosphate–ribose backbone of the dinucleoside phosphate, each of the seven 1,N^6-ethenoadenosine dinucleoside phosphates is drawn in Figure 7 in the proposed conformation of the dinucleoside phosphate *as if it were part of the 11-fold RNA helix described by Arnott* (Arnott, 1971; Bugg *et al.*, 1971). We recognize that we are not supplying data that support singular stacked conformations, but we shall look for consistency with these representative sectional conformations. Heavily hydrated crystals of GpC and ApU have been shown by X-ray analysis to be similar in conformation to those proposed for RNA-11 (Rosenberg *et al.*, 1973; Day *et al.*, 1973). The conformations shown for the 1,N^6-ethenoadenosine dinucleoside phosphates based on the RNA-11 helix are taken as reasonable working approximations for these compounds in solution, at the same time bearing in mind that the dinucleoside phosphates have greater freedom of orientation *either* toward more extensive overlap *or* out of the helical confor-

mations pictured in Figure 7. As is suggested in Figure 7, the ribose-phosphate-ribose backbone allows greater interaction between the 1,N^6-ethenoadenosine and its neighboring nucleoside in ϵApϵC and ϵApU than in ϵCpϵA and UpϵA, respectively, and this greater allowed interaction would account for the internal association observed. The slightly greater degree of association observed for GpϵA over that of ϵApG, based on the RNA-11 model, could be due to the positioning of the polar 2-amino group of guanosine over the 1,N^6-ethenoadenosine ring suggesting a stronger interaction (Bugg *et al.*, 1971) than the overlap of the 1,N^6-ethenoadenosine ring with the guanosine in ϵApG. The sequence effect on base-base interactions observed among the seven fluorescent modified dinucleoside phosphates follows the order observed for their unmodified counterparts, *i.e.*, GpA > ApG, ApC > CpA, and ApU > UpA (Warshaw and Tinoco, 1966). Since the values for the degrees of association of ϵApϵA and of ϵAde-C_3-ϵAde, in which the trimethylene bridge joining the two 1,N^6-ethenoadenines allows the bases to be near plane parallel, are essentially the same in aqueous solution, the ribose-phosphate-ribose backbone prescribes as much base-base interaction as does the trimethylene bridge (see Browne *et al.*, 1968).

The circular dichroism and the degree of intramolecular association obtained from fluorescence data for ϵCpϵA indicate a very low degree of base-base interaction in this 1,N^6-ethenoadenosine dinucleoside phosphate. Although the proposed helical conformation pictured in Figure 7 suggests that the ribose-phosphate-ribose backbone may restrict the overlap of the two ring systems, this type of conformation does not account for the almost total lack of interaction between the purine-like 3,N^4-ethenocytidine and the 1,N^6-ethenoadenosine. Since we have ruled out the possibility that the phosphodiester linkage is 2′ to 5′ or 5′ to 5′ instead of 3′ to 5′, perhaps the conformation of ϵCpϵA in aqueous solution is not helical but instead is similar to those observed in crystalline UpA in which the two rings do not overlap (Rubin *et al.*, 1972; Sussman *et al.*, 1972). The adoption of this type of conformation in ϵCpϵA, which is only about 0.5 kcal/mol higher in energy than the lowest energy helical conformation according to potential energy calculations (for UpA: Brodye *et al.*, 1974), may be invoked to account for the minimal interaction between the bases in ϵCpϵA.

Although extension of the results obtained for the modified fluorescent dinucleoside phosphates to predict the degree of internal association present in their unmodified counterparts is not strictly valid, the degrees of association found for the seven 1,N^6-ethenoadenosine dinucleoside phosphates probably represent an *upper limit* of the extent of complex formation in the corresponding unmodified compounds. Moreover, the fluorescence technique has permitted these results to be obtained for extremely dilute solutions (5×10^{-5} M). The results can be used directly in studies involving chloroacetaldehyde-treated oligonucleotides and tRNA. The quenching of the 1,N^6-ethenoadenosine fluorescence due to stacking interactions in the chloroacetaldehyde-treated tRNA promises to be useful in monitoring the disruption of tertiary structure. These results are valuable in our further application of the chloroacetaldehyde modification reaction to tRNAs.

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References

Arnott, S. (1971), *Progr. Biophys. Mol. Biol. 22,* 181.

Barrio, J. R., Secrist, J. A., III, and Leonard, N. J. (1972a), *Biochem. Biophys. Res. Commun. 46,* 597.

Barrio, J. R., Secrist, J. A., III, and Leonard, N. J. (1972b), *Proc. Nat. Acad. Sci. U.S. 69,* 2039.

Barrio, J. R., Tolman, G. L., Leonard, N. J., Spencer, R. D., and Weber, G. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 941.

Beardsley, K., and Cantor, C. R. (1970), *Proc. Nat. Acad. Sci. U.S. 65,* 39.

Bernardi, A., and Bernardi, G. (1971), *Enzymes, 3rd Ed., 4,* 329.

Brahms, J., Maurizot, J. C., and Michelson, A. M. (1967), *J. Mol. Biol. 25,* 481.

Brdar, B., and Reich, E. (1972), *J. Biol. Chem. 247,* 725.

Brodye, S. B., Stellman, S. D., Hingerty, B., and Langridge, R. (1974), *Biopolymers* (in press).

Browne, D. T., Eisinger, J., and Leonard, N. J. (1968), *J. Amer. Chem. Soc. 90,* 7302.

Bugg, C. E., Thomas, J. M., Sundaralingam, M., and Rao, S. T. (1971), *Biopolymers 10,* 175.

Cantor, C. R., and Tinoco, I., Jr. (1965), *J. Mol. Biol. 13,* 65.

Churchich, J. E. (1963), *Biochim. Biophys. Acta 75,* 274.

Davis, R. C., and Tinoco, I., Jr. (1968), *Biopolymers 6,* 223.

Day, R. O., Seeman, N. C., Rosenberg, J. M., and Rich, A. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 849.

Friest, W., Maelicke, A., Sprinzl, M., and Cramer, F. (1972), *Eur. J. Biochem. 31,* 215.

Henderson, R. E. L., Kirkegaard, L. H., and Leonard, N. J. (1973), *Biochim. Biophys. Acta 294,* 356.

IUPAC-IUB Commission on Biochemical Nomenclature (1971), *J. Mol. Biol. 55,* 299.

Janik, B., Sommer, R. G., Kotick, M. P., Wilson, D. P., and Erickson, R. J. (1973), *Physiol. Chem. Phys. 5,* 27.

Kirkegaard, L. H., Johnson, T. J. A., and Bock, R. M. (1972), *Anal. Biochem. 50,* 122.

Kochetkov, N. K., Shibaev, V. N., and Kost, A. A. (1972), *Dokl. Akad. Nauk SSSR 205,* 100.

Laskowski, M., Sr. (1971), *Enzymes, 3rd Ed., 4,* 313.

Lehrach, H., and Scheit, K. H. (1973), *Biochim. Biophys. Acta 308,* 28.

Lynch, D. C., and Schimmel, P. R. (1974), *Biochemistry 13,* 1841, 1852.

Maelicke, A., Sprinzl, M., von der Haar, F., Khwaja, T. A., and Cramer, F. (1974), *Eur. J. Biochem. 43,* 617.

Millar, D. B., and Steiner, R. F. (1966), *Biochemistry 5,* 2289.

Pachmann, U., Cronwell, E., Rigler, R., Hirsch, R., Wintermeyer, W., and Zachau, H. G. (1973), *Eur. J. Biochem. 39,* 265.

Richards, F. M., and Wyckoff, H. W. (1971), *Enzymes,*

3rd Ed., 4, 647.
Rosenberg, J. M., Seeman, N. C., Kim, J. J. P., Suddath, F. L., Nicholas, H. B., and Rich, A. (1973), *Nature (London) 243,* 150.
Rubin, J., Brennan, T., and Sundaralingam, M. (1972), *Biochemistry 11,* 3112.
Secrist, J. A., III, Barrio, J. R., and Leonard, N. J. (1971), *Biochem. Biophys. Res. Commun. 45,* 1262.
Secrist, J. A., III, Barrio, J. R., Leonard, N. J., and Weber G. (1972), *Biochemistry 11,* 3499.
Spencer, R. D. (1970), Ph.D. Thesis, University of Illinois, p 260.
Spencer, R. D., Vaughn, W. M., and Weber, G. (1969), *in* Molecular Luminescence, Lim, E. C., Ed., New York, N.Y., W. A. Benjamin, p 607.
Spencer, R. D., and Weber, G. (1969), *Ann. N. Y. Acad. Sci. 158,* 361.
Spencer, R. D., and Weber, G. (1972), *in* Structure and Function of Oxidation-Reduction Enzymes, Ehrenberg, A., and Akeson, A., Ed., New York, N.Y., Pergamon Press, p 393.
Spencer, R. D., Weber, G., Tolman, G. L., Barrio, J. R., and Leonard, N. J. (1974), *Eur. J. Biochem. 45,* 425.
Steiner, R. F., Kinnier, W., Lunasin, A., and Delac, J. (1973), *Biochim. Biophys. Acta 294,* 24.
Sussman, J., Seeman, N. C., Kim, S.-H., and Berman, H. (1972), *J. Mol. Biol. 66,* 403.
Uchida, T., and Egami, F. (1971), *Enzymes, 3rd Ed., 4,* 205.
Ward, D. C., Reich, E., and Stryer, L. (1969), *J. Biol. Chem. 244,* 1228.
Warshaw, M., and Cantor, C. R. (1971), *Biopolymers 9,* 1079, and references therein.
Warshaw, M., and Tinoco, I., Jr. (1965), *J. Mol. Biol. 13,* 54.
Warshaw, M., and Tinoco, I., Jr. (1966), *J. Mol. Biol. 20,* 29.
Wintermeyer, W., and Zachau, H. G. (1974), *Methods Enzymol. 29,* 667.
Yang, C. H., and Söll, D. (1973), *Arch. Biochem. Biophys. 155,* 70.
Yang, C. H., and Söll, D. (1974a), *J. Biochem. (Tokyo) 73,* 1243.
Yang, C. H., and Söll, D. (1974b), *Biochemistry 13,* 3615.

The Mode of Interaction of Mitomycin C with Deoxyribonucleic Acid and Other Polynucleotides *in Vitro* †

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ABSTRACT: Reductive activation of mitomycin to a partially reduced form rather than to the fully reduced hydroquinone greatly increases the binding affinity of the drug to native and denatured DNA: complexes of up to a few nucleotides per mitomycin molecule are readily formed in a few minutes at room temperature. The partially reduced form of mitomycin is believed to be the semiquinone radical. The complexes exhibit the following properties: same ultraviolet spectrum as apomitomycin derivatives, increased T_m, resistance to enzymatic hydrolysis. Bound mitomycin is not released under a variety of strong conditions known to dissociate noncovalent complexes. Depurination releases the drug and normal amounts of free guanine and adenine. Upon acid hydrolysis of the complex to bases normal base ratios are obtained. Monomeric nucleotides and GpC do not react with activated mitomycin. Poly(dG) binds mitomycin well while binding to poly(dA), -(dT), or -(dC) is negligible. Binding to poly(dG·dC) or poly(dI·dC) is relatively inhibited. Cross-linking is not observed with ds-RNA. The cross-links in DNA are much more stable than bifunctional mustard-induced cross-links. The hydrolysis product of mitomycin lacking the aziridine ring binds well to DNA but exhibits no cross-linking activity. A model is proposed to explain the results. Covalent bond formation is preceded by a noncovalent association, presumably of the intercalative type, between the mitomycin semiquinone and the polynucleotide, which then facilitates covalent bond formation between appropriately situated functional groups of DNA and the antibiotic. The site of covalent bonds is possibly the O^6 position of guanine.

A large variety of antibiotics are known to interact with the nucleic acids of the cell. As these drugs combine with DNA and/or RNA they modify the natural structure of the nucleic acid, resulting in an inhibition of its template function. Frequently this effect is one or even the only fundamental cause of the biological effect of the antibiotic, as, for example, in the case of the most extensively studied nucleic acid modifying antibiotic, actinomycin. Since most antibiotics are complex molecules the structure of the drug–DNA complex is not known with certainty in a single case. In most instances the affinity in such complexes is noncovalent. Covalent association has been recognized, however, in one unique case: the mitomycin antibiotics. (For a recent review of this field and specific references see Goldberg and Friedman, 1971.)

The mitomycins, objects of the present study, are a class of potent antibiotics. They also exhibit high antitumor activity and act as lysogenic inducers, mutagens, and carcinogens. (For specific references, see Szybalski and Iyer, 1967.) The most studied member of the group is mitomycin C (I) although the others, *e.g.*, porfiromycin (II), are very similar, both chemically and biologically. They are small

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