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

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Abstract: The nature of the base-stacking interaction in aqueous solution between the two adenine rings in adenylyl(3′→5′)adenosine (ApA) has been investigated by proton magnetic resonance spectroscopy. The pmr spectrum was studied as a function of concentration, temperature, solution pH, and concentration of added purine. The results of these pmr studies indicate that the stacking interaction between the two adenine rings in ApA 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. In the purine-binding experiments, evidence was obtained for the formation of the purine-intercalated dinucleotide complex in neutral solution, and it was shown that purine intercalation provides a sensitive probe both for the relative strength of the intramolecular stacking interaction and for the relative conformation of the two bases in the dinucleotide. It was also concluded that while the two adenine rings are destacked by protonation of the bases, increasing the temperature to 95° does not result in complete unfolding of the dinucleotide molecule. Finally, ApA was found to self-associate extensively in aqueous solution, and it was shown that the mode of self-association is strongly pH dependent. Despite complications arising from this self-association, the mode of self-association provided additional evidence for the strength and the conformation of the intramolecular stack.

In these laboratories, several studies have been initiated in an effort to accumulate information which will be of value in furthering the understanding of the chemical and physical properties of the biologically important nucleic acids. Of particular interest are the factors which may contribute to the secondary and tertiary structures of these macromolecules. Sufficient evidence has now been accumulated to indicate that the base-stacking interactions of contiguous nitrogenous bases along the polynucleotide chains make a significant contribution to the stability and conformational properties of nucleic acids.[4–16]

It is also now well established that biological bases, nucleosides, and nucleotides associate extensively in aqueous solution, and that they interact principally *via* vertical stacking of the pyrimidine and purine rings. These interactions have been studied by vapor pressure osmometry,[8,15,17] sedimentation equilibrium,[18,19] and by proton magnetic resonance spectroscopy.[17,20–22] The forces which govern the formation of these *intermolecular* complexes are of considerable interest both from a theoretical point of view and with respect to their importance in stabilizing polynucleotide structures as well as polynucleotide–nucleotide complexes.

This series of papers is concerned with the study of the conformational properties of ribose dinucleoside monophosphates in aqueous solution by proton magnetic resonance spectroscopy. The investigation of the dinucleoside monophosphates is a logical extension of our earlier pmr work on the stacking of simple purine and pyrimidine bases, nucleosides, and nucleotides. There is ample reason to expect the intramolecular base-stacking tendencies in the dinucleoside monophosphates to be more representative of the nearest-neighbor base interactions in a polynucleotide. To the extent that *pairwise* nearest-neighbor intercations are of primary importance in determining the structural and conformational properties of a polynucleotide, a study of the intramolecular base-stacking interactions in these simpler units is expected to lead to a better understanding of the sequence-dependent properties of single-stranded polynucleotides as well as double-stranded nucleic acids. It is noteworthy that several groups of workers[23–32] have already

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examined the optical properties of dinucleotides and trinucleotides with the anticipation that information on these model compounds will be useful in elucidating the three-dimensional molecular structure of RNA molecules.

By virtue of the extreme sensitivity of the method to minute structural and conformational changes within molecules and to environmental effects, proton magnetic resonance spectroscopy is particularly ideal for the study of the base-stacking interactions in the dinucleoside monophosphates. The biologically important nucleosides or nucleotides all have ring hydrogens whose proton resonances can be conveniently monitored. In dinucleotides containing at least one adenine or guanine base, the ring-current magnetic anisotropy of the the purine base can influence the magnetic environment of the ring protons of the opposing base, and hence provide a convenient handle for studying the relative conformation of the two bases of the dinucleotide. For example, information about the intramolecular base-stacking interaction can be obtained by monitoring the chemical shifts of the base protons as a function of temperature and pH. In these laboratories, we have recently developed another method[33] to probe the intramolecular stacking interaction between the two bases in a dinucleotide. In this method a probe molecule, such as purine, which can interact with the bases of the dinucleotide by stacking, is introduced into the system, and from an investigation of the mode of binding between the probe molecule and the dinucleotide, it is also possible to infer the relative conformation of the two bases in the dinucleoside monophosphate.

This paper summarizes the application of these methods toward the elucidation of the intramolecular base-stacking interaction in adenylyl(3′→5′)adenosine (hereafter referred to as ApA; Figure 1). We shall show that the results of these studies enable us to make some rather definite conclusions regarding the conformation of the two adenine rings in this dinucleotide.

Experimental Section

Materials. A-grade ApA was obtained from CalBiochem, Los Angeles, Calif., and was used without further purification. It was shown to be homogeneous by paper chromatography and electrophoresis. Chemical analysis showed that it contained 5.11% phosphorus, in agreement with the theoretical phosphorus content of 5.19%. The uv absorption spectrum at pH 2 exhibited λ_{max} at 257 mμ and λ_{min} at 230 mμ.

A column containing Dowex 50W-X8 cation-exchange resin was used to convert the acid form of ApA to the ammonium and sodium salts. All samples were prepared in deuterium oxide (99.7 mol %), supplied by Columbia Organic Chemicals, Columbia, S. C.

Purine was obtained from Cyclo Chemical Corporation, Los Angeles, Calif. The purine was sublimed *in vacuo* before use.

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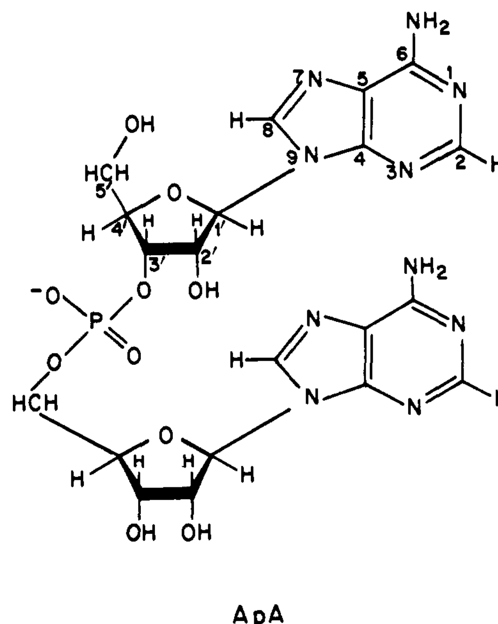

Figure 1. Adenylyl(3′→5′)adenosine (ApA).

Methods. Three nuclear magnetic resonance spectrometers were utilized during the course of this work. The purine-binding experiments at high ApA concentrations were undertaken on a Varian A-60 spectrometer. The pH and concentrations studies were made on a Varian A-60A spectrometer. In these studies, the probe temperature was approximately 40°, and 6% TMS in chloroform was used as the reference compound for monitoring the chemical shifts. The temperature studies and the purine-binding experiments at low ApA concentrations (0.01 *M*) were carried out on a Varian HA-100 spectrometer operating in the frequency-sweep mode. Neat TMS sealed in a capillary was used to provide the field-frequency lock signal and the chemical shifts were measured directly from the lock signal by counting the frequency difference between the reference and sweep oscillators with a Hewlett-Packard 5512A frequency counter. Wherever necessary, a Varian C-1024 time-averaging computer was used to enhance weak signals. Unless specified otherwise, all measurements made on the HA-100 spectrometer were undertaken at the normal probe temperature, which was 31 ± 1°.

Except in the case of the temperature studies the chemical shifts reported in this paper have not been corrected for bulk susceptibility effects. However, it is estimated that this correction would not exceed 1-2 cps in most of our experiments. In the temperature studies, we have attempted to compensate for bulk susceptibility effects and effects arising from changes in the water structure and in the solvent-solute interaction with temperature by referencing the proton resonances to the corresponding proton resonances of adenosine in a 0.0032 *M* adenosine[34] solution at the same temperature.

In the pH studies, the deuterium ion concentration was varied by the addition of small aliquots of aqueous solutions of hydrochloric acid or sodium hydroxide. The sample volumes were not changed significantly during these experiments. The pD of the solutions were monitored by a Leeds and Northrup 7401 pH meter utilizing a miniature (No. 12413) glass electrode. This apparatus was standardized to read pH in H_2O solutions. In this paper, deuterium ion concentrations in the D_2O solutions are indicated by "pD" values, where pD = pH (meter reading) + 0.40.[35]

Results and Discussion

Spectrum and Assignment of Adenine Proton Resonances. The pmr spectrum of ApA in the region of the aromatic protons consists of four distinct resonance peaks which can be assigned to the two adenine H_2 and the two adenine H_8 protons of the dinucleoside monophosphate. Due to asymmetric esterification of the

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Table I. Summary of Chemical Shifts of the Adenine Protons in ApA, ApU, UpA and Related Mononucleosides and Nucleotides at 31°

Molecule	Concn, *M*	Solution pD	Chemical shifts,[a] ppm $H_8(5')$	$H_8(3')$	$H_2(5')$	$H_2(3')$
ApA (sodium salt)	0.01	7–8	8.69	8.65	8.57	8.43
	0.003	7–8	8.70	8.67	8.60	8.47
	Infin dilutn[c]	7–8	8.71	8.68	8.61	8.48
ApU (sodium salt)	0.01	7.7	...	8.77	...	8.63
	0.003	7–8	...	8.80	...	8.66
	0.001	7–8	...	8.80	...	8.67
UpA (sodium salt)	0.01	7–8	8.84	...	8.66	...
	0.003	7–8	8.85	...	8.67	...
3′-AMP	0.01	5.8	...	8.79	...	8.68
	0.01	11	...	8.70	...	8.68
5′-AMP	0.009	6.2	8.92	...	8.68	...
	0.01	10	9.06	...	8.70	...
2′-AMP	0.01	5	8.79		8.68	
Adenosine 3′,5′-cyclic phosphate	0.01	...	8.67[b]		8.67[b]	
Adenosine	0.003	...	8.76		8.68	

[a] Downfield relative to external TMS; not corrected for bulk susceptibility. For discussion of assignment of ApA adenine resonances see text. [b] More exact positions of resonances: 8.674, 8.665. [c] Extrapolated.

sugar moieties of the two nucleosides to the phosphate group (3′- *vs.* 5′-), the two adenine rings of ApA are not geometrically equivalent. The pmr spectrum of ApA clearly indicates that the same protons (H_2 or H_8) on the two adenine rings are not magnetically equivalent.

As in the case of other purine derivatives, the adenine H_8 protons of ApA are readily distinguished from the H_2 protons by deuterium exchange of the H_8 protons upon equilibration in D_2O at elevated temperatures. In this manner, we have been able to assign the two downfield resonances of the ApA adenine spectrum to the H_8 protons. We have not attempted a direct experimental assignment of the two H_8 or the two H_2 resonances by specific deuterium labeling. However, on the basis of (i) Mn(II) ion-binding studies and (ii) temperature studies, we have shown that the adenine proton resonance of the 3′-nucleoside is higher field in each case. In this connection, it is important to emphasize that the difference in the spectral positions of the same protons on the two adenine rings has its origin primarily in their different average spatial relationships with respect to the negatively charged phosphate group and with respect to the opposing adenine ring. In principle, the chemical shifts of the adenine base protons can also be dependent upon the conformation of the base relative to the ribose moiety about the glycosidic bond of each nucleoside unit, which in turn should be sensitive to the conformation of the ribose moiety and the stacking interaction between the two adenine rings. The ribose conformations of the two ribose moieties are not identical because of the asymmetric esterification of the two nucleosides to the phosphate group. However, apparently the difference in the ribose conformations is small. For example, the two $H_{1'}$–$H_{2'}$ coupling constants, which provide some indication of the ribose conformations, are quite similar in ApA over a wide range of temperatures. Hence, unless the base-stacking interaction between the two adenine rings forces the two adenine bases into quite different average conformations relative to their respective ribose moieties (there does not appear to be evidence to support this), the difference in the spectral positions of the same protons on the two adenine rings can only be interpreted in terms of their different average spatial relationships with respect to the negatively charged phosphate group and the respective opposing adenine ring. It should then be possible to assign the two sets of resonances on the basis of their spectral response toward perturbations in the vicinity of the phosphate group and to changes in the interaction between the two adenine rings.

To facilitate our discussion of the specific experiments which led to our assignment of the adenine proton resonances in ApA, we have also examined the chemical shifts of the adenine protons in several related nucleosides, nucleotides, and dinucleotides. In Table I, we have summarized the proton chemical shifts for the adenine H_8 and H_2 resonances in ApA, adenylyl-(3′→5′)uridine (ApU), uridylyl(3′→5′)adenosine (UpA), 2′-adenosine monophosphate (2′-AMP), 3′-AMP, 5′-AMP, adenosine 3′,5′-cyclic phosphate, and adenosine. The constancy of H_2 chemical shift in all of the molecules except ApA is immediately evident, and suggests that the magnetic environment at the H_2 proton is insensitive to the nature of the phosphate substitution and its degree of ionization. By contrast, the spectral position of the H_8 resonance is somewhat more sensitive to the position of the phosphate substitution and its degree of ionization. While the H_8 chemical shifts are similar in adenosine, 2′-AMP, and 3′-AMP under comparable pH conditions, the chemical shift of the H_8 proton in 5′-AMP is noticeably further downfield, by amounts which depend upon the degree of the phosphate ionization. This downfield shift for the H_8 resonance in 5′-AMP no doubt has its origin in the close proximity of the H_8 proton to the phosphate group, and the resultant electrostatic polarization by the negatively charged phosphate. This observation has led us and others[36] to conclude that the adenine base is preferentially oriented in the *anti* conformation with respect to its ribose ring in 5′-AMP. Actually, there is no *a priori* reason not to expect a similar *anti* conformation for the adenine base in 2′-AMP or 3′-AMP, since the conformation of the base is primarily dependent upon conformational energies about the glycosidic bond, and the ribose conformations, as indicated by the $H_{1'}$–$H_{2'}$ coupling constants, are not

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significantly different in these three adenosine monophosphates. There is a similar difference in the chemical shifts of the adenine H_8 protons between ApU and UpA. As expected, the spectral position of the H_8 proton of the 3′-esterified nucleoside is similar to that in adenosine, 2′-AMP, and 3′-AMP, and that of the 5′-esterified nucleoside appears at considerably lower field. The chemical shifts of the adenine protons in the cyclic phosphate are interesting. The H_8 proton resonance appears at considerably higher field than in any of the mononucleotides cited, and this result no doubt reflects the effect of ribose conformation on the chemical shift of this proton. The ribose ring is forced into the 3′-*endo* conformation in the cyclic phosphate, whereas the conformation of the ribose ring in the other mononucleotides is closer to 2′-*endo*. Apparently, the chemical shift of the adenine H_2 resonance is insensitive to the ribose conformation.

Since only the H_8 resonance of the 5′-esterified nucleoside appears to be sensitive to the presence of the phosphate group, it appears that it should be possible to assign the two H_8 resonances in ApA by their spectral response toward perturbations in the vicinity of the phosphate group. With this rationale, we have examined the two H_8 resonances under conditions in which the phosphate is bound to a paramagnetic ion.

Mn(II) ion is known to bind to mononucleotides[37,38] and at low nucleotide concentrations, we have shown that the paramagnetic ion binds primarily to the phosphate group. The paramagnetic ion binding leads to broadening of the proton resonances because of electron-spin, nuclear-spin, dipole–dipole tensor interaction. This dipole–dipole interaction is extremely distance dependent [$\alpha(1/r^6)$], and hence the line broadening may be employed to locate the position of a proton relative to the site of binding. The results of our Mn(II)–ApA ion-binding studies are summarized in Figure 2. For sake of comparison, we have also included the results of a similar study for 5′-AMP and adenosine 3′,5′-cyclic phosphate. The interpretation of the results for the two mononucleotides is straightforward. Clearly, because of the closer proximity of the H_8 proton to the phosphate in the 5′-nucleotide, the H_8 resonance here is broadened to a more appreciable extent than the H_8 resonance of the cyclic adenosine monophosphate, where the phosphate group is fixed at a distance relatively far removed from the H_8 proton. The selective broadening of the lower field H_8 resonance in ApA indicates that it is to be assigned to the H_8 proton of the 5′-nucleoside. Thus, the assignment of the two H_8 resonances of ApA is $H_8(5')$, $H_8(3')$, in the order of increasing field. Finally, we note that the adenine base of the 5′-nucleoside must also be preferentially oriented in the *anti* conformation, since the H_8 resonance would not be sensitive to perturbations in the vicinity of the phosphate group if this were not the case.

We now turn our attention to the assignment of the H_2 resonances. The two H_2 resonances in ApA both appear at significantly higher fields than the spectral position of this proton in ApU, UpA, and the other

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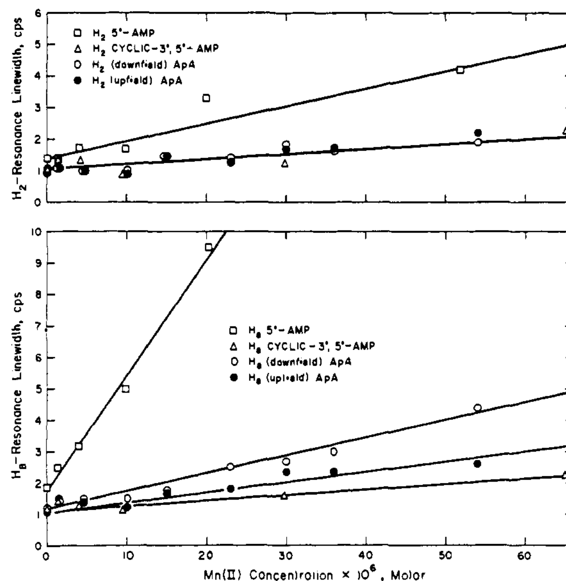


Figure 2. Resonance line widths of adenine protons of 5′-AMP, cyclic 3′,5′-AMP, and ApA as a function of Mn(II) concentration.

adenine mononucleotides (Table I). In view of the lack of sensitivity of the chemical shift of the H_2 proton to the nature of the ribose–phosphate backbone, the upfield shifts in ApA must have their origin in the ring-current magnetic anisotropy of the opposing adenine base. This conclusion immediately indicates that the two adenine bases in ApA are intramolecularly stacked at room temperature, and suggests that it may be possible to assign the H_2 resonances on the basis of their spectral behavior toward alteration of the intramolecular base-stacking interaction with temperature. The chemical shifts of the H_8 protons presumably are also influenced by the ring-current magnetic anisotropy effects of their respective opposing ring. Since the two adenine H_8 proton resonances have already been assigned, the spectral behavior of the two H_8 resonances with increasing temperature can be used to indicate the conformation of the ApA stack, and once this conformation has been ascertained, the H_2 resonances can be assigned on the basis of their temperature behavior.

In Figure 3, we have depicted for illustrative purposes, four possible *stacked* conformations of ApA, as viewed along the axis of the ribose–phosphate backbone in the direction of 5′- to 3′-phosphate esterification. The 3′-*anti*,5′-*anti* conformation is designated by I. The 3′-*anti*,5′-*syn* (II), 3′-*syn*,5′-*anti* (III), and 3′-*syn*,5′-*syn* (IV) stacked conformations have been obtained by rotating either or both of the adenine rings 180° about the respective $C_{1'}$–N_9 glycosidic bond from the DNA-like base orientations. Some twisting of the ribose–phosphate backbone was also necessary to get the two adenine bases oriented as indicated in conformations II and III. In the case of II, the 5′-nucleoside was rotated outward (clockwise as viewed) about the 5′-CH_2–OP phosphoester bond to reduce the steric interaction between the 5′-adenine base with the ribose–phosphate backbone. In the case of III, the 5′-nucleoside was rotated inward (counterclockwise as viewed) about the 5′-CH_2–OP phosphoester bond to gain greater base overlap between the two adenine bases.

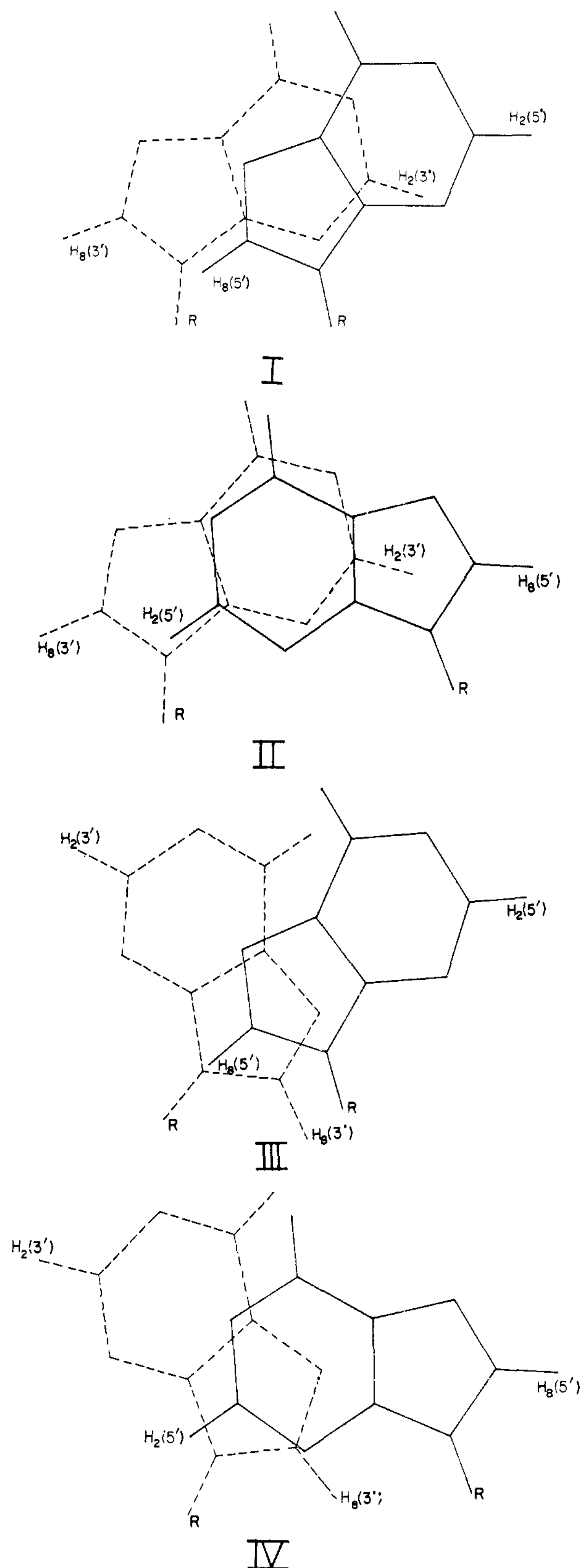


Figure 3. Four possible stacked conformers of ApA.

These illustrations of the various stacked conformations are based on a molecular model of the ApA molecule built from CPK atomic models. The actual average conformations may be somewhat different from those depicted in Figure 3. In any case, the equilibrium dihedral angle of stacking is probably not too well defined, since the potential trough which describes the

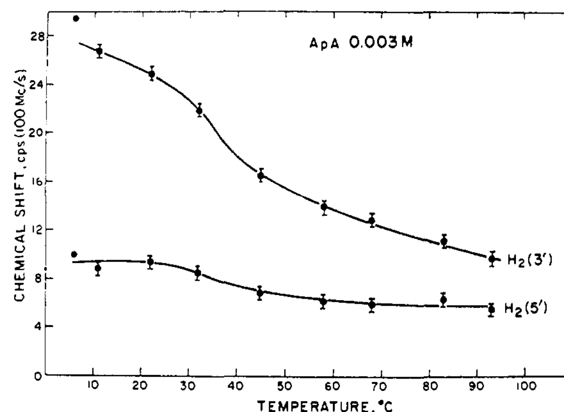


Figure 4a. Variation of the chemical shifts (100 Mcps) of the H_2 resonances of ApA with temperature. The chemical shifts are relative to the H_2 resonance of adenosine at the same temperature and concentration.

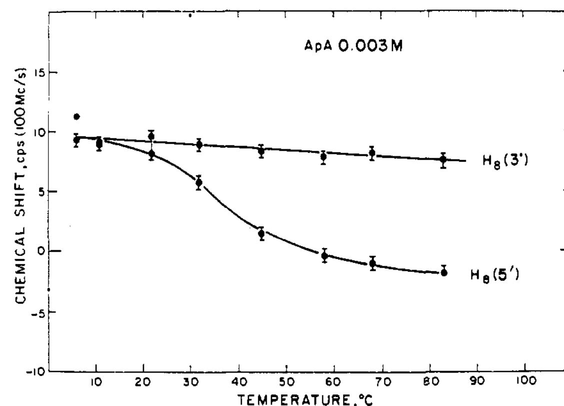


Figure 4b. Variation of the chemical shifts (100 Mcps) of the H_8 resonances of ApA with temperature. The chemical shifts are relative to the H_8 resonance of adenosine at the same temperature and concentration.

interaction between the adenine bases in each of these stacked ApA conformers is relatively broad near the minimum and the amplitude of vibration of the two adenine rings relative to each other is large.

From Figure 3 it is clear that $H_2(3')$ will experience a large ring-current effect from the opposing 5′ base only when the 3′ base is *anti*. Similarly, $H_2(5')$ will experience a sizeable ring-current shift from the 3′ base, only when the 5′-base is *syn*. A similar generalization can be made for the H_8 resonances. Thus $H_8(3')$, and $H_8(5')$ will experience appreciable ring-current shifts from their respective opposing adenine base only when the 3′ base and the 5′ base are *syn* and *anti*, respectively.

In Figure 4, we have summarized the variation of the chemical shifts of the adenine base protons with temperature. For reasons which we shall discuss later, these shifts have been referenced to the shifts of the corresponding protons in adenosine at each temperature. The striking feature of the data is that only one of the H_2 resonances and one of the H_8 resonances exhibit significant downfield shifts, as the two adenine bases are destacked with increasing temperature, namely, $H_8(5')$ and the upfield H_2 resonance. This indicates that both adenine bases are preferentially oriented in the *anti* conformation relative to their respective furanose rings, and that the assignment of the upfield H_2 resonance must be that of the 3′-nucleoside.

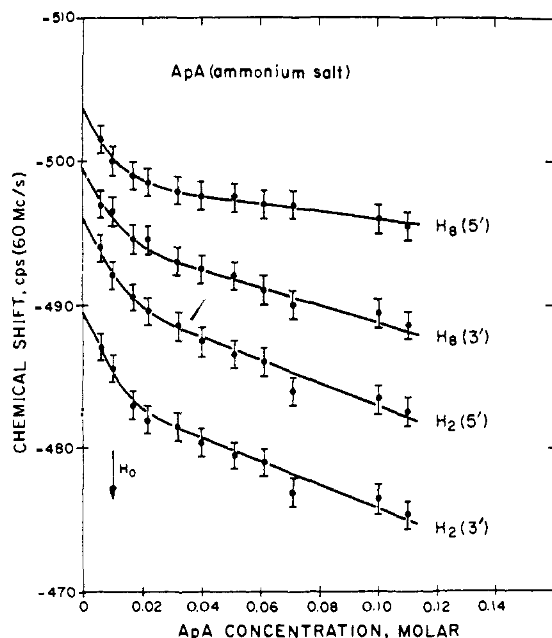


Figure 5. Concentration dependence of the proton chemical shifts of the adenine protons of ApA in aqueous solution at 40°. The chemical shifts (at 60 Mcps) were monitored relative to external TMS (6% in chloroform) and have not been corrected for bulk susceptibility.

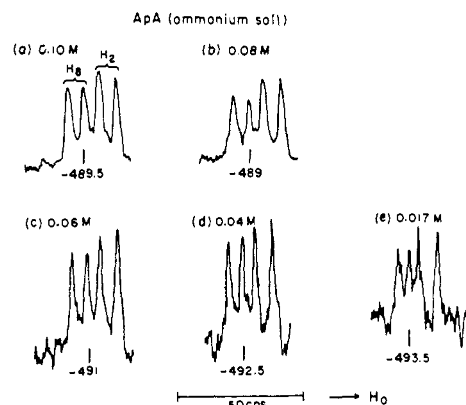
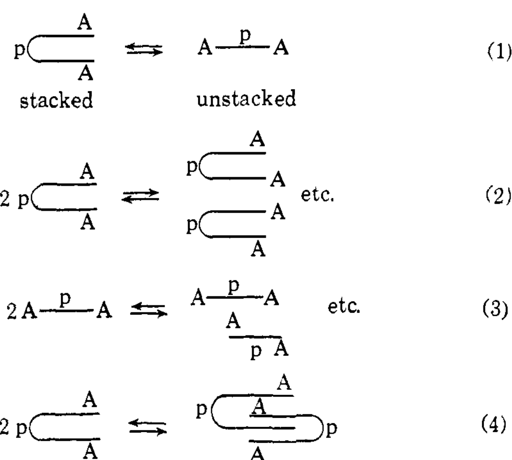


Figure 6. Pmr spectra (60 Mcps) of ApA at various dinucleotide concentrations. Chemical shifts are referred to external TMS (6% in chloroform), temperature ~40°.

The Self-Association of ApA. ApA is expected to self-associate extensively in aqueous solution. To determine the nature and the extent of the molecular aggregation, we have studied the pmr spectrum of the adenine protons of ApA over the concentration range of 0.005–0.11 *M*. A pronounced concentration effect has been observed. The chemical shifts of the four adenine protons of ApA are plotted *vs.* the concentration in Figure 5. The experimental data as displayed have not been corrected for bulk susceptibility. However, in view of the low concentration of the solute even at the highest concentration of ApA studied, bulk susceptibility corrections are extremely small.

The downfield shifts observed for all the proton resonances with decreasing concentration can, of course, be attributed to the breakdown of stacked intermolecular complexes. However, it is interesting that the self-association tendency of ApA is considerably more pronounced than that previously noted for purine.[20] Evidently, a significantly large fraction of ApA molecules exists as stacked "oligomers" even at a concentration as low as 0.05 *M*. This conclusion is consistent with recent osmotic studies of adenosine,[17] which demonstrated that the association constant per step in the stacking multiple equilibria is ~4.5 m^{-1} for this molecule, or about a factor of 2 larger than the value previously reported[15] for purine self-association.

Several modes of intermolecular association are possible. If the dinucleoside is strongly stacked intramolecularly, there can only be "external" stacking of folded ApA molecules. If the two adenine bases are not strongly stacked intramolecularly, then two molecules can base stack intermolecularly on either face of the adenine rings, in which case self-intercalated intermolecular dimers can also be formed. Here, the adenine base of one molecule is inserted between the two bases of another ApA molecule. Evidence for this mode of intermolecular association has been obtained in this laboratory for the purine–pyrimidine (pyrimidine–purine) dinucleoside monophosphates. As in the case of purine intercalation, one would expect the extent of "self-intercalation" to be of lesser importance, the stronger the intramolecular stacking interaction between the two bases of the dinucleotide. We can summarize the different possible modes of self-association in terms of equilibria 1–4.



where A = adenine; p = phosphate-ribose backbone

As mentioned previously, the tendency to form self-intercalated complexes provides some indication of the strength of the intramolecular stacking interaction since intercalation is not expected to be important if the intramolecular base-stacking interaction is strong. To ascertain the relative importance of this mode of self-association, we have studied the line widths of the ApA adenine proton resonances as a function of concentration. In UpA, where self-intercalated dimers are apparently formed,[39] the line widths of the adenine resonances are concentration dependent and are somewhat broadened at high concentrations of the dinucleotide. The source of the adenine line broadening is presumably analogous to that observed for the purine proton resonances when purine is incorporated between the two bases of a dinucleotide. In

(39) J. H. Nelson, B. W. Bangerter, J. H. Prestegard, and S. I. Chan, manuscript in preparation.

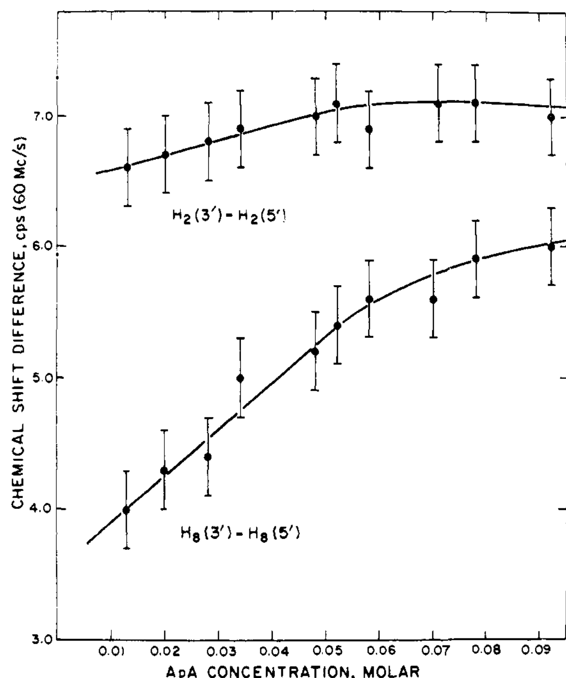


Figure 7. Chemical-shift difference (60 Mcps) of the two H_2 adenine resonances and of the two H_8 adenine resonances of ApA as a function of ApA concentration.

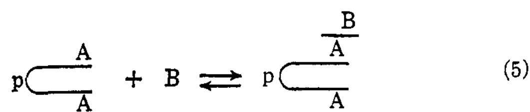
Figure 6, we have reproduced tracings of the pmr spectrum of ApA in the region of the adenine ring protons at various concentrations of the dinucleotide. It is noteworthy that the line widths of the proton resonances are not greatly affected as the concentration of the dinucleotide is increased. Therefore, it may be concluded that ApA exists primarily in the folded conformation with the two adenine bases stacked intramolecularly, and that it self-associates principally *via* external stacking with little base intercalation.

It is interesting to note that the concentration shifts are slightly different for the two adenine H_8 resonances. In Figure 7, we have plotted the concentration dependence of the difference in the chemical shifts of the two adenine H_8 resonances and the corresponding difference of the two H_2 resonances. Whereas the chemical-shift difference for the H_2 resonances remains essentially unchanged over the entire concentration range, the chemical-shift difference for the H_8 protons decreases as the concentration of ApA is lowered. At infinite dilution, the H_8 chemical-shift difference is ~3.5 cps (at 60 Mcps) and that for H_2 is ~6.5 cps. At a concentration of 0.1 M, the difference for the H_8 resonances is ~6 cps and the H_2 difference is ~7 cps. We do not believe that the above concentration-induced change in the chemical-shift difference for the H_8 resonances arises from the formation of self-intercalated dimers. If this were the explanation, we might expect a similar concentration-induced effect for the H_2 resonances, even though it is possible that on the average there can be accidental cancellation of intermolecular and intramolecular ring-current effects when the two adenine bases in an ApA molecule move apart to incorporate an inserting adenine base of another molecule. However, it does seem surprising that the chemical shift difference between the two H_2 resonances should persist at the infinite dilution value over such a wide range of concentrations. A more disturbing aspect of this interpretation is the large concentration-induced change in the difference in chemical shifts of the H_8 resonances, particularly in view of the relative sharpness of all the adenine proton resonances even at high concentrations of ApA. For purine intercalation, the largest observed difference in the purine-induced shifts has not exceeded 5 cps at 60 Mcps. We are more inclined to attribute the observed concentration-induced change in the magnetic nonequivalence of the two H_8 resonances to slightly different exposure of the two H_8 protons to intermolecular ring-current magnetic anisotropy effects of externally stacking ApA molecules. If the conformation of the ApA molecule in solution is similar to that of a dApdA (dA = deoxyadenosine) segment in double-helical DNA [both adenine bases oriented *anti* relative to the ribose moiety about the $C_{1'}$–N_9 glycosidic bond; Figure 3 (I)] then H_8 of the 5′-nucleoside is situated in the "cleft" formed by the adenine rings and the ribose–phosphate backbone, whereas H_8 of the 3′-nucleoside is located near the base of the ribose–phosphate "ridge" away from the phosphate group. Consequently, $H_8(5')$ is expected to be, on the average, somewhat more shielded from the ring-current magnetic anisotropy effects of an ApA molecule stacked externally on the 5′-adenine base due to steric effects of the "ridge" of the ribose–phosphate backbone, when compared with similar ring-current effects on the $H_8(3')$ when an ApA molecule stacks externally on the 3′ base. Therefore, in the self-association of ApA, external base stacking may result in a greater upfield shift for $H_8(3')$ than for $H_8(5')$; hence the magnetic nonequivalence of the H_8 protons should increase with concentration, as observed. Since the adenine H_2 protons are considerably more removed from the ribose–phosphate backbone when the adenine bases are in the intramolecularly stacked conformation discussed above, we expect external self-stacking to result in little change in the magnetic nonequivalence of the H_2 protons.

ApA–Purine Interactions

The binding of purine to intramolecularly stacked ApA molecules can be described in terms of equilibria 5–11 where B denotes any purine species, irrespective of its state of aggregation, *i.e.*, purine monomers, and stacked dimers, trimers, tetramers, etc. In order to indicate that only monomeric purine can intercalate between the bases of the dinucleotide, we have designated monomeric purine also by M. By contrast, purine dimers, trimers, tetramers, as well as monomers, can stack on the external faces of folded ApA molecules.

It is apparent from the results presented in the previous section that the binding of purine to ApA can be complicated by the self-association of the dinucleotide even at quite low dinucleotide concentrations. Irrespective of the mode of self-association, it is clear that these processes will compete with purine binding. While the mere external stacking of folded ApA molecules is not expected to greatly reduce the activity of the dinucleotide toward purine intercalation, it will, however, hamper the external binding by purine stacks. Any appreciable extent of self-intercalation will certainly inhibit the formation of purine-intercalated dinucleotide complexes.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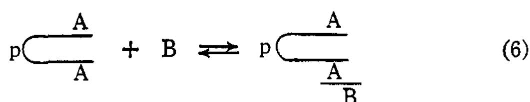
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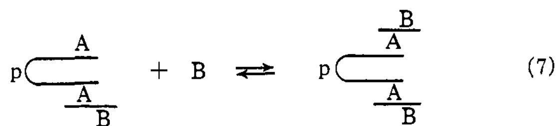
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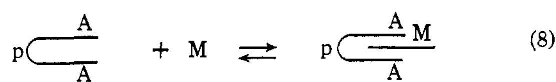
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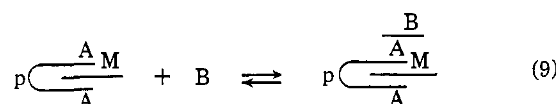
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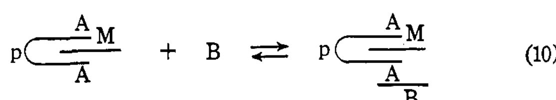
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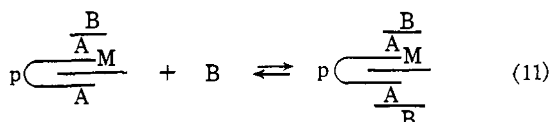
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An additional complication arises in these purine-binding studies due to the self-association of the probe molecule. The extent of purine intercalation clearly depends upon the concentration of monomeric purine in solution. However, due to extensive polymerization of the purine itself, the purine monomer concentration increases only slowly with increasing stoichiometric purine concentration.[15] Thus, if the intercalation tendency is small, it may not be possible to push the equilibrium over to the intercalated complex by increasing the purine concentration. Increasing the dinucleotide concentration will merely increase the fraction of purine which is intercalated, without greatly altering the fraction of the dinucleotide existing as the intercalated complex. Furthermore, if the dinucleotide concentration is sufficiently high, there can be further reduction in the purine activity toward intercalation due to competition by external purine stacking. In the case of the binding of purine to ApA, where the interaction between an adenine and a purine base is expected to be relatively strong, this reduction in the purine monomer concentration by external binding can be quite significant even at ApA concentrations as low as 0.10 *M*.

These considerations clearly suggest that the purine-binding experiments be done at low dinucleotide concentrations, both to eliminate complications arising from ApA intermolecular effects, and to favor purine intercalation as much as possible at low purine concentrations. The concentration of monomeric purine is $\sim$0.25 *M* at a purine stoichiometric concentration of 1.0 *M* at room temperature.[15] Thus, it is desirable that the dinucleotide concentration in the purine-binding studies should not be much higher than 0.01 *M*. From sensitivity considerations, it is possible to reduce the dinucleotide concentration by another factor of 10. However, the longer data collecting time and problems connected with computer time averaging of weak signals in the vicinity of strong purine resonances make it impractical.

Evidence for purine intercalation comes from two different experimental observations. First, the stacking of purine on the adenine rings of the dinucleotide results in high-field shifts for the two sets of adenine proton resonances. As was demonstrated in our earlier study of the binding of purine to the pyrimidine–pyrimidine dinucleoside monophosphates,[33] the purine-induced shifts are in general quite different for each of the two chemically similar protons on each ring. This difference in the purine-induced shifts has been attributed to the formation of the purine–dinucleotide intercalated complex.[33] Due to geometric nonequivalence of the two bases in the dinucleotide, the ring-current magnetic anisotropy of the inserted purine base is expected to result in slightly different induced chemical shifts for the analogous protons of the two bases. In the case of ApA, where the two adenine rings may be strongly stacked intramolecularly, a small change in magnetic nonequivalence may also be expected for the H_8's due to external purine binding. This purine-induced change in the magnetic nonequivalence of the H_8 protons from external purine stacking is similar to the concentration-induced effect observed for these protons and arises from the close proximity of the H_8 protons to the ribose–phosphate backbone. Since the adenine base also possesses a ring-current magnetic anisotropy, we would also expect some contribution to the intercalation shifts from destacking of the two adenine rings, if they are stacked intramolecularly prior to purine intercalation.

Secondly, evidence for purine intercalation is also provided by the effect of the dinucleotide on the proton magnetic resonance spectrum of the purine protons. In the presence of the dinucleotide, the purine proton resonances are significantly broadened particularly at low purine–dinucleotide concentration ratios where the fraction of incorporated to unbound purine is highest. Such line broadening of the purine resonances has been observed when purine is complexed with pyrimidine–pyrimidine and purine–pyrimidine (pyrimidine–purine) dinucleoside monophosphates.[33,39] The effect is extremely stereospecific. For example, the purine H_6 and H_8 resonances are broadened to a considerably greater extent than the H_2 resonance. The effect is also strongly sequence dependent. For instance, in our earlier purine-binding experiments with TpT, dUpT, and TpdU, the line broadening was most pronounced for TpdU and least evident for dUpT, with TpT showing intermediate behavior.[33] The origin of the purine line broadening is considered to be the result of magnetic dipolar interaction between the purine protons and the ribose protons of the 3′-esterified nucleoside. This observed line-broadening phenomenon has been discussed in our earlier communication,[33] but we shall have occasion to elaborate on this discussion more fully in a later paper.

It is clear that the purine-induced differential shifts and the purine line-broadening results complement each other, since the two properties monitored are sensitive to different stages of the purine-intercalation equilibrium. The purine-induced differential shifts

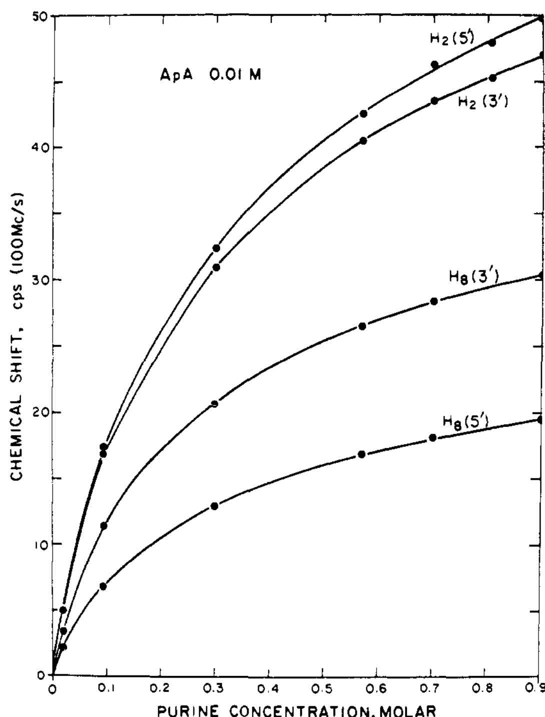

Figure 8. Purine-induced chemical shifts (100 Mcps) observed for the adenine ring protons of ApA (sodium salt), ApA concentration 0.01 *M*.

are largest when the equilibrium is shifted as far to the intercalated complex as possible. The purine line widths are, however, broadest at low purine concentrations, where the fraction of incorporated to unbound purine is highest. Increasing the purine concentration increases the fraction of the dinucleotide existing as the intercalated complex at the expense of more unbound purine and since the observed purine line widths represent a weighted average between bound and free purine due to rapid exchange, the purine line widths sharpen up with increasing purine–dinucleotide ratio.

We have studied the complexing of purine to ApA at both low and high ApA concentrations. The results of these studies are summarized below.

a. Purine Binding at Low ApA Concentrations. The purine-induced shifts for the four adenine proton resonances of a 0.01 *M* ApA solution are summarized in Figure 8. These data have been obtained at 100 Mcps on the Varian HA-100 spectrometer with computer time averaging. It is interesting to note the difference in the purine-induced shifts observed for the H_2 protons. We feel that this is strong evidence for purine intercalation between the two adenine bases in ApA. In contrast to what has been observed for the protons of TpT and CpC, and for that matter, the H_8 protons of ApA, the purine-induced shift for H_2 of the 5′-esterified nucleoside is larger than that observed for the H_2 resonance of the 3′-nucleoside. This result may seem surprising, but it can be readily rationalized if prior to purine intercalation ApA exists preferentially in a folded conformation, with the bases oriented *anti* relative to their respective ribose moieties. In this folded conformation, the two adenine bases are stacked in a partial overlap manner with the $H_2(3')$ located directly below the center of the 5′-adenine ring [Figure 3 (I)]. $H_2(3')$ therefore experiences a large ring-current magnetic anisotropy effect due to the 5′ base. On the other hand, the ring-current magnetic anisotropy of the 3′-adenine base at the position of the $H_2(5')$ is small. Hence, the purine-induced shifts for $H_2(5')$ can reflect little of the intramolecular conformational changes which must accompany the purine intercalation process, whereas such structural changes are certainly reflected in the position of the $H_2(3')$ resonance. The purine-induced shifts for $H_2(3')$ therefore represent a compensation of changes in both the intramolecular and intermolecular ring-current effects. The two H_2 protons are probably not equally exposed to the ring-current magnetic anisotropy of the incorporated purine base in the intercalated complex. The effect of this ring-current magnetic anisotropy is generally larger for the ring protons of the 3′ base. Apparently, the differences are not large enough to compensate for the larger decrease in the intramolecular ring-current effect of the 5′-adenine base on the $H_2(3')$ proton when the two adenine rings move apart to incorporate the purine base.

The difference in the purine-induced shifts for the H_8 protons is almost 12 cps at 100 Mc at a purine concentration of 1 *M*, with the induced shift larger for $H_8(3')$ than $H_8(5')$. This is the largest purine-induced differential shift that we have observed. On the basis of our earlier observed concentration-induced differential shifts for these protons, we estimate that as much as 5 cps of this presently observed purine-induced differential shift may be accounted for in terms of external purine stacking on the adenine bases of folded ApA molecules and on the adenine bases of intercalated purine-ApA complexes. The remaining 7 cps is presumably due to purine intercalation. This intercalation-induced differential shift for the H_8 protons still appears to be unusually large when it is compared with the differential purine shift of only 3–4 cps between the H_8 protons in ApU and UpA under the same conditions.[39] Again, this large intercalation-induced differential shift can be understood in terms of the conformational changes in the ApA molecule during the intercalation process. In the 3′-*anti*,-5′-*anti* stacked conformation of ApA, $H_8(5')$ would experience a sizeable magnetic anisotropy from the 3′-adenine base since this hydrogen would be situated almost directly above this base in the folded conformation. $H_8(3')$ on the other hand, experiences essentially no ring-current magnetic anisotropy effects from the 5′-adenine base. As in the case of $H_2(3')$, the purine-induced shift for $H_8(5')$ therefore reflects both the ring-current magnetic anisotropy effects of the incorporated purine base and the corresponding reduction in the intramolecular ring-current effect of the opposing adenine base when the two adenine rings move apart in the formation of the intercalated complex. The observation that the purine-induced shifts for the adenine H_8 resonance in UpA is significantly larger ($\sim$15 cps)[39] than the corresponding shift for $H_8(5')$ in ApA reinforces the above interpretation. It should be noted that the phenomenon discussed above is accentuated since the effect of the ring-current magnetic anisotropy of the incorporated purine base is somewhat larger at $H_8(3')$ than at $H_8(5')$, a result which follows simply from geometric considerations.

A purine-induced differential shift was also observed

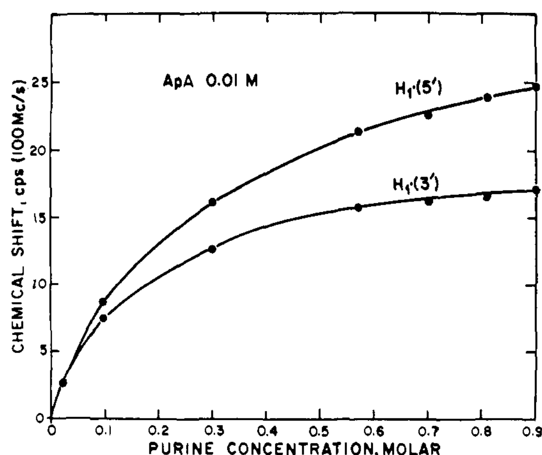

Figure 9. Purine-induced chemical shifts (100 Mcps) observed for the H_1' ribose protons of ApA (sodium salt), ApA concentration 0.01 *M*.

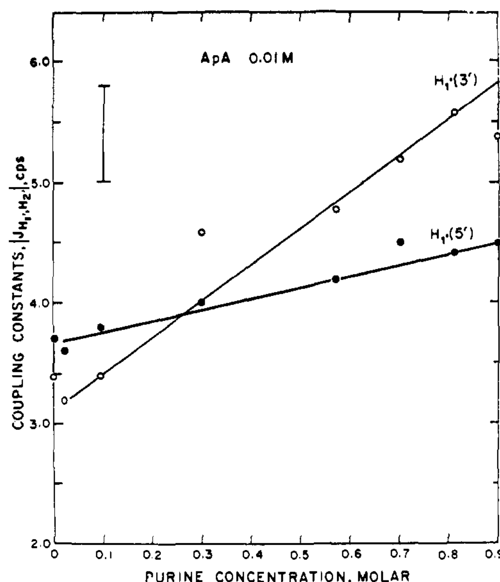

Figure 10. Variation of the $|J_{H_1',H_2'}|$ coupling constants with purine concentration. (Experimental uncertainty indicated by error bar.)

for the H_1'-ribose protons (Figure 9). This induced differential shift is about 8 cps at a purine concentration of 0.9 *M*. We have not made a definite assignment of the resonances of these ribose protons. However, on the basis of a similar purine-binding study of GpA and ApG (G = guanosine), where the $H_{1'}$-proton resonances have been assigned, we have tentatively assigned the lower field doublet of the two sets of ribose $H_{1'}$ resonances of ApA to the 5′-esterified nucleoside. In the purine-binding studies of ApG, GpA, and several other dinucleoside monophosphates, we have noted that the purine-induced chemical shift of the 5′-esterified nucleoside is always greater than the corresponding shift of the $H_{1'}$-proton of the 3′-nucleoside under equivalent experimental conditions. From geometric considerations, it appears that purine-intercalation would account for most of the purine-induced differential shifts observed for these $H_{1'}$ protons.

It is interesting to note the variation of the $H_{1'}$–$H_{2'}$ coupling constants with purine addition. Each of the $H_{1'}$ ribose protons is coupled to the $H_{2'}$ proton in the same furanose ring, yielding the two pairs of doublets observed for the $H_{1'}$ protons. In the absence of purine, the coupling constants are both ~3.5 cps. This value may be compared with a $|J_{H_1',H_2'}|$ of 5.6 cps for 5′-adenosine monophosphate (pD 6.2) and 5.9 cps for 3′-adenosine monophosphate (pD 5.8) at a concentration of ~0.01 *M*. The coupling constants for 3′-AMP and 5′-AMP are both slightly concentration dependent. At a concentration of 0.2 *M* these coupling constants are 4.6 (pH 6.0) and 4.5 cps (pH 6.5), respectively.[40,41] In any case, $|J_{H_1',H_2'}|$ is noticeably smaller for ApA than for its adenosine nucleotides. This suggests that the intramolecular stacking interaction of the two adenine bases in ApA changes the ribose conformation of both nucleosides of the dinucleotide from that found in the simple nucleotides (from 2′-*endo* toward 3′-*endo*).[41,42]

The variation of the ($H_{1'}$–$H_{2'}$) coupling constants with concentration of added purine is illustrated in Figure 10. The uncertainty in these coupling constants is large, since they were measured with difficulty due to poor sensitivity at the low concentration of dinucleotide employed in these measurements (0.01 *M*). At high purine concentrations, the measurements were further complicated by the overlapping of the two pairs of spin multiplets. However, despite the poor quality of the experimental data, the measurements do establish a definite trend, and it is possible to conclude that $|J_{H_1',H_2'}|$ increases with purine concentration for both nucleosides of the ApA molecule. At a purine concentration of 0.9 *M*, $|J_{H_1',H_2'}|$ is ~4.5 cps for the downfield doublet and ~5.5 cps for the upfield doublet. It thus appears that the ribose conformation of the two furanose rings of the ApA dinucleotide changes when purine binding occurs. We believe the origin of this conformational change is the result of purine intercalation. When the two adenine bases move apart to incorporate a purine base, there is apparently a tendency for the ribose conformation to be restored to that of the mononucleotides.

b. Purine-Binding at High ApA Concentrations. The purine resonances were not extensively broadened in the purine-binding experiments discussed above even at the lowest purine concentration investigated. This is not unexpected, since the proportion of unbound purine has to be higher to attain a significant degree of purine intercalation at low dinucleotide concentrations. In order to investigate the intercalation process *via* the purine line-broadening method, we have therefore also studied the binding of purine to ApA at high ApA concentrations. To facilitate the comparison of these line-broadening results with those previously reported for the pyrimidine–pyrimidine dinucleoside monophosphates, the concentration of ApA in these experiments was chosen to be comparable to those used in our purine-binding experiments for TpT, TpdU, and dUpT. While the purine-induced shifts at high ApA concentrations are complicated by the self-association of the dinucleotide, the interpretation of the purine line widths is probably still straightforward, since the purine line-broadening still provides a direct indication of the extent of purine intercalation.

(40) C. D. Jardetzky and O. Jardetzky, *J. Am. Chem. Soc.*, **82**, 222 (1960).
(41) C. D. Jardetzky, *ibid.*, **84**, 62 (1962).
(42) C. D. Jardetzky, *ibid.*, **82**, 229 (1960).

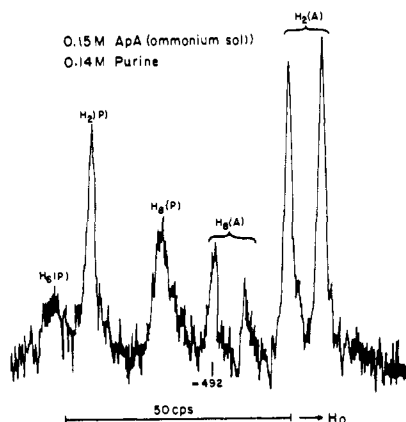

Figure 11. Pmr spectrum (60 Mcps) of the base protons in a mixture of 0.15 *M* ApA (ammonium salt) and 0.14 *M* purine. The chemical shifts were monitored relative to external TMS (6% in chloroform).

The purine-induced shifts observed upon the addition of purine to a 0.15 *M* solution of the ammonium salt of ApA in D_2O are summarized in Table II. One striking feature of the data is that the purine-induced shifts are essentially parallel for the chemically similar protons of the geometrically different adenine rings. Hence, under the conditions of the experiments, a relatively small fraction of ApA exists as the purine-intercalated dinucleotide complex. A small purine-induced differential shift (~2 cps) is observed for the H_8 protons. However, we feel that the source of this induced differential shift is primarily purine external stacking.

Table II. Effect of Purine on the Chemical Shifts of the Adenine Protons of 0.15 *M* ApA (Ammonium Salt)

Purine concn, *M*	Chemical shift difference[a,c] $H_8(3')-H_8(5')$	$H_2(3')-H_2(5')$	Purine-induced shifts[b,c] $H_8(5')$	$H_8(3')$	$H_2(5')$	$H_2(3')$
0.0	7.0	7.5	0.0	0.0	0.0	0.0
0.14	7.5	7.5	3.0	3.5	5.0	5.0
0.29	8.0	7.5	4.0	5.0	7.5	7.5
0.48	8.5	7.5	5.0	6.5	9.5	9.5
0.56	8.5	7.5	5.5	7.0	11.0	11.0
0.68	8.5	7.0	7.5	9.0	13.5	13.0
0.76	8.1	7.2	8.5	9.5	14.0	14.0
0.83	8.1	7.4	10.5	11.5	15.5	15.5
1.11		7.4	10.5		18.0	18.0
1.23	8.5	6.9	10.0	11.5	18.0	17.5
1.36	8.9	7.1	9.5	11.5	18.5	18.0
1.50		6.6	9.5		19.0	18.0
1.62	8.5	7.1	9.5	11.0	19.0	18.5

[a] ±0.2 cps. [b] ±1 cps. [c] In cycles per second at 60 Mcps.

The limited purine-induced shifts given in Table II are significantly smaller than those observed in the earlier purine-binding studies at the lower dinucleotide concentration. These smaller shifts no doubt reflect the breakdown of the ApA molecular aggregates by the purine during the accompanying purine-binding process.

Some line broadening of the purine resonances is observed in these purine-binding experiments. However, under comparable conditions, the observed line broadening of the purine resonances appears to be considerably smaller here than in the case of the other dinucleoside monophosphates which we have studied. The purine line width data are summarized in Table III, and the effect is illustrated in Figure 11. Again, the line broadening is most pronounced for the purine H_6 and the least evident for the purine H_2.

Table III. Line Widths[a] of Purine Resonances in ApA–Purine Solutions[b]

Purine concn, *M*	H_6	H_2	H_8
0.14	6.0	2.5	4.0
0.29	4.0	2.2	3.0
0.48	4.0	2.2	3.0
0.56	3.8	2.2	3.0
0.68	4.0	2.3	3.0
0.76	4.1	2.0	2.9
0.83	4.2	2.3	3.5
1.11	3.8	1.9	2.5
1.23	3.3	1.8	2.7
1.36	3.0	1.6	2.3
1.62	3.5	2.3	2.6
0.125[c]	0.8	1.0	1.0

[a] Full width at half-intensity in cycles per second. [b] 0.15 *M* ApA (ammonium salt). [c] In the absence of ApA.

Effects of pH

The evidence presented thus far indicates that the two adenine bases in ApA are intramolecularly stacked at room temperature. It is natural, then, to study the system under conditions which might alter the extent of this base-stacking. Perhaps the most obvious of these are the temperature and the pH of the solution. We discuss here the dependence of the intramolecular base-stacking interaction on the pD of the solution.

As the pD of the solution is lowered, protonation of the bases of the dinucleoside monophosphate occurs. The bases are then positively charged and one would expect the intramolecular stacking interaction (as well as *inter*molecular stacking) to be reduced from electrostatic considerations. As we shall show, the apparent pK_a for protonation of the adenine rings of ApA corresponds to a pD value of 3.7 (pH meter readings of 3.3). Since the pK_a for protonation of the purine ring in purine is about 2.4,[43] it may be possible to adjust the pD of an ApA solution to a value corresponding to sufficient destacking of the adenine rings to make purine intercalation more favorable upon the addition of purine to the system. With this idea in mind, we have completed a protonation study of ApA, and have examined in detail the binding of purine to ApA (sodium salt) at a pD of 3.7.

a. Protonation of ApA. For ApA the highest pK_a for protonation should correspond to protonation of the adenine rings. Reported values[44] for the pK_a of the adenine ring of 3′-AMP range from 3.56 to 3.70. Similarly, pK_a values for 5′-AMP have been reported[44] in the range 3.74–3.98. It is thus not unreasonable to expect protonation of at least one of the two adenine bases of ApA over the pD range 3–4. Since the free energy of the intramolecular base-stack-

(43) A. Albert and D. J. Brown, *J. Chem. Soc.*, 2060 (1954); also, B. W. Bangerter and S. I. Chan, unpublished work.
(44) D. O. Jordan, "The Chemistry of Nucleic Acids," Butterworth, Inc., Washington, D. C., 1960, p 137.

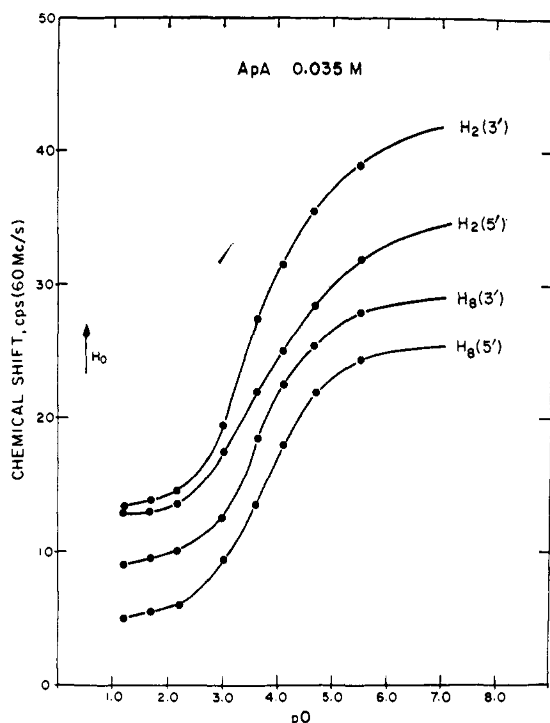

Figure 12. Variation of the chemical shifts (60 Mcps) of the adenine protons of 0.035 M ApA with solution pD.

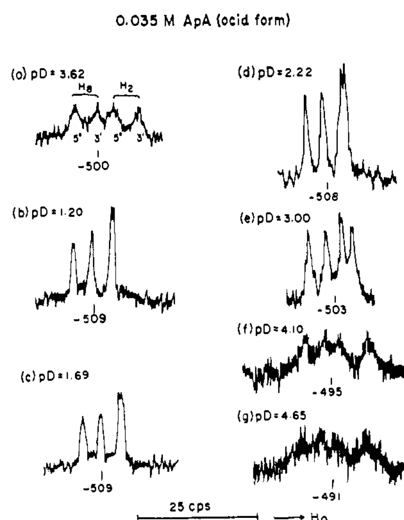

Figure 13. Pmr spectra (60 Mcps) of the adenine protons of 0.035 M ApA at various solution pD's. The chemical shifts were monitored relative to external TMS (6% in chloroform).

ing interaction is small compared to the free energy of protonation, the pK_a for protonation of the second adenine ring of a monoprotonated ApA molecule should not be greatly different from the pK_a for protonation of the first adenine base. At pH ~4, the predominant species is then expected to be monoprotonated ApA ($ApAH^+$ and H^+ApA), and below pH ~3, both adenine bases of the dinucleotide would most likely be protonated. Protonation of the phosphate group is not expected in this pH region, since the pK_a for phosphate protonation is ~1.

Stoichiometry suggests that each adenine ring is only monoprotonated in the pH range 1.5–4.0. The site of protonation is, however, uncertain and it is possible that several monoprotonated adenine species are in multiple equilibrium with one another, as in the case of purine.[45] The most likely sites for protonation are the three basic ring nitrogens of adenine, *i.e.*, N_1, N_3, and N_7.

The effect of pH on the four ring proton resonances of ApA is depicted in Figure 12. As expected, all of the resonances are shifted downfield with decreasing pD. This downfield shift is good evidence for ring protonation. Since all of the adenine resonances are shifted downfield by comparable amounts, it is probable that several monoprotonated adenine species are in equilibrium. From the profiles of the chemical shift curves of Figure 12, it appears that the pK_a of the apparent acid involved is in the vicinity of 3.7. However, the curves do not represent a simple protonation process, since several equilibria including monoprotonated and diprotonated species of both stacked and unstacked ApA molecules are involved. These equilibria have been discussed by Simpkins and Richards[46] in their spectrophotometric and potentiometric studies of the protonation of dinucleotides.

(45) J. M. Read, Jr., and J. H. Goldstein, *J. Am. Chem. Soc.*, 87, 3440 (1965); also B. W. Bangerter and S. I. Chan, unpublished work.
(46) H. Simpkins and E. G. Richards, *Biochemistry*, 6, 2513 (1967).

Protonation shifts observed for heterocycles are generally attributed to: (i) changes in the π-charge densities upon protonation, and (ii) alteration of the magnetic anisotropy from the lone-pair electrons of the nitrogen which is protonated. In this case, part of the observed downfield shifts arises from the breakdown of intermolecular ApA stacks upon protonation and from changes in the relative conformation of the two adenine bases.

It is interesting to note the merging of the two H_2 proton resonances at low pD. Since these protons are far removed from the point of phosphate attachment, we expect them to be magnetically equivalent in the absence of the ring-current magnetic anisotropy effect of a neighboring adenine base. It is then not unreasonable that the magnetic nonequivalence observed at neutral pD is removed when the two adenine rings are destacked by protonation. The observed magnetic nonequivalence of the H_2 protons therefore clearly arises from intramolecular stacking of the geometrically nonequivalent adenine bases.

However, the magnetic nonequivalence of the two H_8's is still 4 cps at 60 Mcps at a pD of 1.2. The two adenine rings are destacked and the dinucleotide unfolded under these conditions. This magnetic nonequivalence must therefore be inherent in the asymmetric attachment of the phosphate to the two ribose moieties.

In the pD studies, we have also noticed some interesting line-width changes in the ApA spectrum. In Figure 13a we have reproduced a tracing of the spectrum of a solution of 0.035 M ApA (acid form) to which sufficient HCl (0.3 N) had been added to decrease the pD of the solution from its original value of 3.90 to 3.62. Compared to the spectrum of a solution of comparable concentration but at higher pD's, the resonance peaks of the adenine protons are significantly broader at this pD value. With the addition of more HCl, the resonances sharpened considerably. The spectrum of the adenine protons at a pD of 1.2 is illustrated in Figure 13b. At this point, 0.4 N NaOH

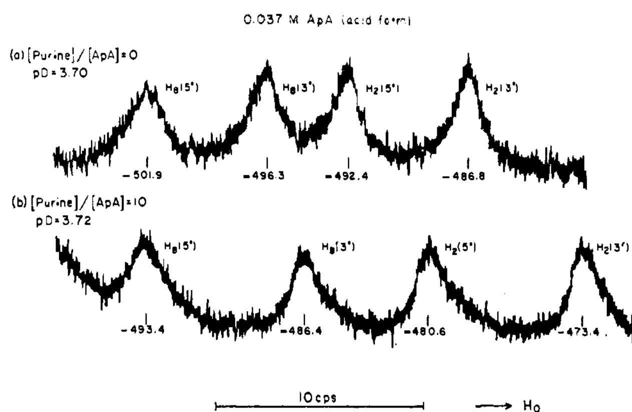

Figure 14. Pmr spectra (60 Mcps) of the adenine protons of 0.037 *M* ApA at [purine]/[ApA] ratios of (a) 0 and (b) 10. The solution pD was maintained at ~3.7. The chemical shifts were monitored relative to external TMS (6% in chloroform).

was added to the solution mixture and the pD of the solution was raised in several gradual steps. The resulting spectra at pD's of 1.69, 2.22, 3.00, 4.10, and 4.65 are reproduced in Figures 13c–g. The line broadening is apparently reversible, and the onset of the line broadening appears to occur at a pD of ~3.5.

We have attributed the relatively broad resonance peaks observed for the adenine protons of ApA in the pD range 3.5–4.5 to the formation of self-intercalated dimers. Apparently, self-intercalation of ApA molecules becomes a more probable mode of self-association when one of the adenine bases is protonated. It has been shown that the monoprotonated form of ApA is unstacked.[46] Thus, the formation of self-intercalated dimers is probably not totally unexpected under these conditions. Lowering the pD of the solution beyond ~3.5 would increase the number of diprotonated molecules (each adenine base monoprotonated), and this presumably would result in the breakdown of the intermolecular stacks. The adenine proton resonances should therefore be sharp at low pD's as observed.

The possibility of intermediate proton chemical exchange between protonated and nonprotonated adenine bases was also considered as an alternative explanation for the observed broadening of the adenine resonances. However, the resonance broadening was found to be absent at low dinucleotide concentrations, and this concentration dependence would appear to rule out this interpretation of the observations.

b. ApA–Purine Interactions at pD ≈3.7. The results of the above protonation study of ApA suggest that one might expect purine intercalation to proceed with comparative ease when the binding of purine to ApA is studied at pD ≈ 3.7. Table IV summarizes the purine-induced shifts observed for the adenine protons when purine was progressively added to a solution of 0.037 *M* ApA while the pD of the solution was maintained at approximately 3.7. We note that these experiments were undertaken at a reasonably high ApA concentration, where the dinucleotide would normally be appreciably self-associated under neutral pH conditions.

Table IV. Purine-Induced Shifts for the Adenine Protons of ApA at pD ≈3.7[a]

Purine: dinucleotide	pD	Purine-induced shifts[b,c] $H_8(5')$	$H_8(3')$	$H_2(5')$	$H_2(3')$
0	3.70	0.0	0.0	0.0	0.0
1	3.75	2.7	2.8	3.0	3.5
2	3.80	3.6	4.5	5.3	6.2
5	3.65	4.4	5.4	6.2	7.0
10	3.72	8.5	9.9	11.8	13.4

[a] ApA concentration = 0.037 *M*. [b] In cycles per second at 60 Mcps. [c] ±0.2 cps.

It is gratifying to note the purine-induced differential shifts observed, particularly for the H_2 protons. This result is illustrated in Figure 14, where the spectrum of a solution containing no purine is compared with that of a solution in which the purine to dinucleotide concentration ratio is 10:1. We feel that this dramatic result is strong evidence for purine intercalation. As in the case of the pyrimidine dinucleotides, *e.g.*, TpT and CpC, the purine-induced shifts here are also larger for the protons of the 3′ base than for those of the 5′ base. The direction of the purine-induced differential shifts observed for the H_2 protons is therefore opposite to that reported earlier for the system at neutral pH and at an ApA concentration of 0.01 *M*. The absolute magnitude of the induced differential shifts for these protons is also significantly larger under the present experimental conditions. For the H_8 protons, the direction of the induced differential shift is not affected by lowering the pD. The magnitude of the purine-induced differential shift is considerably larger at neutral pD than at pD ≈3.7. It is possible to rationalize the apparent differences in the results obtained at the two pD values in terms of differences in the relative conformation of the two adenine bases prior to the formation of the intercalated complex. The nature of the intramolecular stacking interaction between the adenine bases at neutral pD has already been discussed. At pD ≈3.7, the data suggest that the two bases are somewhat "uncoiled" initially, and the intercalated complex is formed by interaction of a neutral purine molecule with the two adenine bases in a manner similar to that previously discussed[33] for TpT.

Further evidence for the formation of purine-intercalated complexes under the present conditions comes, of course, from the line widths of the purine resonances, particularly at low purine–dinucleotide concentration ratios where the highest fraction of the purine molecule would exist as the intercalated complex. In Figure 15, we have reproduced three spectra in the region of the purine and adenine proton resonances for purine–dinucleotide ratios of 1:1, 2:1, and 10:1. The purine line broadening is much more pronounced at pD ≈3.7 than at the higher pD's.

Temperature Studies

In an effort to obtain some quantitative information concerning the energetics of the stacking interaction, we have studied the resonance positions of the adenine protons as a function of temperature. In the course of this study, we have found that the temperature data vary greatly, depending upon the choice of the reference compound used to reference the chemical shifts. Changes in the bulk susceptibility of the solution with temperature can be adequately accounted for by referencing the chemical shifts relative to that of an "inert" reference compound in the same solvent and at

the same temperature. However, the proton chemical shifts of both the reference compound and the dinucleotide molecule of interest are subject to solvent effects. The temperature shifts therefore may also reflect changes in the solvent–solute interactions and the solvent structure with temperature in addition to changes in the stacking interactions. Hence, the reference compound should be chosen with some care, if the temperature data are to be meaningful. In the present temperature study, we have monitored the chemical shifts of the ApA H_2 and H_8 resonances relative to the corresponding proton resonances in adenosine at the same temperature, solution pH, and roughly the same concentration. It is hoped that this choice of reference compound will tend to minimize the contribution of solvent effect changes to the temperature shifts. The concentration of the adenosine in the reference solution was 0.0032 *M*. Even though the adenosine base proton chemical shifts are strongly concentration dependent, the concentration shifts relative to their infinite dilution values amount to no more than 1–2 cps at 100 Mcps at this concentration. Furthermore, since the temperature measurements for the dinucleotide and adenosine were made at roughly the same concentration, changes in the intermolecular stacking with temperature are also somewhat compensated.

The temperature shifts observed for the adenine protons of ApA (0.0030 *M*) have already been summarized in Figure 4. These measurements were made over the temperature range of 5–95° As expected, increasing the temperature results in downfield shifts for all of the adenine spectral peaks. The magnitude of the temperature shifts varies greatly with the base proton. For example, the total change in the chemical shift of the $H_2(3')$ resonance over the temperature range investigated is ~18 cps, whereas the corresponding change for $H_2(5')$ is only ~4 cps. Similarly, the temperature shift observed for $H_8(5')$ is ~12 cps, while the corresponding shift for $H_8(3')$ is much smaller.

As mentioned previously, it is reasonable to assume that these temperature shifts arise from changes in the relative conformation of the two adenine bases with temperature. Since the H_2 protons are far removed from the phosphate linkage and the ribose moieties, the temperature shifts observed for these protons must arise almost entirely from changes in the ring-current magnetic anisotropy of the opposing adenine base. At ~5°, $H_2(3')$ and $H_2(5')$ are respectively 28 and 10 cps upfield relative to the H_2 resonance in adenosine. Hence, in the stacked conformation, $H_2(3')$ experiences a significantly larger ring-current magnetic anisotropy effect from the opposing base than $H_2(5')$. This immediately suggests that the two adenine bases in ApA are stacked with each of the rings preferentially oriented in the *anti* conformation relative to the ribose moiety. However, the magnitude of the ring-current shift for $H_2(3')$ due to the opposing base appears to be smaller than that which one might anticipate on the basis of the ring-current effect of an adenine ring, while at the same time, it appears that $H_2(5')$ is shifted unusually far upfield relative to H_2 in adenosine. We are therefore also led to conclude that other stacked conformations which differ from conformer I by a 180° rotation of either or both adenine rings about the respective

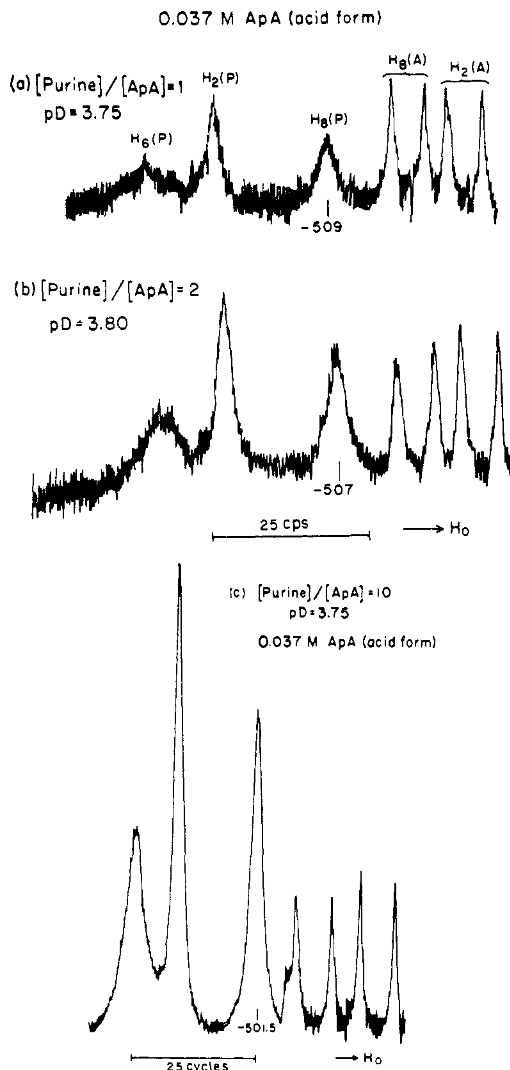

Figure 15. Illustrations of the broadening of the purine resonances observed in ApA–purine mixtures at pD ≈3.7, ApA concentration 0.037 *M*. [purine]/[ApA] concentration ratios: (a) 1, (b) 2, (c) 10.

glycosidic linkages must also be populated in this temperature range. There are two conformers in which $H_2(3')$ will experience a large ring-current effect from the opposing 5′ bases, namely, I and II. In the other two conformations, $H_2(3')$ should experience essentially no ring-current shift from the opposing adenine base. Similarly, it is clear that in two of these conformers, namely II and IV, $H_2(5')$ will experience a sizeable ring-current shift from the 3′ base, while in conformers I and III, this ring-current effect would be negligible. Thus the limiting chemical shifts observed at low temperatures for both $H_2(3')$ and $H_2(5')$ relative to adenosine can presumably be interpreted in terms of the relative populations of the four stacked conformers.

The interpretation of the temperature shifts observed for the H_8 protons is not quite so straightforward. First, the chemical shift of each H_8 resonance is a function of the conformation of the adenine base relative to the ribose at the glycosidic linkage, and it is not clear to what extent the stabilization of the two possible base conformations as a result of the intramolecular base stacking would change the chemical shift of these protons from the rotational mean value observed for adenosine.

The $H_8(3')$ resonance appears 10 cps upfield from the adenosine H_8 resonance. The origin of this 10-cps difference in the chemical shifts is not exactly clear; however, part of the upfield shift of $H_8(3')$ might be due to the existence of stacked conformers in which the base of the 3′-nucleoside is *syn*. The shift for $H_8(5')$ is further complicated by the phosphate effect, due to its proximity to the phosphate attachment when the 5′ base is oriented in the *anti* conformation. The effect of the phosphate group is to deshield this proton relative to its chemical shift in adenosine. For example, H_8 in 5′-AMP is ~16 cps downfield from H_8 in adenosine; and in ApU and UpA, the magnetic nonequivalence between the H_8 protons is ~9 cps, with $H_8(5')$ downfield. At 5°, $H_8(5')$ in ApA is ~10 cps upfield from H_8 in adenosine, and is in fact accidentally equivalent magnetically with $H_8(3')$. Hence, $H_8(5')$ must experience an *average* ring-current shift of +20 cps or more at this temperature. The temperature shifts observed for this proton resonance also indicate that the position of this resonance is influenced by the ring-current magnetic anisotropy of the 3′-adenine base. The $H_8(5')$ resonance moves *downfield* with increasing temperature, and at ~90°, this resonance is located 2 cps downfield from H_8 in adenosine. This downfield shift clearly arises from changes in the ring-current effect due to partial destacking of the adenine rings with increasing temperature.

It is interesting to note that even at 95°, the H_2 resonances of ApA are still at significantly higher field than the H_2 resonance of adenosine [+10 cps for $H_2(3')$ and +6 cps for $H_2(5')$]. This persistence of the ring-current shifts indicates that there is still residual base–base interaction at this temperature. The potential well which describes the interaction between the adenine bases is thus relatively deep compared to kT. It is likely that the temperature shifts arise primarily from changes in the mean distance between the adenine rings and in the average dihedral angle of stacking.

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.[30] Regardless of the details of this or any other model which may be proposed to describe the intramolecular base-stacking process, however, some general features are expected for the pmr chemical shift *vs.* temperature plots. 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 detailed shape of the temperature profiles in the transition region is clearly sensitive to the details of the intramolecular base-stacking interaction, depending both on the energetics of the process as well as on the variation of the property monitored with the extent of stacking. In the two-state model, however, the sigmoidal curves, when plotted *vs.* the reciprocal absolute temperature, are necessarily symmetrical about the apparent "transition temperature" located at the point of maximum slope, halfway between the high- and low-temperature asymptotic limits of the property monitored.

Two of the temperature curves in Figure 4 are roughly sigmoidal in shape with asymptotic limits at both high and low temperatures, and may be compared with similar temperature curves obtained from optical rotatory dispersion,[28,47] circular dichroism,[27,29–31] and hypochromism[9,12,47] measurements by other workers. The point of maximum slope in these chemical shift *vs.* temperature plots appears to be in the neighborhood of 37°. This "transition temperature" is somewhat higher than the values generally obtained by the other methods. It is not surprising that the pmr method will yield a higher "transition temperature" since the ring-current shift, which is used to monitor the extent of stacking is sensitive over a wider range of unstacking than the optical properties.

When sigmoidal temperature curves are interpreted in terms of the two-state model (stacked *vs.* unstacked), it is customary to extract thermodynamic information about the energetics of the stacking process from a van't Hoff plot. A value of −11 kcal/mol can be obtained for the apparent $\Delta H°$ of the stacking process if our pmr temperature data for $H_2(3')$ were treated in this manner. However, it is our contention that this $\Delta H°$ value probably does not possess any thermodynamic significance. Since the quantity measured is an ensemble average of an observable over the accessible quantum states, a two-state system is not really defined, unless the expectation value of this observable changes abruptly between two sets of quantum states beyond a certain cutoff energy. Even then, this cutoff energy may depend upon the property monitored, in which case the thermodynamic parameters extracted from the temperature data will also be a function of the property. In our case, the ring-current shift changes only gradually with the degree of unstacking. Thus, the two-state model is not strictly applicable. In any case, the apparent enthalpy of destacking extracted from the temperature data should be higher here compared with those extracted from ORD, circular dichroism, and hypochromism data, since the ring-current shift is probably sensitive to a wider range of unstacking than these optical properties. Values of $\Delta H°$ for the unstacking of ApA as determined from studies of optical properties have ranged from 5 to 10 kcal/mol depending on the optical property.

Summary

The pmr spectrum of adenylyl(3′→5′)adenosine has been studied as a function of concentration, solution pH, temperature, and concentration of added purine. The chemical shifts of the adenine ring protons and the ribose $H_{1'}$ protons, the coupling constants between the $H_{1'}$ and $H_{2'}$ protons, and the line widths of the adenine proton resonances as well as those of the added purine were monitored.

ApA was found to self-associate extensively in aqueous solution, and the chemical shifts of the adenine ring protons are strongly concentration dependent. The mode of self-association was shown to be strongly pH dependent. At neutral pH, the intramolecular

(47) R. C. Davis and I. Tinoco, Jr., private communication.

stacking interaction beteeen the two adenine rings is relatively strong so that ApA self-associates primarily by external stacking of folded ApA molecules. However, upon protonation of one of the adenine rings, the intramolecular stacking interaction between the adenine rings is apparently reduced sufficiently so that self-intercalation also becomes probable.

In the purine-binding studies, evidence was obtained for the formation of the purine-intercalated dinucleotide complex. These purine-binding experiments were undertaken over a wide range of experimental conditions: neutral pH, low pH, and low and high dinucleotide concentrations. It was shown that purine intercalation provides a sensitive probe both for the relative strength of the intramolecular stacking interaction and for the relative conformation of the two bases in the dinucleotide. Purine intercalation was monitored by the purine-induced chemical shifts observed for the adenine H_2 and H_8 proton resonances and the $H_{1'}$ ribose resonances, the variation of the coupling constants between the $H_{1'}$ and $H_{2'}$ ribose protons with purine concentration, and the line broadening of the purine resonances. At neutral pH, the observed purine-induced shifts of the adenine protons were shown to arise not only from the ring-current magnetic anisotropy effect of the purine base (whether externally bound or intercalated), but also from modification of the intramolecular ring-current effect of the opposing adenine ring when purine intercalation results in changes in the relative conformation of the two adenine rings. From an analysis of these purine-induced shifts, it was then possible to infer the relative conformation of the two adenine rings in ApA at neutral pH prior to purine intercalation, and it was concluded that the adenine rings are stacked with the bases preferentially oriented as in a similar dApdA segment in double helical DNA. Purine-binding studies at low pH's, where the adenine rings are protonated but the purine base is uncharged, indicate that the two bases in ApA are uncoiled under these conditions and the intercalated dinucleotide complex is formed by interaction of a neutral purine with the two adenine bases.

While the dinucleotide is destacked upon protonation of the adenine rings, increasing the temperature to 95° does not result in complete unfolding of the dinucleotide molecule. Thus, it appears that the potential well describing the stacking interaction is relatively deep compared to kT. It is probable that the bottom of this potential trough is also quite flat, so that the two adenine rings undergo large amplitude vibrations relative to each other. Again, it was possible to conclude from the temperature shifts that the two adenine rings in ApA are stacked with each of the bases preferentially oriented in the DNA-like conformation (3'-*anti*,5'-*anti*) relative to its ribose moiety. However, an examination of the chemical shifts of the ApA adenine ring protons relative to those of adenosine suggests that the other stacked conformers are also somewhat populated.

In conclusion, the pmr results indicate that ApA is strongly stacked at room temperature and at neutral pH. This conclusion is consistent with that reached by other workers based upon investigations of the optical properties of this dinucleotide (hypochromism, circular dichroism, and optical rotatory dispersion). However, in this work, it was also possible to establish the preferred conformation of the adenine bases in the intramolecular stack.

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