Proton Magnetic Resonance Studies of Ribose Dinucleoside Monophosphates in Aqueous Solution. II. The Nature of the Base-Stacking Interaction in Adenylyl-(3′ → 5′)-cytidine and Cytidylyl-(3′ → 5′)-adenosine[1]

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Abstract: The nature of the intramolecular base-stacking interaction in aqueous solution between the adenine and cytosine bases in adenylyl-(3′→5′)-cytidine (ApC) and cytidylyl-(3′→5′)-adenosine (CpA) has been investigated by proton magnetic resonance spectroscopy. The pmr spectra of both dinucleoside monophosphates were studied as a function of concentration and temperature. The results of these pmr studies indicate that the intramolecular base-stacking interaction between the adenine and cytosine bases in these dinucleoside monophosphates is rather strong, and that the stacking tendencies are comparable for the two sequence isomers. A comparison of the data obtained in this work for ApC and CpA with those previously reported for adenylyl-(3′→5′)-adenosine (ApA) suggests that the intramolecular stacking interaction is somewhat stronger in ApA than in ApC and CpA. The chemical shifts of the cytosine H_5 and adenine H_2 protons, and their variations with temperature, were shown to be consistent with stacked conformations in which both bases of the dinucleoside monophosphates are preferentially oriented in the *anti* conformation as in similar dApdC and dCpdA (dA ≡ deoxyadenosine; dC ≡ deoxycytidine) segments of double-helical DNA. The intramolecular stacking interaction was found to have a pronounced effect on the conformation of the ribose moieties, and these conformational changes are discussed. This work also reveals a small magnetic anisotropy for the cytosine base, which had not been detected in previous work. The concentration studies indicate extensive self-association of these dinucleoside monophosphates, and analysis of the concentration data facilitated determination of the dimerization constant for the association process as well as the nature of the intermolecular complexes.

This paper is the second in the series on proton magnetic resonance studies of the conformation of ribose dinucleoside monophosphates in aqueous solution. In the first paper in this series,[4] an investigation of the base-stacking interaction between the two adenine rings in adenylyl-(3′→5′)-adenosine (ApA) by proton magnetic resonance spectroscopy was reported. The pmr spectrum of ApA was studied as a function of concentration, temperature, and concentration of added purine. From the results of these studies, it was possible to make some rather definite conclusions regarding the conformation of the two adenine rings in this dinucleotide. It was shown that the stacking interaction between the two adenine bases is relatively strong and that the adenine rings are stacked with each of the bases preferentially oriented in the *anti* conformation as in a similar dApdA (dA = deoxyadenosine) segment in double-helical DNA.

This paper summarizes the results of a similar study for two other ribose dinucleoside monophosphates: adenylyl-(3′→5′)-cytidine (ApC; Figure 1) and cytidylyl-(3′→5′)-adenosine (CpA). In ApC and CpA, the stacking interaction is between a purine base and a pyrimidine base; hence, we expect the intramolecular stack here to be less stable thermodynamically than that in ApA, where the stacking interaction is between two purine bases. Since ApC differs from CpA only in esterification of the phosphate group to the ribose moieties of the two nucleosides, this structural difference may lead to important sequence-dependent effects, and it is therefore of interest to compare the results obtained for the two sequence isomers.

Experimental Section

Materials. A-grade ApC, CpA, adenosine, and cytidine were obtained from Calbiochem, Los Angeles, Calif., and were used without further purification. A column of Dowex 50W-X8 cation exchange resin was used to convert ApC and CpA from the acid forms to their respective sodium salts. All samples were prepared in D_2O (99.7 mol %), which was supplied by Columbia Organic Chemicals, Columbia, S. C. All measurements were made at neutral pH and solutions were not buffered.

Instrumentation. The 100-Mcps pmr spectra were obtained on a Varian HA-100 nmr spectrometer operated in the frequency-sweep mode. The normal probe temperature was 29 ± 1°. In the variable-temperature studies, the sample temperature was controlled to ±1° by means of a Varian V-4343 variable-temperature controller and was determined using the methanol and ethylene glycol samples and calibration curves supplied by Varian. Tetramethylsilane (TMS) in a sealed capillary was used as an external reference and provided the field-frequency-lock signal. Chemical shifts were measured to ±0.1 cps by counting the frequency difference between the reference and sweep oscillators with a Hewlett-Packard 5512-A frequency counter. Except where specified otherwise, the chemical shifts reported are referred to the TMS capillary. A Varian C-1024 time-averaging computer was used to enhance weak signals.

Results and Discussion

Spectra and Assignment of Proton Resonances. The 100-Mcps pmr spectra at 29° of 0.010 *M* solutions of the sodium salts of ApC and CpA are presented in Figures 2 and 3. The region of the adenine H_8 and H_2 and cytosine H_6 protons is shown in Figure 2a,b, and the region of the cytosine H_5 and ribose $H_{1'}$ protons is depicted in Figure 3a,b. For comparison, the spectra

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(4) S. I. Chan and J. H. Nelson, *J. Am. Chem. Soc.*, **91**, 168 (1969).

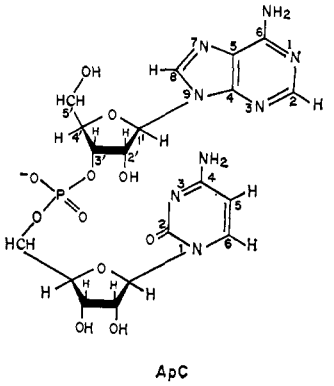


Figure 1. Adenylyl-(3′→5′)-cytidine.

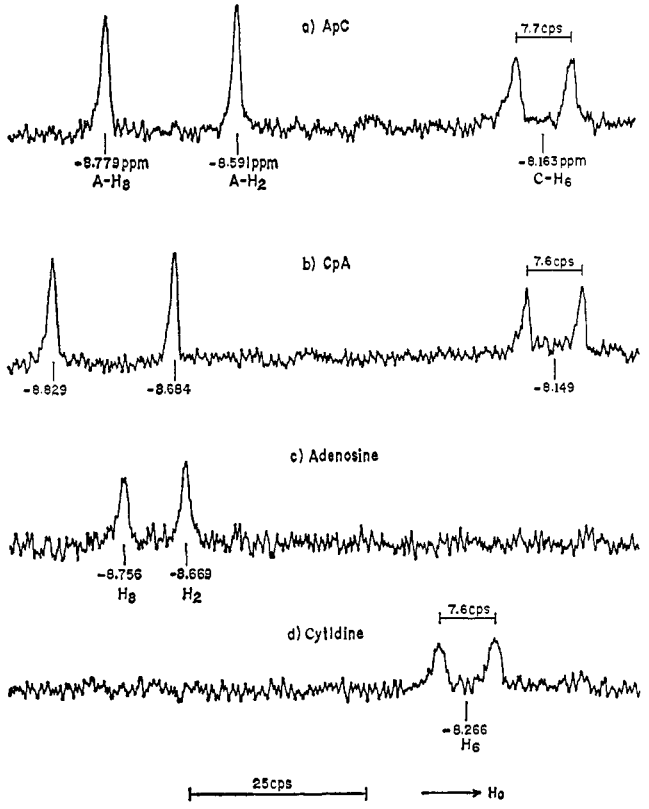


Figure 2. Time-averaged 100-Mcps pmr spectra at 29° of 0.010 *M* solutions of (a) ApC (sodium salt), (b) CpA (sodium salt), (c) adenosine, and (d) cytidine, in the region of the adenine H_2, H_8 and cytosine H_6 protons. Each spectrum represents the accumulation of 31 scans.

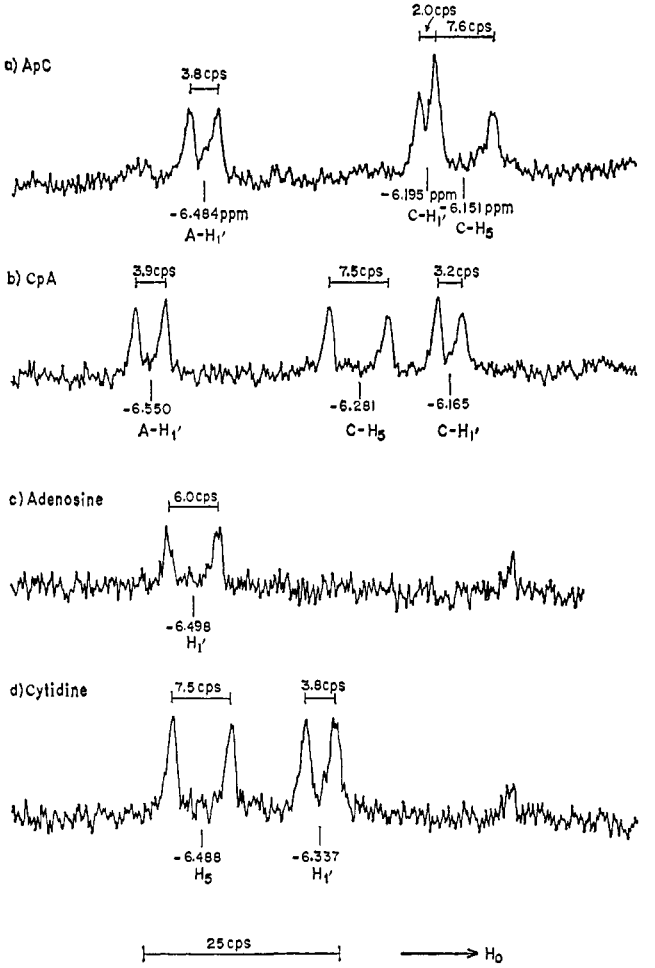


Figure 3. Time-averaged 100-Mcps pmr spectra at 29° of 0.010 *M* solutions of (a) ApC (sodium salt), (b) CpA (sodium salt), (c) adenosine, and (d) cytidine, in the region of the adenosine $H_{1'}$ and cytidine H_5, $H_{1'}$ protons. Each spectrum represents the accumulation of 31 scans.

of the adenosine and cytidine nucleosides in the same spectral regions under similar experimental conditions are shown in Figures 2c,d and 3c,d.

The assignment of the dinucleotide resonances depicted in Figures 2 and 3 is straightforward from comparison with the nucleoside spectra. As in the case of other purine derivatives, the adenine H_8 protons of ApC and CpA are readily distinguished from the H_2 protons by deuterium exchange of the H_8 protons upon equilibration in D_2O at elevated temperatures.[5] In this manner, the resonance at lowest field has been assigned to the H_8 proton in both ApC and CpA. The cytosine H_6 and H_5 protons are spin–spin coupled to give doublets, with the coupling constant, $|J_{H_5-H_6}| = 7.6$ cps, in both ApC and CpA as well as in the monomeric cytidine nucleoside. The ribose $H_{1'}$ resonances are doublets from coupling with the $H_{2'}$ protons. The coupling constants, $|J_{H_{1'}-H_{2'}}|$, however, change markedly in going from the nucleosides to the dinucleotides. In both ApC and CpA, the lower field $H_{1'}$ doublet can be assigned to the adenosine nucleoside. Because of the ring-current magnetic anisotropy of the adenine base, the $H_{1'}$ ribose resonances of the purine nucleosides are expected to appear at *lower* fields compared to those of the pyrimidine nucleosides.

In addition to the resonances shown in Figures 2 and 3, the ribose $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, and $H_{5''}$ protons give rise to resonances in the spectral region from −4.20 to −5.20 ppm. There is extensive spin–spin coupling among these protons, and the resonances due to the 3′- and 5′-esterified nucleosides do not coincide. As a result of these complexities, this spectral region was not investigated to any extent. Finally, the hydroxyl and amino group protons do not give distinct resonances because of rapid chemical exchange of these protons with the solvent, D_2O.

Discussion of the Proton Chemical Shifts. All of the monitored resonances of ApC and CpA are shifted from their spectral positions in the component nucleo-

(5) M. P. Schweizer, S. I. Chan, G. K. Helmkamp, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **86**, 696 (1964).

sides. This is due in part to the effect of the singly charged phosphate group, and resonances of analogous protons on 3′- and 5′-esterified nucleosides are affected differently because of the difference in phosphate attachment. The influence of the phosphate group on the chemical shifts of the base protons is primarily a consequence of its negative charge and resulting electric field. The properties of the neighboring base can also have an effect on the chemical shifts of protons of a particular base in a dinucleotide. The adenine base in both ApC and CpA possesses a large ring-current magnetic anisotropy which can produce upfield shifts in the proton resonances of the neighboring cytosine base. The cytosine base, however, appears to have a negligibly small magnetic anisotropy from previous work.[6]

Recently, Prestegard and Chan[7] demonstrated that the chemical shifts of the adenine H_8 proton and the uracil H_6 proton are dependent upon the rotational conformation of the base about the glycosidic bond in the nucleosides and mononucleotides. This work also showed that the base conformation about the glycosidic bond can influence the conformation of the ribose ring to which the base is attached. In the dinucleotides, the intramolecular stacking interaction between the two bases can affect both the rotational conformation of each base relative to its ribose ring and the conformations of the ribose moieties. Hence, the factors which affect the chemical shifts of the adenine and guanine H_8 protons and those of the uracil and cytosine H_6 protons can be quite complex in the dinucleotides.

The effect of the phosphate group on the chemical shifts of the cytidine H_6, H_5, and $H_{1'}$ protons of ApC and CpA can approximately be canceled out by referring the chemical shifts of these protons to those of the corresponding 3′- or 5′-esterified cytidine nucleosides of CpC.[8] In Table I are summarized the chemical shifts of these cytidine protons in 0.01 *M* ApC, CpA, and CpC (all sodium salts), and the differences in the chemical shifts resulting from the substitution of adenine for cytosine as the adjacent base in ApC and CpA are indicated. The cytidine H_6, H_5, and $H_{1'}$ resonances in ApC and CpA can be seen to be shifted appreciably upfield from their corresponding spectral positions in CpC.

Table I. Chemical Shifts (in ppm) of Cytidine Proton Resonances Resulting from the Substitution of Adenine for Cytosine as the Adjacent Base in a Dinucleoside Monophosphate (Concentration 0.010 *M*)

	H_6		H_5		$H_{1'}$	
	3′	5′	3′	5′	3′	5′
ApC		−8.163		−6.151		−6.195
CpA	−8.149		−6.281		−6.165	
CpC	−8.308	−8.358	−6.400	−6.408	−6.242	−6.331
Shift	+0.159	+0.195	+0.119	+0.257	+0.077	+0.136

The upfield shifts observed for the cytidine H_5 resonances in ApC and CpA relative to CpC must have their origin primarily in the ring-current magnetic anisotropy of the adjacent adenine base, since the resonances of these protons in the pyrimidine nucleosides and nucleotides have been shown[7] to be relatively insensitive to the conformation of the ribose ring and the rotational conformation of the base about the glycosidic bond. On the basis of the work by Prestegard and Chan, however, one would expect the chemical shifts of the cytidine H_6 protons in ApC and CpA to be influenced by the conformation of the cytosine base relative to the ribose ring, as well as by the ring-current magnetic anisotropy of the neighboring adenine base.

In this work, we have adopted the nomenclature of Donohue and Trueblood[9] in describing the relative orientation of the planar purine or pyrimidine ring with respect to the sugar moiety. The angle of rotation about the glycosidic bond between $C_{1'}$ of the ribose and N_9 of a purine (or N_1 of a pyrimidine) is specified by the torsion angle ϕ_{CN}, which is defined as the dihedral angle between the plane of the base and the plane formed by the $C_{1'}$–$O_{1'}$ bond of the furanose ring and the $C_{1'}$–N_9 (or $C_{1'}$–N_1) glycosidic bond. The torsion angle, ϕ_{CN}, is 0° when C_8 of a purine base (or C_6 of a pyrimidine base) is eclipsed with the ether oxygen ($O_{1'}$) of the furanose ring, and positive angles are measured when the base is rotated in the clockwise direction when viewing from N_9 (or N_1) to $C_{1'}$. The rotational conformation of the base about the glycosidic bond is generally categorized as either *syn* or *anti*. The *anti* conformation describes a range of torsion angles centered at $\phi_{CN} \sim -30°$, and the *syn* conformation denotes the range of torsion angles centered at $\phi_{CN} \sim +150°$. For steric reasons, the base of a pyrimidine nucleoside is expected to be preferentially oriented in the *anti* conformation.[10] The base conformation in purine nucleosides can be either *anti* or *syn*, though the *anti* conformation is again expected to be more stable.

Prestegard and Chan found that the uridine H_6 resonance shifted upfield as the ribose $H_{1'}$–$H_{2'}$ coupling constant, $|J_{H_{1'}-H_{2'}}|$, became smaller with the addition of salt to a solution of the nucleoside or nucleotide. A decrease in $|J_{H_{1'}-H_{2'}}|$ is indicative of a change in average ribose conformation from 2′-*endo* toward 3′-*endo* ring puckering.[11] Consideration of Corey–Pauling–Koltun molecular models indicates that the conformation of the pyrimidine base relative to the ribose ring is quite restricted when the ribose conformation is 2′-*endo*, with the H_6 proton quite close to the ether oxygen of the furanose ring ($\phi_{CN} \sim -10$ to $-40°$). A change in ribose conformation toward 3′-*endo* permits a greater range of allowed torsion angles ($\phi_{CN} \sim -10$ to $-90°$) about the glycosidic bond for the base, and the H_6 proton would on the average be further from the ether oxygen. The change in the chemical shift of the H_6 proton with changing ribose conformation was attributed to the electric field effect of the ether oxygen. It was felt by Prestegard and Chan that H_6 is deshielded when it is in close proximity to the ether oxygen, so that the H_6 resonance shifts downfield as the base–ribose conformation is restricted to average torsion angles of smaller negative values.

In the dinucleotides, the intramolecular stacking interaction between the two bases is expected to play

(6) M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **87**, 5241 (1965).
(7) J. H. Prestegard and S. I. Chan, *ibid.*, **91**, in press.
(8) B. W. Bangerter and S. I. Chan, manuscript in preparation.
(9) J. Donohue and K. N. Trueblood, *J. Mol. Biol.*, **2**, 363 (1960).
(10) A. E. V. Haschemeyer and A. Rich, *ibid.*, **27**, 369 (1967).
(11) C. D. Jardetzky, *J. Am. Chem. Soc.*, **84**, 62 (1962).

an important role in determining the base conformations. Examination of CPK molecular models of these molecules, for example, indicates that, for effective base–base overlap, the pyrimidine bases must assume rotational conformations with ϕ_{CN} near 0 to $-10°$, regardless of the conformation of the ribose rings. Hence, if base–base overlap were to serve as the important criterion for the extent of the base-stacking interaction, we would expect the H_6 proton to be on the average more nearly eclipsed with the ether oxygen of the ribose ring, the stronger the stacking interaction between the two bases. There is indeed experimental evidence to support this. In CpC,[8] where the two cytosine bases have also been shown to be intramolecularly stacked at room temperature, the cytosine H_6 resonance of the 3′-esterified nucleoside was found to be 0.06 ppm *downfield* from the H_6 resonance in the monomeric cytidine nucleoside, and this resonance was found to shift upfield with increasing temperature, approaching the chemical shift in the monomeric nucleoside at high temperatures. Since the cytosine base is known to possess only a small (or negligible) magnetic anisotropy, these observations can only be interpreted in terms of the effect of the intramolecular base-stacking interaction on the orientation of the cytosine base about the glycosidic bond.

The intramolecular base-stacking interaction is expected to be stronger in ApC and CpA than in CpC. On the basis of the conformational effects discussed above, we would then expect the cytosine H_6 resonances in ApC and CpA to appear at *lower* fields relative to the corresponding resonances in CpC. As indicated by the data in Table I, however, the H_6 resonances are at appreciably *higher* fields in ApC and CpA than in CpC at 29°. It is evident, then, that the cytidine H_6 protons of both ApC and CpA are also exposed to the ring-current magnetic anistropy of the adjacent adenine base to a large extent. The appreciable ring-current shifts observed for both the cytosine H_5 and H_6 resonances would seem to indicate that ApC and CpA tend to assume conformations in which the adenine and cytosine bases are intramolecularly stacked to a significant extent at 29° in aqueous solution.

The factors affecting the chemical shifts of the $H_{1'}$ (C) protons in ApC and CpA are also complex. As indicated above, we expect effects arising directly from the location of the phosphate attachment to be compensated for when the chemical shifts of these protons in ApC and CpA are referred to those of the corresponding protons in CpC. From geometrical considerations, the ring-current magnetic anisotropy of the adjacent adenine base is expected to shift the cytidine $H_{1'}$ resonance further upfield in ApC than in CpA, if the bases are intramolecularly stacked to a comparable extent. This trend is clearly indicated in the chemical shift data for these protons.

However, Prestegard and Chan[7] pointed out that the chemical shifts of these ribose $H_{1'}$ protons should also be sensitive to the conformation of the ribose ring and the conformation of the base about the glycosidic bond. The dependence of the chemical shift on the ribose conformation is essentially a consequence of the proximity of the $H_{1'}$ proton to the 2′-hydroxyl group. Prestegard and Chan observed that the 2′-hydroxyl group of the ribose ring exerts a shielding effect on $H_{1'}$. Since the $H_{1'}$ proton is closer to the 2′-hydroxyl group when the ribose ring is in the 3′-*endo* conformation than when it is in the 2′-*endo* conformation, the $H_{1'}$ resonance is expected to shift upfield when the ribose conformation changes from 2′-*endo* to 3′-*endo*. The dependence of the $H_{1'}$ chemical shift on the base conformation relative to the ribose ring arises from the proximity of the $H_{1'}$ proton to the keto group of the pyrimidine base. The keto group appears to deshield the $H_{1'}$ proton, and since the intramolecular base stacking in the dinucleotide is expected to restrict the conformation of the base about the glycosidic bond so that the 2-keto group is rotated away from the $H_{1'}$ proton, the effect of the base-stacking interaction on the conformation of the bases is seen to lead to upfield shifts for the $H_{1'}$ resonance.

In ApC, part of the large upfield shift observed for the cytidine $H_{1'}$ resonance relative to CpC is undoubtedly due to the ring-current effect of the adjacent adenine ring. The ribose conformations of the 5′-esterified cytidine nucleosides in ApC and CpC are almost identical ($|J_{H_{1'}-H_{2'}}|$ = 2.0 cps in ApC and 2.2 cps in CpC); hence, differences in the $H_{1'}$ chemical shifts due to the 2′-hydroxyl group are small. Since the ring-current effect on the chemical shift of the $H_{1'}$ proton in ApC is not expected to be large, the remaining part of the observed upfield shift must therefore be accounted for in terms of differences in the average conformation of the cytosine base about the glycosidic bond between ApC and CpC.

The upfield shift observed for the $H_{1'}$ resonance of the 3′-esterified cytidine nucleoside in CpA relative to CpC is smaller than the corresponding difference for the 5′-nucleoside between ApC and CpC. The stronger intramolecular base-stacking interaction in CpA relative to CpC will tend to shift the $H_{1'}$ (C) resonance in CpA upfield relative to CpC, primarily because of its influence on the rotational conformation of the cytosine base about the glycosidic bond, since the effect of the ring-current magnetic anisotropy of the adjacent adenine base on the chemical shift of this proton is small (see below). Here the difference in the ribose conformation is larger, the ribose conformation being somewhat more 3′-*endo* in CpC[8] ($|J_{H_{1'}-H_{2'}}|$ = 3.2 cps in CpA and 2.3 cps in CpC). This difference in the ribose conformation will result in deshielding of the $H_{1'}$ (C) proton in CpA relative to CpC and could account for the smaller upfield shift observed for CpA compared with ApC when the chemical shifts of these protons are referred to those for CpC.

The effect of a neighboring cytosine base on the chemical shifts of the adenosine protons of ApC and CpA can be assessed by comparing the chemical shifts of these protons with those of the corresponding protons in adenosine 3′-monophosphate and adenosine 5′-monophosphate. The chemical shifts of the adenosine protons in these molecules are strongly concentration dependent, and hence it is necessary to compare them at extremely low concentrations. The infinite-dilution chemical shifts for the H_8, H_2, and $H_{1'}$ protons of 3′-AMP and 5′-AMP (sodium salts, pD 5.9) have been reported by Schweizer, *et al.*[12] and are compared with the infinite-dilution chemical shifts

(12) M. P. Schweizer, A. D. Broom, P. O. P. Ts'o, and D. P. Hollis, *J. Am. Chem. Soc.*, **90**, 1042 (1968).

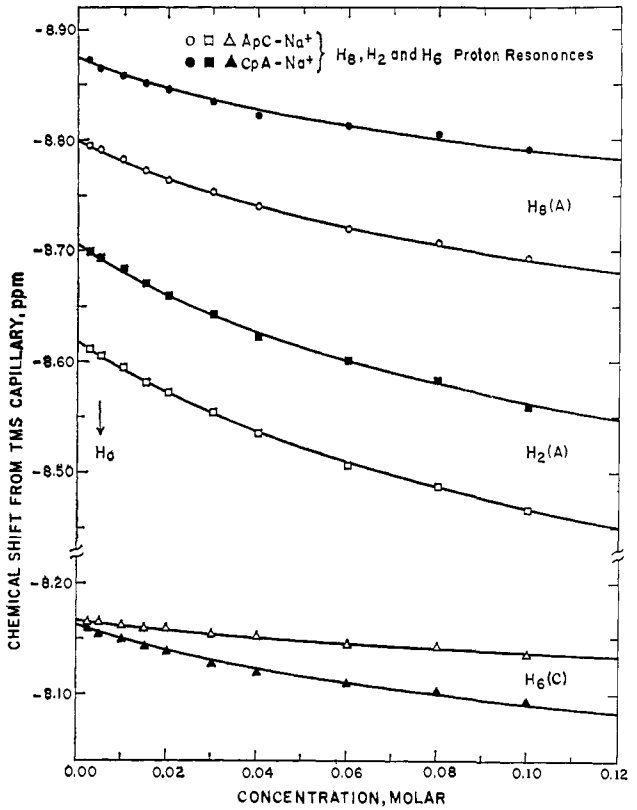


Figure 4. Concentration dependence of the adenine H_8, H_2 and cytosine H_6 proton chemical shifts of ApC and CpA in aqueous solution at 29°.

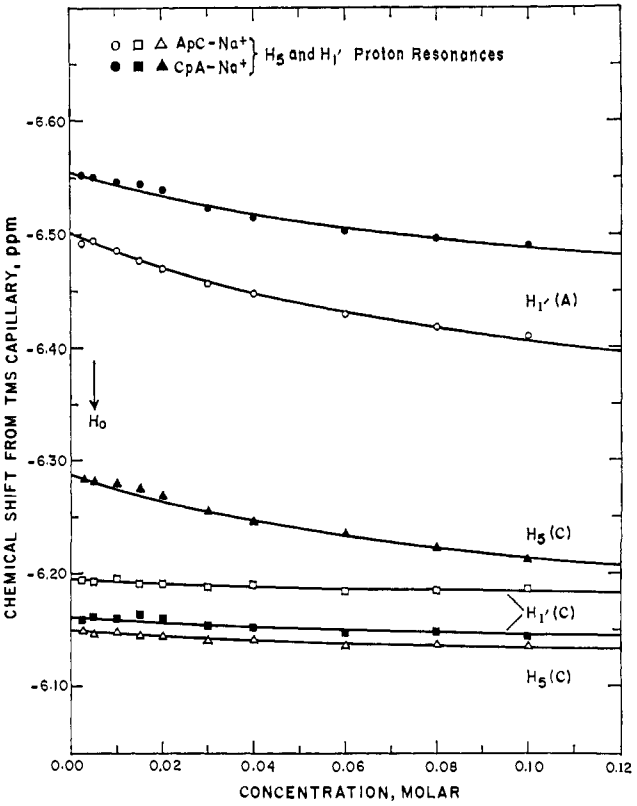


Figure 5. Concentration dependence of the adenosine $H_{1'}$ and cytidine H_5, $H_{1'}$ proton chemical shifts of ApC and CpA in aqueous solution at 29°.

obtained in this work for ApC and CpA in Table II. The data indicate that esterification of cytidine to 3′-AMP and 5′-AMP causes the H_2 resonance of ApC and the H_8 resonance of CpA to be shifted upfield by about 0.08 ppm, but has only a small effect on the other monitored adenosine resonances. A similar chemical

Table II. Chemical Shifts (in ppm) of Adenosine Proton Resonances Resulting from the Esterification of Cytidine to Adenosine 3′- and 5′-Monophosphates

	H_8	H_2	$H_{1'}$
ApC	−8.800	−8.619	−6.501
3′-AMP-Na^+ [a]	−8.805	−8.697	−6.530
Shift	+0.005	+0.078	+0.029
CpA	−8.874	−8.706	−6.556
5′-AMP-Na^+ [a]	−8.957	−8.705	−6.578
Shift	+0.083	−0.001	+0.022

[a] pD 5.9.

shift difference for the adenine H_8 protons between the dinucleotide UpA and 5′-AMP (pD 6.2) has also been noted[4] and might suggest that the phosphate group is on the average constrained further away from the adenine base in the dinucleoside monophosphate than in the nucleotide. The sizeable upfield shift observed for the adenine H_2 proton in ApC relative to 3′-AMP is surprising in view of the lack of sensitivity of the adenine H_2 resonance to the nature of the ribose backbone and the generally accepted notion that the cytosine base has a negligibly small magnetic anisotropy. The present observations would seem to indicate that the cytosine base can have a small influence on the magnetic environment of a proton located in its immediate vicinity.

Concentration Dependence. The effect of concentration on the pmr spectra of ApC and CpA was studied in order to determine the extent of intermolecular association of these molecules and to ascertain the effects of this association on the chemical shifts of the various protons. Monomeric bases,[13] nucleosides,[6,14] and nucleotides[12] have been shown to associate extensively by base stacking in aqueous solution, and it would be expected that the dinucleoside monophosphates would exhibit similar behavior. Knowledge of the extent and the nature of the intermolecular association process is important in assessing the results of experiments designed to investigate only the *intra*molecular interaction between the two bases of a dinucleotide.

The concentration dependence of the chemical shifts of the monitored protons of ApC and CpA was studied over the concentration range 0.0025 to 0.100 *M* at 29°, and the results are summarized in Figures 4 and 5. All the resonances are shifted to higher fields with increasing dinucleotide concentration, with the adenine base protons exhibiting the largest shifts. The extrapolated infinite-dilution chemical shifts and the concentration shifts at 0.10 *M* are summarized in Table III. Changes in the bulk magnetic susceptibility of the solution with concentration are extremely small over the concentration

(13) S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, *J. Am. Chem. Soc.*, **86**, 4182 (1964); O. Jardetzky, *Biopolymers Symp.*, 1, 501 (1964).

(14) A. D. Broom, M. P. Schweizer, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **89**, 3612 (1967).

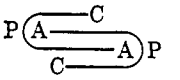
Table III. Extrapolated Infinite Dilution Chemical Shifts and Concentration Shifts at 0.10 *M* (in ppm) for ApC and CpA Proton Resonances at 29°

Proton	Chemical shift at infinite dilution		Concentration shift at 0.10 *M*	
	ApC	CpA	ApC	CpA
H_8 (A)	−8.800	−8.874	0.107	0.082
H_2 (A)	−8.619	−8.706	0.152	0.146
H_6 (C)	−8.167	−8.163	0.031	0.069
$H_{1'}$ (A)	−6.501	−6.556	0.091	0.066
H_5 (C)	−6.149	−6.287	0.013	0.075
$H_{1'}$ (C)	−6.194	−6.161	0.008	0.016

range investigated (~0.2 cps), and no corrections have been applied to these data.

The upfield shifts observed with increasing dinucleotide concentration indicate that ApC and CpA self-associate intermolecularly by base stacking. Since the cytosine base has only a negligible magnetic anisotropy and the proton chemical shifts of the monomeric cytidine nucleoside are not concentration dependent despite the intermolecular base stacking of cytidine demonstrated in osmotic studies,[15] the observed concentration shifts in ApC and CpA must be a consequence of the ring-current magnetic anisotropy of the adenine base. The large concentration shifts exhibited by the adenine ring protons indicate considerable A–A base stacking in the intermolecularly associated species. In the case of ApC, the cytosine base protons are only slightly affected by the intermolecular association, implying that little A–C base stacking is involved in the complex or complexes formed. With CpA, on the other hand, a considerably greater degree of intermolecular A–C base stacking would appear to be indicated by the larger concentration shifts observed for the cytosine H_6 and H_5 protons. This sequence-dependent behavior is rather surprising, and a consideration of the several possible types of intermolecular complexes may be helpful in understanding these results.

If the dinucleotide is strongly stacked intramolecularly, it will self-associate primarily by base stacking of the external or exposed faces of the bases of the folded molecule. In both ApC and CpA, a large tendency for A–A base stacking is to be expected, followed by A–C base stacking, which in turn is more favorable than C–C base stacking.[15] The concentration shifts observed for the cytosine H_6 and H_5 resonances may be in part due to A–C stacked intermolecular complexes of this type, with the differences in the concentration shifts of these protons between ApC and CpA reflecting differences in the A–C base-stacking tendencies as a result of geometric effects and steric restrictions on A–C base overlap in the associated species. While this rationalization of the sequence-dependent behavior is not unreasonable, it is surprising to note the negligibly small concentration shift for the $H_{1'}$(C) proton in CpA, since the concentration shift observed for this proton is expected to be comparable to that for the H_6 (C) proton. For example, the cytidine H_5, H_6, and $H_{1'}$ proton resonances of the monomeric cytidine nucleoside are shifted upfield by 0.074, 0.046, and 0.031 ppm, respectively, in the presence of 0.1 *M* CpA, and by 0.096, 0.066, and 0.054 ppm in the presence of 0.1 *M* adenosine. If the two bases in ApC and CpA are not strongly stacked intramolecularly, however, self-intercalated intermolecular dimers might also be formed.[4] In such complexes, a base of one molecule would be inserted between the two bases of another dinucleotide molecule. Again, because of the greater tendency for A–A base stacking, we expect the intercalated intermolecular complexes of ApC or CpA to involve adenine insertion primarily. Such a self-intercalated ApC dimer may be represented schematically as



The formation of these intermolecular complexes can also lead to concentration shifts for the cytidine proton resonances. These shifts will reflect both the ring-current magnetic anisotropy effects of the incorporated adenine base and the accompanying *reduction* in the intramolecular ring-current effect of the adjacent base as a result of the formation of the intercalated complex. Since the cytidine protons in ApC experience a larger ring-current effect from the adjacent adenine base in the intramolecular stack than in CpA, and since the incorporated adenine base in the intercalated complex is expected to shift the cytidine proton resonances in both dinucleotides by similar amounts, the larger concentration shifts for the cytidine resonances in CpA may not be unexpected.

On the basis of the concentration data, it is not possible to ascertain the relative importance of the various intermolecular complexes which may contribute to the observed cytidine proton concentration shifts. However, it is probable that both types of intermolecular complexes considered here are present to some extent in these solutions.

Although the dinucleoside monophosphates, like the simpler bases and nucleosides, may associate intermolecularly to form dimers, trimers, tetramers, and higher associated species in aqueous solution,[15,16] only the dimer would probably be of significance at dinucleotide concentrations below 0.10 *M*. This is particularly true of the purine–pyrimidine or pyrimidine–purine dinucleoside monophosphates because of the favored purine–purine interactions and the relatively weak pyrimidine–pyrimidine interactions. Accordingly, we have interpreted the concentration shifts in terms of a dimer equilibrium. Within the framework of this model, the concentration shifts of a given proton are given by eq 1, where δ_{obsd} is the

$$\delta_{obsd} - \delta_M = (\delta_D - \delta_M) \times \left\{\frac{(4Km + 1) - (1 + 8Km)^{1/2}}{4Km}\right\} \quad (1)$$

observed chemical shift; δ_M is the chemical shift in the monomer (infinite dilution value); δ_D denotes the chemical shift in the dimer; K is the association constant in l./mol; and m denotes the stoichiometric molar concentration of the dinucleotide. As is well known, it is not possible to distinguish between the formation of one *vs.* several dimer species in this analysis due to rapid chemical exchange. Where several dimer species

(15) P. O. P. Ts'o, I. S. Melvin, and A. C. Olson, *J. Am. Chem. Soc.*, **85**, 1289 (1963).
(16) K. E. Van Holde and G. P. Rossetti, *Biochemistry*, **6**, 2189 (1967).

are involved, as is probably the case here, the over-all association constant K is the sum of the formation constants for all the dimer species, and the dimer shift $\delta_D - \delta_M$ represents a weighted mean over all these species; *i.e.*,

$$K = \sum_i K_i \tag{2}$$

$$\delta_D - \delta_M = \frac{\sum_i (\delta_{D_i} - \delta_M) K_i}{\sum_i K_i} \tag{3}$$

where δ_{D_i} and K_i denote the dimer shift and the formation constant for the ith dimer species.

A computer least-squares fit of the concentration data to eq 1 yielded the results given in Table IV. In this

Table IV. Values of Dimerization Constant K (l./mol)[a]

	ApC			CpA		
Proton	Best K	Best av K	Dimerization shift, ppm	Best K	Best av K	Dimerization shift, ppm
H_8 (A)	2.7	2.5	0.40	4.0	2.7	0.30
H_2 (A)	2.4		0.57	2.5		0.51
$H_{1'}$ (A)	3.3		0.35	2.2		0.24
H_6 (C)			0.11	4.5		0.26
H_5 (C)			0.05	1.6		0.26
$H_{1'}$ (C)			0.04			0.05

[a] Giving the best fit to the concentration data for the various ApC and CpA protons. The best average value for a set of protons and the dimerization shifts calculated using the best average dimerization constant are also given.

analysis, the best fit for a particular value of K was determined by treating $\delta_D - \delta_M$ as an adjustable parameter, and K was varied from 0.5 to 8.0 l./mol in increments of 0.1 l./mol. The value of K giving the lowest mean-square deviation for each proton was selected as the "best K." The δ_M values used were obtained for a given proton by extrapolating the chemical shifts to infinite dilution (Table III). Only fitting of the adenosine H_8, H_2, and $H_{1'}$ shifts of ApC and CpA and the cytidine H_6 and H_5 shifts of CpA was attempted, as the remaining resonances were not shifted appreciably with concentration and the relative experimental error would be large. The value of K giving the lowest *total* mean-square deviation for a particular set of protons (adenosine protons and cytidine protons) was also determined, and is termed the "best average" K. In the case of ApC, the "best average" K does not differ appreciably from the best K for each proton. Although the K giving the best fit varies a great deal depending on the proton resonance considered in the case of CpA, the "best average" K values for the adenosine and cytidine moieties are quite consistent. The dimer shifts reported in Table IV are those obtained using the "best average" K for all protons.

The dimerization constants of 2.5 l./mol for ApC and 2.7 l./mol for CpA obtained by the above procedure indicate that the intermolecular self-association tendencies of these two dinucleoside monophosphates are very similar. These values are to be compared with the self-association constants of 4.5 m^{-1} for adenosine[14] and 0.9 m^{-1} for cytidine,[15] obtained previously by osmotic studies. Since the dimerization of ApC and CpA involves primarily A–A stacking, a factor of ~2 reduction in the dimerization constant from the value for the adenosine nucleoside is to be expected, since only one face of the adenine base would be accessible for intermolecular A–A base stacking if the dinucleotide is intramolecularly stacked. Finally, the dimer shifts obtained for the adenine proton resonances of ApC and CpA are comparable to those reported by Chan, *et al.*,[13] for purine: H_6, 0.59 ppm; H_2, 0.68 ppm; H_8, 0.47 ppm. Thus, the treatment of the intermolecular self-association presented here yields qualitatively reasonable results.

This study of the concentration dependence of the pmr spectra of ApC and CpA clearly shows an appreciable tendency for intermolecular association by these dinucleoside monophosphates. At a concentration of ~0.10 M, for example, 25–30% of these dinucleotides are dimerized. Obviously, studies aimed at determining the *intra*molecular base-stacking interactions must be done at low concentrations, so that the effects due to the intramolecular interaction will not be overshadowed by effects due to the intermolecular processes.

Temperature Study of ApC and CpA. In order to study the intramolecular base-stacking interactions in ApC and CpA, the proton magnetic resonance spectra of these dinucleoside monophosphates were studied as a function of temperature. In connection with these experiments there are two important experimental considerations. The first of these involves the necessity of working at as low a dinucleoside monophosphate concentration as is feasible, to minimize the intermolecular base-stacking effects demonstrated in the concentration study. The intermolecular association process is certainly temperature dependent and could completely mask the intramolecular base stacking of interest here if the concentration of dinucleoside monophosphate were appreciable. On the other hand, the nmr method is not very sensitive, and a compromise between the elimination of intermolecular effects and a reasonable spectrum accumulation time is necessary. A concentration of 0.010 M for the dinucleoside monophosphate was selected as representing a reasonable compromise between these two requirements. At this concentration, the accumulation of about 30 successive scans is necessary to obtain a spectrum with a reasonably good signal-to-noise ratio. Less than 5% of the dinucleoside monophosphate are dimerized at 0.01 M, and the chemical shifts of the various ApC and CpA protons differ from the infinite-dilution values by less than 3 cps at 29°.

The second experimental consideration is the choice of an appropriate reference compound for the chemical shift measurements. It might appear likely that any internal standard would suffice to compensate for changes in bulk diamagnetic susceptibility differences between the D_2O solution and the TMS capillary (which provides the lock signal) with temperature. However, various possible reference compounds exhibit different temperature dependences in D_2O solution with respect to the TMS capillary. This behavior suggests that changes in solute–solvent interaction with temperature may influence the chemical shift of a proton of the solute molecule. Since the purpose of the temperature studies of the dinucleoside monophosphates is to examine only

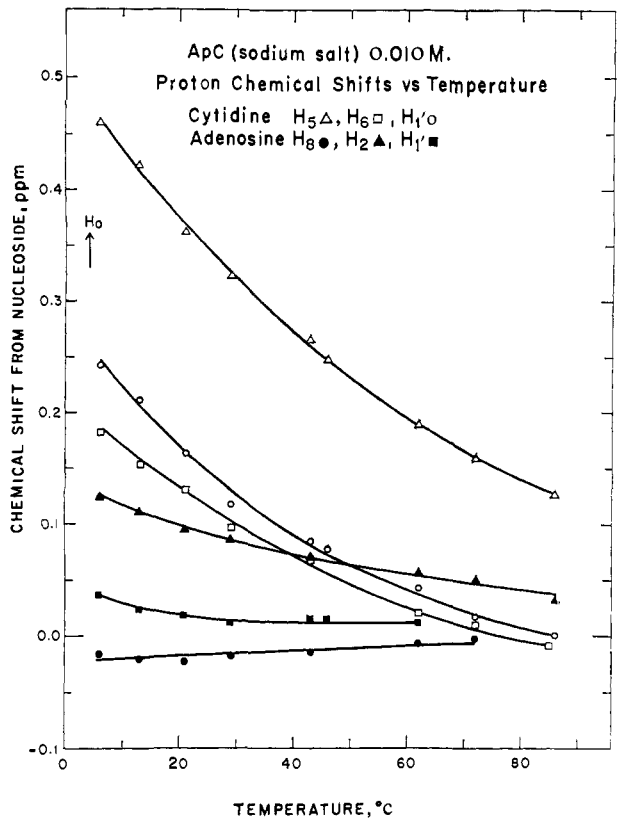


Figure 6. Variation of the chemical shifts (at 100 Mcps) of the various monitored proton resonances of ApC with temperature. The chemical shift of each proton is referenced to the analogous proton of the corresponding nucleoside at the same temperature.

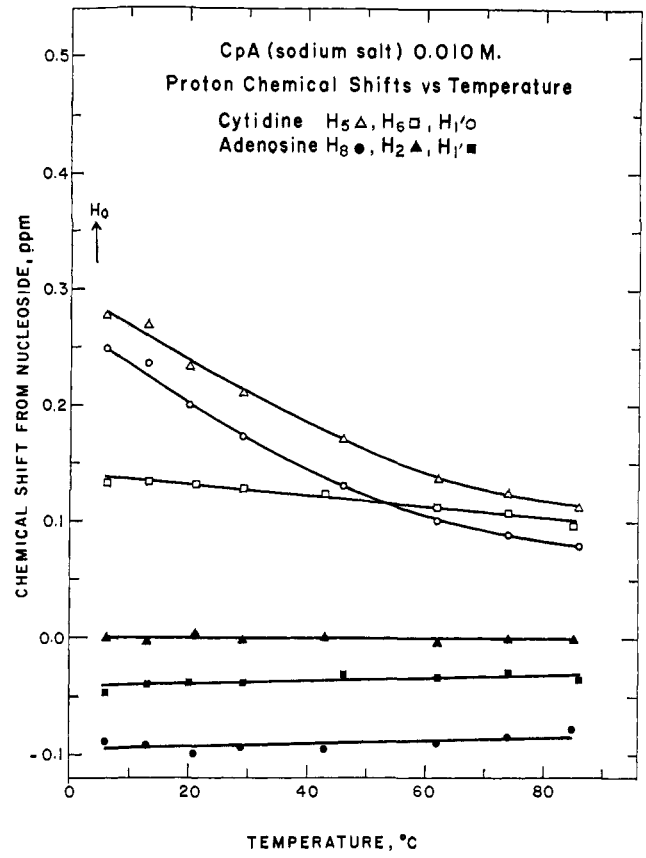


Figure 7. Variation of the chemical shifts (at 100 Mcps) of the various monitored proton resonances of CpA with temperature. The chemical shift of each proton is referenced to the analogous proton of the corresponding nucleoside at the same temperature.

the effect of the intramolecular base-stacking interaction on the conformation of these molecules, it is therefore important to choose a reference compound which would interact with the D_2O solvent in the same manner as the dinucleoside monophosphates. For this reason, the monomeric nucleosides were selected to serve as references for the chemical shift measurements. In this study, a particular proton of ApC or CpA is referenced to the analogous proton of the corresponding nucleoside at the same temperature.

The effect of temperature on the chemical shifts of the adenosine H_8, H_2, and $H_{1'}$ protons from the TMS capillary was determined for a 3.2×10^{-3} *M* adenosine solution,[17] and these data served as a reference for the adenosine H_8, H_2, and $H_{1'}$ resonances of ApC and CpA. The low adenosine concentration in the reference solution rendered intermolecular A–A base-stacking effects unimportant over the entire temperature range investigated. A 0.030 *M* solution of cytidine was studied as a function of temperature, and the chemical shifts of the H_6, H_5, and $H_{1'}$ protons were measured relative to internal $N(CH_3)_4^+$. The use of $N(CH_3)_4Cl$ in the ApC and CpA solutions made possible a comparison of cytidine chemical shifts in the dinucleoside monophosphates and the cytidine nucleoside, with the proton resonance of $N(CH_3)_4^+$ serving as an intermediate reference. As the C–C base-stacking interaction does not affect the chemical shifts of the cytidine protons, it was possible to use a rather high cytidine concentration in the reference solution. It was not feasible to add adenosine and cytidine to the ApC and CpA solutions to serve as direct internal standards, because the spectra of the nucleosides and the dinucleoside monophsophates overlap.

The temperature dependence of the chemical shifts observed for the adenosine H_8, H_2, and $H_{1'}$ and cytidine H_6, H_5, and $H_{1'}$ protons of ApC and CpA relative to corresponding protons of the nucleosides are summarized in Figures 6 and 7. These measurements were made over the temperature range of 6 to 86°. As expected, all of the monitored cytidine resonances in ApC and CpA are shifted downfield relative to the cytidine nucleoside as the temperature is increased. Over the temperature range investigated, the cytidine H_5, H_6, and $H_{1'}$ proton resonances are shifted downfield by 0.33, 0.19, and 0.24 ppm, respectively, in ApC, and 0.16, 0.03, and 0.18 ppm, respectively, in CpA. With the exception of the H_2 resonance in ApC, the monitored adenosine resonances in these dinucleotides exhibit almost no shifts relative to the adenosine nucleoside with temperature. This H_2 resonance shifts downfield by 0.09 ppm as the temperature is increased from 6 to 86°.

It is reasonable to assume that these shifts arise from changes in the relative conformation of the two bases of the dinucleotide with temperature. However, care should be taken not to interpret these temperature shifts solely in terms of the magnetic anisotropy of the neighboring base. Changes in the intramolecular base-stacking interaction can lead to changes in the

(17) J. H. Prestegard, J. H. Nelson, B. W. Bangerter, and S. I. Chan, unpublished data.

conformations of the ribose rings and the conformation of the bases about their glycosidic bonds, and these conformational variations can also alter the chemical shifts of the protons under consideration. It is therefore necessary to consider these conformational changes in order to interpret the temperature shifts correctly.

A sensitive indication of the conformation of the ribose ring is provided by the $H_{1'}$–$H_{2'}$ coupling constants, and values of $|J_{H_{1'}-H_{2'}}|$ for both the adenosine and cytidine nucleosides in ApC and CpA at a number of temperatures are summarized in Table V. These

Table V. Temperature Dependence of the Spin–Spin Coupling Constants between $H_{1'}$ and $H_{2'}$ Ribose Protons for the Two Ribose Moieties

	$\lvert J_{H_{1'}-H_{2'}}\rvert$, cps[a]			
	ApC		CpA	
Temp, °C	A	C	A	C
6	~2.5	~1.8	2.8	2.1
13	3.1	~2.0	~3.0	2.5
20			3.4	2.7
21	3.3	~2.4		
29	3.9		3.8	3.3
43	4.1	~2.6		
46	4.3	2.8	4.4	3.6
62	4.5	~3.5	4.7	4.2
72	4.7	~3.0		
74			4.8	~3.7
86	4.8	*b*	4.8	4.4

[a] Accuracy of measurement ±0.1 cps except where an approximate value is given (± ~0.3 cps). [b] Overlaps the H_5 (C) resonance.

coupling constants are seen to be strongly temperature dependent, essentially doubling in magnitude between 6 and 86°. Since the $H_{1'}$–$H_{2'}$ coupling constants are virtually independent of temperature in the mononucleosides and mononucleotides,[17] the observed variations in these ribose coupling constants of ApC and CpA with temperature must clearly result from changes in the intramolecular base-stacking interaction. The temperature data indicate that the ribose conformations of both the adenosine and cytidine nucleosides become more 3′-*endo*[11] when the dinucleotide is intramolecularly stacked.

In view of the large conformational changes induced in the ribose–phosphate backbone by the intramolecular stacking interaction, and possible additional effects on the conformation of the bases about their glycosidic bonds, it appears that only the temperature shifts observed for the cytosine H_5 and adenine H_2 protons can safely be interpreted solely in terms of the magnetic anisotropy of the neighboring base. These protons are located somewhat further away from the ribose–phosphate backbone than are H_6 of cytosine and H_8 of adenine, and the spectral positions of the resonances for these protons do not appear to be particularly sensitive to the nature of the ribose–phosphate backbone. Thus, the temperature shifts observed for these protons may be used to infer information about the intramolecular interaction between the two bases directly.

The temperature shift observed for the H_5(C) resonance in ApC is seen to be quite large. Since this H_5(C) resonance is 0.46 ppm upfield from that of the cytidine nucleoside at 6°, the large downfield shift observed with increasing temperature is not unexpected. The ring current of a neighboring adenine base is not expected to produce an upfield shift much greater than 0.5–0.6 ppm for the protons of the cytosine base with the most favorable base–base overlap in the intramolecular stack.[6,13] It may therefore be concluded that the two bases in ApC are rather strongly stacked at low temperatures (~75% stacked at 6°). The temperature shift observed for the H_5(C) proton of CpA is somewhat smaller than that for ApC, apparently reflecting the smaller ring-current shift experienced by this proton (0.28 ppm at 6°).

The observed shifts of the adenine H_2 proton in ApC with temperature would seem to be indicative of a small magnetic anisotropy for the cytosine base which had not been detected in previous work.[6] However, no temperature shifts were observed for the adenine H_2 proton in CpA.

It is interesting to note that at 86° the cytosine H_5 resonances of ApC and CpA are still at significantly higher fields than that of the cytidine nucleoside (~0.15 ppm for both ApC and CpA). These observations indicate that both ApC and CpA are not completely destacked at 86° and that there is still residual base–base interaction at this temperature.

The differences between ApC and CpA in the chemical shifts of the cytosine H_5 and adenine H_2 protons relative to the mononucleosides and the corresponding differences in the temperature shifts of these protons most likely arise from differences in the conformations of these molecules. The temperature shifts for these protons are much smaller for CpA than ApC, and a comparison of the temperature data for the cytosine H_6 protons indicates a similar behavior for these protons as well. There are two possible explanations for this sequence-dependent behavior. First of all, it may be that the intramolecular A–C base-stacking interaction is stronger in ApC than in CpA, so that at a given temperature ApC is stacked to a greater degree than is CpA. This could result from the possibility of more favorable base overlap in ApC than in CpA as a consequence of the difference in the phosphate attachment in the two sequence isomers. Secondly, it may be that, regardless of the relative tendencies of ApC and CpA toward intramolecular base stacking, the cytosine protons and the adenine H_2 proton of CpA experience the effect of the magnetic anisotropy of the adjacent base to a lesser degree than do those of ApC in the stacked environment.

A consideration of CPK molecular models of ApC and CpA is helpful in resolving this question. In Figure 8 we have depicted for illustrative purposes the most probable stacked conformations of ApC and CpA as viewed along the axis of the ribose–phosphate backbone in the direction of 5′- to 3′-phosphate esterification. In these model stacked conformations, we have maximized the extent of A–C base overlap, while avoiding strong repulsive nonbonded interactions between the base of the 5′-nucleoside and the ribose–phosphate–ribose backbone. Both the adenine and cytosine bases in these models are in the more stable *anti* conformations with respect to their ribose moieties, and though not shown, the ribose–phosphate–ribose backbones are in a conformation approximating that found in double-helical DNA. In order to obtain effective

parallel A–C base overlap and to minimize strong repulsive nonbonded interactions, however, it is necessary that the two ribose rings be in the 3′-*endo* conformation (see below). For steric reasons, the base of the cytidine nucleoside can only exist in the *anti* conformation; however, it appears that the intramolecular base-stacking interaction can also stabilize the less favorable *syn* conformation in the case of the adenosine nucleoside.

In the stacked ApC model, the cytosine base is able to overlap the six-membered ring of the adenine base quite well, and the cytosine protons (particularly H_5) are situated directly above the adenine ring where they would be exposed to a rather strong magnetic field from the adenine ring current. In the stacked CpA model, only partial overlap of the cytosine and adenine bases is possible in this conformation, and the cytosine H_5 and H_6 protons are well away from the adenine base and would thus experience a smaller magnetic field due to the adenine ring current. Rotation of the adenine base by 180° about the glycosidic bond to give the *syn* conformation would allow a greater adenine–cytosine base overlap, but the cytosine protons would still not be exposed to the adenine ring-current effect to the extent that they are in ApC. We note that, were it possible for the cytosine base to assume the *syn* conformation, the cytosine base protons in CpA would experience a sizeable ring-current effect from the adjacent adenine base irrespective of whether the adenine base orientation is *anti* or *syn*. Also, in these hypothetical stacked conformations of ApC and CpA, only the adenine H_2 proton of ApC is sufficiently close to the cytosine base to be affected by its small magnetic anisotropy. Thus it appears quite likely that the observed differences in the temperature shifts for the cytosine H_5 and adenine H_2 protons between ApC and CpA reflect steric and geometric factors which affect the exposure of these protons to the magnetic anisotropy of the neighboring base, although a difference in the intramolecular A–C base-stacking tendencies between ApC and CpA is also possible. However, a comparison of the $H_{1'}$–$H_{2'}$ coupling constant data between ApC and CpA would seem to indicate that the stacking tendencies between the adenine and cytosine bases are rather comparable in the two sequence isomers.

The interpretation of the temperature shifts for the cytidine H_6 and $H_{1'}$ protons is not quite so straightforward, since, in addition to the ring-current magnetic anisotropy of the neighboring adenine base, other factors can contribute to these temperature shifts. The chemical shifts of the H_6(C) protons, for example, are also dependent upon the rotational conformation of the cytosine base about the glycosidic bond, and if the cytosine base can rotate away from the conformation stabilized by the intramolecular stacking interaction when the dinucleotide becomes destacked, an upfield shift with increasing temperature would be expected for this contribution. Thus, the observed temperature shifts for these protons may reflect partial compensation of effects due to changes in the relative conformation of the adenine and cytosine bases by changes in the conformation of the cytosine base about its glycosidic bond. As noted above, the temperature shifts observed for the H_6(C) protons are much larger in ApC than

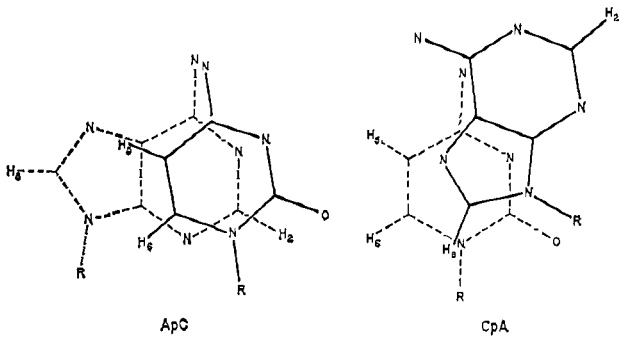


Figure 8. Possible stacked conformations of ApC and CpA.

CpA. This, we feel, arises primarily from a difference in the exposure of these protons to the ring-current magnetic anisotropy of the neighboring adenine base in the intramolecular stack. Finally, we note that although the H_6(C) resonance of CpA is still 0.12 ppm upfield from the H_6(C) resonance of cytidine at 86°, the H_6(C) protons of ApC and the cytidine nucleoside are almost magnetically equivalent at this temperature. This should not be taken to imply that ApC is stacked to a lesser degree than is CpA at this temperature, since in the absence of intramolecular base stacking, the H_6(C) resonance of ApC should appear ~0.08 ppm downfield from that of the cytidine nucleoside because of the effect of the phosphate group.

The temperature shifts observed for the $H_{1'}$(C) protons of ApC and CpA are quite large and do not exhibit the sequence dependence manifested by the other cytidine protons. The resonances of these protons are appreciably upfield from that of the $H_{1'}$ proton in cytidine at low temperature. This may appear to be surprising, since the effect of the ring-current magnetic anisotropy of the adjacent adenine base on these protons is not expected to be large, neither of these protons being located close to the regions of large ring-current fields from the adenine base in the intramolecular stack. The cytidine $H_{1'}$ proton in the stacked CpA molecule, in particular, is well removed from the adenine base. On the basis of our earlier discussion of the chemical shifts of these protons, it seems reasonable to attribute the observed temperature shifts to the observed conformational changes in the ribose rings (less 3′-*endo* with increasing temperature) and to possible changes in the conformations of the cytosine bases about the glycosidic bonds when the two bases in the dinucleotide become unstacked with increasing temperature. Both of these contributions are expected to lead to deshielding of the $H_{1'}$ protons as the temperature is increased.

In view of the smallness of the temperature shifts observed for the remaining proton resonances, it would not be appropriste to discuss them in detail. The adenine H_8 resonances in both ApC and CpA are shifted slightly upfield, the total shifts amounting to no more than 0.02 ppm over the temperature range investigated. Similarly, the temperature shift observed for the adenosine $H_{1'}$ resonance in CpA is small and upfield. The corresponding shift observed for the adenosine $H_{1'}$ proton in ApC is somewhat larger (0.04 ppm) but downfield. As in the case of the cytidine $H_{1'}$ resonances, the adenosine $H_{1'}$ chemical shifts are affected both by the ribose conformation and the

average conformation of the adenine base about the glycosidic bond.

The intramolecular stacking interaction between the two bases of the dinucleoside monophosphates has generally been discussed in terms of a two-state model, in which an equilibrium exists between "stacked" and "unstacked" forms of the molecule.[18] The limitations of this two-state model have been discussed by Chan and Nelson in the first paper of this series.[4] Regardless of the details of this or any other model which may be proposed to describe the intramolecular base-stacking process, some general features are expected for the pmr chemical shift *vs.* temperature plots for the cytidine protons of ApC and CpA. Specifically, a sigmoidal variation is expected, with the two asymptotic limits at low and high temperatures denoting the chemical shifts of the proton in the stacked and unstacked environments, respectively. Since the intramolecular base-stacking process in the dinucleoside monophosphates is not expected to be a cooperative phenomenon, with a well-defined "melting" over a narrow temperature region, the transition between the stacked and unstacked states may extend over a wide temperature range.

The data presented in Figures 6 and 7 clearly indicate that only the intermediate- and the high-temperature portions of the sigmoidal temperature profile are observed for ApC and CpA over the available temperature range of $\sim$0 to $\sim$100° in D_2O solution. These observations can be compared with those reported for ApA, where part of the low-temperature region was apparently also observed. Thus, ApA appears to be more strongly stacked than either ApC or CpA at low temperatures.

The width of the transition between the "stacked" and "unstacked" states also appears to be somewhat broader in ApC and CpA than in ApA. In the two-state model, the sigmoidal curves, when plotted *vs.* the reciprocal of the absolute temperature, are necessarily symmetrical about the apparent "transition temperature" located at the point of maximum slope, half way between the high- and low-temperature asymptotic limits, and the width of the transition is determined primarily by the apparent enthalpy change associated with the stacking process. On this basis, one might therefore be inclined to conclude that the apparent enthalpy of stacking is somewhat larger in magnitude in ApA than in ApC and CpA. The apparent "transition temperature" between the stacked and unstacked states in ApC and CpA (as given by the point of maximum slope in a chemical shift *vs.* $1/T$ plot) appears in the neighborhood of $\sim$10 to 20° and is somewhat lower than the apparent "transition temperature" of $\sim$35° previously noted for ApA. Again, one might argue that this is evidence for a stronger intramolecular stacking interaction in ApA than in ApC and CpA. While these conclusions are not necessarily unreasonable, we point out that the detailed shape of the temperature profile in the transition region is sensitive to the details of the intramolecular base-stacking interaction and depends both on the energetics of the process as well as on the variation of the property monitored with the extent of stacking. In particular, the point of maximum slope need not be located half way between the low- and high-temperature asymptotic limits of the property monitored.

A more complete knowledge of the chemical shift *vs.* temperature profiles would allow an estimation of the thermodynamic parameters for the intramolecular base-stacking process within the framework of the two-state model. The use of a concentrated salt solution as the solvent for the dinucleoside monophosphates, such as 4.7 *M* KF used by Brahms, *et al.*,[18] allows the temperature range to be extended to $\sim -20°$. However, it is probable that the intramolecular base-stacking interaction is quite sensitive to the ionic strength of the solution, and the thermodynamic parameters obtained for the system in 4.7 *M* KF might differ greatly from those appropriate for the system at *ca.* zero ionic strength.

Finally, we wish to comment on the conformational changes induced in the ribose rings by the intramolecular base-stacking interaction. The effect of this interaction on the conformations of the ribose rings in the dinucleoside monophosphates has been noted by Chan and Nelson[4] in paper I of this series and independently also by Hruska and Danyluk[19] in the case of ApA. These observations complement the pmr chemical shift results in cases where chemical shift changes are observed, although the coupling constant data offer practically no information about the detailed conformation of the intramolecular stack. In cases where chemical shift changes are small or negligible, and not readily interpretable, such as in the pyrimidine–pyrimidine dinucleoside monophosphates, the ribose conformational changes are useful as an indication of the strength of the intramolecular stacking interaction between the two bases.[8] Although it is reasonable to expect that changes in the degree of intramolecular base-stacking interaction would be accompanied by changes in the conformation of the ribose–phosphate–ribose backbone, it would seem appropriate to speculate on the causes of these conformational changes induced in the ribose rings. We note that in all the dinucleoside monophosphates which we have investigated thus far, the intramolecular stacking interaction between the two bases invariably favors the 3′-*endo* conformation. Examination of CPK molecular models indicates that if the ribose conformation of the 3′-nucleoside is 2′-*endo*, the two bases of the dinucleoside monophosphate cannot overlap because of nonbonded repulsion of the >CHOH (2′) group of the 3′-esterified nucleoside by the base and the ether oxygen of the 5′-esterified nucleoside. This steric repulsion is relieved when the ribose conformation of the 3′-nucleoside becomes 3′-*endo*. Similar nonbonded interactions probably are also responsible for the conformational changes in the ribose ring of the 5′-nucleoside, though the source of the steric interaction is less obvious here. However, from the CPK molecular models, it appears that there is less steric interaction between the base and the 5′-CH_2 group of the 5′-nucleoside in the stacked conformation when the conformation of the ribose ring is more 3′-*endo*.

Conclusions

The results of the present pmr study of the conformational properties of ApC and CpA indicate

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

(19) F. E. Hruska and S. S. Danyluk, *J. Am. Chem. Soc.*, **90**, 3266 (1968).

that the adenine and cytosine bases in these dinucleoside monophosphates are quite strongly stacked at room temperature, and that the intramolecular base-stacking tendencies are quite comparable for the two sequence isomers. A comparison of the results obtained in this work for ApC and CpA with those previously reported by Chan and Nelson[4] for ApA suggests that the intramolecular stacking interaction is probably somewhat stronger in ApA than in ApC and CpA. These conclusions are in general agreement with those reached by other workers,[18,20,21] based upon optical studies of these systems, at least with regard to the general classification of these dinucleoside monophosphates as being "stacked." The present pmr study also yielded information concerning the conformation of the intramolecular stacks and the conformational changes in the ribose–phosphate–ribose backbone accompanying the intramolecular base-stacking interaction. The chemical shift data were shown to be consistent with stacked conformations in which both bases of the dinucleoside monophosphates are preferentially oriented in the *anti* conformation, as in similar dApdC and dCpdA (dA ≡ deoxyadenosine; dC ≡ deoxycytidine) segments of double-helical DNA. The average ring conformation of the two ribose moieties has been found to become more 3′-*endo* as a result of the intramolecular base-stacking interaction. Finally, the concentration dependence of the pmr spectra of ApC and CpA indicates that these dinucleotides undergo extensive intermolecular self-association, pointing out the necessity of working at a low concentration of dinucleotide in order to obtain meaningful results regarding the intramolecular process.

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Synthesis of Peptide Analogs of the N-Terminal Eicosapeptide Sequence of Ribonuclease A. XII. Synthesis of des-Lys^1-[Orn^{10}]-, des-Lys^1,Glu^2-[Orn^{10}]-, and des-Lys^1,Glu^2,Thr^3-[Orn^{10}]-S-peptides[1,2]

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Abstract: Syntheses are described of three analogs of S-peptide in which the arginyl residue, in position 10, has been replaced by ornithine and one, two, or three of the residues of the 1–3 sequence are respectively removed. The stereochemical homogeneity of these peptides, *i.e.*, des-Lys^1-[Orn^{10}]-, des-Lys^1,Glu^2-[Orn^{10}]-, and des-Lys^1,Glu^2,-Thr^3-[Orn^{10}]-S-peptide, was assessed by digestion with aminopeptidase M followed by quantitative amino acid analysis. The enzymic properties of the three synthetic analogs were checked, with RNA, after recombination with S-protein. Both the des-Lys^1-[Orn^{10}]-S-peptide and the des-Lys^1,Glu^2-[Orn^{10}]-S-peptide form an about 50% active partially synthetic ribonuclease at a molar ratio of 1:1 with S-protein, while further removal of threonine in position 3 is accompanied by a significant decrease of the potential activity of the des-Lys^1,Glu^2,Thr^3-[Orn^{10}]-S-peptide. The des-Lys^1,Glu^2,Thr^3-[Orn^{10}]-RNase S′ shows 20% of the ability of RNase S′ to catalyze the depolymerization of RNA.

Much information is presently available about the importance of the different amino acid side chain residues in the S-peptide sequence in connection with the specific noncovalent binding responsible for the formation of the complex between S-peptide and S-protein.

Structure–function studies, carried out in our laboratory by the synthetic approach,[3] have shown that

(1) The peptides and peptide derivatives mentioned have the L configuration. For a simpler description the customary L designation for individual amino acid residues is omitted. The following abbreviations are used: (a) [IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **241**, 2491 (1966)]: Z = benzyloxycarbonyl, Boc = *t*-butyloxycarbonyl, OMe = methyl ester, OEt = ethyl ester, OBu^t = *t*-butyl ester, ONp = *p*-nitrophenyl ester, DMF = dimethylformamide, TFA = trifluoroacetic acid, TCA = trichloroacetic acid. (b) [F. M. Richards, *Proc. Natl. Acad. Sci. U. S.*, **44**, 162 (1958)]: RNase A, the principal chromatographic component of beef pancreatic ribonuclease; RNase S, subtilisin-modified RNase A; S-peptide, the eicosapeptide obtained from RNase S; S-protein, the protein component obtained from RNase S; RNase S′, the reconstituted enzyme obtained by mixing equimolar amounts of S-peptide and S-protein. According to (c) M. S. Dosher and C. H. W. Hirs, *Federation Proc.*, **25**, 527 (1966), natural S-peptide is a mixture of at least (1–20)-S-peptide and (1–21)-S-peptide.

(2) Some of the results recorded in this paper have been presented at the IXth European Peptide Symposium, Orsay, France, April 15, 1968; E. Scoffone, F. Marchiori, L. Moroder, R. Rocchi, and A. Scatturin in "Peptides 1968," E. Bricas, Ed., North-Holland Publishing Co., Amsterdam, Netherlands, 1968, p 325.

(3) (a) E. Scoffone, R. Rocchi, F. Marchiori, L. Moroder, A. Marzotto, and A. M. Tamburro, *J. Am. Chem. Soc.*, **89**, 5450 (1967); (b) R. Rocchi, L. Moroder, F. Marchiori, E. Ferrarese, and E. Scoffone, *ibid.*, **90**, 5885 (1968); (c) F. Marchiori, R. Rocchi, L. Moroder, A. Fon-