STUDIES OF THE CONFORMATIONAL PROPERTIES OF DINUCLEOSIDE MONOPHOSPHATES AND OLIGORIBONUCLEOTIDES BY PROTON MAGNETIC RESONANCE

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Dedicated to the memory of my father.

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ABSTRACT

The structural and conformational properties of several dinucleoside monophosphates and oligonucleotides in aqueous solution have been studied by proton magnetic resonance spectroscopy. Specifically, the role of intramolecular base-stacking interactions and other intramolecular forces in the determination of the conformational properties of these molecules has been investigated; and the results of these studies are discussed in relation to the structural properties of biologically significant nucleic acids.

The proton magnetic resonance spectrum of adenylyl-(3'→5')-adenosine (ApA) was studied as a function of concentration, temperature, solution pH, and concentration of added purine. The results of these 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 concentration studies, ApA was found to self-associate extensively in aqueous solution via vertical stacking of the planar bases. The purine-binding studies indicate that ApA-purine complexes are formed via purine-adenine base-stacking and evidence was obtained for the formation of a purine-intercalated dinucleotide complex in neutral solution.

Similar proton magnetic resonance studies of adenylyl-(3'→5')-guanosine (ApG) and guanylyl-(3'→5')-adenosine (GpA) indicate that each

of these dinucleotides is strongly stacked intramolecularly at 30° and neutral pH with the bases preferentially oriented in the anti conformation. The base-stacking interaction in these molecules is somewhat weaker than the adenine-adenine base-stacking in ApA. These studies also provided information concerning conformational changes which occur in the ribose rings of the dinucleotides as a result of changes in the intramolecular base-stacking; and this phenomenon is discussed in detail.

The concentration dependence of the proton magnetic resonance spectra of adenylyl-(3'→5')-uridine (ApU) and uridylyl-(3'→5')-adenosine (UpA) has been used to determine the mode of self-association of these dinucleotides and the dimerization equilibrium constant of the self-association process. The binding of 6-methylpurine to ApU was used in additional studies of the nature of purine-dinucleotide complexes and the effect of complex formation on the spectra of these molecules.

Finally, the intramolecular base-stacking interactions in ApApA and ApApApA were shown to be comparable to those in ApA. The study of these molecules indicates that each assumes a molecular configuration similar to an analagous segment of double helical DNA.

The results of these studies explicitly demonstrate the importance of base-stacking interactions in determining the conformational properties of nucleic acids and their derivatives in aqueous solution.

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I. HISTORICAL INTRODUCTION

A. Nucleic Acids in Living Organisms

1. Introduction. Since the discovery of nucleic acids by Miescher (1) almost a century ago, continued efforts in nucleic acid research have gradually demonstrated that these macromolecules provide some of the most essential functions in the cells of living organisms. They are critically important in the processes of growth, reproduction, and heredity; and, in addition, they play an important role in mutagenesis and carcinogenesis. In the cells of all living organisms are two types of nucleic acids--ribonucleic acid or RNA and deoxyribonucleic acid or DNA.

2. Biological Role of DNA. Several important advances have been made toward the elucidation of the particular biological significance of DNA since the discovery by Avery et al. (2) in 1944 that the genetic material is deoxyribonucleic acid. Nine years later, Watson and Crick (3) proposed their double helical structure for DNA. This structural model provided for the first time a plausible mechanism by which the DNA molecule could effect the replication and transcription of genetic information. Accordingly, the Watson-Crick hypothesis also provided tremendous impetus for increased research activity devoted to the determination of the exact mechanism by which the genetic information stored in DNA is manifested in the structural properties and the genetic characteristics of a living cell as well as the precise

mechanism responsible for the transfer of hereditary messages from one generation to the next.

3. Structure of DNA. In the B lattice configuration, DNA consists of two polymer chains coiled plectonemically around the same axis to form a double helix. Each chain is a linear polymer in which consecutive monomeric nucleoside residues are linked together by phosphate groups resulting in the formation of 3', 5' phosphodiester bridges between the pentoses of adjacent nucleosides. The two strands are of opposite polarity so that the linkage in one strand is 3'→5' while in the other it is 5'→3'. The polynucleotide chains are comprised of four different nucleotides, each of which contains a deoxyribose residue, a phosphate group, and a purine or a pyrimidine base. The pyrimidine bases are thymine (T) and cytosine (C) and the purine bases are adenine (A) and guanine (G). The structures of these DNA constituents are illustrated in Figure 1. The bases are arranged on the inside of the helix so that a pyrimidine base of one chain is paired to a purine base of the other. Moreover, this base-pairing is extremely specific in that adenine nearly always pairs with thymine and guanine is almost always base-paired to cytosine. The physical basis of this specific complementarity is found in the cooperation of several intermolecular forces between the constituent nucleotides of DNA. There are ten base pairs for every turn of the helix and the bases tend to stack with their planes perpendicular to the axis of the helix and approximately 3.4 Å apart. Since the specific base-pairing requires that the bases of the two strands are complementary, the base sequence of one strand determines that of the other, and it is this characteristic that enables DNA

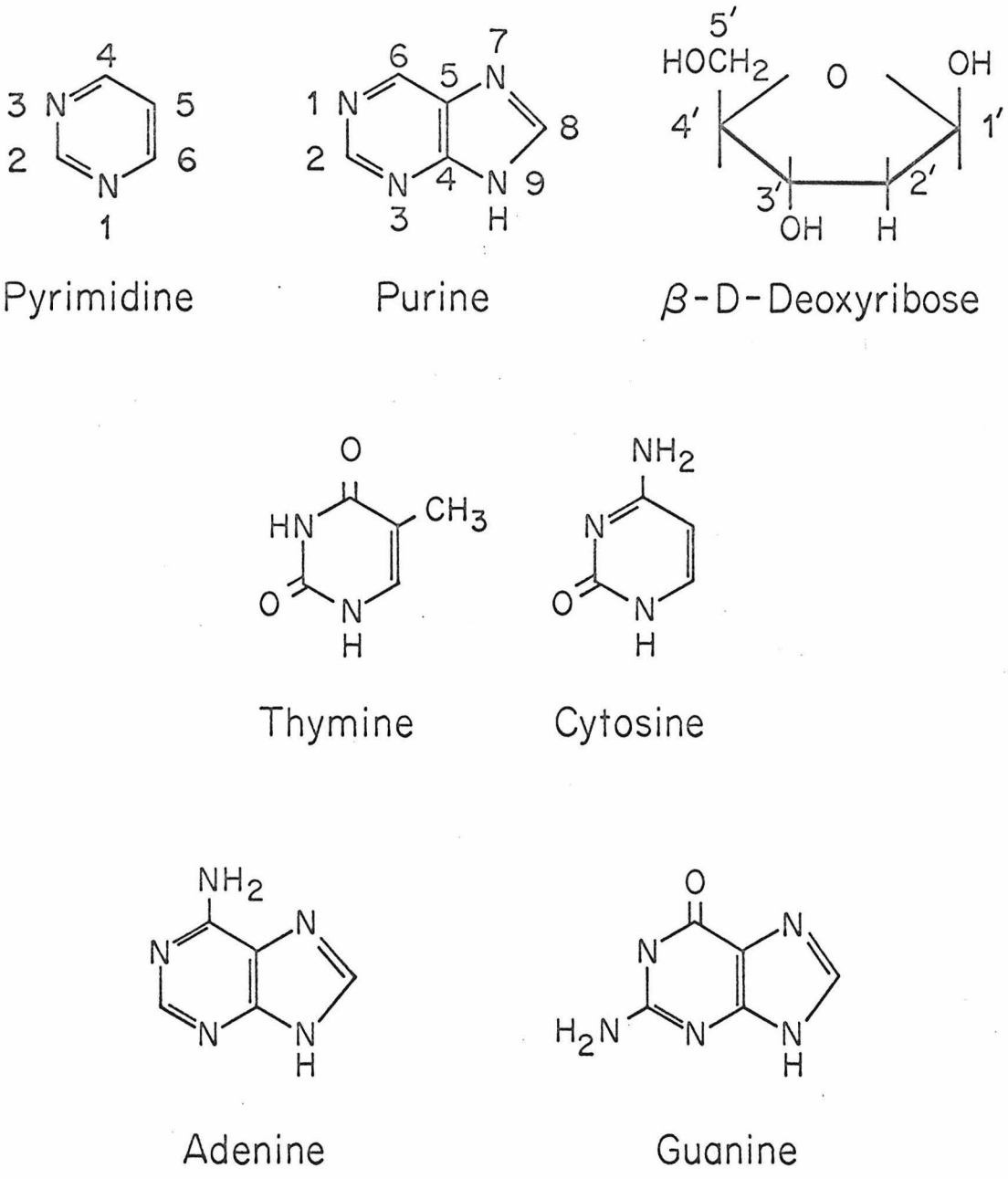


Figure 1. Structures of some deoxyribonucleic acid constituents.

to duplicate itself during replication. The process of replication apparently involves the unwinding of the two strands of the double helix followed by the formation of hydrogen-bonded base pairs between the bases of each strand and the respective complementary monomer units. These monomer units are then enzymatically linked by the formation of phosphodiester bonds to synthesize a new complementary polynucleotide chain on each of the original strands. Complete untwisting of the DNA duplex is not necessary before synthesis of the new strands begins. It is evident that the unique chemical and physical properties of DNA make it particularly suitable for the replication of genetic information.

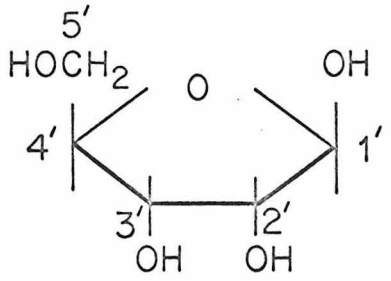
4. Biological Role of RNA. Since the genetic characteristics of a living organism are largely determined by the nature of the cellular proteins, much of the biochemical research during the last decade has been directed toward the determination of the exact mechanism by which protein synthesis is governed by DNA. The general principles involved in protein synthesis have been established by many workers, and these principles have led to the formulation of one of the central dogmas of molecular biology (4). This states that the genetic information stored in DNA is transcribed to a messenger ribonucleic acid (mRNA) which associates with several ribosome particles to form polysomes in which it performs its role as a stable template in the biosynthesis of the protein.

A different type of ribonucleic acid, transfer RNA (tRNA), serves as a molecular carrier in the cell in that it transports the

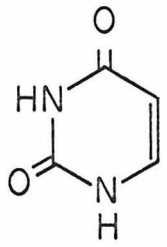
amino acids to the site of protein synthesis on the ribosomes. Transfer RNA's are relatively small molecules, consisting of approximately 80 nucleotides (M. W. $\approx$ 25,000). These RNA molecules are enzymatically aminoacylated to specific amino acids; and they transport the amino acids to a specific site on the mRNA template. The amino acids are then incorporated into the growing protein chain by the appropriate enzymes. There are several tRNA molecules in the molecular apparatus of a cell and each is specific for a particular amino acid. A sequence of three nucleotides (codon) on the mRNA selects the proper tRNA and hence the appropriate sequence of amino acids is incorporated into the protein. The codon of the template selects the appropriate tRNA not because of the structure of the amino acid itself, but because there is a complementary coding site on the tRNA molecule. This specificity in the interaction between tRNA and mRNA was originally advanced in the adaptor theory of Crick (5) which has subsequently received significant experimental verification.

In contrast to DNA which determines the amino acid composition and sequence in proteins, RNA, therefore, participates directly in the chemical processes and mechanisms by which protein synthesis occurs. These processes involve the complex molecular interactions of tRNA, mRNA, ribosomal RNA, and the structural proteins of ribosomes and enzymes. These mechanisms have not yet been completely determined, but it is likely that the macromolecular structures of the RNA molecules contribute to their functionality in these processes. Therefore, the study of the structures and the forces which determine the structures of ribonucleic acids is of primary importance.

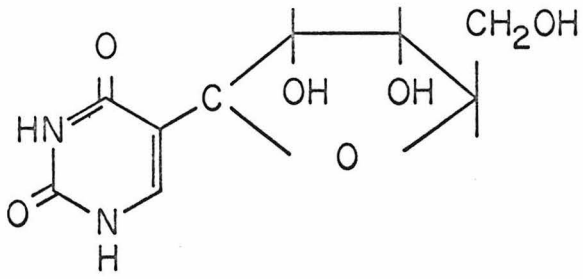
5. Structure of RNA. Like DNA, RNA is also a polynucleotide, consisting of a long sequence of nucleosides linked together by phosphoric acid. However, the pentose sugar is ribose rather than deoxyribose and uracil is substituted for thymine. In addition, small amounts of pseudouridine and other nucleosides have also been found in several RNA's (Figure 2). The experimental evidence presently accumulated indicates that RNA in solution is structurally much different from double helical DNA except for a few replicative forms of viral RNA and the complementary homopolyribonucleotides. The RNA molecules are apparently single-stranded flexible chains which possess secondary structure in the form of helical regions. This secondary structure of RNA apparently arises from the formation of sequences of intramolecularly hydrogen-bonded base pairs. Intramolecular base-stacking interactions between adjacent purine and pyrimidine bases along the RNA chain can also stabilize the secondary structure of these molecules. Non-hydrogen-bonded or weakly base-stacked residues can loop out of the helical regions so that the properly bonded complementary base pairs fall into place (6-8). In addition to this secondary structure, RNA molecules may also have a specific tertiary structure in solution and it is possible that the tertiary structure enables the RNA molecules to recognize and interact with specific enzymes and other cellular constituents and thereby perform their important role in protein synthesis (9-11). At present, relatively little is known about the three-dimensional topology of RNA and this area is currently one of very active investigation.



β-D-Ribose



Uracil



Pseudouridine

Figure 2. Structures of some ribonucleic acid constituents.

B. Molecular Forces and Nucleic Acid Structures

Due to the tremendous importance of both DNA and RNA in biological systems and the relationship between the structure of these molecules and their functions in living cells, several studies have been initiated in an effort to determine the forces which stabilize nucleic acid conformations in solution and impart to them their unique physical and chemical properties.

Several different types of intramolecular interactions can contribute to the conformational stability of nucleic acids in solution (12). These include such effects as hydrogen bonding, hydrophobic interactions, van der Waals interactions, London dispersion forces, charge-transfer complexation, and other electrostatic interactions. All of these effects except hydrogen bonding can contribute to the formation of stable vertical base-stacks along a polynucleotide chain. The precise effect responsible for base-stacking is not yet fully understood, but sufficient evidence has now been accumulated to indicate that the base-stacking interactions of contiguous nitrogenous bases along the polynucleotide chains of nucleic acids have a profound influence on the stability and conformational properties of the nucleic acids (13-19). Accordingly, a summary of studies pertinent to base-stacking interactions is presented here.

C. Synthetic Polynucleotides as Models for the Study of Base-Stacking

A number of synthetic polynucleotides have been useful in the study of specific base-stacking interactions along polymer strands (20). The heterogeneity of the base composition of native nucleic acids makes

it difficult to study the interactions between specific bases and their influence on the secondary and tertiary structure of these molecules. However, homopolynucleotides are particularly suitable for studying specific forces involved in maintaining polynucleotide structures in aqueous solution.

Under conditions of neutral pH, polyadenylic acid (poly A) is a one-stranded polymer with considerable secondary structure at temperatures near 0°C (21, 22). Studies employing optical spectroscopic techniques (23-25) have shown that single-stranded poly A melts gradually over a broad temperature range, and the most reasonable hypothesis in agreement with this observation is that the secondary structure is the result of the formation intramolecular base stacks which are disrupted noncooperatively with increasing temperature. Nuclear magnetic resonance measurements of poly A (26, 27) yielded results which are consistent with a secondary structure stabilized by base-stacking. Optical rotatory dispersion studies of polycytidylic acid (poly C) have shown that, at neutral pH, it also exists in an ordered secondary structure which is stabilized by base-stacking (28). Conversely, it has been demonstrated by optical rotatory dispersion (29-31) and nuclear magnetic resonance (26, 32) that polyuridylic acid (poly U) has little secondary structure at neutral pH and 25°. Under these conditions poly U appears to be a relatively disordered randomly coiled polymer. It may be concluded from these results that adenine and cytosine have a greater tendency to form base stacks under conditions of neutral pH and 25°C than uracil. Hence, these bases could be expected to make a greater contribution to the stability of ordered

native nucleic acid structures than uracil.

Synthetic polynucleotides also provide excellent models for studying the specific interactions (hydrogen-bonding and base-stacking) in the binding of monomers, oligomers, and polymers to polynucleotide chains. These binding experiments are particularly interesting in view of the fact that the biosynthesis of nucleic acids and the binding of tRNA to mRNA involve similar interactions. Many such studies have been summarized in the review of Felsenfeld and Miles (20).

D. Base-Stacking Interactions of Bases, Nucleosides, and Nucleotides

1. Introduction. The observation that different bases evidently make significantly different contributions to the properties of polynucleotides suggests the expediency of studying the properties of the individual constituent bases, nucleosides, and nucleotides in solution. Several such studies have been completed and have yielded greater insight into the nature of the base-stacking interactions of these molecules, their relative energetics, and their relative importance with respect to the well-established hydrogen-bonding interactions.

2. Osmotic Studies. Studies by Ts'o et al. of the osmotic properties of purine, 6-methyl purine, and fourteen pyrimidine and purine nucleosides have shown that these compounds associate extensively in aqueous solution (33-36). Analysis of the thermodynamic data showed that the association proceeds beyond the dimer stage to a higher degree of polymerization, and that purine bases have a greater tendency to self-associate than pyrimidine bases. Equilibrium

constants obtained for these compounds have been summarized by Ts'o (36), and they range from 0.6 molal^{-1} for uridine to 22.2 molal^{-1} for N-6-dimethyladenosine.

3. Sedimentation Equilibrium and Calorimetry. Van Holde and Rossetti have utilized sedimentation equilibrium studies to investigate the intermolecular association of purine (37) and of adenosine-5'-monophosphate (38) under pH conditions in which the phosphate group has a single negative charge. Although they obtained a larger equilibrium constant for the self-association of purine (K = 2.8 molal^{-1} vs K = 2.1 molal^{-1}), their results are in qualitative agreement with the results of the osmotic studies. In addition to these investigations, Gill et al. (39, 40) have extracted thermodynamic parameters for the self-association of several purine derivatives in water utilizing calorimetric techniques.

4. Nuclear Magnetic Resonance Spectroscopy. The studies discussed above offer conclusive evidence that the bases, nucleosides, and nucleotides of nucleic acids associate extensively in aqueous solution. However, these experimental techniques are incapable of determining the mode of the intermolecular association. There is some indirect evidence that horizontal association through hydrogen bonding is not the primary binding process because all of the bases, nucleosides and nucleotides studied associate much more readily than urea (41), which is a very strong hydrogen-bonding agent, and also because methylation of the bases at the site of hydrogen bond donor atoms enhances rather than diminishes the intermolecular interaction.

More direct experimental evidence for the mode of intermolecular association of these planar molecules can be obtained from nuclear magnetic resonance (NMR) spectroscopy. The electronic shielding of magnetic nuclei is very sensitive to intermolecular and intramolecular interactions. In fact, the resonance frequencies of the purine and pyrimidine protons of biological bases, nucleosides, and nucleotides are influenced in a much different manner by vertical base-stacking interactions and horizontal hydrogen-bonding interactions.

The change which occurs in the electronic distribution in the neighborhood of a hydrogen donor atom upon intermolecular association via hydrogen-bonding results in lower field chemical shifts for these protons (42). Specific guanine-cytosine and thymine (uracil)-adenine base-paired complexes have been detected in organic solvents by monitoring the downfield shifts of the hydrogen-bonded amino and ring protons upon admixture of the hydrogen-bonding species (43, 44). The resonance frequencies of the other protons of these molecules were not significantly changed when intermolecular complexes were formed in the organic solvents. In these studies, hydrogen-bonded complexes formed between the complementary base pairs of the Watson-Crick hydrogen-bonding scheme were more stable than complexes involving other base pairs. There is some question, however, as to whether this specificity of interaction demonstrated in organic solvents contributes to the specificity observed in native nucleic acids. The application of these results toward the elucidation of forces which contribute to the stability of polynucleotides in living systems is, therefore, somewhat questionable. It is unfortunate that in aqueous solution

the hydrogen-bonded protons of the complementary base pairs cannot be observed by NMR spectroscopy. These protons exchange rapidly with the water protons, and the result is a single average resonance absorption signal.

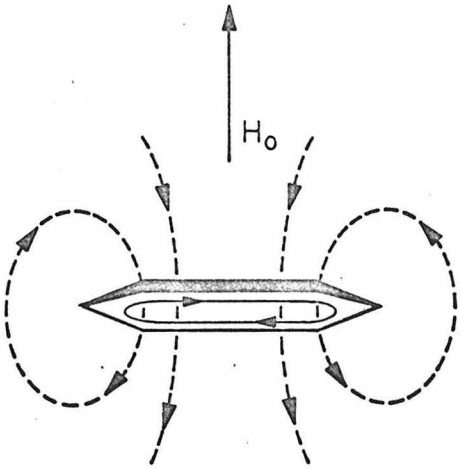
As noted above, the formation of hydrogen-bonded complexes results in relatively large downfield shifts for the resonances of the bond-forming hydrogens and only negligible shifts (if any) for the other base protons. In contrast, vertical base-stacking causes the resonances of the base protons to shift to higher field provided the ring of the base opposite the protons has sufficient aromaticity.

If the π-electrons of a molecular ring are sufficiently mobile, a large diamagnetic current is induced in the plane of the ring by an externally applied magnetic field when the direction of the applied field is perpendicular to the plane of the molecule (45). The precessional current of the π-electrons results in a secondary magnetic field which opposes the primary field in the general region directly above or below the molecular plane and reinforces it around the periphery of the ring (Figure 3a). Calculations by Johnson and Bovey (46) on the ring-current magnetic anisotropy of the benzene molecule have shown that the diamagnetic region (the region of opposing secondary field) is somewhat larger than the paramagnetic region (the region of reinforcing secondary field). The two areas are depicted in Figure 3b for a quadrant of a plane passing normally through the center of the benzene ring. The curve shown in Figure 3b represents the cross-section of the nodal surface separating the region of diamagnetic shielding from the paramagnetic region. Outside the dimensions of the molecule this curve

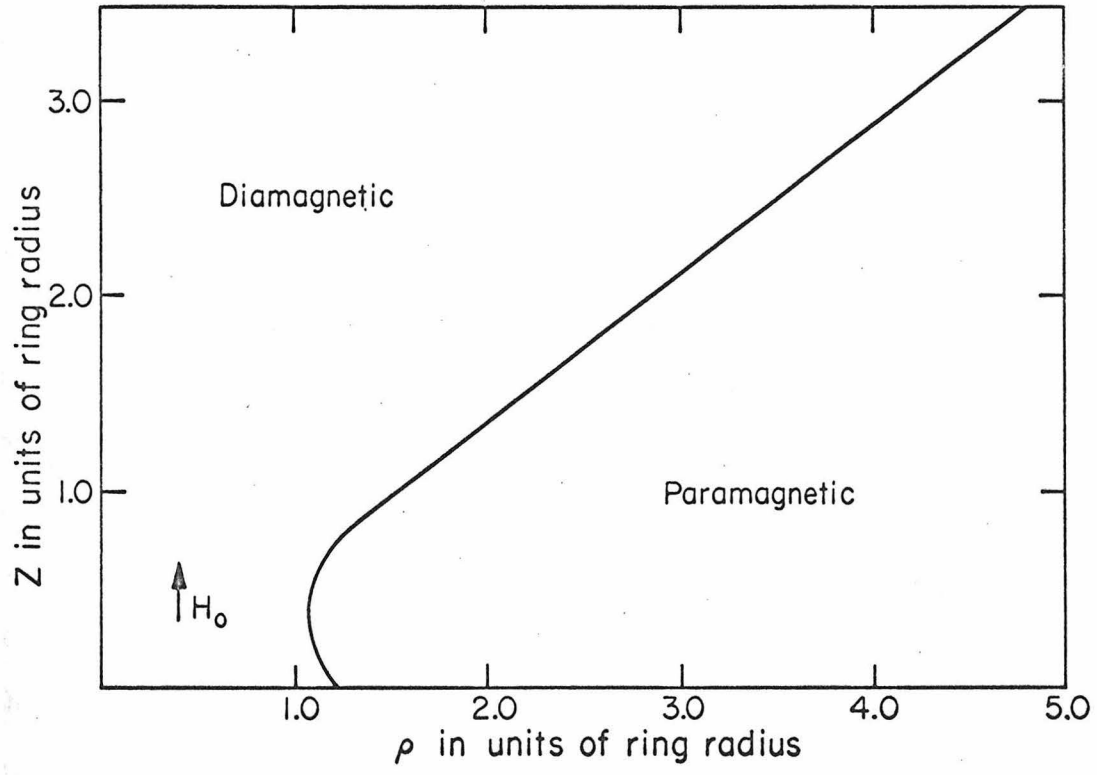
Figure 3. (a) Current of π-electrons and the resulting lines of magnetic force induced in benzene by an applied magnetic field, H_O.

(b) The diamagnetic and paramagnetic regions (in a quadrant of a plane passing normally through the center of a benzene ring) resulting from the ring-current of the π-electrons induced by the applied field, H_O. Z and ρ are the usual cylindrical coordinates. The diamagnetic region is that region in which the applied magnetic field is opposed by the secondary field of the circulating π-electrons. The applied field is reinforced by this secondary field in the paramagnetic region. [Johnson and Bovey (46)]

(a)


H_o

(b)


3.0
2.0
1.0
Z in units of ring radius
Diamagnetic
Paramagnetic
H_o
1.0
2.0
3.0
4.0
5.0
ρ in units of ring radius

is very nearly linear and makes an angle of approximately 35° with the molecular plane.

When stable base stacks are formed, the base protons are on the average more exposed to the diamagnetic shielding of the neighboring base. Consequently, upfield shifts would be expected for the resonances of the base protons in stacked complexes. In the absence of vertical stacking, it is likely that the ring-current anisotropy would shift the base proton resonances downfield in horizontal hydrogen-bonded complexes. The discussion presented here demonstrates that nuclear magnetic resonance spectroscopy is a powerful tool for studying the mode of intermolecular associations of the planar biological bases.

Chan and co-workers (47, 48) studied the proton magnetic resonance spectra of purine and 6-methylpurine as a function of concentration, temperature, solvent, and solution pH. These workers attributed the higher field chemical shifts which accompany increased concentration to the association of these molecules by vertical stacking of the rings in a partially overlapping manner. The temperature, solvent, and pH studies substantiated this conclusion, and model calculations indicated that the average distance between the planes of the stacking molecules is 3 to 4 Å. Jardetzky (49) observed progressive upfield shifts with increased concentration for the base protons and some of the ribose protons of several bases, nucleosides, and nucleotides of nucleic acids. He also concluded that these shifts are due to vertical stacking of the rings. The possibility of hydrogen bonding was not eliminated, however, since stacking of hydrogen-bonded base pairs

could also result in upfield shifts; but no direct evidence for hydrogen-bonded complexes was found in these experiments.

More recently, Helmkamp and Kondo (50) have extended these NMR experiments in an attempt to elucidate the preferred orientations of the purine monomer units in base stacks. Their results are consistent with a model in which the hexagonal rings are preferentially stacked face to face. This results in larger concentration shifts and larger temperature shifts for the H_2 and H_6 protons of purine relative to H_8 (Figure 1).

NMR spectroscopy has also been applied to the study of the base-stacking properties of several purine nucleosides (35). These studies have shown that adenosine derivatives interact more strongly than unsubstituted purine. The mode of interaction in aqueous solution was established as base-stacking and some specificity of interaction was indicated. Two models were proposed for the preferred orientation of the stacks and in each of these the six-membered rings participate more directly in the stacking interaction, accounting for the larger concentration dependent shifts of the H_2 purine protons relative to the H_8 protons. The tendencies of self-association of these various nucleosides appear to correlate reasonably well with the polarizability of the molecules, but not with dipole moment values.

The self-association of several pyrimidine nucleosides has also been studied by NMR spectroscopy (51). Very small concentration shifts were observed for the base proton resonances of uridine, cytidine, and thymidine although uridine and cytidine have been shown to associate in aqueous solution by osmotic pressure lowering (33). The

pyrimidine bases evidently do not support a significant π-electron ring current so that the intermolecular interactions of these pyrimidines and their derivatives cannot be as conveniently monitored as those of the purines by NMR spectroscopy. It was possible to detect a reasonably strong association between purine and the pyrimidine nucleosides, uridine, thymidine, and cytidine. Purine-pyrimidine base-stacking interactions appear to be stronger than pyrimidine-pyrimidine interactions and weaker than purine-purine interactions.

The results of NMR studies also indicate that the mononucleotides of adenosine form intermolecular base stacks in spite of the charged phosphate group (52). However, the effect of the phosphate appears to reduce the stacking interaction about 30-40% relative to adenosine.

E. Base-Stacking Interactions in Dinucleotides and Larger Oligonucleotides

1. Introduction. A study of the dinucleoside phosphates and larger oligonucleotides is a logical extension of the earlier work on the simple bases, nucleosides, and nucleotides. There is ample reason to expect the intramolecular base-stacking tendencies in the dinucleoside phosphates and larger oligonucleotides to be more representative of the nearest-neighbor base interactions in a polynucleotide. To the extent that pairwise nearest-neighbor interactions 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 oligonucleotides is expected to

lead to a better understanding of the sequence-dependent properties of single-stranded polynucleotides as well as the double-stranded nucleic acids.

2. Hypochromism. The methods of ultraviolet spectroscopy have proved invaluable in the study of dinucleotides and oligonucleotides. Many of the characteristics of the ultraviolet absorption spectrum of adenylyl-(3'→5')-adenosine (ApA) were first reported by Michelson (53) in 1959, and a few years later the hypochromism of the homologous series of adenine ribonucleotides of chain length from two to eleven was noted by Singer et al. (54). Michelson (55) was the first to point out that the formation of internal hydrogen bonds between the bases of oligonucleotides such as ApA is very unlikely, and, accordingly, the observed hypochromism is due to some other intramolecular effect. He suggested that the hypochromism of oligonucleotides results from the stacking of the planar purine and/or pyrimidine bases; and that the interaction of the π-electrons of adjacent bases not only stabilizes the oligomer conformation, but also results in a new electronic species which has an ultraviolet absorption spectrum peculiar to the entire molecule, rather than the summation of the independent absorptions of the component mononucleotides. Leng and Felsenfeld (24) measured the hypochromism of a series of oligoadenylates as a function of temperature and concluded that the temperature dependence of the hypochromism in these molecules arises from changes in the pair-wise stacking between adjacent bases along the chain. The unstacking of the adenine bases in these molecules proceeds by a noncooperative

process similar to the temperature denaturation reported above for poly A.

3. Circular Dichroism. Base-stacking interactions in the simple model compounds have also been demonstrated by circular dichroism (CD). This spectroscopic technique was initially applied to the study of the thermodynamic and conformational properties of ApA (56), (3'→5') adenylate oligomers (57), and (3'→5') oligocytidylic acids (58). In each of these studies, it was concluded that the secondary structure of these molecules may be attributed to base-stacking interactions between adjacent bases. A two-state (stacked or unstacked) model was utilized to estimate the thermodynamic parameters of the base-stacking interactions. At neutral pH and 0°C, a standard state enthalpy change of 8 kcal per mole of nucleotide residue and a ΔF^{o} of 1 kcal / mole were obtained for the adenine-adenine interactions in each adenylate oligomer. The standard state free energy change at 0°C is also about 1 kcal / mole in favor of stacking in the oligocytidylates. Cytidylate and adenylate oligomers larger than the heptamer form hydrogen-bonded double helical complexes at pH values less than 4.

The studies of the cytosine derivatives were done in an unusual solvent system. A solution of 4.7 M KF was employed in order to make experimental observations at temperatures as low as -20°C. Perhaps more meaningful biological significance could be derived from studies of solutions of lower salt concentration. Preliminary studies of model compounds in solutions containing no ionic salts or solutions

of low salt concentration have now been reported. The effects of temperature and pH on the circular dichroism of several oligonucleotides of transfer RNA in aqueous solution have been studied by the Russian workers, Zavil'gel'skii and Li (59). These studies are interesting because they employ oligomers of varying base composition and demonstrate that base-stacking interactions are dependent on the base sequence.

Dinucleoside phosphates are of particular interest because they provide a means of studying the base-stacking interaction between two specific bases. A variety of (3'→5') and (2'→5') dinucleoside monophosphates have been studied by Brahms, Maurizot, and Michelson (60) using circular dichroic spectra in the temperature range -20° to +80°C and in 4.7 M KF at pH 7.5. Evidence was obtained for stacked conformations of the (3'→5') dinucleotides at low temperatures and neutral pH; and thermodynamic parameters were obtained for the process, stacked $\rightleftharpoons$ unstacked, utilizing a simplified two-state model. ΔH° was of the order of 6 to 7.5 kcal/mole for all of the dinucleotides studied. The biological significance of these thermodynamic parameters is somewhat questionable due to the solvent system employed and the intrinsic difficulties associated with the application of the oversimplified two-state model (61). The (2'→5') derivatives of ApA, ApC, CpC, and (3'→5') dGpdG (dG = deoxyguanosine) are essentially in a disordered form in the temperature range -20 to + 80°C in 4.7 M KF. An internal hydrogen bond between the 2' OH group and a phosphate oxygen was postulated to account for the stability of the stacked (3'→5') dinucleotides relative to the (2'→5') dinucleotides and to (3'→5')dGpdG.

The Russian workers (59) have also studied specific base-stacking interactions in various dinucleotides in aqueous solution (no salt) by CD. They report that base-stacking is important in determining the conformation of the dinucleotides in solution and that the relative base-stacking strength in sequence isomers is often dependent on the sequence of the bases. Dinucleotides containing adenine have considerably greater asymmetry than pairs containing guanine (GpG shows almost no CD effect at all at pH 5-6). Very little base-stacking is reported for UpU and TppT, but the intramolecular interaction between the bases of TpT is reported to be of the same order as that in ApA and ApΨp (Ψ = pseudouridine). The hydrophobic denaturing agents, ethanol and formaldehyde, decrease the strength of the TpT base stacks. The greater stability of the thymine-thymine intramolecular interactions relative to UpU base-stacking is in agreement with the observation that methylated monomer derivatives have a greater tendency to form intermolecular base stacks (36). One of the most interesting observations made in these dinucleotide studies is that the bases of ApΨp stack in such a manner as to form the elements of a left-handed helix while all the other dinucleotides form right-handed helical structures. Since various tRNA's have significant quantities of pseudouridine, it is possible that this nucleoside plays a decisive role in the secondary structure of these molecules.

4. Optical Rotatory Dispersion. One of the spectroscopic tools which has been most successfully applied to the study of intramolecular base-stacking is optical rotatory dispersion (ORD). Warshaw, Bush,

and Tinoco (62) interpreted the ORD of ApA in terms of the vertical interaction between the two adenine bases. These studies were extended to the oligoadenylates (63, 64) with the conclusion that the intramolecular base-stacking interactions are responsible for the effects observed in the ORD. Several trinucleotides of varying base composition and sequence have also been studied by ORD (65-67). The results of these experiments have shown that only nearest neighbor base-stacking interactions are significantly manifested in the ORD and hypochromism and that different results are obtained for isomers which differ only in base sequence. The pH of the solution has a profound effect on the base-stacking tendencies of the molecules. At neutral pH, trinucleotides with a central uridine residue, consecutive uridine residues, or consecutive guanosine residues appear to form the least stable intramolecular base stacks.

As in the case of the circular dichroism studies, the nature of the interaction between two specific bases can be studied most conveniently by ORD and hypochromism in the dinucleoside phosphates; and all the possible dinucleoside monophosphates (except GpG) of A, U, G, and C have been investigated by ORD at 25° and an ionic strength of 0.1 (68, 69). In addition, the base-stacking in a number of dinucleoside monophosphates has been studied as a function of temperature in 25.2% LiCl by ORD and hypochromism (61). These experiments support the hypothesis that base-stacking interactions determine the structural and conformational properties of the dinucleoside monophosphates in aqueous solution. The pH of the solution, and consequently, the electronic charge of each of the bases has a significant

effect on the tendency of the dinucleotide to stack. If both bases of the molecule are similarly charged, destacking occurs. At pH 1, G^+pC^+ and C^+pG^+ may be exceptions to this observation. Adenine, cytosine, and guanine appear to form base stacks while uracil does not. The exceptions are that at neutral pH, UpG and CpU are stacked; at pH 11.5, CpU^- stacks and ApG^- does not; and at pH 1, UpG^+ and G^+pU form intramolecular base stacks. It should be noted that the criteria for classifying a dinucleotide as stacked or unstacked is somewhat arbitrary. In the classification discussed above, states with hypochromism less than or equal to 3% are considered unstacked; all other molecules were assumed to be stacked. The optical rotation measurements were in general agreement with the absorption results. Sequence-dependence was observed in the ORD and hypochromism of the dinucleotides and it is interesting that the dinucleotide of the structure purine ribotide (3'→5') pyrimidine riboside has a higher value of hypochromism than its sequence isomer.

5. Theoretical Calculations. If only nearest neighbor interactions are considered, some of the optical properties of oligonucleotides and polynucleotides can be predicted from the results of the dinucleoside monophosphates and the mononucleosides. Tinoco et al. have applied the data obtained for these simpler molecules to the theoretical calculation of some optical properties of the 64 trinucleoside diphosphates (70) of A, U, C, and G and some ribonucleic acid models (71).

Theoretical calculations of the van der Waals-London interactions between stacked purines and pyrimidines have also been

reported by Pullman and co-workers (72). They calculated the interaction energies in vacuum, ΔH_v, between the two bases of the single-stranded homodinucleotides, ApA, GpG, UpU, and CpC utilizing the monopole approximation. The calculated interaction energies between the bases of the 3'-anti, 5'-anti conformations (geometry of the DNA helix) of ApA and GpG were -9.46 kcal/mole and -7.19 kcal/mole, respectively. However, much weaker interactions were indicated for similar conformations of CpC (+0.24 kcal/mole) and UpU (-3.32 kcal/mole). A 180° rotation (about the glycosidic linkage) of one of the bases of these pyrimidine-pyrimidine dinucleotides increased the C-C interaction (-7.27 kcal/mole) and decreased the U-U interaction (-0.76 kcal/mole). Since these interaction energies were calculated neglecting solvent effects, their physical and biological significance is difficult to evaluate.

6. Nuclear Magnetic Resonance Spectroscopy. The experimental techniques discussed above have demonstrated the importance of base-stacking in dinucleotides and oligonucleotides. However, they offer very little information concerning the details of the stacking interactions or the specific conformations of these molecules in solution. It should be noted that the crystal and molecular structure of adenylyl-(2'→5')-uridine has been determined by X-ray methods (73). The planes of the bases are parallel and about 3.4 Å apart, but they overlap only to a small extent.

The intrinsic capability of NMR spectroscopic to probe the specific electronic and magnetic environments at multiple atomic

sites in molecules and molecular complexes and the extreme sensitivity of the method to minute structural and conformational changes within the molecules render it particularly suitable for the study of the details of the base-stacking interactions in and the conformational properties of dinucleoside monophosphates and the larger oligonucleotides.

In dinucleotides the resonance frequencies of the protons of each nucleoside may be influenced by the magnetic anisotropies associated with the neighboring base. This provides a convenient handle for studying the relative conformation of the two bases of these molecules. 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 (74) and pH. In these laboratories, another method has been developed 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 vertical stacking, is introduced into the system, and from an investigation of the mode of binding between the probe molecule and the dinucleotide, it is possible to infer the relative conformation of the two bases. As was demonstrated in a recent proton magnetic resonance study of the binding of purine to several pyrimidine-pyrimidine dinucleotides (TpT, TpdU, dUpT; dU = deoxyuridine), the addition of purine can lead to the formation of a purine-dinucleotide complex in which the purine base is intercalated in a sandwich-like fashion between the two bases of the dinucleotide (75). Presumably, the purine intercalation tendencies can be used to provide

some crude measure of the intramolecular base-stacking tendencies in the dinucleoside monophosphates. If the intramolecular base-stacking interaction is strong, it will inhibit the formation of the purine-intercalated complex, and the mode of binding of the purine to the dinucleotide will be primarily stacking of the purine bases on the external faces of the bases of the folded dinucleotide molecule.

NMR spectroscopy of the dinucleotides also offers a unique opportunity to study the specific effect of a particular base on the chemical shift of the base protons of the neighboring nucleoside. For example, in the series, ApU, ApG, ApC, and ApA [ApU = adenylyl-(3'→5')-uridine, etc.], the influence of the various 5'-esterified nucleosides on the magnetic environment of the adenosine protons can be effectively studied. As has been observed with other spectroscopic techniques, the base-sequence is important in determining the characteristics of the NMR spectra of the dinucleotides; the spectra of the base protons of ApG and GpA are significantly different, for example (Chapter III, this thesis). The accumulation of values of the chemical shifts of the base protons in various dinucleotides can be helpful in interpreting the NMR results of larger oligo- and polynucleotides. Such methods have been applied in the determination of the relative frequency of occurrence of purines and pyrimidines at the 5'-neighbor position of thymine in several single-stranded DNA's by studying the resonance frequencies of the thymine methyl protons in various magnetic environments (18). Similar frequency determinations may also be possible in the synthetic co-polymers. The utilization of NMR spectroscopy in the identification of and the determination of the base

sequence in oligonucleotides obtained in the degradation of tRNA's may also be possible.

In order for the chemical shifts of the protons of dinucleotides and oligonucleotides to be interpreted only in terms of intramolecular phenomena, very dilute solutions of these molecules must be used. Otherwise, the effects of intermolecular associations will be manifested in the experimental data and intramolecular effects will not be correctly elucidated. Extrapolated infinite dilution shifts are desirable, but the results of solutions 0.01 M or less have proved to be adequate. Unfortunately, some recent proton magnetic resonance studies on the base-stacking interactions in dinucleotides (76) and trinucleotides (77) at relatively high concentrations have been reported in which the possible effects of intermolecular associations have been completely overlooked. However, some meaningful NMR results have been recently reported by Hruska and Danyluk on the base-stacking interactions in dinucleotides (74, 78).

During the past few years, proton magnetic resonance studies of the base-stacking interactions in several systems of biological interest have been pursued in our laboratories. Bangerter and Chan have extensively investigated the nature of the intramolecular base-stacking in aqueous solutions of ApC, CpA, CpC, and UpU (79, 80). They have also studied the binding of purine to each of these dinucleotides and to ApU (79, 80). More recently, these workers have reported some very interesting results on the binding of purine and adenosine to poly U (32, 79, 81).

This thesis summarizes the results of proton magnetic resonance studies of the conformational properties of and the base-stacking interactions in the following dinucleoside monophosphates and oligoadenylates: ApA (Chapter II); ApG and GpA (Chapter III); ApU and UpA (Chapter IV); and ApApA and ApApApA (Chapter V).

The advantage of beginning the NMR studies of the properties of oligonucleotides in aqueous solution with the dinucleotides is inherent in their relative simplicity and the unique opportunity they offer to study the possible interaction between two specific bases at consecutive nucleotide sites along an oligo- or polynucleotide chain. The study of pairs of sequence isomers also makes it possible to elucidate the effect of the base sequence on the properties of these nucleic acid derivatives.

Adenylyl-(3'→5')-adenosine was a logical system for our initial NMR studies of the dinucleotides from several different viewpoints. At the time we began our studies relatively little information about the dinucleotides and oligonucleotides had been published. The results of some experiments on the optical properties of ApA had appeared in the literature, however (53, 54, 56, 62, 68); and these offered the opportunity of comparing our NMR results with those of other methods. More recently, several other papers on ApA and some oligoadenylates have appeared, as noted previously in this introduction; and the results of these studies have been compared with our results in Chapter II.

Associated with each ring of ApA is a sizable ring-current magnetic anisotropy. This characteristic makes possible the monitoring of the effects of intramolecular base-stacking at several sites on the molecule. Each adenine ring would, for example, be expected to affect

the chemical shifts of at least some of the protons of the neighboring nucleoside in intramolecular base stacks. Such effects would likely be somewhat less in dinucleotides derived from the other common bases of nucleic acids since the guanine ring current is considerably less pronounced than that of adenine and the ring currents of uracil, thymine, and cytosine are even weaker. Accordingly, more information about the specifics of the base-stacking were anticipated in the NMR studies of ApA. The adenine ring has an additional advantage because it has no substituent keto-oxygens. These atoms can serve as centers of localized magnetic anisotropy, and the effect of such anisotropies on the resonance frequencies of the nuclei of neighboring atoms is frequently very difficult to determine. Consequently, such effects could complicate the interpretation of the NMR data.

In addition, each adenine ring has two protons (H_2, H_8) whose resonances can be conveniently monitored. These protons are located at opposite ends of the adenine ring so that the effects of the intramolecular base-stacking interaction can be probed at widely varying sites on the bases. As is indicated in Chapter II, this characteristic of ApA proved to be very useful in determining the conformations of the respective adenine bases in the folded dinucleotide.

The preliminary studies on the monomers indicated that the purine derivatives have a greater tendency to form intermolecularly stacked complexes than the pyrimidines. Correspondingly, ApA would be expected to form relatively stable intramolecular base-stacks; and it was assumed that a strongly stacked dinucleotide would provide a better system for testing the efficacy of the application of NMR

spectroscopy to the study of the dinucleotide conformations than the more weakly stacked molecules.

The choice of ApA as the initial system for our NMR studies proved to be a fortunate one because we have been able to determine many of the specifics of the intramolecular base-stacking in this molecule.

One of the disadvantages of studying a homodinucleotide such as ApA is that it is not possible to study the effects of base-sequence on the conformational properties of the dinucleotides. In an effort to determine these effects we have also studied two sets of sequence isomers: ApG-GpA and ApU-UpA.

The structural similarity of guanine and adenine motivated the choice of the ApG-GpA isomer set as a system for study. Because of the similarity of the structure and geometry of these two bases, any differences in the intramolecular base-stacking in the adenine-guanine dinucleotides and ApA could possibly be attributed to intrinsic differences in the stacking tendencies of the two planar bases. It was assumed that a study of ApG and GpA would provide some measure of such stacking tendencies.

The study of ApG and GpA is also interesting from another point of view. The tendency of guanine derivatives to form molecular aggregates in aqueous solution is well known. However, relatively little is understood concerning the exact mode of intermolecular association, although it has been attributed, in some cases, to the hydrogen-bonding capabilities of guanine. We initiated the ApG-GpA studies with the additional hope of determining some of the base-stacking properties of

guanine and the possible implications of these properties in the unusual tendency of guanine derivatives to form intermolecular aggregates.

Finally, an investigation of the properties of the p. m. r. spectra of ApG and GpA provide a useful method for checking the results obtained for ApA.

In all of the studies reported in this introduction, uracil has been characterized by its weak base-stacking tendency. Therefore, of the series of dinucleotides containing adenine, it was postulated that ApU and UpA would form the least stable intramolecular base stacks. On the other hand, experimental evidence has shown that the adenine-adenine interactions in ApA are probably the strongest base-stacking interactions in this series of dinucleotides. To test this hypothesis and possibly compare the spectral properties of the strongest and weakest stacking dinucleotides, we have also studied the proton magnetic resonance spectra of ApU and UpA.

With the completion of the dinucleotide studies reported here, Bangerter's work on ApC, CpA, and ApU (79, 80, 83), and Prestegard's temperature studies of ApU (83) we have completed an investigation of the conformational properties of each of the seven dinucleoside monophosphates containing the adenine base. A discussion of these results as well as Bangerter's results on CpC and UpU (79, 80) is found in this thesis and in other publications of the research group of Professor Sunney I. Chan.

The base-stacking interactions in ApA were more extensively studied than those of the other dinucleotides reported in this thesis. It was only natural, then, to extend the NMR studies of the dinucleotides to

the oligonucleotides of adenine. The proton magnetic resonance spectra of the trinucleotide, ApApA, and the tetranucleotide, ApApApA, have been studied to elucidate the conformational properties of these molecules in solution. The base-stacking interactions in these molecules are particularly interesting because they are probably more representative of the base-stacking interactions along polynucleotide chains. Again, the fact that each adenine ring accommodates a large ring-current anisotropy contributes to the detailed information which can be obtained from the NMR studies of these oligoadenylates.

II. THE NATURE OF THE BASE-STACKING INTERACTION IN ADENYLYL-(3'→5')-ADENOSINE

A. Introduction

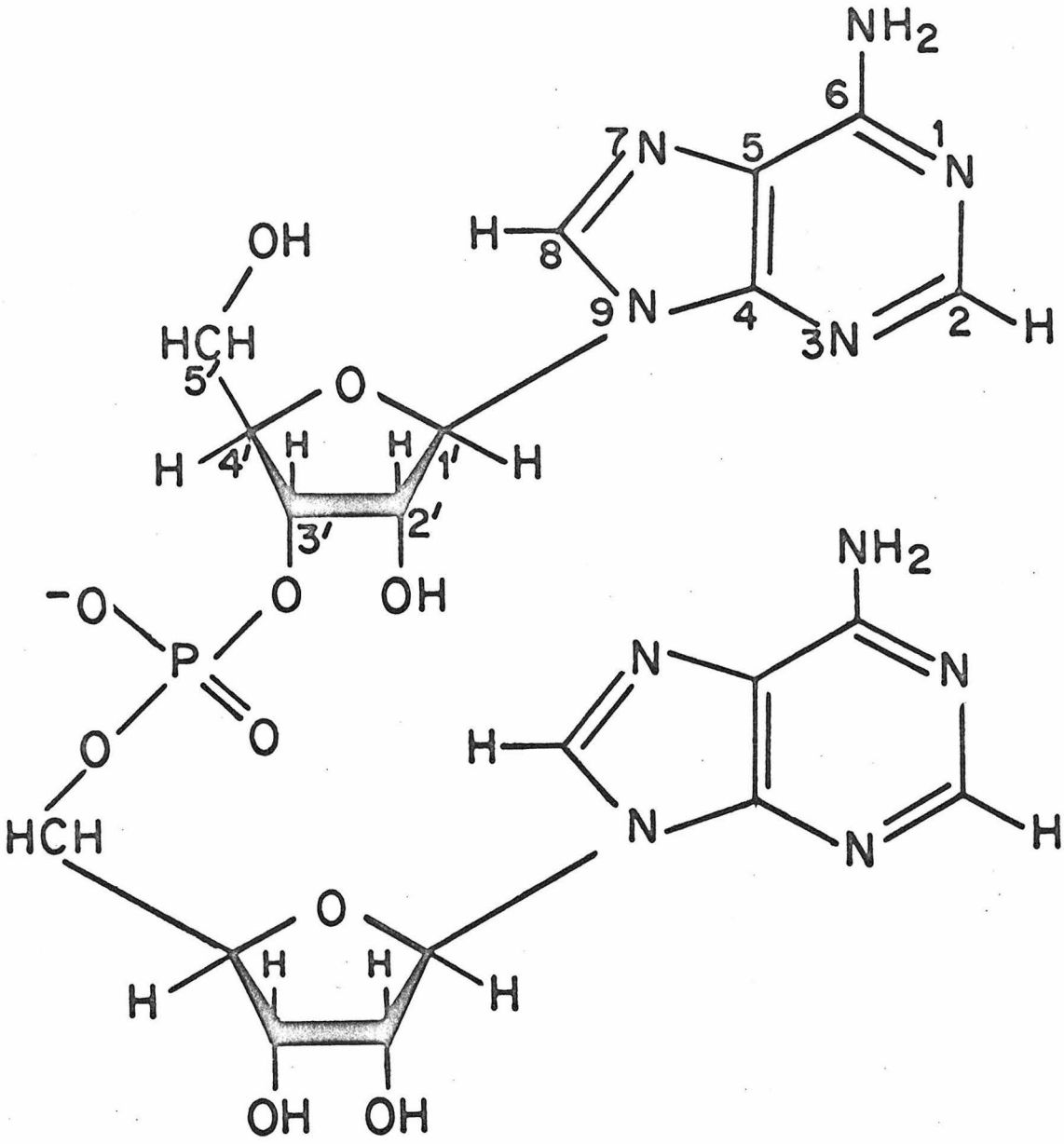
This chapter summarizes the application of proton magnetic resonance (p. m. r.) spectroscopy towards the elucidation of the intramolecular base-stacking interaction in adenylyl-(3'→5')-adenosine (hereafter referred to as ApA; Fig. 4). We shall show that the results of these studies enable us to make some rather definite conclusions regarding the conformation of the two adenine rings in this dinucleoside monophosphate.

B. Experimental

1. Materials. A-grade ApA was obtained from Calbiochem, Los Angeles, California, 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 u.v. 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 mole %), supplied by Columbia Organic Chemicals, Columbia, South Carolina.

Purine was obtained from Cyclo Chemical Corporation, Los



ApA

Figure 4. Adenylyl-(3'→5') Adenosine.

Angeles, California. The purine was sublimed *in vacuo* before use.

2. Methods and Instrumentation. 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 concentration studies were made on a Varian A-60A spectrometer. In these studies, the probe temperature was approximately 40°C, 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°C.

In the variable temperature studies, the sample temperature was controlled to ± 1°C with a Varian V-4343 variable temperature controller. The probe temperature was determined using methanol and ethylene glycol samples and calibration curves supplied by Varian.

Except in the case of the temperature studies the chemical shifts reported in this chapter 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 (84) 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 chapter, deuterium ion concentrations in the D_2O solutions are indicated by pD values, where pD = pH (meter reading) + 0.40 (85).

C. Spectrum and Assignment of Adenine Proton Resonances

The p.m.r. 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 sugar moieties of the two nucleosides to the phosphate group (3'- versus 5'-), the two adenine rings of ApA are not geometrically equivalent. The p.m.r. spectrum of ApA depicted in Figure 5 clearly indicates that the same protons (H_2 or H_8) on the two adenine rings are not magnetically equivalent.

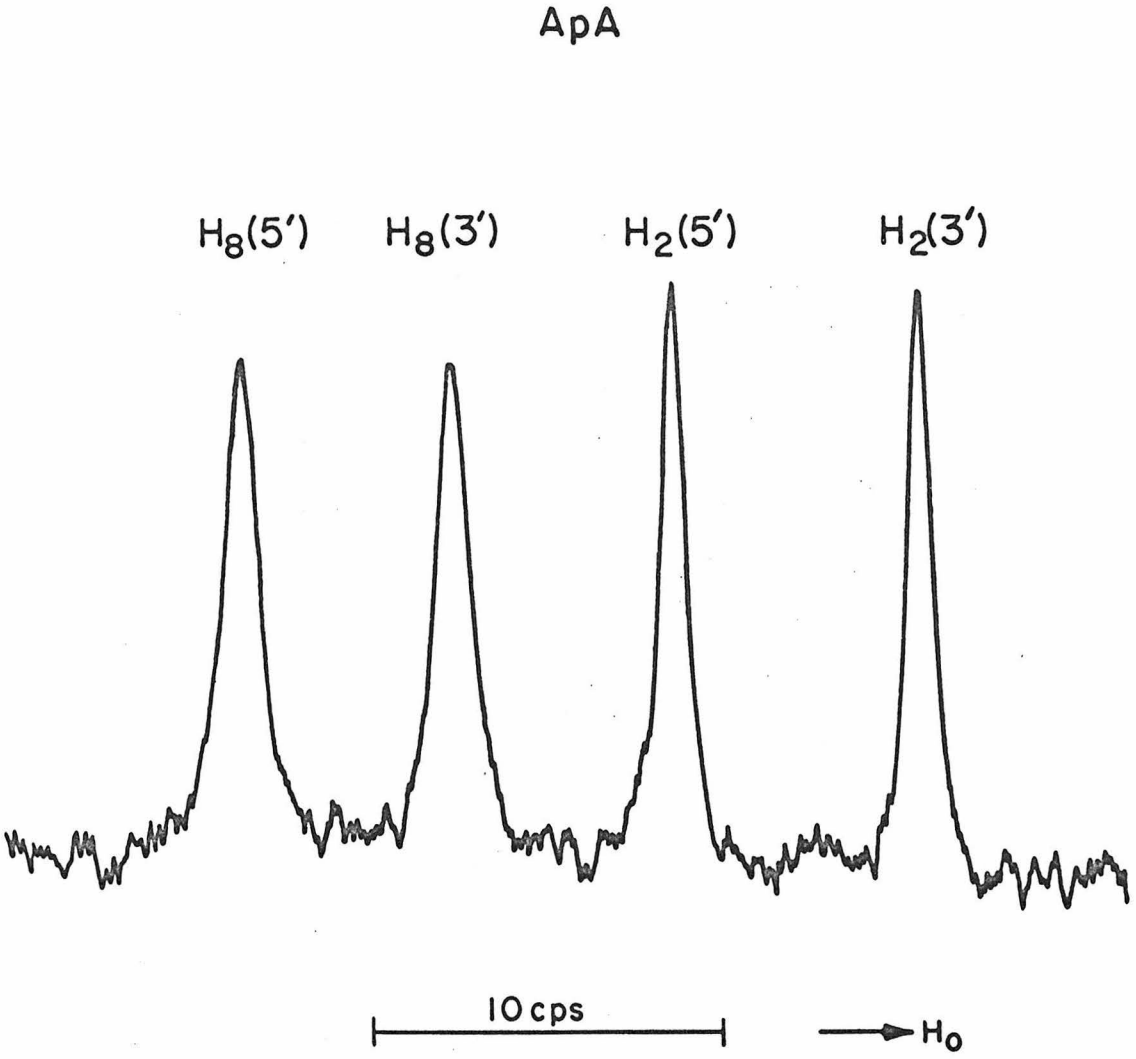


Figure 5. Proton magnetic resonance spectrum of the adenine ring protons of 0.1 M. ApA (ammonium salt) at 60 Mcps. The spectral assignments are indicated; $H_8(5')$, for example, designates the H_8 proton of the 5'-esterified nucleoside. The assignment of the spectrum is discussed in the text.

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 labelling. 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. (It is our convention to refer to the 3'-nucleoside of the dinucleoside monophosphates as that nucleoside which is esterified to the phosphate through the 3'-hydroxyl group of its ribose residue.) 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 towards perturbations in the vicinity of the phosphate group and to changes in the interaction between the two adenine rings.

To facilitate the 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 of 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

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

Molecule	Concentration 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
	Infinite dilution[c]	7-8	8.71	8.68	8.61	8.48
ApU (sodium salt)	0.01	7-8	- -	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.

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 (52, 86) 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 significantly different in these three adenosine monophosphates.

The nomenclature of Donohue and Trueblood (87) is used to describe the conformation of the adenine base with respect to the ribose ring. These workers have defined a torsional angle, ϕ_{CN}, as the dihedral angle between the plane of the base and the plane common to the $C_{1'}$-$O_{1'}$ bond of the ribose ring and the $C_{1'}$-N_9 glycosidic bond. This torsional angle is arbitrarily defined as 0° when the C_8 atom is eclipsed with the ribose ether oxygen and positive angles are measured in a clockwise direction when viewing along the glycosidic bond from $C_{1'}$ to N_9. Relatively stable conformations may be assumed by the adenine base in two general ranges of ϕ_{CN}. When the plane of the base is oriented in the general range around -30°, the base conformation is

said to be anti. The syn-conformation range is centered at about +150°. For the purpose of discussion we shall assume that changes in base conformation from syn to anti or vice versa result from 180° rotations about the glycosidic bond.

There is a difference in the chemical shifts of the adenine H_8 protons of ApU and UpA. 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 due to the deshielding effect of the phosphate group. 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 (3'-endo means that $C_{3'}$ is located on the same side of the plane defined by the atoms $C_{1'}$, $O_{1'}$, and $C_{4'}$ as the $C_{4'}$-$C_{5'}$ bond) 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 towards 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 (88, 89) 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 ($\alpha \frac{1}{r^6}$), 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 6. For purpose of comparison, we have also included the results of similar studies for 5'-AMP and adenosine-3', 5'-cyclic phosphate. The line-broadening effect of Mn(II) on the p. m. r. spectra of the adenine protons of 5'-AMP and of ApA is illustrated for several different Mn(II) concentrations in Figures 7 and 8, respectively.

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 be preferentially oriented in the anti-conformation,

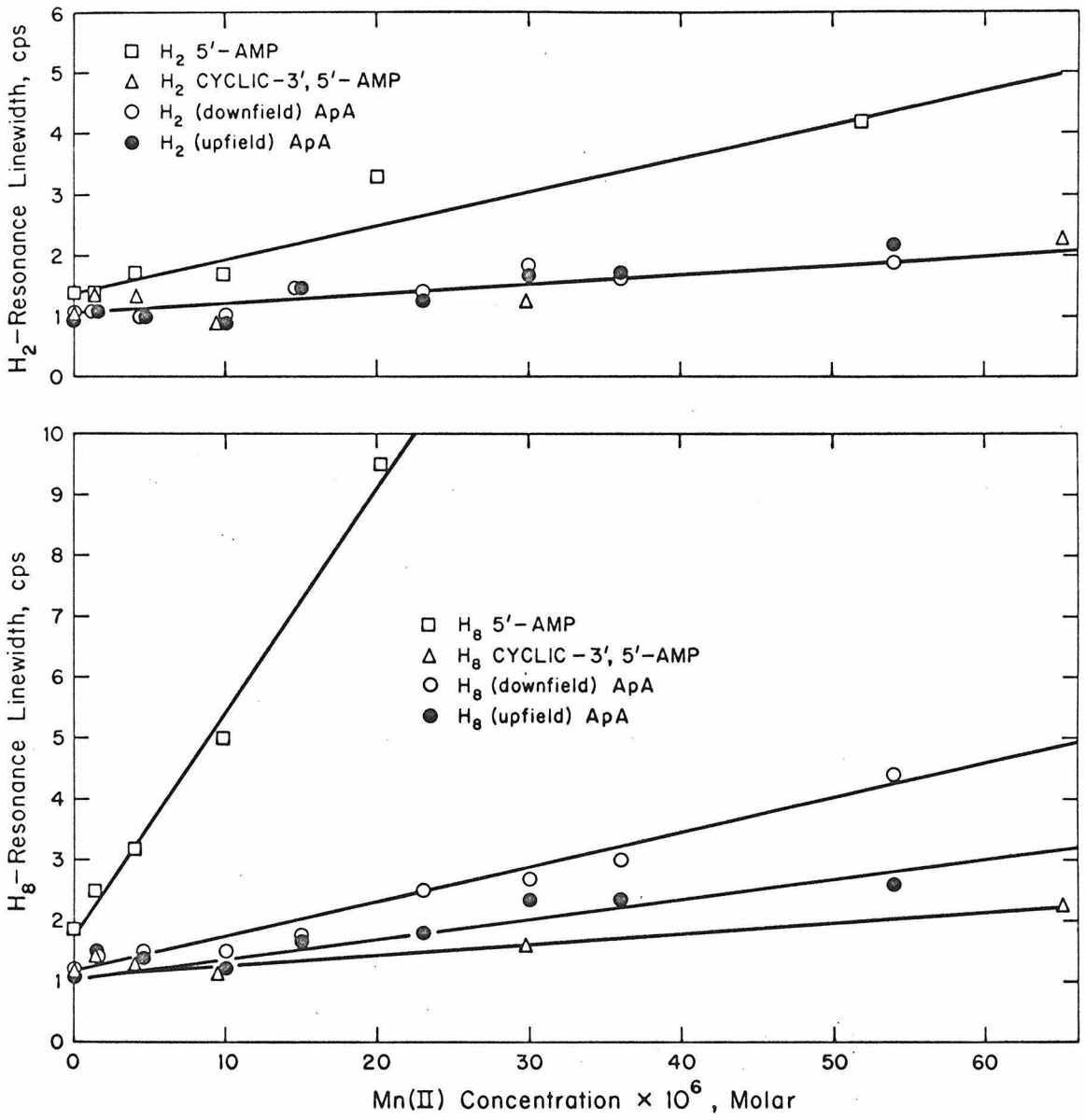


Figure 6. Resonance linewidths of the adenine protons of 5'-adenosine monophosphate, adenosine-3',5'-cyclic monophosphate, and adenylyl-(3'→5')-adenosine as a function of Mn(II) concentration.

Figure 7. The line-broadening effect of Mn(II) on the adenine resonances of 5'-AMP (5'-AMP concentration = 0.01 M). The spectra were time-averaged and the chemical shifts were monitored relative to external TMS.

LINEWIDTHS OF ADENINE RESONANCES

0.01M. Adenosine-5′-Monophosphate

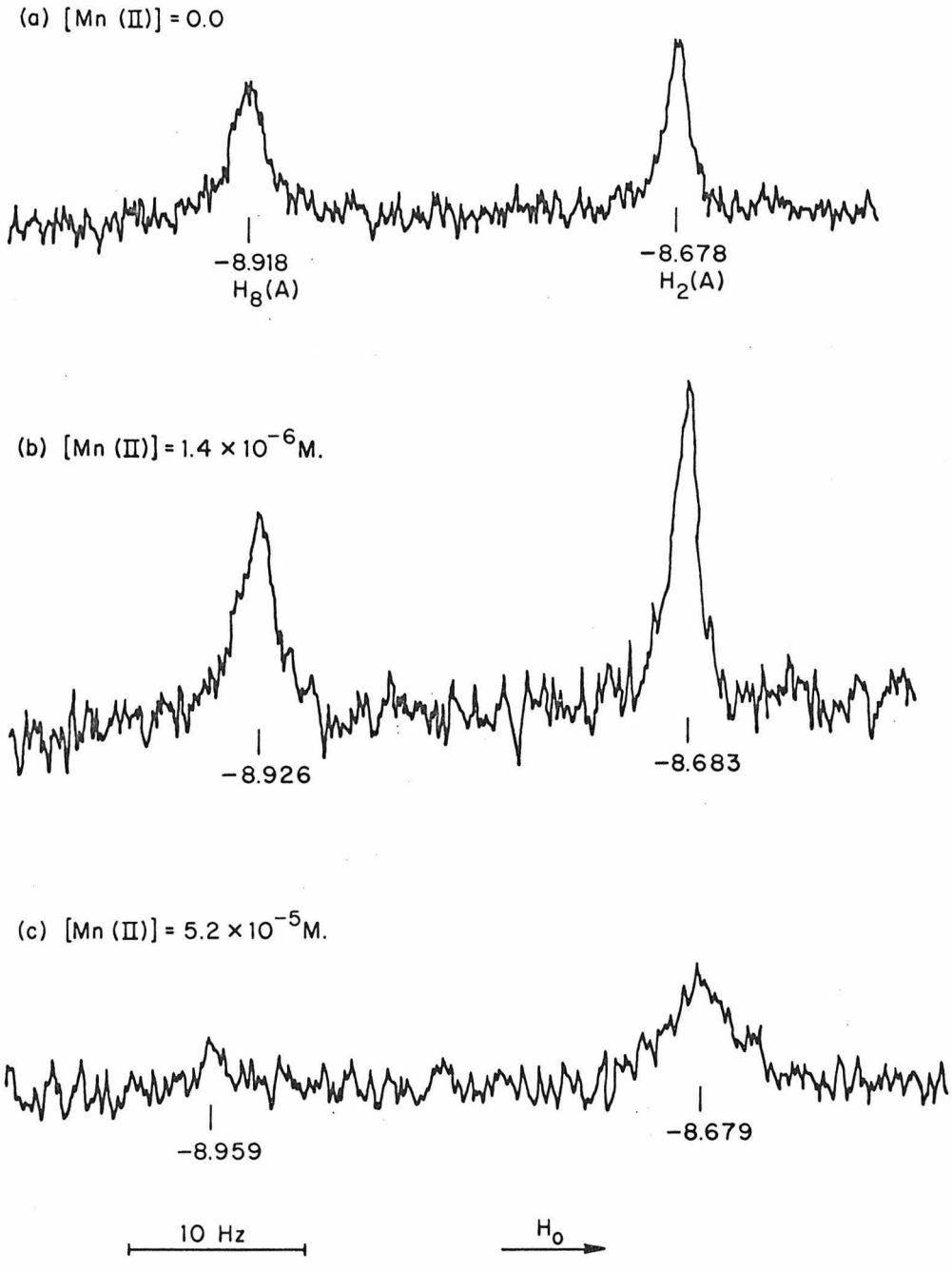
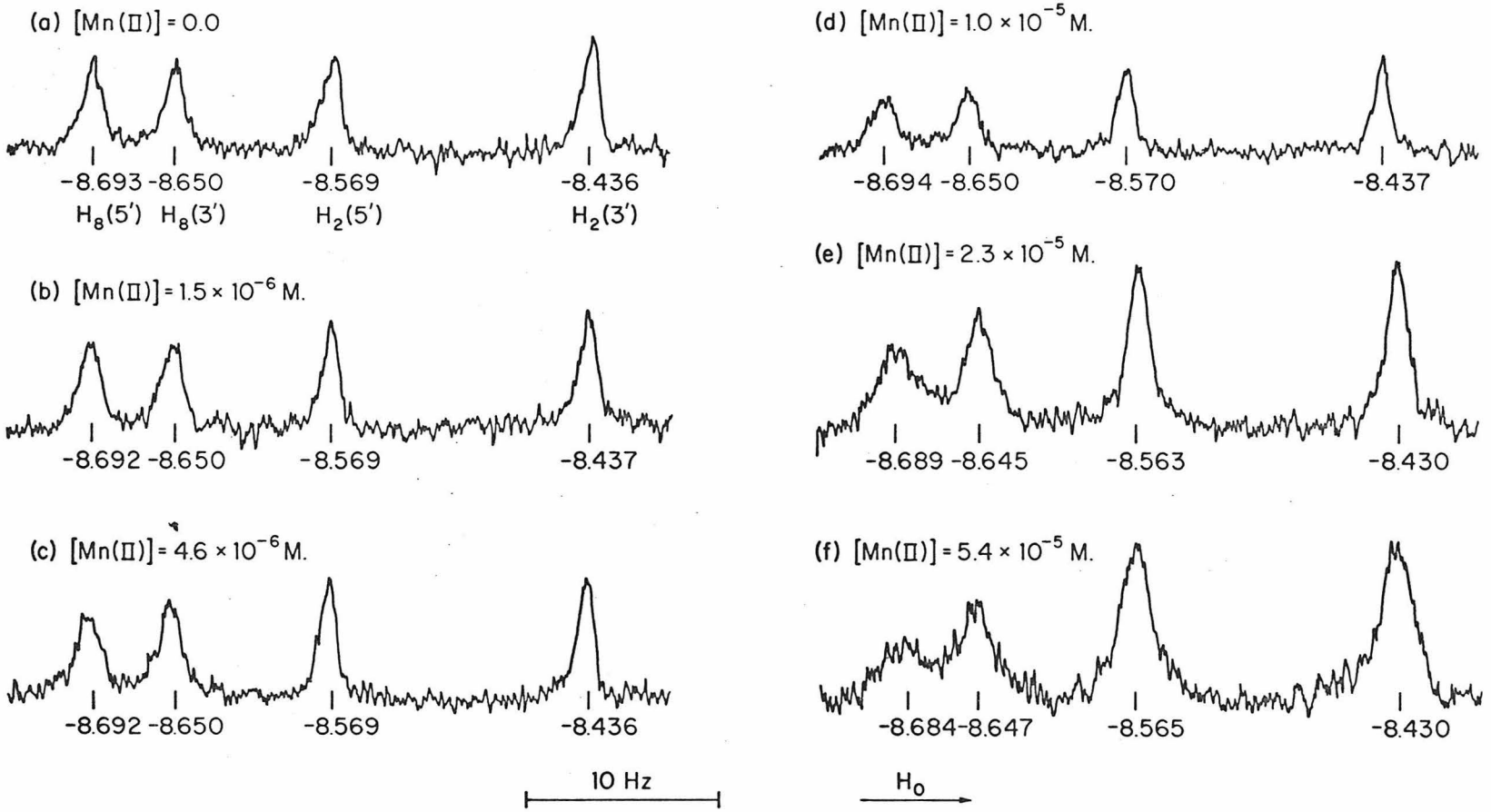


Figure 8. The line-broadening effect of Mn(II) on the adenine resonances of ApA (ApA concentration = 0.01 M). The spectra were time-averaged and the chemical shifts were monitored relative to external TMS.

LINEWIDTHS OF ADENINE RESONANCES

0.01 M. Adenylyl-(3′→5′)-Adenosine



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 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 towards 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 Figures 9-12, we have depicted, for illustrative purposes, four possible <u>stacked</u> conformations of ApA, as viewed along the axis of the ribose-phosphate backbone in the direction of 5'- to 3'-phosphate esterification. The 3'-<u>anti</u>, 5'-<u>anti</u> conformation is designated by I. The 3'-<u>anti</u>, 5'-<u>syn</u> (II), 3'-<u>syn</u>, 5'-<u>anti</u>(III), and 3'-<u>syn</u>,

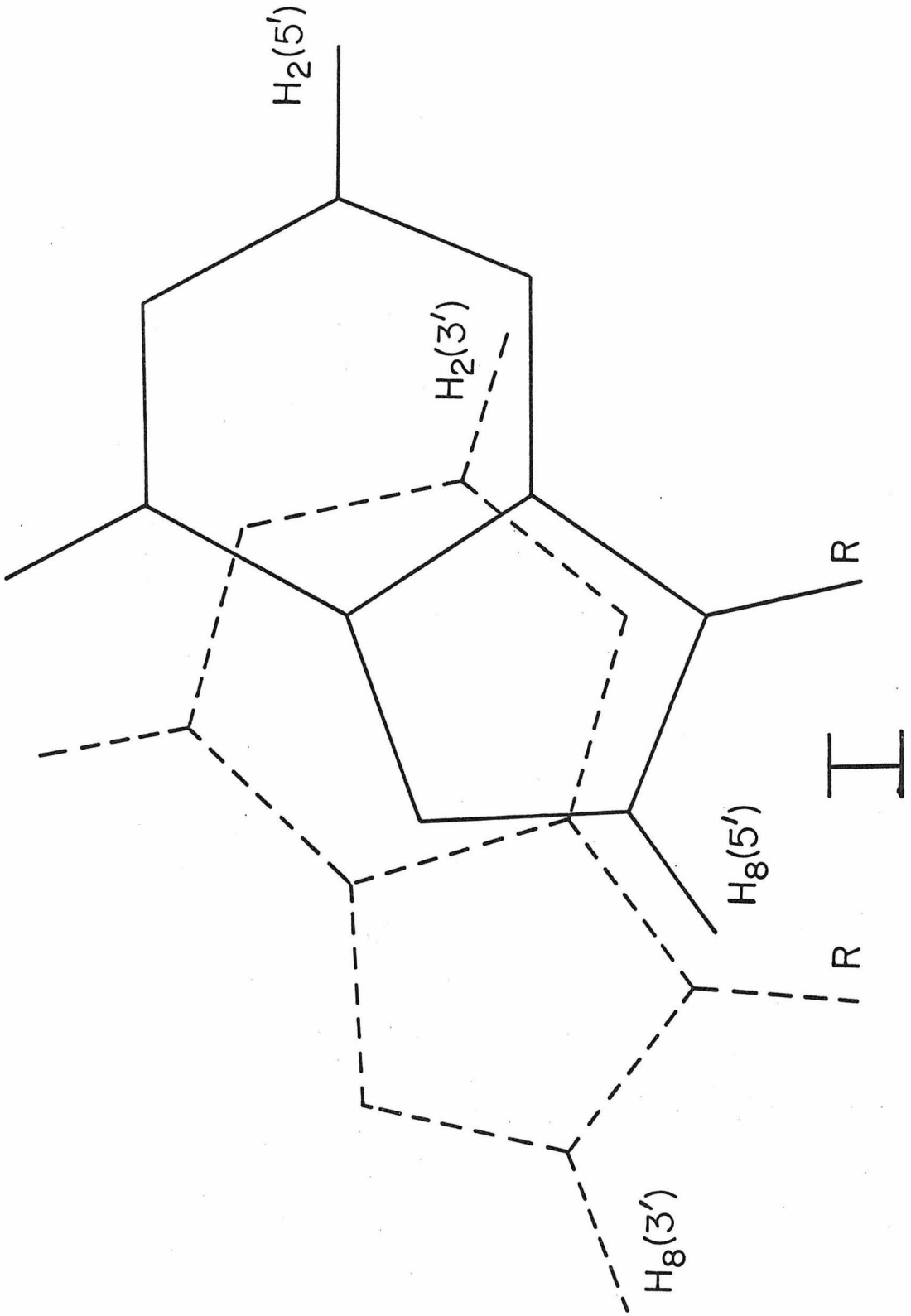
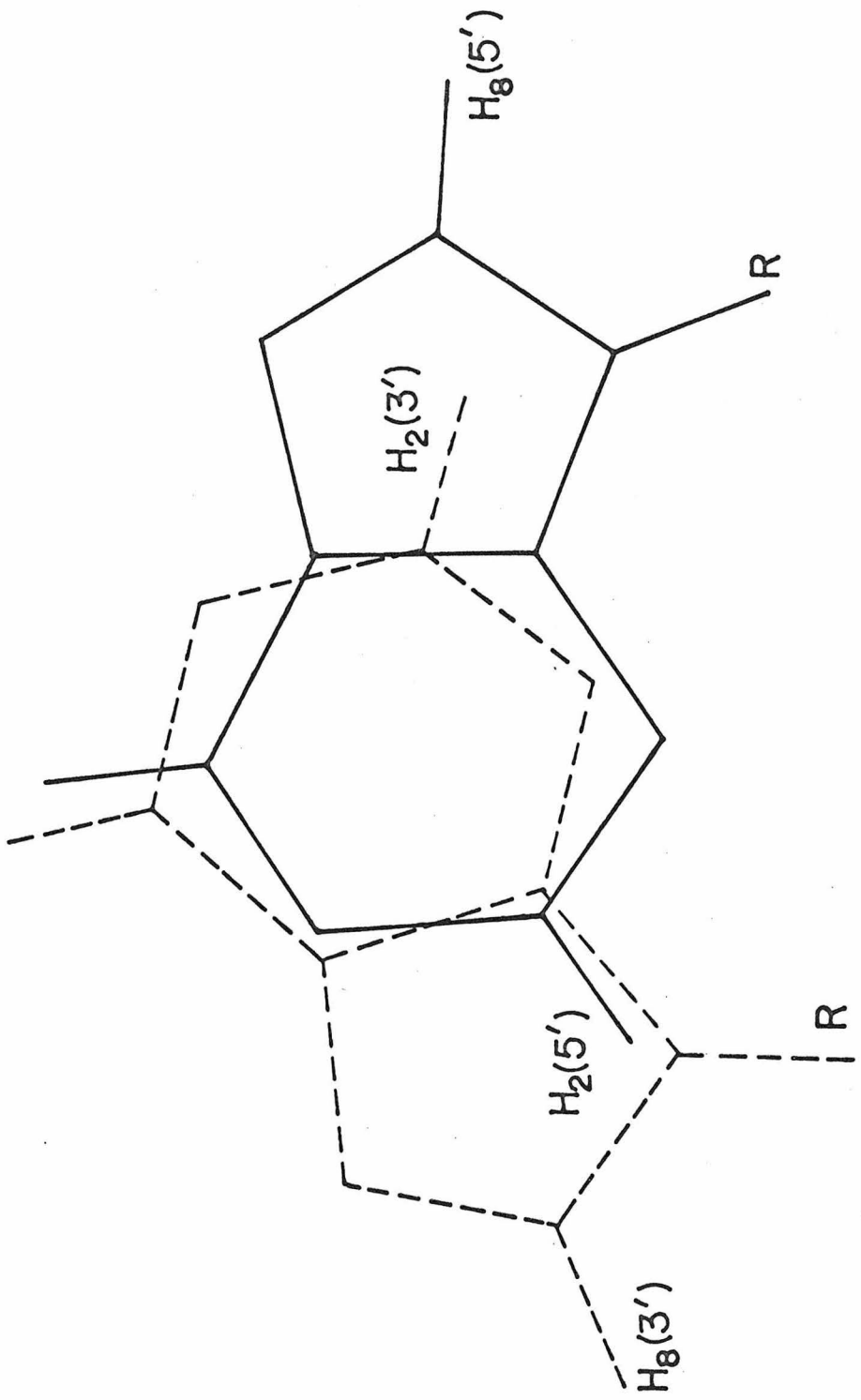


Figure 9. The 3'-anti, 5'-anti conformation of ApA.



II

Figure 10. The 3'-anti, 5'-syn conformation of ApA.

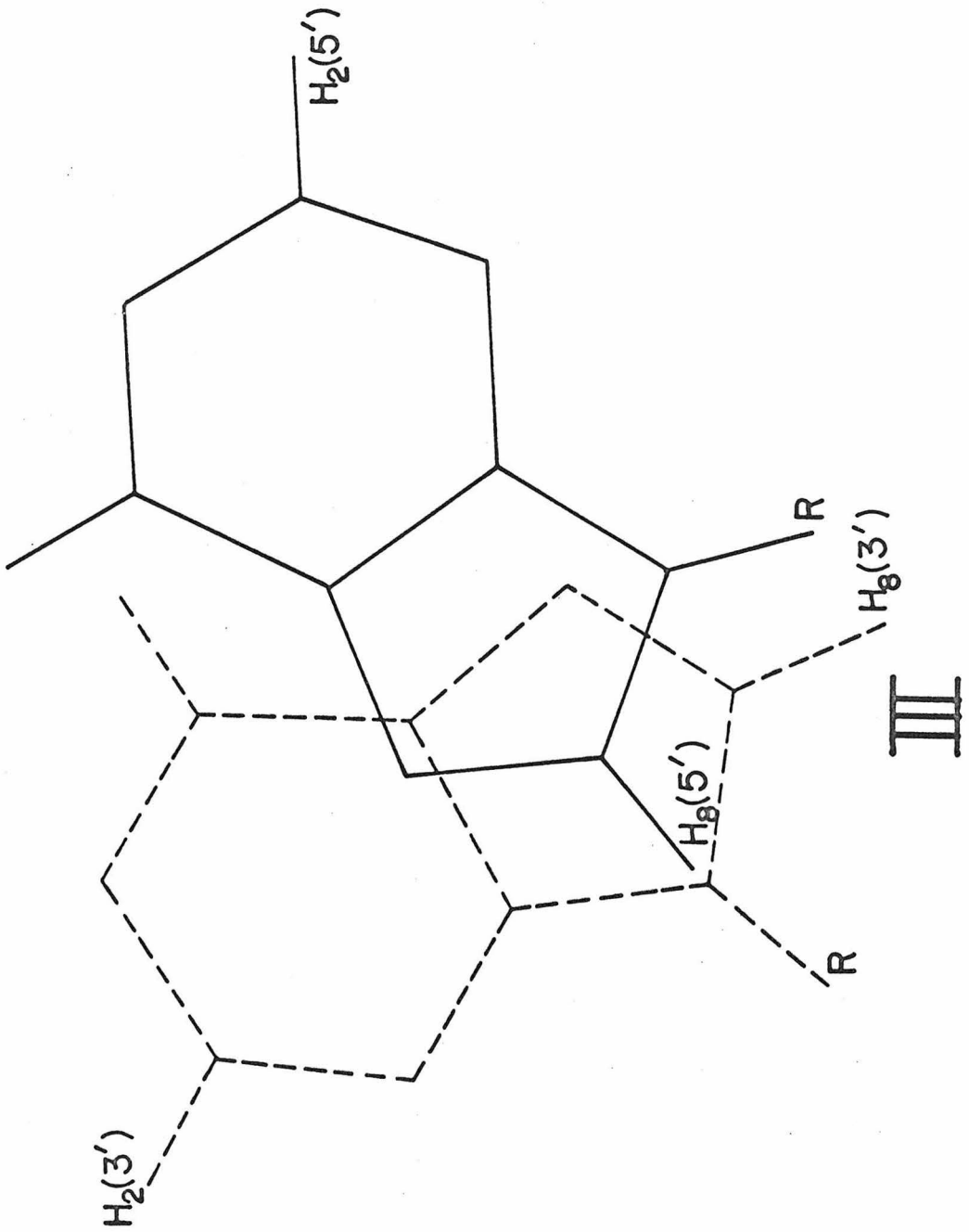


Figure 11. The 3'-syn, 5'-anti conformation of ApA.

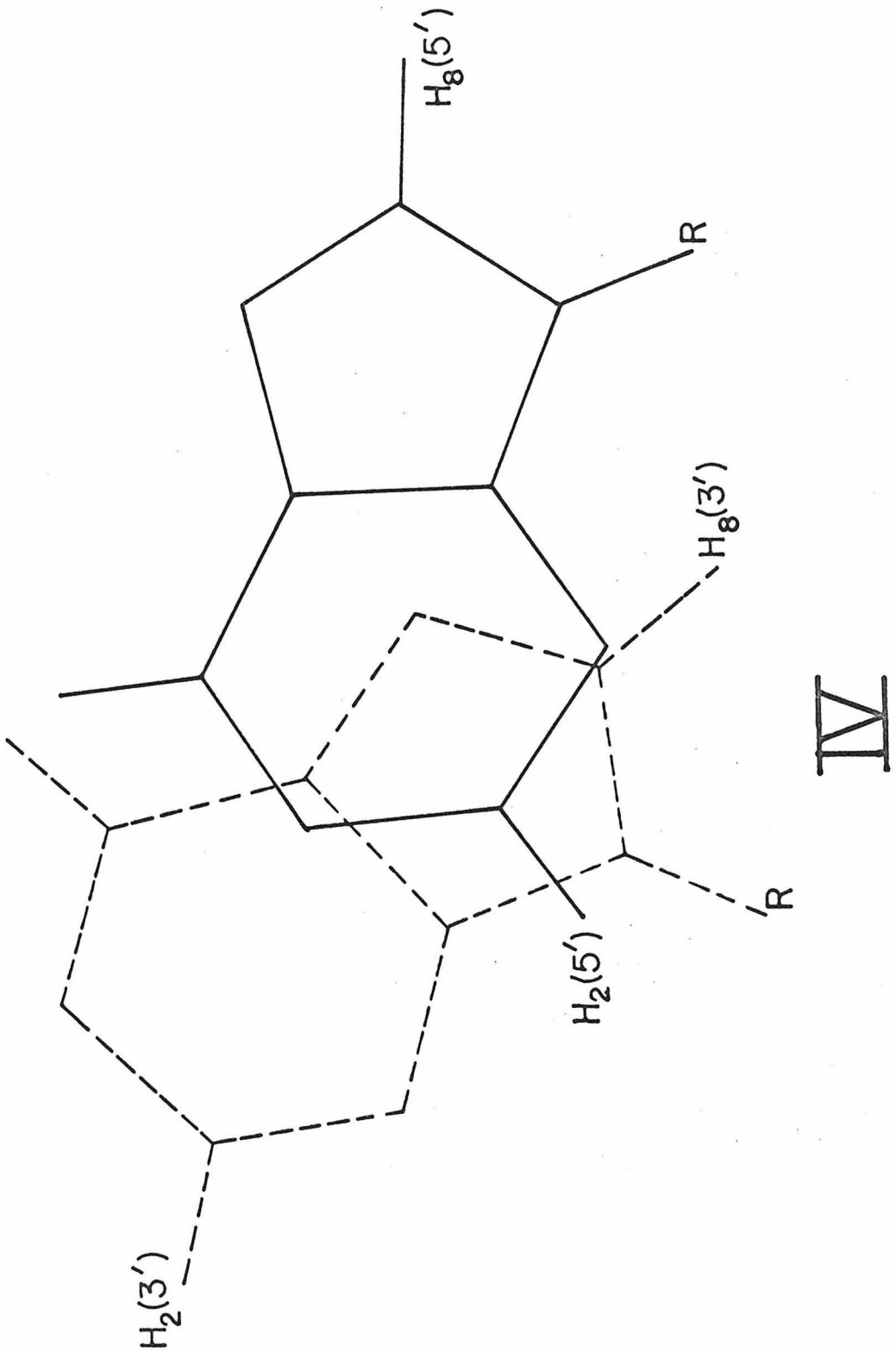


Figure 12. The 3'-syn, 5'-syn conformation of ApA.

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. 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 Figures 9-12. In any case, the equilibrium dihedral angle of stacking is probably not too well defined, since the potential trough which describes the 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 Figures 9-12 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 sizable 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 bases only when the 3'-base and the 5'-base are syn and anti, respectively.

In Figures 13 and 14, 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.

D. 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 p.m.r. spectrum of the adenine protons of ApA over the concentration range of 0.005 M to 0.11 M. A pronounced concentration effect has been observed. The chemical shifts of the four adenine protons of ApA are plotted versus the concentration in Figure 15. 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.

Figure 13. Variation of the chemical shifts (100 Mcps) of the H_2 resonances of 0.003 M ApA with temperature. The chemical shifts are plotted relative to the H_2 resonances of adenosine at the same temperature and concentration.

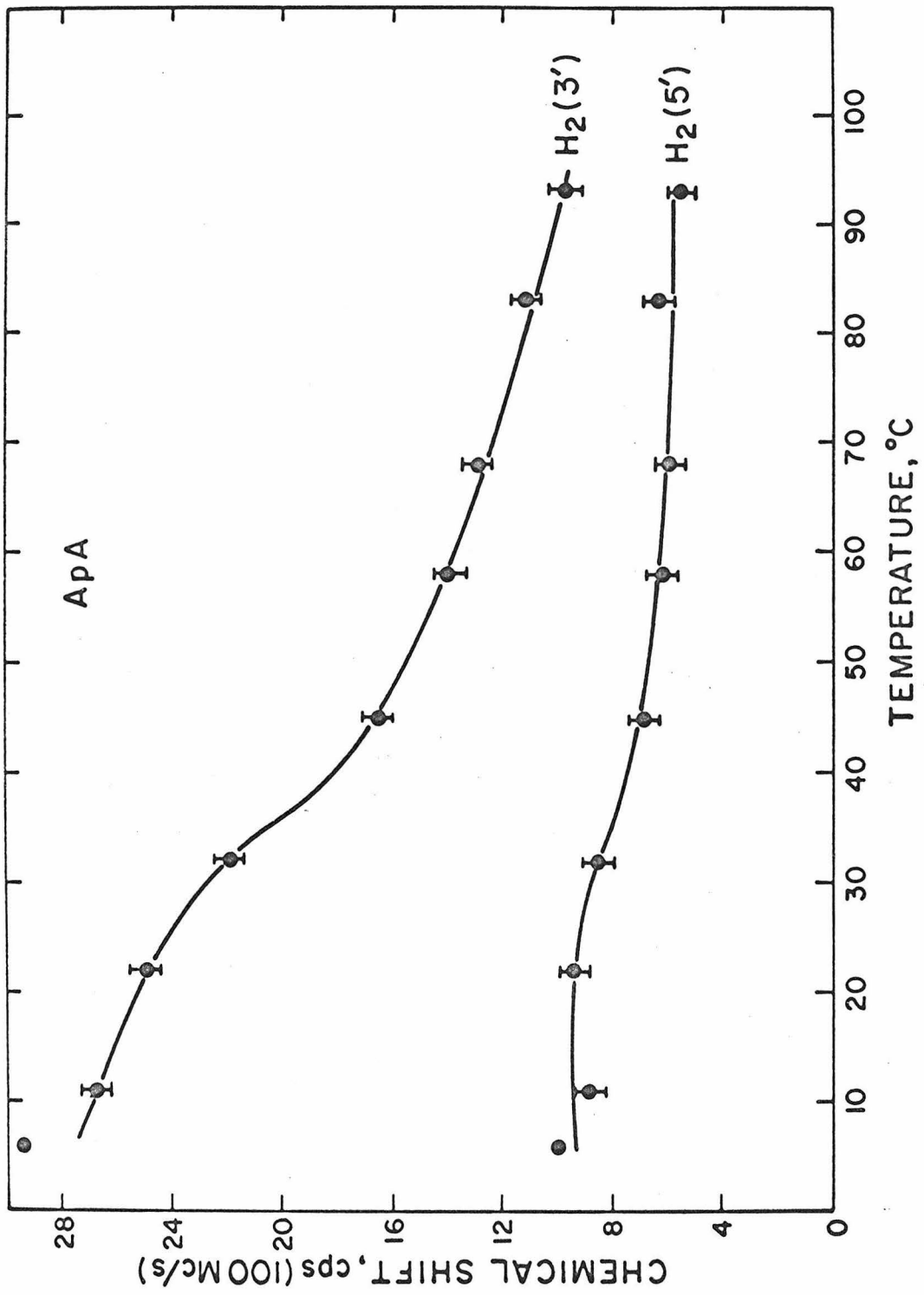
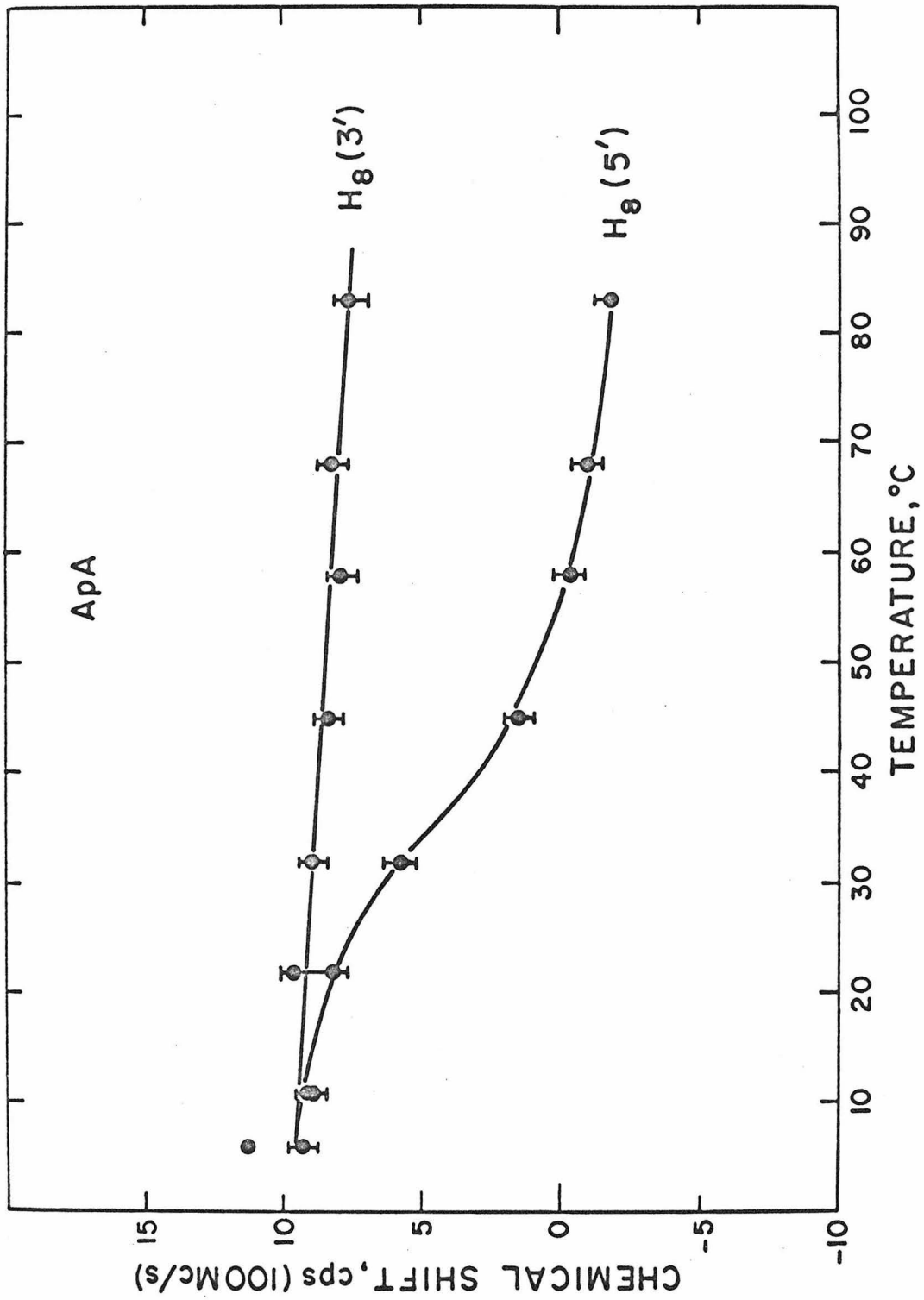

ApA
H2(3')
H2(5')
CHEMICAL SHIFT, cps (100 Mc/s)
TEMPERATURE, °C
0
4
8
12
16
20
24
28
10
20
30
40
50
60
70
80
90
100

Figure 14. Variation of the chemical shifts (100 Mcps) of the H_8 resonances of 0.003 M ApA with temperature. The chemical shifts are plotted relative to the H_8 resonances of adenosine at the same temperature and concentration.


ApA
$H_8(3')$
$H_8(5')$
CHEMICAL SHIFT, cps (100Mc/s)
TEMPERATURE, °C
15
10
5
0
-5
-10
10
20
30
40
50
60
70
80
90
100

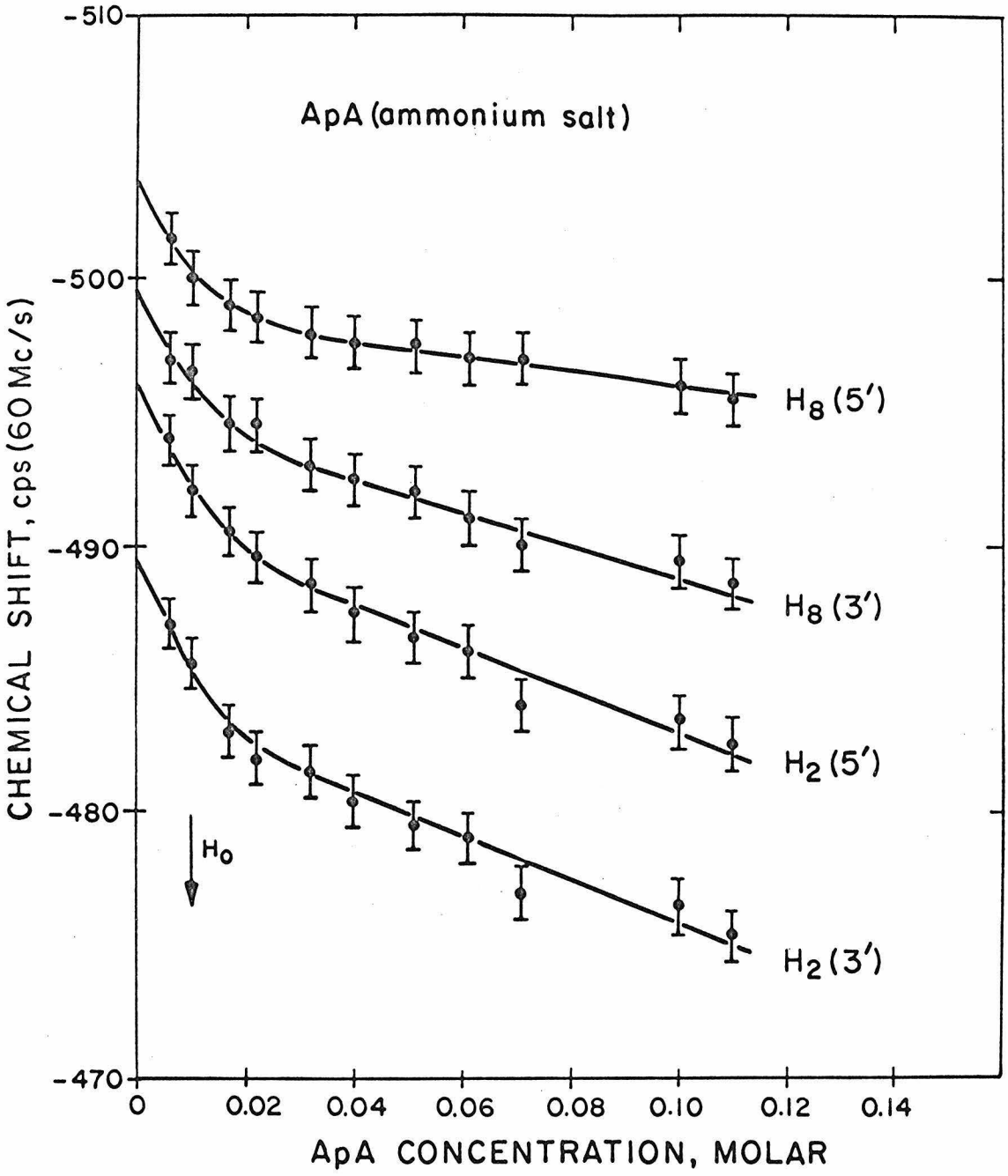
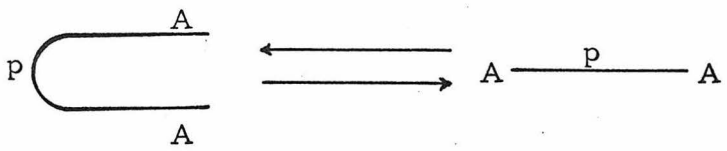


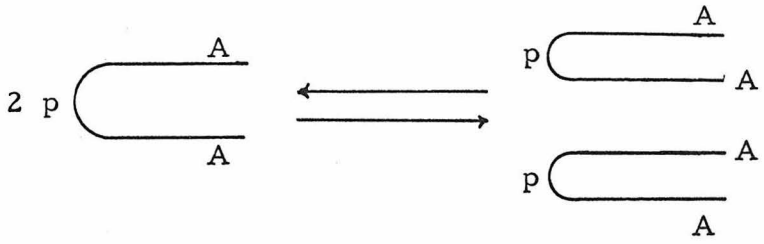
Figure 15. Concentration dependence of the proton chemical shifts of the adenine protons of ApA in aqueous solution at 40°C. The chemical shifts (at 60 Mcps) were monitored relative to external TMS (6% in chloroform) and have not been corrected for bulk susceptibility.

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 (48). 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 (35), which demonstrated that the association constant per step in the stacking multiple equilibria is ~ 4.5 molar^{-1} for this molecule, or about a factor of 2 larger than the value previously reported (34) for purine self-association.

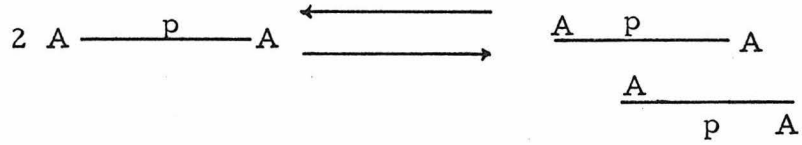
Several modes of intermolecular association are possible. If the dinucleoside is strongly stacked intramolecularly, there can only be external stacking of folded ApA molecules. If the two adenine bases are not strongly stacked intramolecularly, then two molecules can base-stack intermolecularly on either 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 schematically as follows:



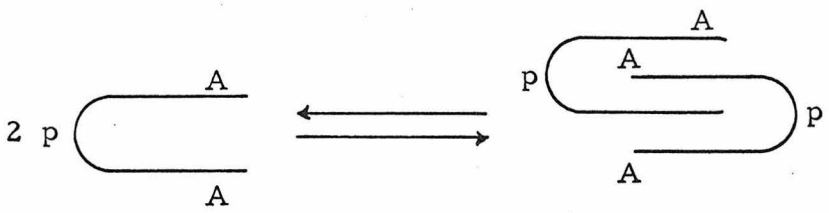
(II-1)



etc. (II-2)



etc. (II-3)



(II-4)

where A = adenine base

p = phosphate-ribose backbone

The tendency to form self-intercalated complexes provides some indication of the strength of the intramolecular stacking interaction since self-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 linewidths of the ApA adenine proton resonances as a function of

concentration. In UpA, where self-intercalated dimers are apparently formed (83, Chapter IV, this thesis), the linewidths 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 Figure 16, we have reproduced tracings of the p.m.r. spectrum of ApA in the region of the adenine ring protons at various concentrations of the dinucleotide. It is noteworthy that the linewidths 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 17, 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-

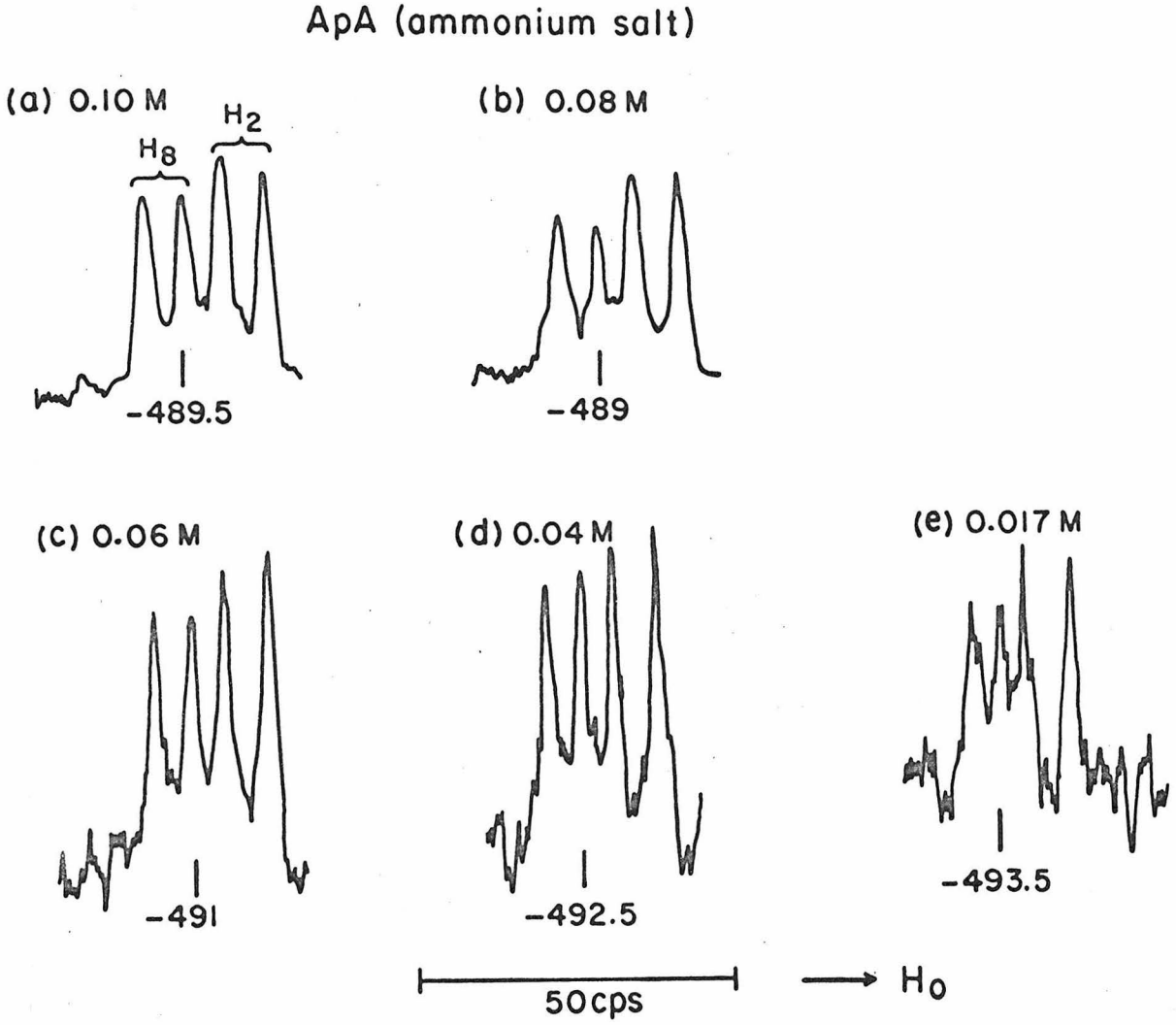


Figure 16. Proton magnetic resonance spectra (60 Mcps) of the adenine protons of ApA at various dinucleotide concentrations. Chemical shifts are referred to external TMS (6% in chloroform). Temperature ~ 40°C.

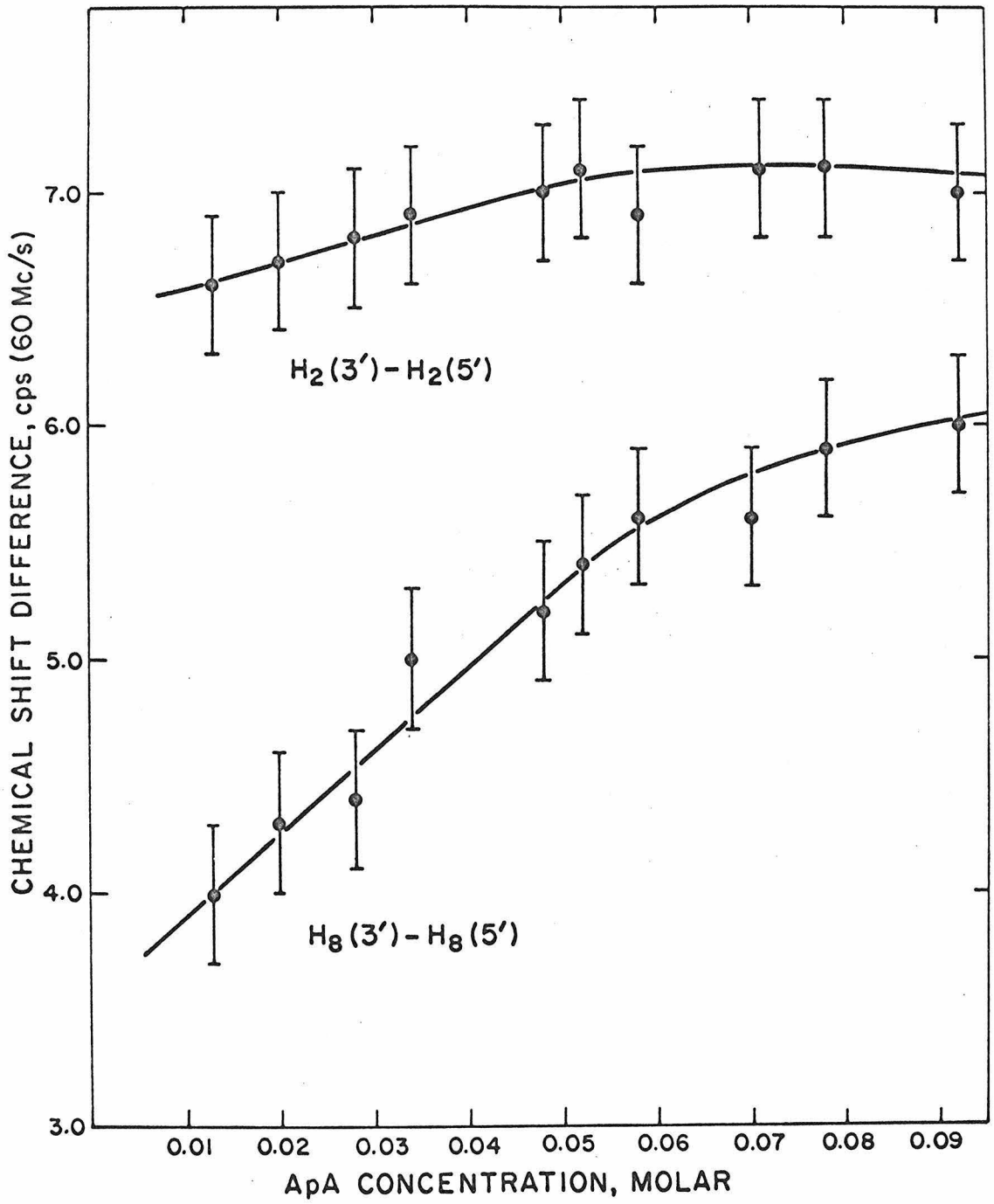


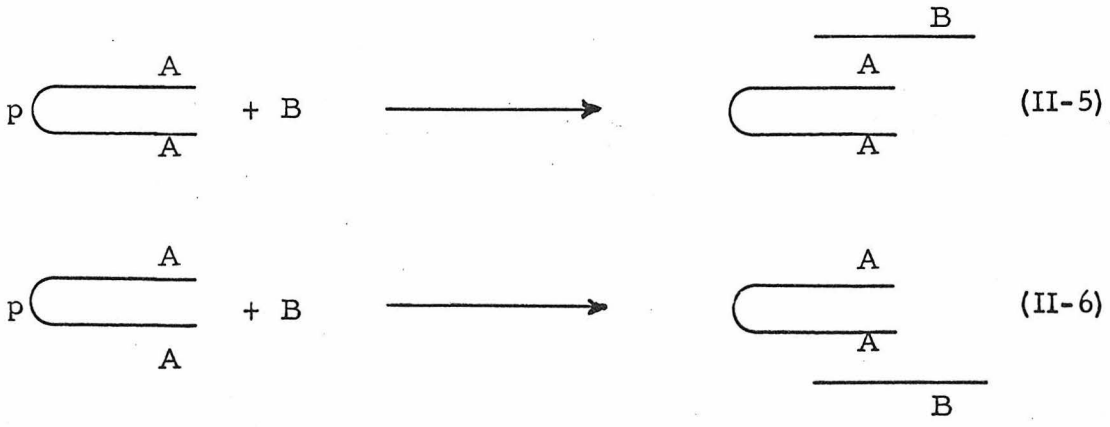
Figure 17. Chemical shift difference (60 Mcps) of the two H_2 adenine resonances and of the two H_8 adenine resonances of ApA as a function of ApA concentration.

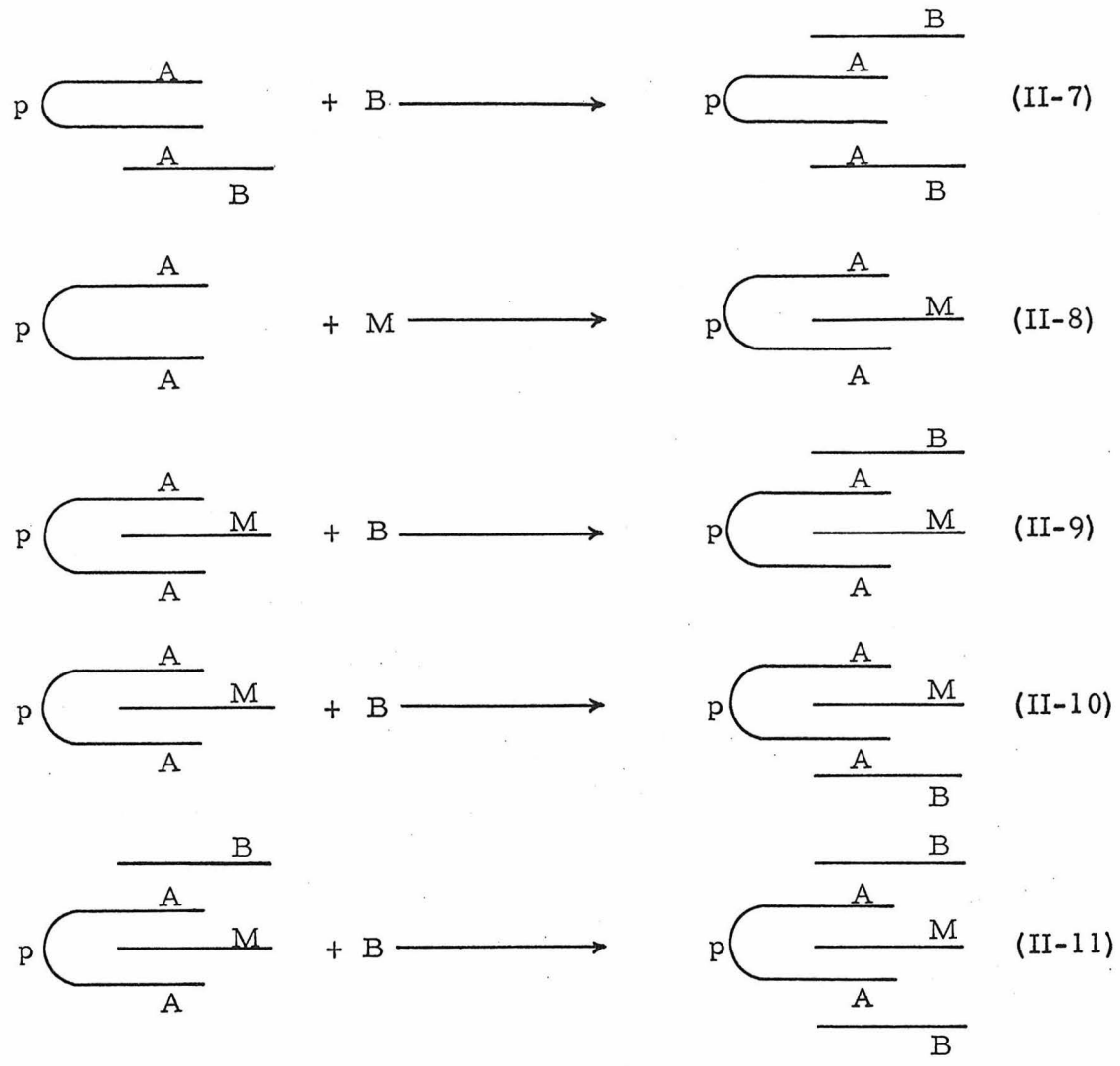
shift difference of the H_8 resonances arises from the formation of self-intercalated dimers. If this were the explanation, we might expect a similar concentration-induced effect for the H_2 resonances, even though it is possible that on the average there can be accidental cancellation of intermolecular 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 <u>anti</u> relative to the ribose moiety about the $C_{1'}$-N_9 glycosidic bond; Figure 9) 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 difference in the chemical shifts of the H_8 protons should increase with concentration, as observed. Since the adenine H_2 protons are considerably further 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 chemical shift difference of the H_2 protons.

E. ApA - Purine Interactions

1. Introduction. The binding of purine to intramolecularly stacked ApA molecules can be described in terms of the following multiple equilibria:





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 towards 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.

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 (34). 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 towards 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 ~ 0.25 M at a purine stoichiometric concentration of 1.0 M at room temperature (34). 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 an earlier study of the binding of purine to the pyrimidine-pyrimidine dinucleoside monophosphates, 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 (75). 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 (75, 79, 83). 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 the 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. 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 an earlier communication (75).

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 are largest when the equilibrium is shifted as far to the intercalated complex as possible. The purine linewidths 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 linewidths represent a weighted average between bound and free purine due to rapid exchange, the purine linewidths 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.

2. Purine-binding at Low ApA Concentrations. The purine-induced shifts of the four adenine proton resonances of a 0.01 M ApA solution are summarized in Figure 18. 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

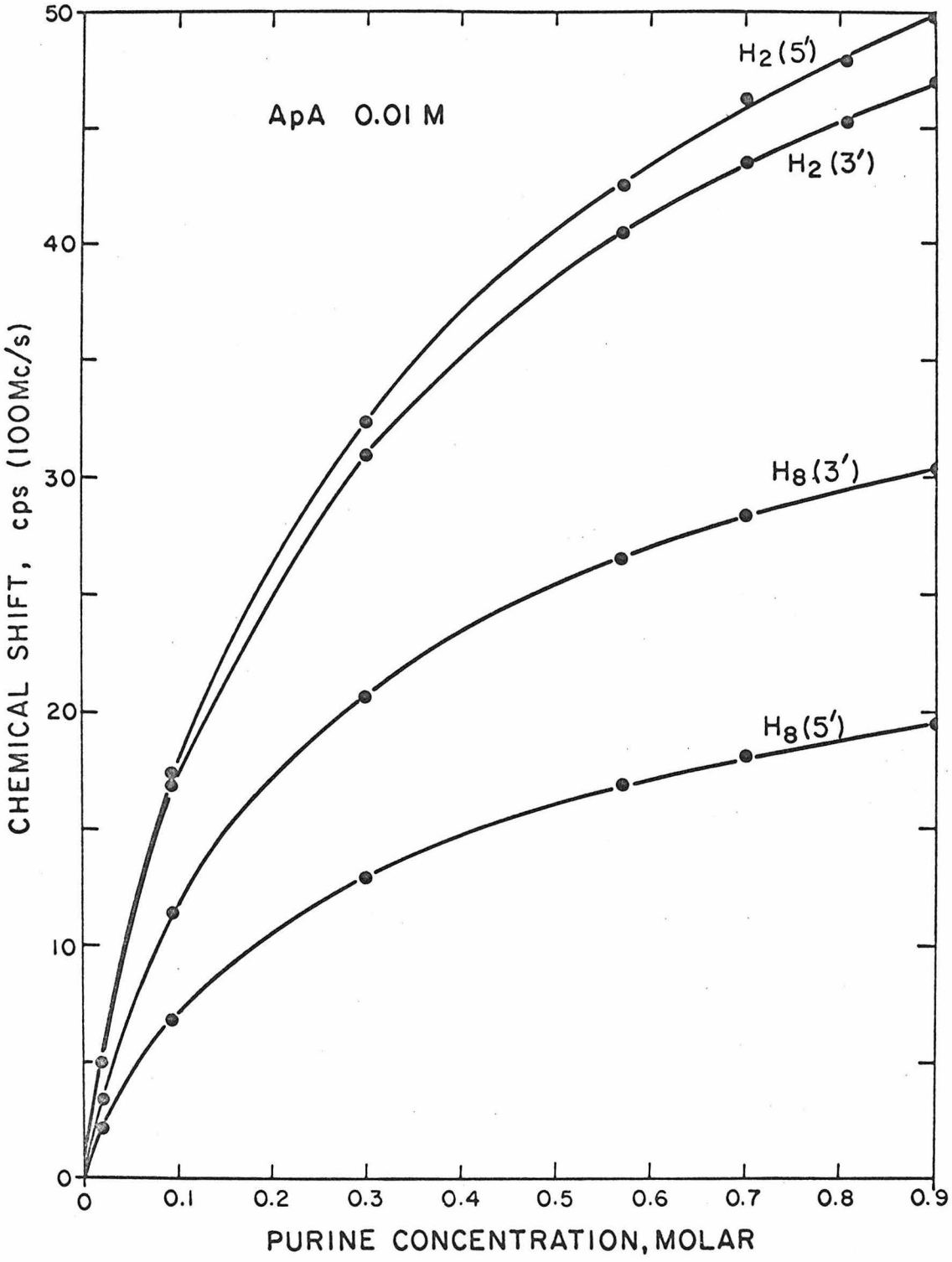


Figure 18. Purine-induced chemical shifts (100 Mcps) of the adenine ring protons of 0.01 M ApA (sodium salt).

contrast to that which 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 9). $H_2(3')$ 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 of $H_2(5')$ 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 of the H_8 protons is almost 12 cps at 100 Mcps 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 of 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 (79, 83). 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 (~ 15 cps) (79, 83) 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 for the $H_{1'}$-ribose protons (Figure 19). 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 (Chapter III, this thesis), 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

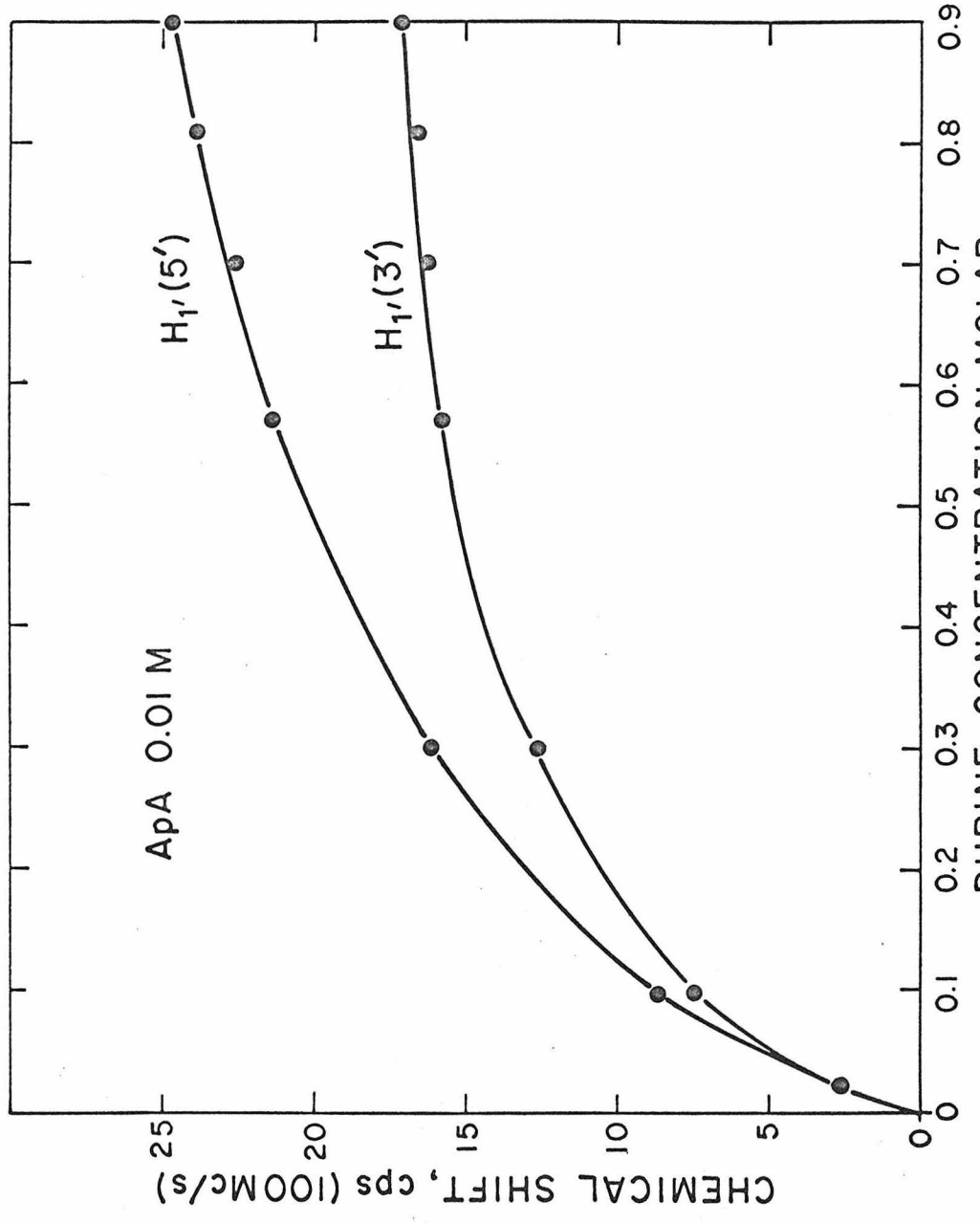


Figure 19. Purine-induced chemical shifts (100 Mcps) of the $H_{1'}$ ribose protons of 0.01 M ApA (sodium salt).

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 (90, 91). 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 towards 3'-endo) (91, 92).

The variation of the ($H_{1'}$-$H_{2'}$) coupling constants with concentration of added purine is illustrated in Figure 20. 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

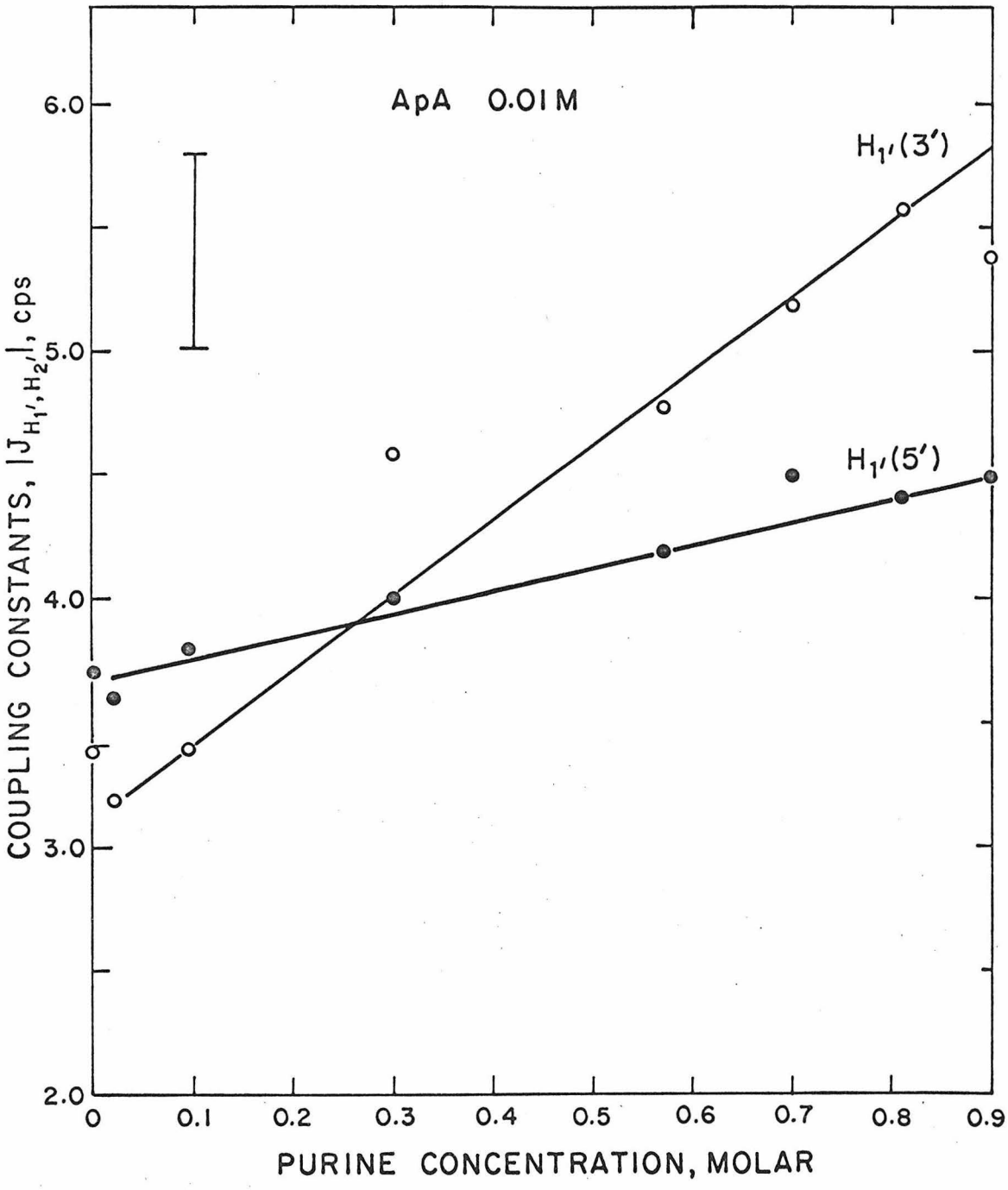


Figure 20. Variation of the $|J_{H_{1'}-H_{2'}}|$ coupling constants of ApA with purine concentration. The experimental uncertainty is indicated by the error bar in the upper left-hand corner.

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.

3. 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 the earlier 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 linewidths is probably still straightforward, since the purine line-broadening still provides a direct indication of the extent of purine intercalation.

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

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

Purine Concentration M	Chemical Shift Difference[a] cps at 60 Mcps		Purine-induced Shifts[b] cps at 60 Mcps			
	$H_8(3')-H_8(5')$	$H_2(3')-H_2(5')$	$H_8(5')$	$H_8(3')$	$H_2(5')$	$H_2(3')$
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.

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.

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 linewidth data are summarized in Table III, and the effect is illustrated in Figure 21. Again, the line-broadening is most pronounced for the purine H_6 and the least evident for the purine H_2.

F. The Effects of pH

1. Introduction. The evidence presented thus far indicates that the two adenine bases in ApA are intramolecularly stacked at room temperature. It was natural, then, to study the system under conditions which might alter the extent of this base-stacking. Perhaps the

TABLE III. Linewidths[a] of Purine Resonances in ApA-Purine Solutions[b]

Purine Concentration (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 M Purine in the absence of ApA	0.8	1.0	1.0

[a]Full width at half-intensity in cps.

[b]0.15 M ApA (ammonium salt).

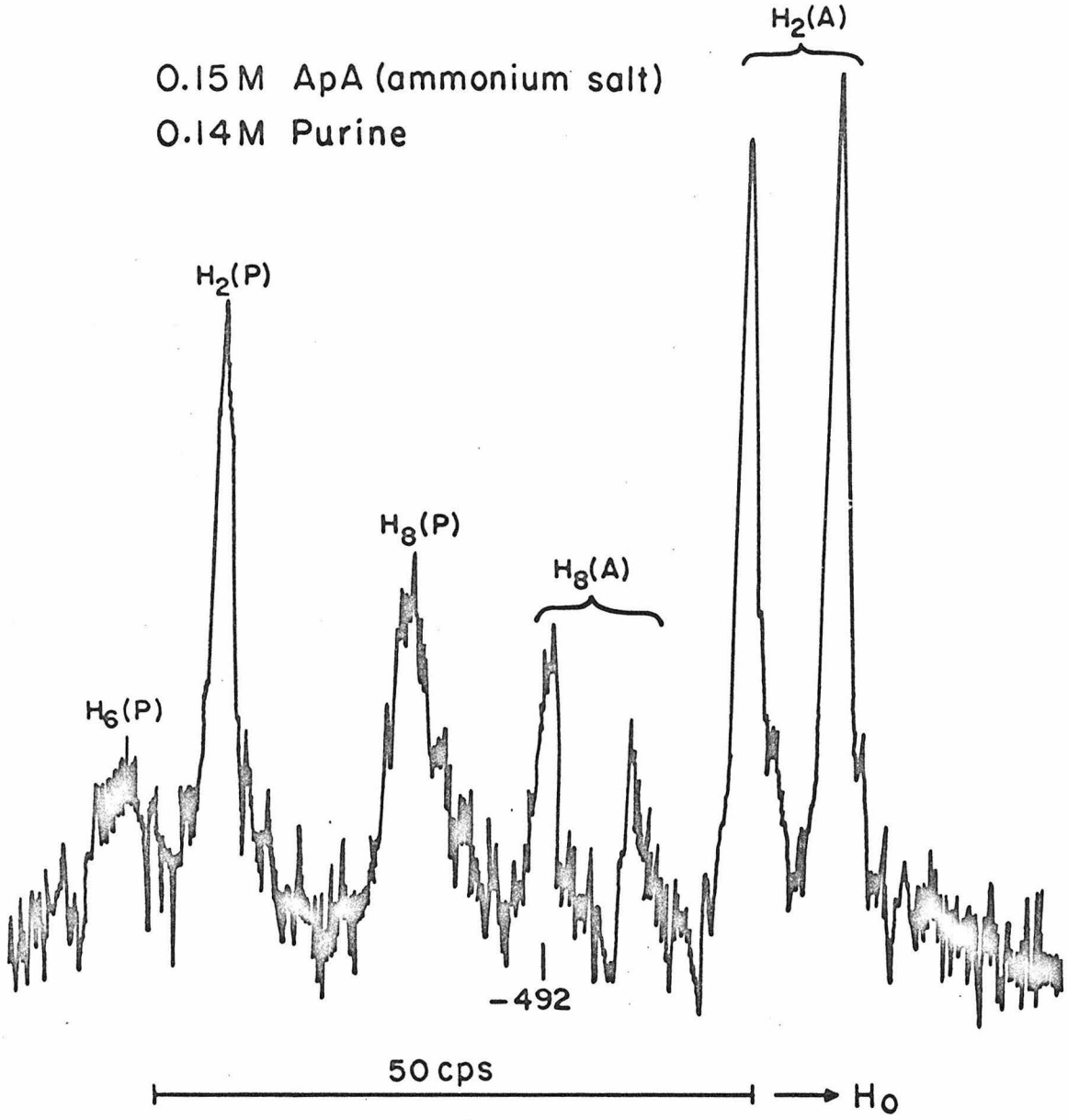


Figure 21. Proton magnetic resonance spectrum (60 Mcps) of the base protons in a mixture of 0.15 M ApA (ammonium salt) and 0.14 M purine. The chemical shifts were monitored relative to external TMS (6% in chloroform). The diminished intensity of the H_8 adenine resonances is due to deuterium labelling which results from proton exchange with the solvent, D_2O.

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 intermolecular 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 = 3.3). Since the pK_a for protonation of the purine ring in purine is about 2.4 (93), 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.

2. Protonation of ApA. For ApA the highest pK_a for protonation should correspond to protonation of the adenine rings. Reported values (94) 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 (94) in the range 3.74 to 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 to 4. Since the free energy of the intramolecular base-stacking 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 to 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 (95). 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 22. 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 22, 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 (96) in their spectrophotometric and potentiometric studies of the protonation of dinucleotides.

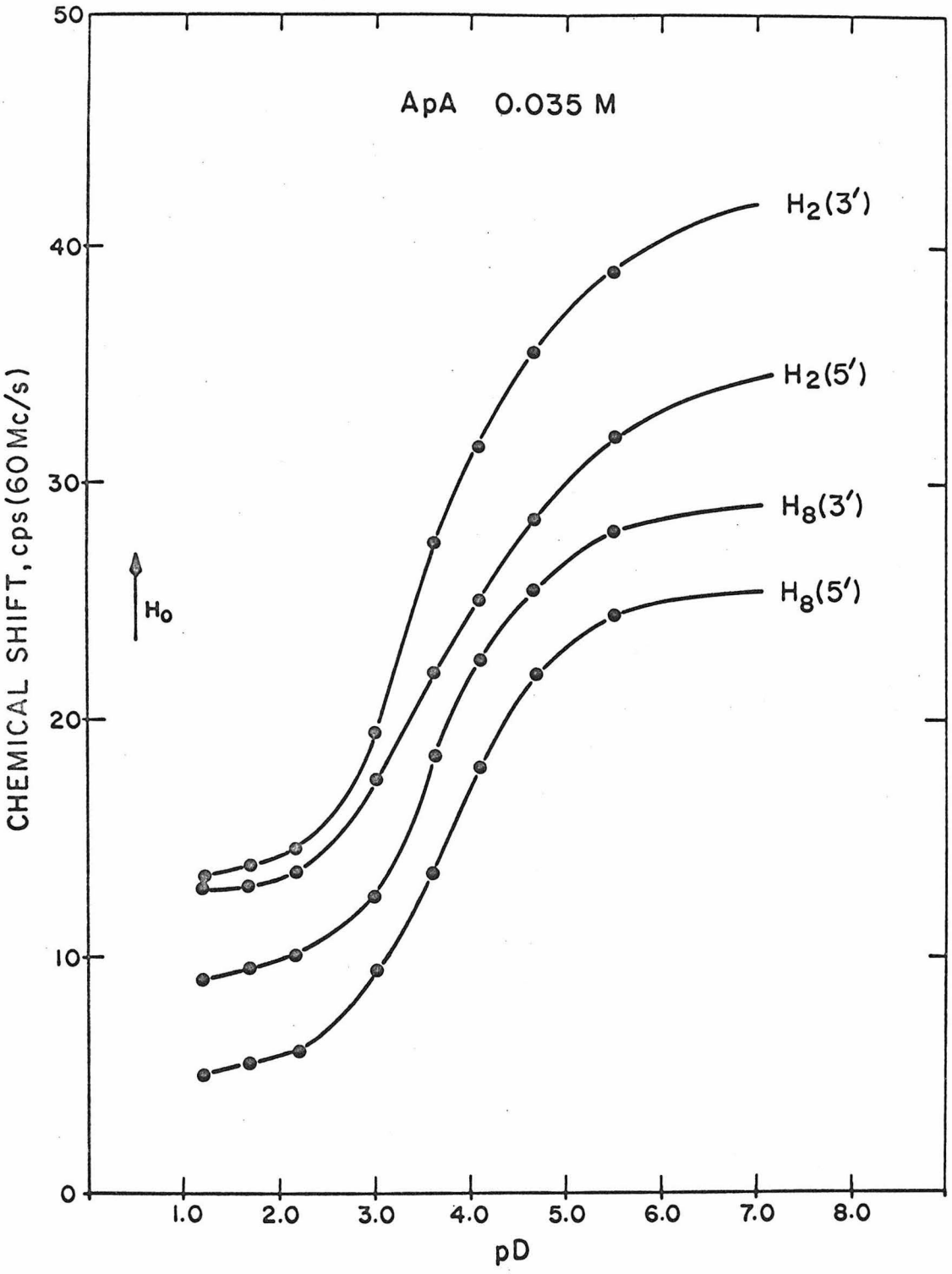


Figure 22. Variation of the chemical shifts (60 Mcps) of the adenine protons of 0.035 M ApA with solution pD.

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 arise 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 linewidth changes in the ApA spectrum. In Figure 23(a) 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 23(b). At this point, 0.4 N NaOH 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 23(c)-(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 (96). 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

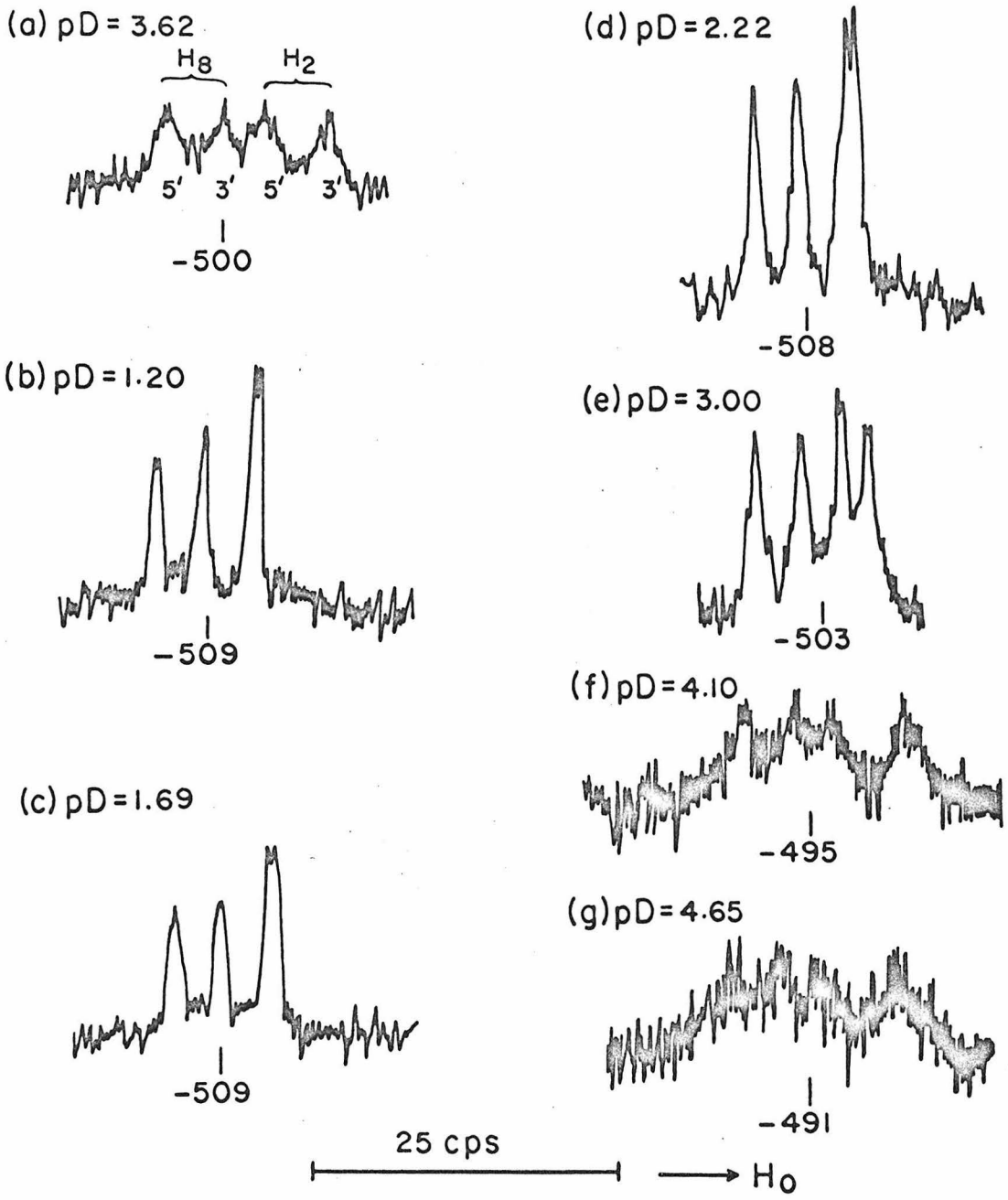


Figure 23. Proton magnetic resonance spectra (60 Mcps) of the adenine protons of 0.035 M ApA at various solution pD's. The chemical shifts were monitored relative to external TMS (6% in chloroform).

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.

3. ApA-Purine Interactions at pD $\approx$ 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 a pD $\cong$ 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 $\approx$ 3.7.[a]

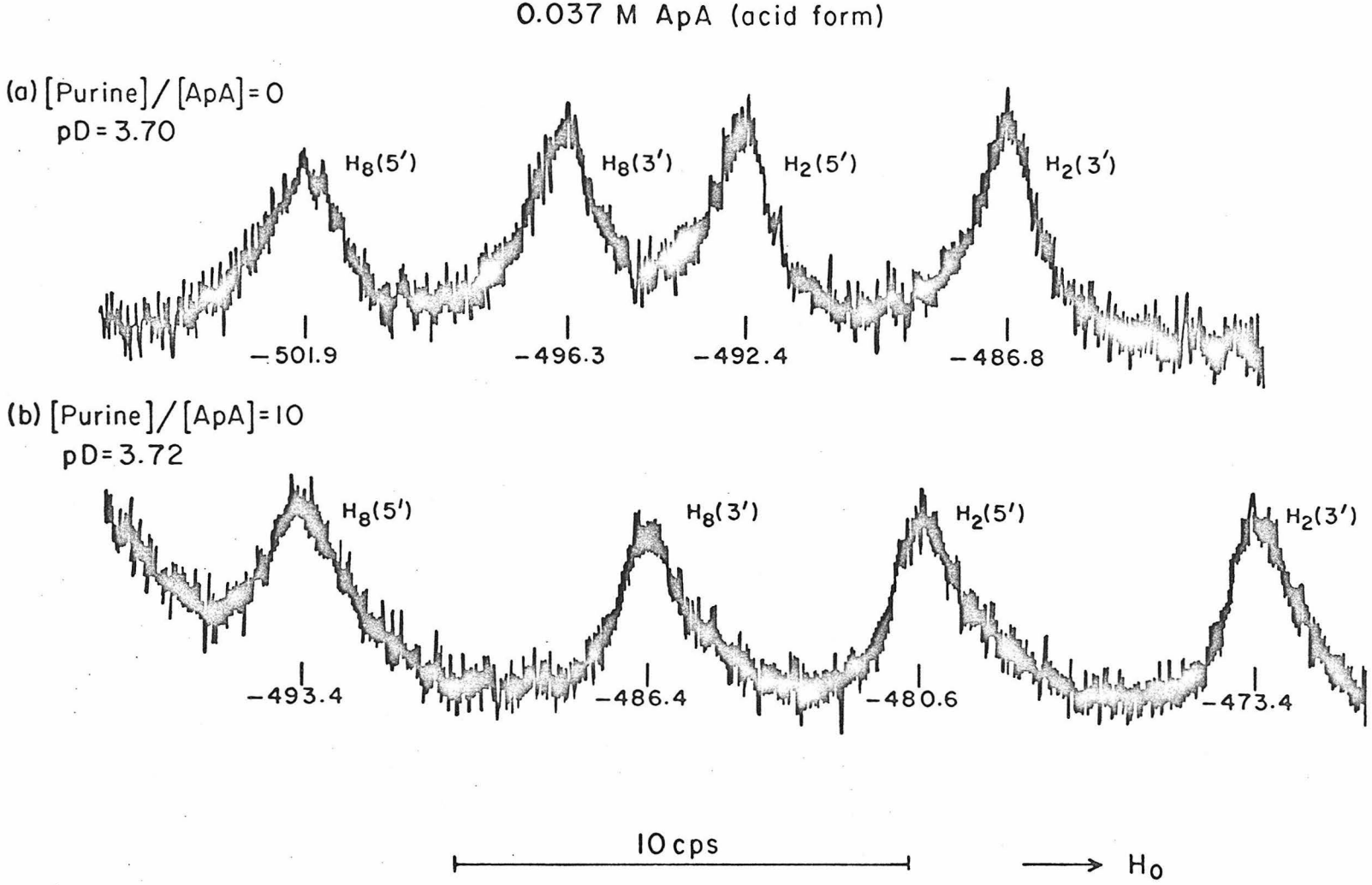
Purine/ Dinucleotide	pD	Purine-induced shifts cps at 60 Mcps[b]			
		$H_8(5')$	$H_8(3')$	$H_2(5')$	$H_2(3')$
0	3.70	0.0	0.0	0.0	0.0
1	3.75	2.7	2.8	3.0	3.5
2	3.80	3.6	4.5	5.3	6.2
5	3.65	4.4	5.4	6.2	7.0
10	3.72	8.5	9.9	11.8	13.4

[a] ApA concentration = 0.037 M.

[b] $\pm$ 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 24, 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 $\approx$ 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 $\approx$ 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 for TpT (75).

Figure 24. Proton magnetic resonance 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).


0.037 M ApA (acid form)
(a) [Purine] / [ApA] = 0
pD = 3.70
H8(5′)
H8(3′)
H2(5′)
H2(3′)
−501.9
−496.3
−492.4
−486.8
(b) [Purine] / [ApA] = 10
pD = 3.72
H8(5′)
H8(3′)
H2(5′)
H2(3′)
−493.4
−486.4
−480.6
−473.4
10 cps
H0

Further evidence for the formation of purine-intercalated complexes under the present conditions comes, of course, from the linewidths 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 Figures 25 and 26, 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 $\approx$ 3.7 than at the higher pD's.

G. 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 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 interaction 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.

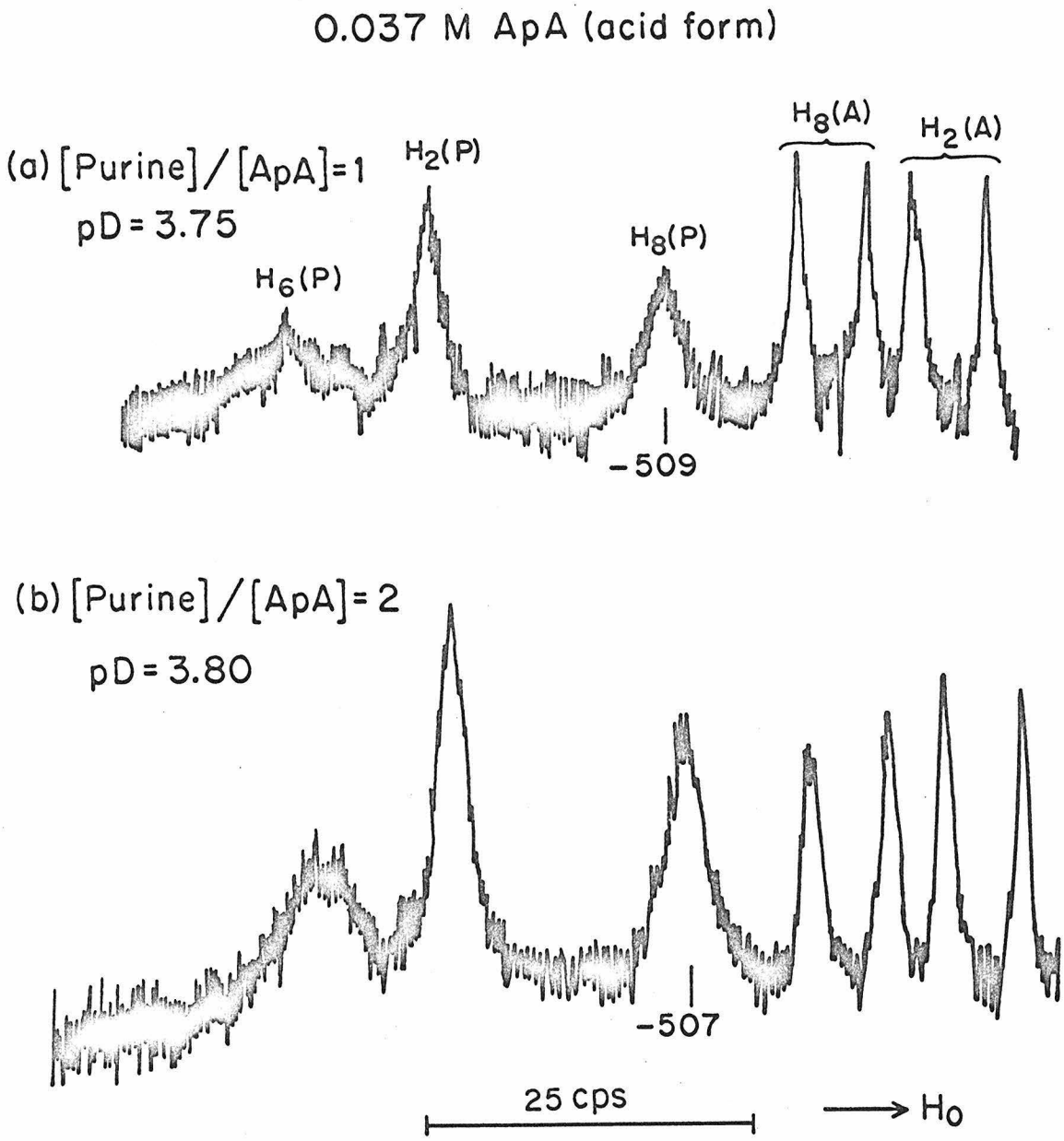


Figure 25. Illustrations of the broadening of the purine resonances observed in ApA-purine mixtures at pD $\approx$ 3.7. The ApA concentration was 0.037 M and the [purine]/[ApA] concentration ratios were (a) 1 and (b) 2.

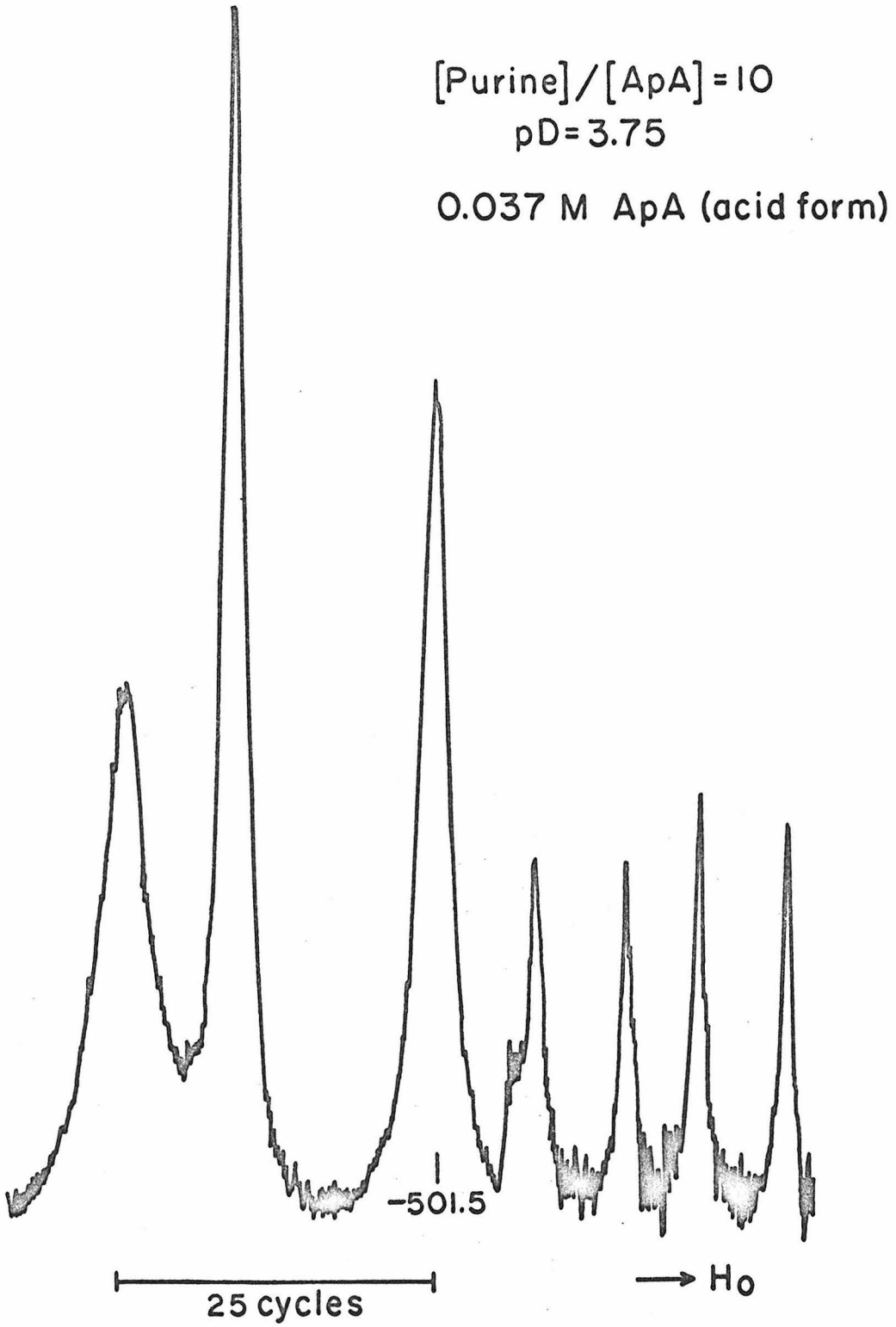


Figure 26. Illustration of the broadening of the purine resonances in an ApA-purine mixture at pD $\approx$3.75. The ApA concentration was 0.037 M and the [purine]/[ApA] ratio was 10.

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 at 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 Figures 13 and 14. These measurements were made over the temperature range of 5^{o} to 95^{o}C. 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 $\sim 5^{\circ}C$, $H_2(3')$ and $H_2(5')$ are respectively 28 cps 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 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 <u>syn</u>. 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 <u>anti</u> 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°C, $H_8(5')$ 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 <u>average</u> 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°C, 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°C, the H_2 resonances of ApA are still 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 (60). 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 p. m. r. chemical shift versus 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 versus 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 Figures 13 and 14 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 (61, 64), circular dichroism (56-58, 60), and hypochromism (22, 24, 61) measurements by other workers. The point of maximum slope in our sigmoidal chemical shift versus temperature plots appears to be in the neighborhood of 37°C. This "transition temperature" is somewhat higher than the values generally obtained by the other methods. It is not surprising that the p. m. r. 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 versus 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/mole can be obtained for the apparent ΔH^0 of the stacking process if our p. m. r. temperature data for $H_2(3')$ were treated in this manner. However, it is our contention that this ΔH^0 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 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 ΔH^0 for the unstacking of ApA as determined from studies of optical properties have ranged from 5-10 kcal/mole depending on the optical property. This wide range of standard state enthalpy values results, in part, from the difficulty in determining the asymptotic limits (at high and low temperatures) of the various experimental curves. However, it also reflects

the fact that the two-state model is not strictly applicable to the base-stacking interaction in ApA.

H. Summary

The p. m. r. 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 linewidths 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. Despite complications arising from this self-association, it was shown that the mode of interaction under various conditions provided information about both the strength and the conformation of the intramolecular stack. The mode of self-association was shown to be strongly pH dependent. At neutral pH, the intramolecular stacking interaction between 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. This dependence of the mode of self-association upon the solution pH was readily monitored by the linewidths of the adenine ring proton resonances. At neutral pH, these resonances were sharp and were not significantly broadened even at high ApA concentrations.

Around pH ~4, the adenine resonances were, however, significantly broadened. The origin of this line-broadening is presumably analogous to the broadening of the purine resonances observed upon purine intercalation. At lower pH's, where both the adenine rings become protonated, the intermolecular stacks are broken down, and the adenine resonances are sharp.

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, 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 protons, 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, it was shown that the extent of purine-intercalation is not large; the intramolecular stacking interaction between the two adenine rings must therefore be relatively strong under these conditions. The observed purine-induced shifts 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. Accordingly, the energetically most favorable stacked conformer of ApA is I (3'-anti, 5'-anti). A CPK molecular model of ApA stacked in the preferred 3'-anti, 5'-anti conformation is depicted in Figure 27. The model is viewed along the axis of the ribose-phosphate backbone in the direction of 5'- to 3'-phosphate esterification. Two other views of the energetically favorable conformer of ApA are shown in Figures 28 and 29. In Figure 28 the molecule is also viewed along the axis of the backbone, but in the opposite direction so that the 3'-base is in the foreground. The relative effect of the ring-current magnetic anisotropies of the two adenine rings of the 3'-anti, 5'-anti conformer of ApA on the chemical shifts of the various ring protons may be inferred from Figures 27 and 28. The dinucleotide is viewed along an axis perpendicular to the axis of the molecular backbone in Figure 29. The planar adenine bases have been moved apart as they would be when purine intercalation occurs.

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 in a manner similar to that previously conjectured for the pyrimidine-pyrimidine dinucleoside monophosphates.

Figure 27. A CPK molecular model of ApA stacked in the energetically favorable 3'-anti, 5'-anti conformation. The model is viewed along the axis of the ribose-phosphate backbone in the direction of 5'- to 3'-phosphate esterification.

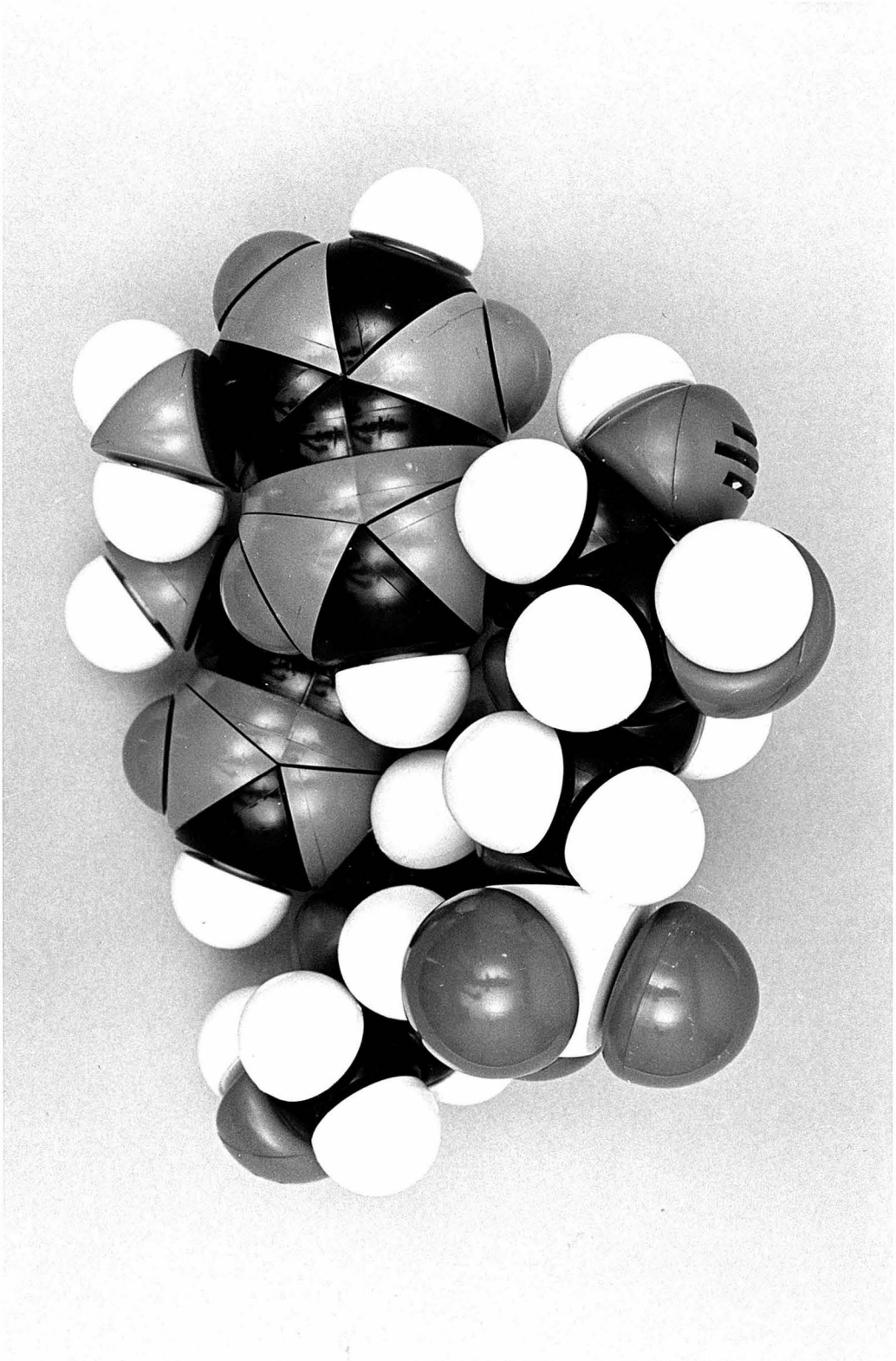


Figure 28. A CPK molecular model of ApA stacked in the energetically favorable 3'-anti, 5'-anti conformation. The model is viewed along the axis of the ribose-phosphate backbone in the direction of 3'- to 5'-phosphate esterification.

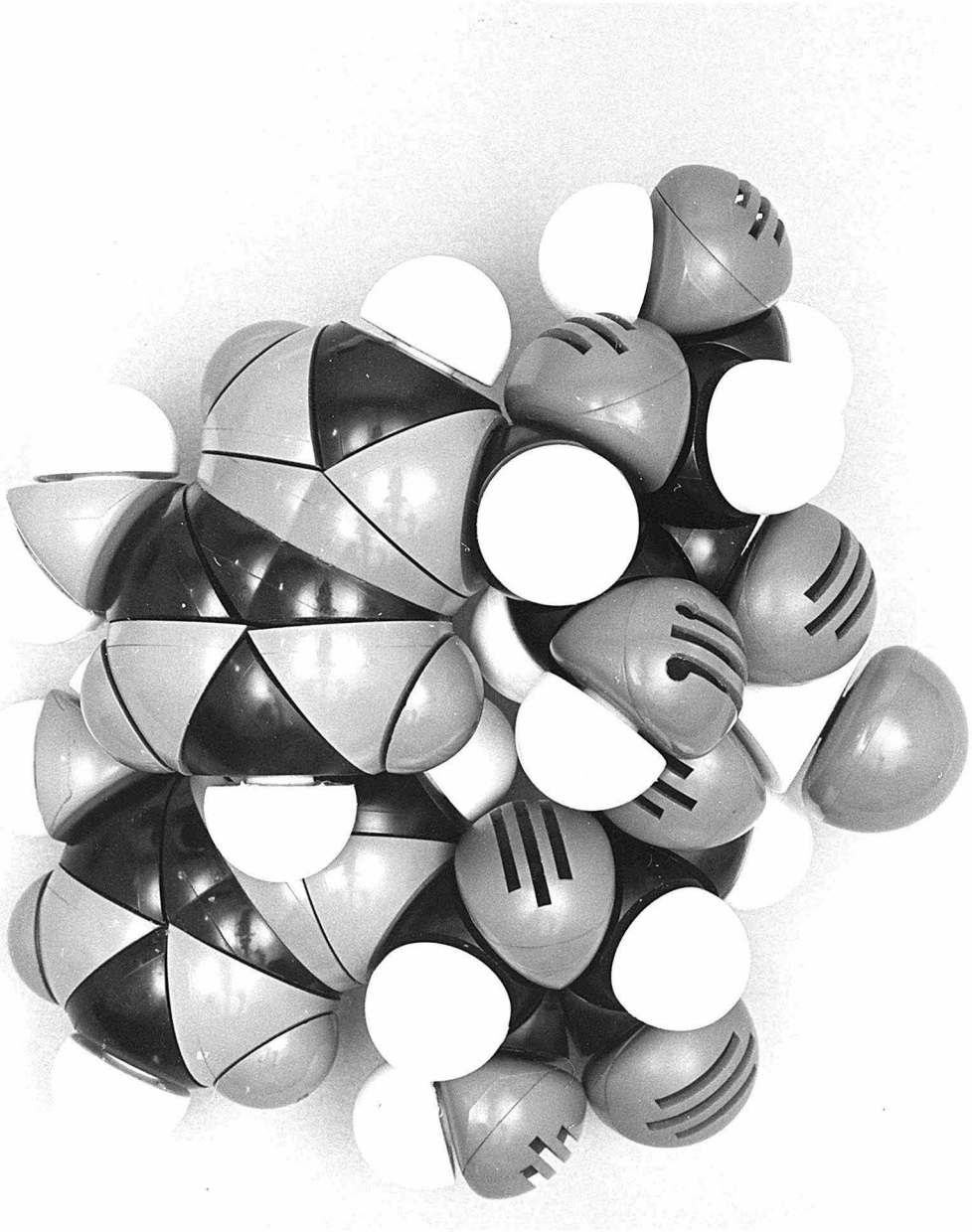
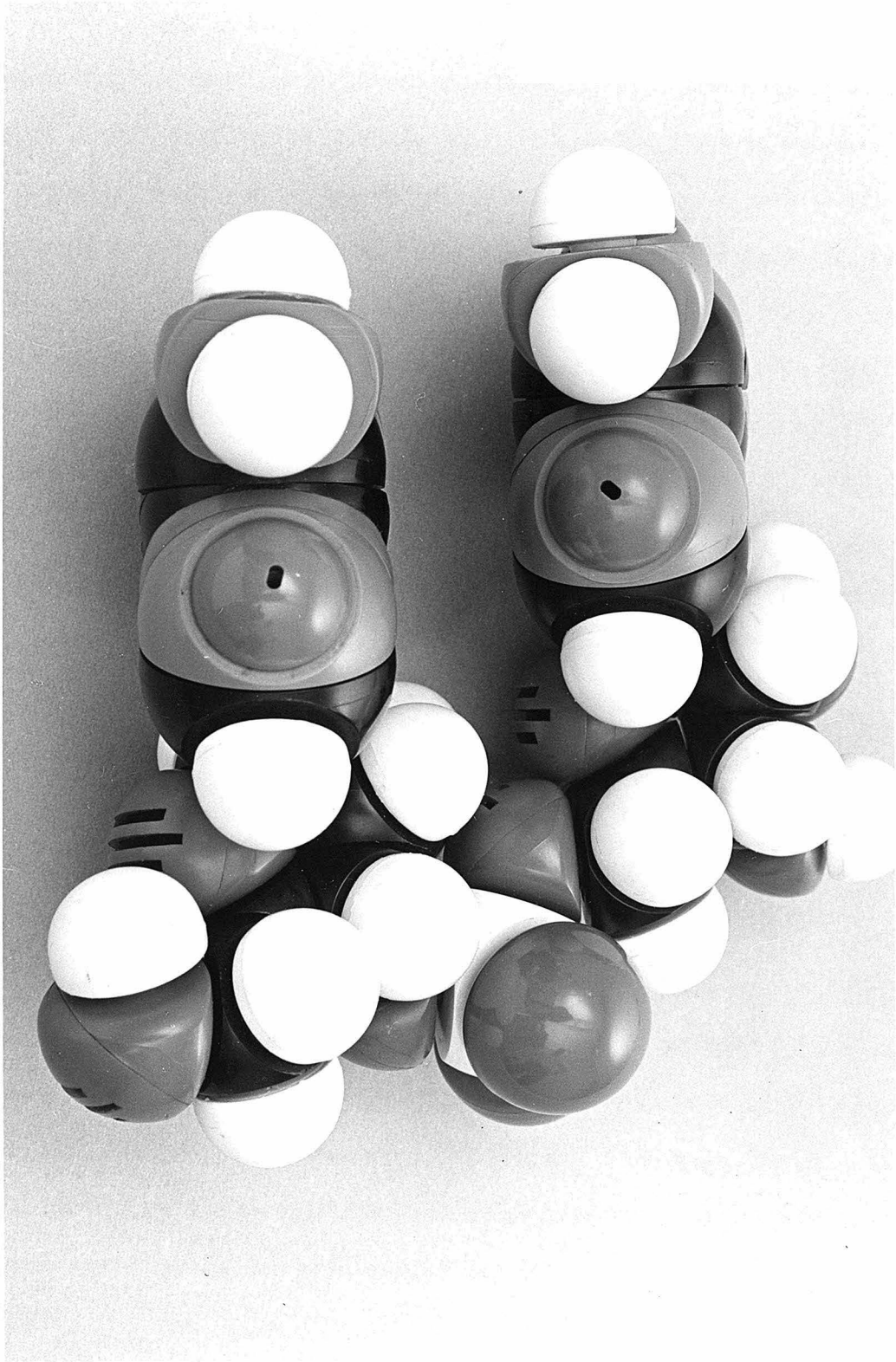


Figure 29. A CPK molecular model of ApA stacked in the energetically favorable 3'-anti, 5'-anti conformation. The model is viewed from a direction perpendicular to the ribose-phosphate backbone.



While the dinucleotide is destacked upon protonation of the adenine rings, increasing the temperature to 95°C 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. This indicates that the free energy difference between the 3'-anti, 5'-anti stacked conformation and the other conformations which result from a 180° rotation of one or both of the adenine bases about the glycosidic linkage is not large.

In conclusion, the p. m. r. 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.

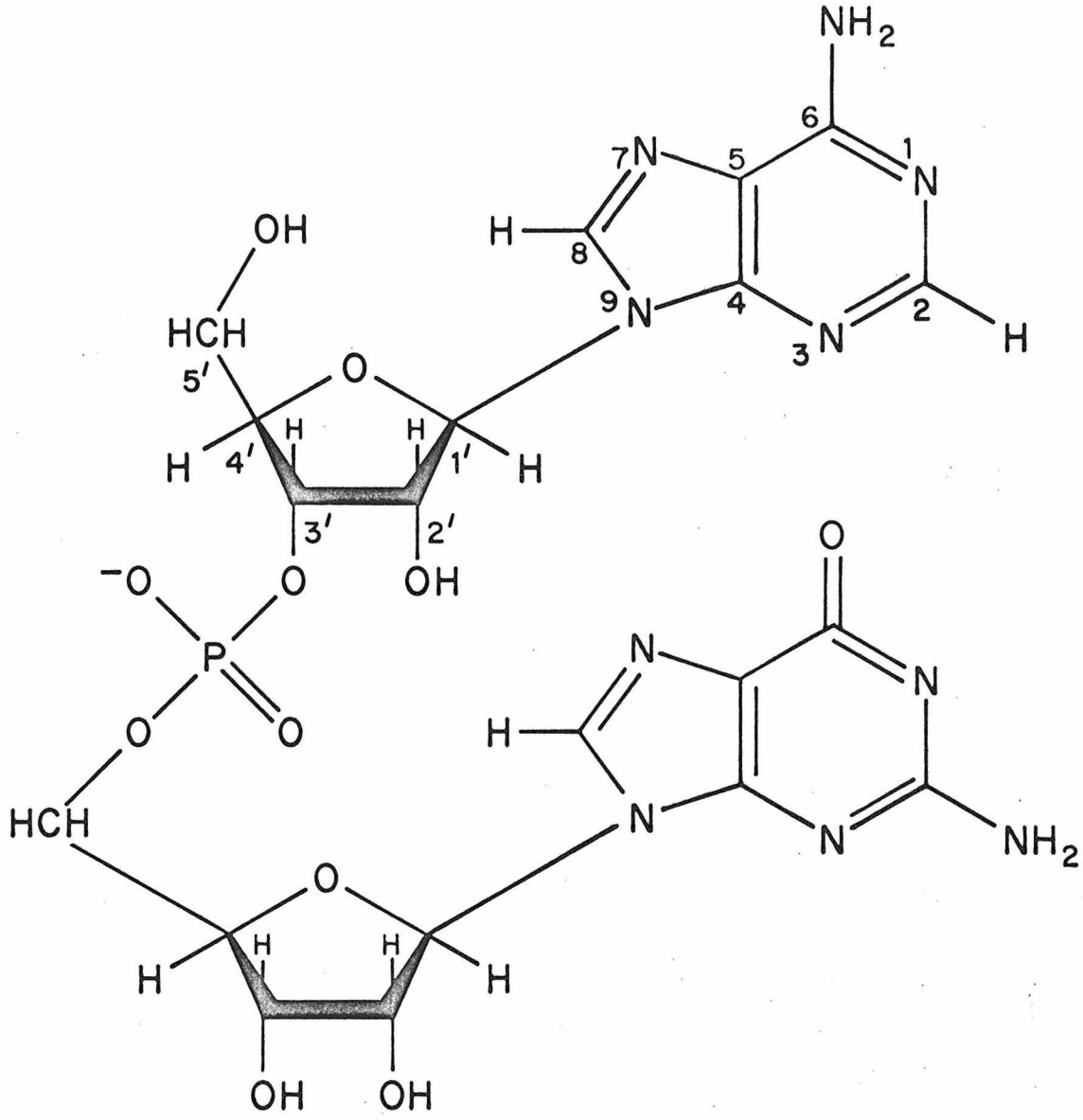
III. THE NATURE OF THE BASE-STACKING INTERACTIONS IN ADENYLYL-(3'→5')-GUANOSINE AND GUANYLYL-(3'→5')-ADENOSINE

A. Introduction

In Chapter II, we reported proton magnetic resonance studies of the conformational properties of adenylyl-(3'→5')-adenosine (ApA) in aqueous solution. In these studies it was shown that the base-stacking interaction between the two adenine bases is relatively strong and that the adenine rings are preferentially oriented in the anti conformation (82).

This chapter summarizes the results of similar studies of adenylyl-(3'→5')-guanosine (ApG) and guanylyl-(3'→5')-adenosine (GpA). Since adenine and guanine are both purine bases, ApG and GpA are structurally and dimensionally very similar to ApA. Therefore, a comparison of the adenine-guanine stacking interactions in these molecules with the intramolecular base-stacking in ApA provides an opportunity to study the effect of base composition on the conformational properties of dinucleoside monophosphates in aqueous solution.

ApG differs from GpA only with respect to the base sequence along the ribose-phosphate backbone. In ApG the phosphate is esterified to the guanosine nucleoside at the 5'-hydroxyl group of the ribose residue and to the 3'-hydroxyl group of adenosine (Figure 30). The sites of phosphate esterification on the two nucleosides are reversed in the case of GpA (Figure 31). It is our convention to refer to the



ApG

Figure 30. Adenylyl-(3'→5')-Guanosine

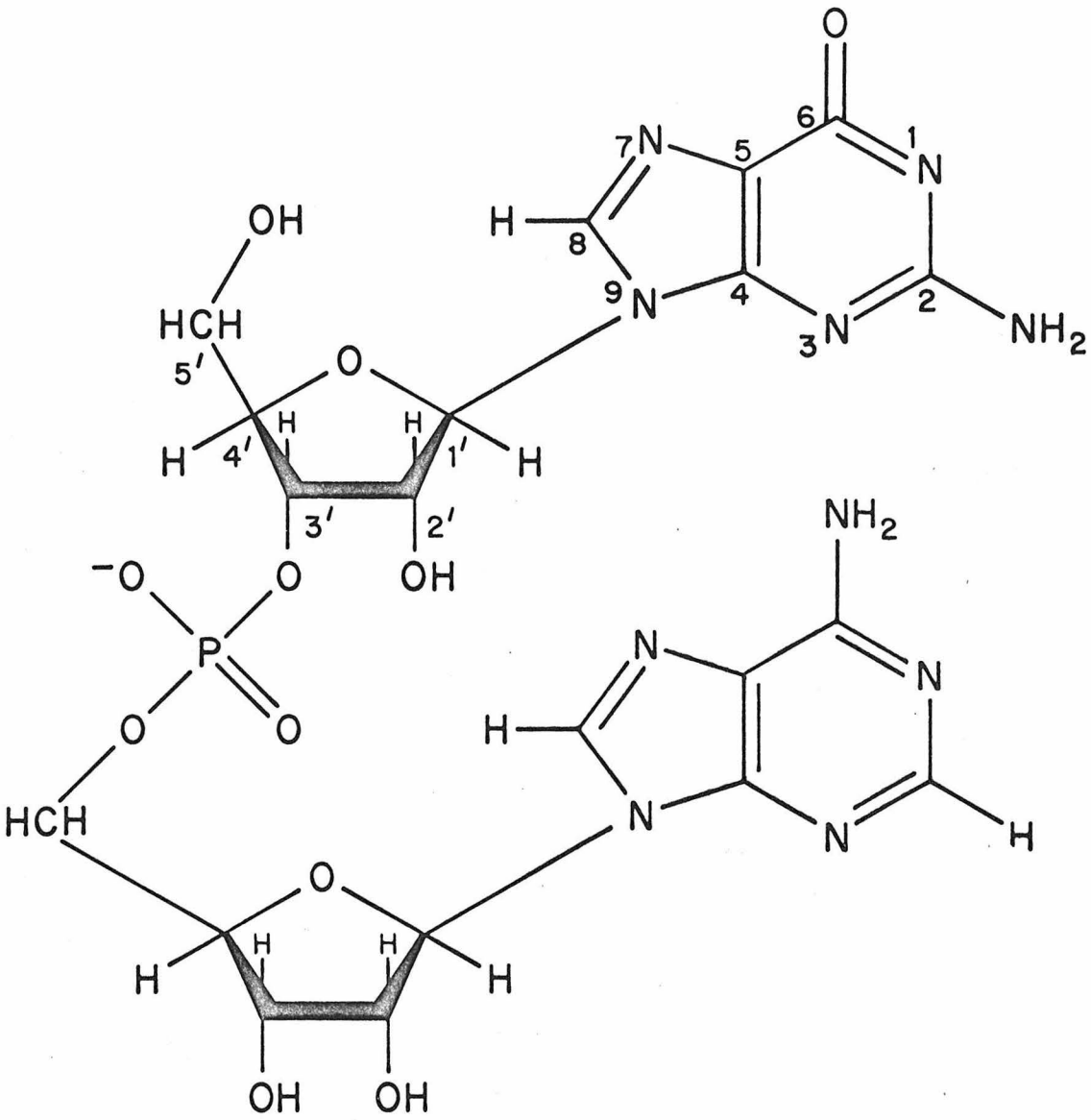


Figure 31. Guanylyl-(3'→5')-Adenosine

adenosine nucleoside of ApG as the 3'-nucleoside since it is linked to the phosphate at the 3'-site of the ribose residue. The structural difference between ApG and GpA may result in important base-sequence-dependent effects on the conformational properties of these molecules. Accordingly, it is of particular interest to compare the results of the studies of these two sequence isomers.

B. Experimental

1. Materials. A-grade adenosine, guanosine, 5'-adenosine monosphosphate, 5'-guanosine monophosphate, and GpA were obtained from Calbiochem, Los Angeles, California. ApG was purchased from Sigma Chemical Company, St. Louis, Missouri. These materials were used without further purification.

A column containing Dowex 50W-X8 cation exchange resin was used to convert the dinucleoside monophosphates from the acid forms to their respective sodium salts. All samples were prepared in D_2O (99.7 mole %) which was supplied by Columbia Organic Chemicals, Columbia, South Carolina.

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

2. Instrumentation. A Varian HA-100 spectrometer operating in the frequency sweep mode was used to record the spectra of these studies. Neat TMS sealed in capillary tubing provided the field/frequency lock signal and the chemical shifts were measured directly from the lock signal by counting the difference between the reference and sweep oscillators with a Hewlett-Packard 5512A frequency counter.

When necessary, weak signals were enhanced by time-averaging with a Varian C-1024 time-averaging computer. The probe temperature in these studies was 29 ± 1°C unless specified otherwise.

In the variable temperature studies, the probe temperature was controlled to ± 1°C with a Varian V-4343 variable temperature controller. This temperature was determined using methanol and ethylene glycol samples and the calibration curves supplied by Varian Associates.

C. Spectra and Assignment of Proton Resonances of ApG and GpA

The 100 Mcps p.m.r. spectra of the base protons of 0.01 M ApG and 0.02 M GpA are presented in Figure 32. For comparison the spectra of adenosine-5'-monophosphate (5'-AMP) and guanosine-5'-monophosphate (5'-GMP) in the same spectral regions under similar experimental conditions are also shown. In addition, chemical shifts of the $H_2(A)$, $H_8(A)$, $H_8(G)$, $H_{1'}(A)$, and $H_{1'}(G)$ protons of ApG, GpA, ApA, adenosine, adenosine-5'-monophosphate, guanosine, and guanosine-5'-monophosphate at low concentrations are summarized in Table V.

The assignment of the H_2 adenine resonance in each of the adenine-guanine dinucleotides is straightforward. The H_8 protons of the adenine and guanine rings exchange with the solvent (D_2O) deuterium atoms at elevated temperatures, leaving only the H_2 resonances in the base proton spectral region. By such deuterium labelling we have assigned the central resonance in each of the base proton spectra of ApG and GpA to the adenine H_2 [$H_2(A)$] proton.

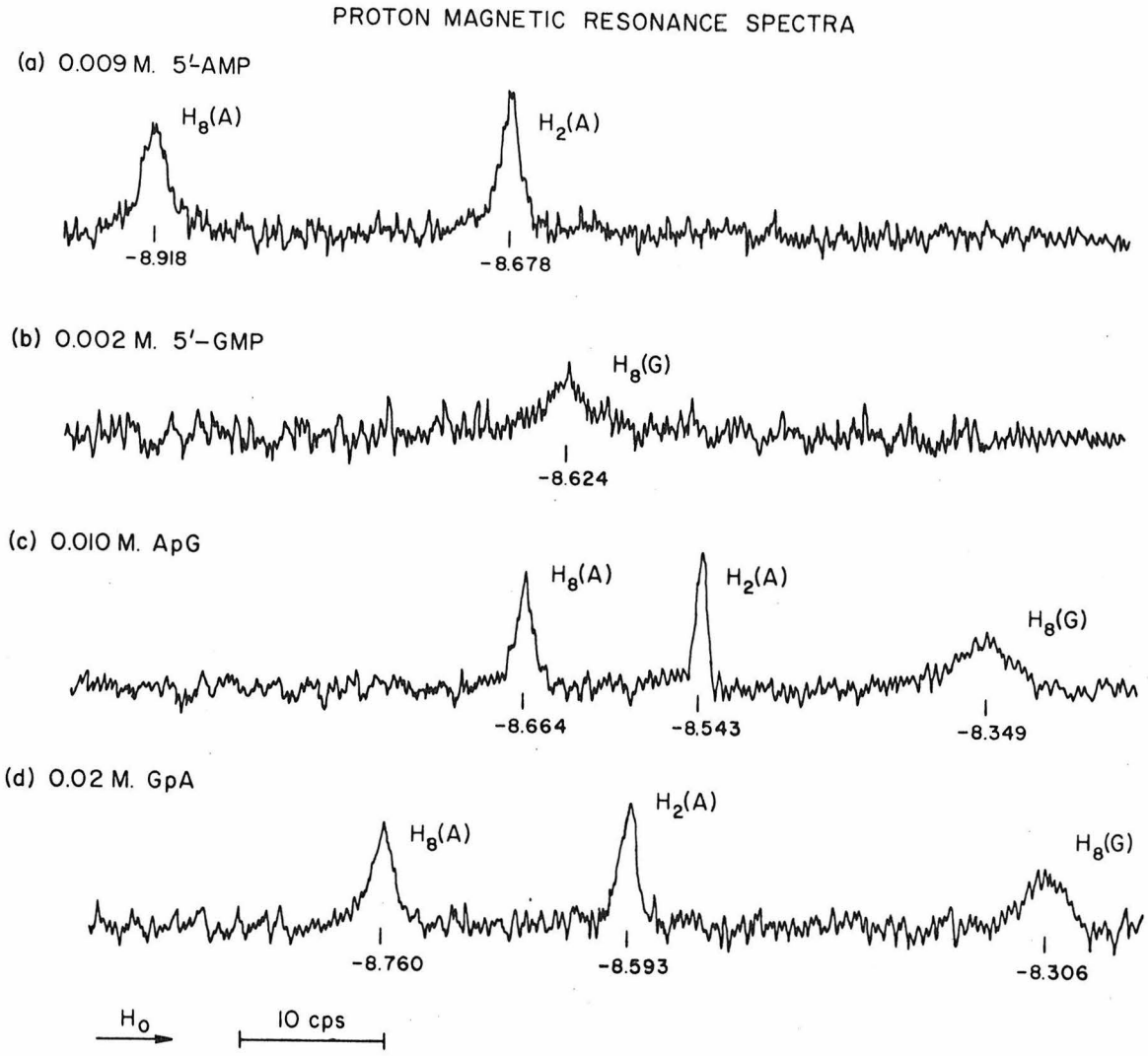


Figure 32. Proton magnetic resonance spectra (100 Mcps) of the base protons of (a) 0.009 M 5'-AMP, pD = 6.2; (b) 0.002 M 5'-GMP, pD = 8.3; (c) 0.010 M ApG (sodium salt), pD = 7-8; and (d) 0.02 M GpA (sodium salt), pD = 7-8. The spectra were recorded at a probe temperature of 29° ± 1°C. The chemical shifts are referenced with respect to external TMS and have not been corrected for bulk susceptibility.

TABLE V. Summary of the Chemical Shifts of the Adenine, Guanine, and Ribose-$H_{1'}$ Protons of ApG, GpA, ApA, 5'-AMP, 5'-GMP, Adenosine, and Guanosine at 29°C

Molecule	Solution pD	Concentration (Molar)	Chemical Shifts[a] (ppm)					
			$H_8(5')$[b]	$H_8(3')$	$H_2(5')$	$H_2(3')$	$H_{1'}(5')$	$H_{1'}(3')$
ApG (sodium salt)	7-8	0.005	8.37	8.70	- -	8.59	6.25	6.39
		Infinite Dilution[c]	8.37	8.71	- -	8.61	6.25	6.39
GpA (sodium salt)	7-8	0.01	8.77	8.33	8.62	- -	6.52	6.13
		Infinite Dilution	8.79	8.35	8.65	- -	6.53	6.14
ApA (sodium salt)	7-8	0.01	8.69	8.65	8.57	8.43	6.42	6.30
		0.003	8.70	8.67	8.60	8.47	- -	- -
		Infinite Dilution	8.71	8.68	8.61	8.48	- -	- -
Adenosine	- -	0.003	8.76		8.68		6.53	
5'-AMP	6.2	0.009	8.92		8.68		6.59	
	10	0.01	9.06		8.70		- -	
Guanosine	- -	0.002	8.45				6.36	
5'-GMP	6.2	0.002	8.56				- -	
	8.3	0.002	8.62				6.37	

[a] Downfield relative to external TMS; not corrected for bulk susceptibility.

[b] $H_8(5')$ designates the H_8 proton of the nucleoside which is esterified to the phosphate at the 5'- position of its ribose residue.

[c] Extrapolated.

The H_2(A) resonances of ApG and GpA both appear at higher fields than the corresponding proton resonances of the adenine mononucleotides. In view of the lack of sensitivity of the chemical shift of the H_2 proton to the nature of the ribose-phosphate backbone, these upfield shifts must have their origin in the ring-current magnetic anisotropy of the opposing guanine base. This conclusion immediately indicates that ApG and GpA are intramolecularly stacked at 29°C. The resonance frequency of each of the H_2 protons of ApA is at higher field than the corresponding frequency of the H_2(A) protons of ApG and GpA. This could be attributed to either the smaller ring-current magnetic anisotropy of the guanine ring or less stable intramolecular base-stacking interactions in the adenine-guanine dinucleotides. We shall discuss these two effects in greater detail in a later section of this chapter.

Finally, at infinite dilution the chemical shift of the H_2(A) proton of GpA is lower field than that of the adenine H_2 proton of ApG. This is consistent with a model in which each of the adenine bases of these dinucleotides is preferentially oriented in the anti conformation.

The assignment of the H_8 resonances of the guanine and adenine rings of the dinucleotides has been made through a comparison of the chemical shifts of these protons with the H_8 proton resonances of the nucleosides, adenosine and guanosine. The data summarized in Table V show that the H_8 resonance of guanosine is approximately 0.3 ppm upfield from the H_8 adenosine resonance. The lower field resonance frequency of the adenosine proton is likely the result of the stronger π-electron ring-current of the adenine ring. In planar aromatic

systems where π-electrons are free to flow around the conjugated loop, the interatomic currents of the π-electrons generate a secondary magnetic field when the system is located in a strong magnetic field (45; Chapter I, this thesis). The direction of the current is such that this secondary magnetic field reinforces the primary field at the site of the aromatic protons attached to the ring and opposes the primary field directly above or below the molecular plane. Since the 6-substituted oxygen atom of the guanine ring is primarily a keto-oxygen under the conditions of our experiments (97, 98), the ring-current in the six-membered ring of guanine is probably not very pronounced and most of the ring-current magnetic anisotropy of this molecule is localized in the five-membered imidazole ring (99). In comparison, the electronic structure of adenine is such that each of the rings may accommodate a significant ring current and, therefore, this base has a larger over-all ring-current magnetic anisotropy than guanine. Consequently, the adenine H_8 proton resonates at a lower field than the corresponding guanine proton. It is assumed that the ribose ring of each of the nucleosides has a similar effect on the chemical shift of the respective H_8 protons.

The resonance frequency of the H_8 proton of both adenosine and guanosine is shifted to lower field with phosphate substitution at the 5'-hydroxyl of the respective ribose residues. This downfield shift no doubt has its origin in the close proximity of the H_8 proton of each nucleoside to the phosphate group, and the resultant electrostatic polarization by the negatively charged phosphate. From this observation, it may be concluded that the bases of 5'-AMP and 5'-GMP are

preferentially oriented in the anti-conformation. In each of these monophosphates, the magnitude of the downfield shift is dependent on the degree of phosphate ionization. The secondary phosphate pK of 5'-AMP and 5'-GMP corresponds to a pD value of approximately 6.5 (21). At solution pD values significantly greater than this, the phosphate is doubly charged, and the downfield shift of the H_8 proton of the nucleotides is also larger. The data summarized in Table V show that under comparable experimental conditions the phosphate deshielding effect is somewhat greater for the H_8 proton of 5'-AMP. It is possible that the syn conformation of 5'-GMP is stabilized by the formation of an intramolecular hydrogen bond between one of the 2-amino protons of the guanine base and a phosphate oxygen atom.

From the data of Table V it is obvious that the chemical shift of one of the H_8 protons in each of the adenine-guanine dinucleotides is similar to the chemical shift of the guanosine H_8 proton while the remaining H_8 has almost the same resonance frequency as H_8 of adenosine. Accordingly, we have assigned the lowest field resonance of the aromatic spectral region of each dinucleotide to H_8(A) and the highest field resonance to H_8(G). For both ApG and GpA, the spectral assignment of the base protons is, in the order of increasing field: H_8(A), H_2(A), and H_8(G).

It is of interest to note that the intrinsic linewidth of the H_8 proton resonance of either guanosine or 5'-GMP is measurably greater than the corresponding H_8 resonance linewidth of adenosine or 5'-AMP. This effect is illustrated for the nucleotides in Figure 32. Greater resonance linewidths are also observed for the H_8 guanine protons in the spectra

of ApG and GpA (Figure 32). In each case the resonance of the H_8 adenine proton is much sharper. This provides good experimental evidence that our assignment based on a comparison with the nucleosides is justified.

From a similar comparison with the nucleosides, we have assigned the $H_{1'}$ ribose protons of ApG and GpA. The resonance of each of these protons appears as a doublet in the p. m. r. spectrum due to nuclear spin-spin coupling with the $H_{2'}$ proton of the same furanose ring. This coupling constant is, in all cases, approximately 4 cps at 29°C and 0.01 M. The $H_{1'}$ resonance of adenosine is approximately 0.14 ppm downfield from that of guanosine. Therefore, we have assigned the downfield resonance of the two $H_{1'}$ doublets to the adenosine nucleoside in each of these dinucleoside monophosphates. This assignment is not quite as obvious as the assignment of the H_8 protons because the chemical shifts of the $H_{1'}$ protons vary significantly depending on the site of phosphate esterification on the nucleoside and on the ribose ring conformations. We shall discuss these chemical shifts and coupling constants in greater detail in a later section of this chapter.

In addition to the protons discussed previously, the p. m. r. spectra of ApG and GpA also include the resonances of the $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, and $H_{5''}$ protons of each ribose ring. These protons resonate 4.0 to 5.2 ppm downfield from external TMS, but because of complicated nuclear spin-spin coupling patterns and inadequate resolution of these resonances, we have not extensively studied the spectra of these ribose protons. The protons of the amino and hydroxyl groups rapidly exchange with the solvent, D_2O, and are, therefore, also of little interest in these studies.

D. Self-Association of ApG

1. Introduction. The self-association of several purine and pyrimidine nucleosides and nucleotides has been studied by proton magnetic resonance spectroscopy and by other methods (Chapter I, this thesis). In Chapter II an investigation of the self-association of ApA by p. m. r. has been reported. As expected on the basis of evidence obtained in the earlier studies of the simpler molecules, ApA self-associates extensively in aqueous solution and the mode of interaction is intermolecular stacking of the intramolecularly folded dinucleoside monophosphate. As a consequence of the formation of these intermolecular stacks, the chemical shifts of the base protons of ApA are concentration-dependent. Increased concentration results in upfield shifts for the adenine proton resonances due to the ring-current anisotropy effects of the intermolecularly stacked molecules.

In this chapter the chemical shifts of H_2(A), H_8(A), H_8(G), $H_{1'}$(A), and $H_{1'}$(G) of ApG are reported as a function of concentration. Upfield shifts with increasing concentration were also observed for the resonances of each of these protons in the concentration range 0.2 M to infinite dilution. The results are illustrated in Figures 33 and 34. The chemical shifts of the same protons of GpA are also shown at two different concentrations, 0.02 M and 0.01 M. The concentration shifts of the proton resonances of ApG and GpA also result from the formation of intermolecular stacks as in the case of ApA. The total concentration shifts of the purine base protons and the ribose protons of ApG are compared with the corresponding shifts of ApA, ApU, and UpA in Table VI.

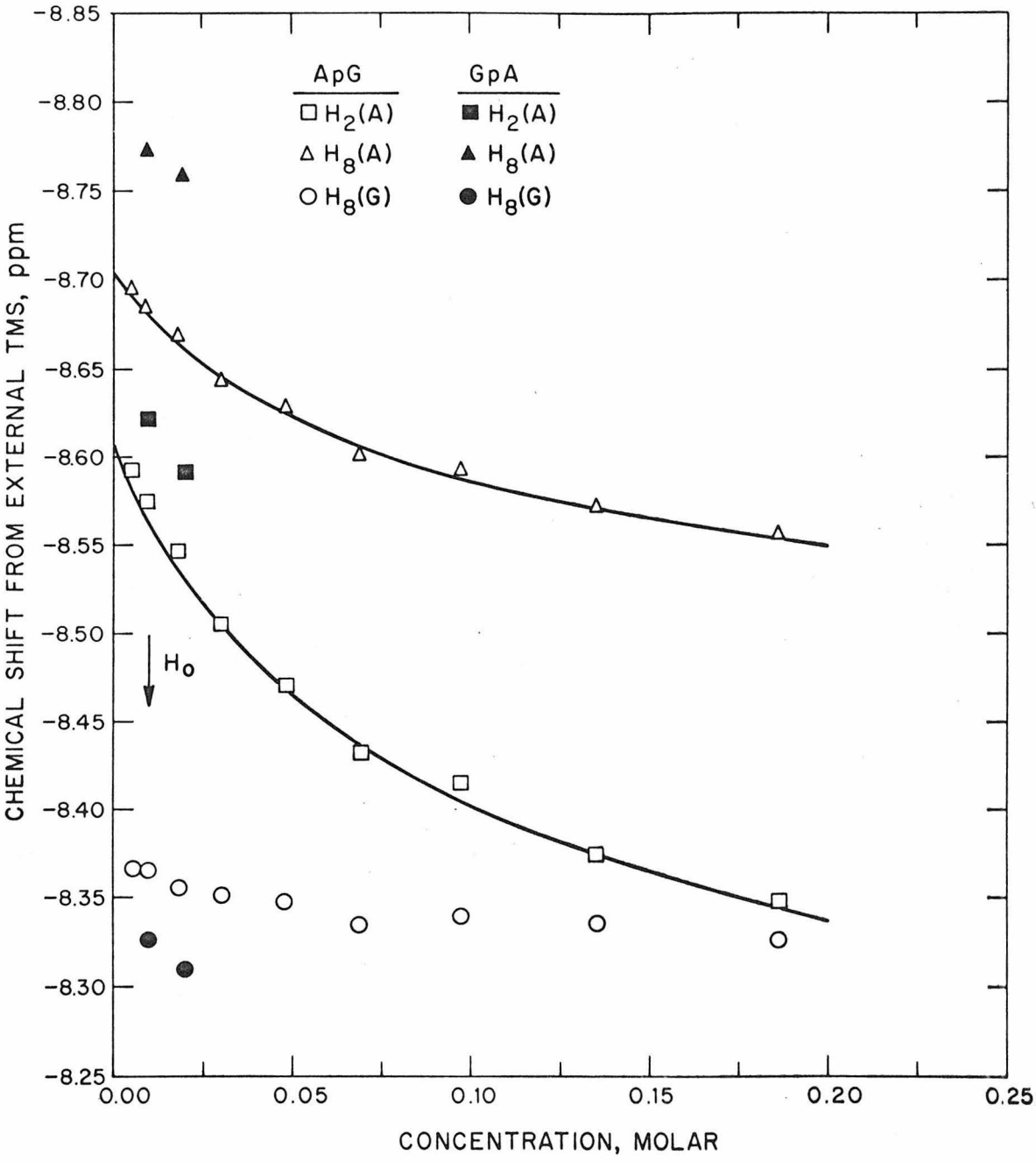


Figure 33. Variation of the chemical shifts of the base protons of ApG and GpA with dinucleotide concentration. The chemical shifts are referenced with respect to external TMS and have not been corrected for bulk susceptibility.

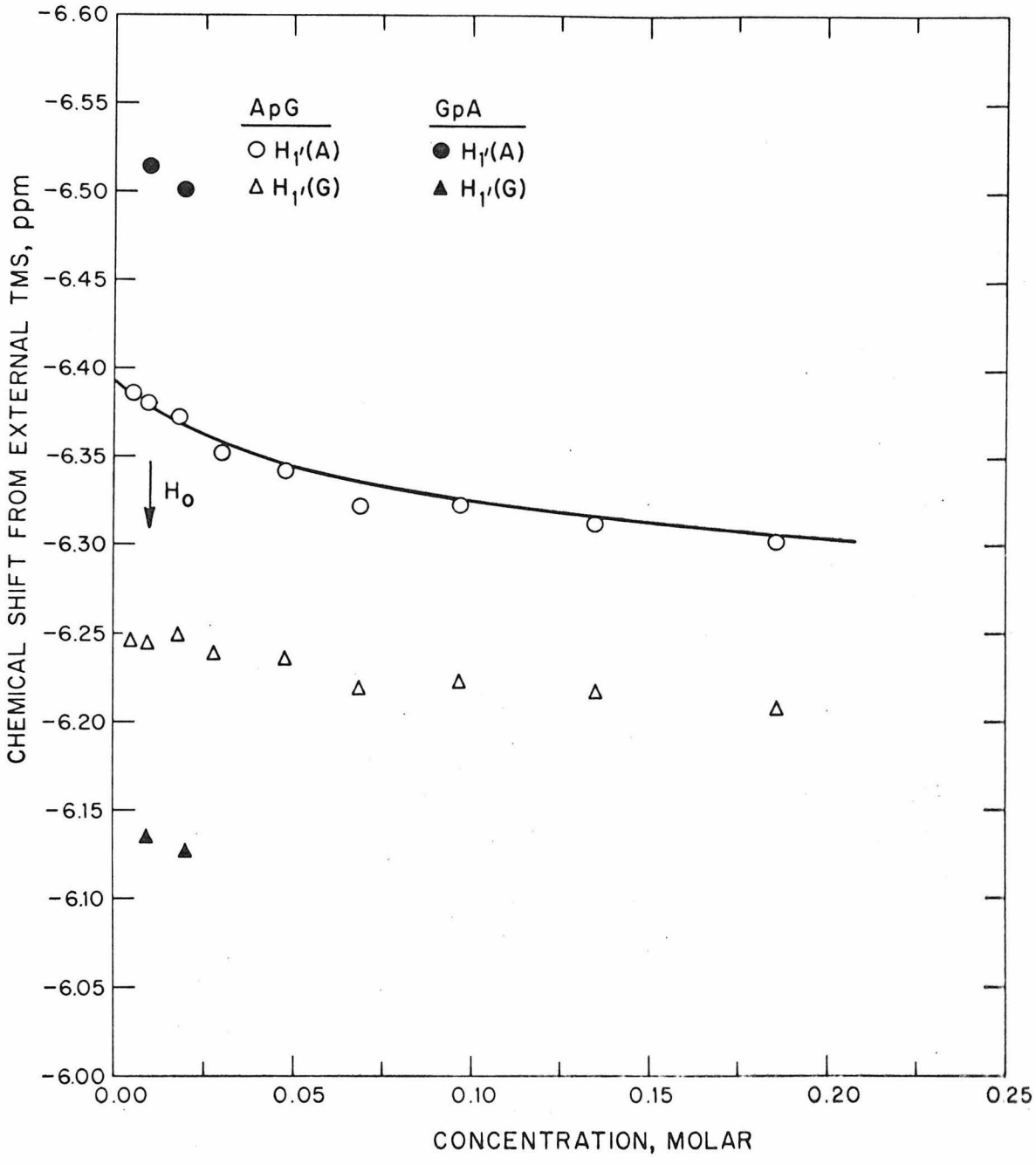


Figure 34. Variation of the chemical shifts of the ribose $H_{1'}$ protons of ApG and GpA with dinucleotide concentration. The chemical shifts are referenced with respect to external TMS and have not been corrected for bulk susceptibility.

TABLE VI. Net Shifts of the Purine Base Proton and Ribose Proton Resonances of Adenosine,[a] ApA, ApG, ApU, and UpA Over Equivalent Concentration Ranges

Molecule	Net Shift[b] (ppm)											
	Infinite Dilution[c] to 0.11 M						Infinite Dilution to 0.20 M					
	$H_8(5')$	$H_8(3')$	$H_2(5')$	$H_2(3')$	$H_1'(5')$	$H_1'(3')$	$H_8(5')$	$H_8(3')$	$H_2(5')$	$H_2(3')$	$H_1'(5')$	$H_1'(3')$
Adenosine[a]	0.121		0.247		0.115		- - -		- - -		- - -	
ApA	0.115	0.158	0.224	0.232	- -	- -	- -	- -	- -	- -	- -	- -
ApG	0.030	0.126	- - -	0.213	0.02	0.07	0.044	0.154	- - -	0.260	0.04	0.09
ApU	- - -	0.094	- - -	0.156	0.00	0.08	- - -	0.128	- - -	0.212	0.00	0.11
UpA	0.064	- - -	0.136	- - -	0.06	0.00	0.087	- - -	0.187	- - -	0.07	-0.01

[a]Data of Reference (35); concentration range 0–0.10 m.

[b]Upfield shift with increasing concentration; not corrected for bulk susceptibility.

[c]Extrapolated.

2. Concentration Shifts of the Purine Base Protons. The net concentration shifts of the H_2 proton of adenosine and the H_2(A) protons of both ApA and ApG are reasonably similar although the ApG resonance is shifted somewhat less with increasing concentration than the other H_2(A) resonances. These similar shifts indicate that the extent of intermolecular self-association of each of these molecules is quite comparable in the concentration range 0—0.1 M. This is not surprising since each of the bases of these molecules is a purine derivative. The H_2(A) resonances of both UpA and ApU are shifted considerably less with concentration than those of ApA and ApG (Table VI). The smaller concentration shifts of the H_2-adenine protons of ApU and UpA reflect the smaller tendency of these two dinucleotides to form intermolecular aggregates. The specific details of the concentration shifts of ApU and UpA are discussed in Chapter IV. However, it is pertinent to note that in the dimerization of ApU or UpA via base-stacking, the most stable complexes are those which result from the formation of adenine-adenine base-stacks. Therefore, further polymerization of these dinucleotides is limited because the formation of adenine-uracil base-stacks would be required, and the base-stacking interactions of uracil are relatively weak. On the other hand, polymerization of ApA or ApG beyond dimerization would result in the formation of adenine-adenine or adenine-guanine base-stacks which are apparently relatively stable. The larger H_2(A) concentration shifts of ApA and ApG are a direct result of the greater capacity of these dinucleotides to form trimers, tetramers, and larger intermolecular aggregates. The somewhat smaller concentration shift of H_2(A) of ApG compared to H_2(3') of ApA may be attributed to the

smaller ring-current anisotropy of the guanine ring or to a smaller stacking tendency of guanine.

The concentration shifts of the H_8(A) resonances of ApA, ApG, ApU, and UpA follow a pattern similar to that of the H_2(A) shifts. The H_8(3') proton resonance of ApA is shifted further upfield with increasing concentration than either H_8 of adenosine or H_8(A) of ApG; and H_8(A) of ApU experiences even a smaller concentration-induced chemical shift (Table VI). Consistent with the H_2(A) concentration shifts is the observation that the H_8(5') resonance of ApA is shifted much more than the H_8(5') UpA resonance. The limited concentration data of GpA do not permit a realistic comparison with the other dinucleotides although it is evident from Figure 33 that the adenine proton resonances of this molecule experience sizable concentration shifts. As in the case of the adenine H_2 resonances, the different concentration shifts of the H_8(A) protons indicate that the self-association of ApA is somewhat larger than that of ApG; and that ApU and UpA have even a smaller tendency to form intermolecular aggregates, especially polymers beyond the dimer stage.

In each of these dinucleotides and in adenosine, the H_2(A) resonances experience a much greater concentration shift than H_8(A). The larger H_2 shift in adenosine may be attributed to steric shielding of the H_8 proton by the ribose ring and the accompanying specific adenine-adenine orientations in the base-stacks. In Chapter II the larger concentration shifts of the H_2 protons of ApA were attributed to different exposure of the base protons to ring-current magnetic anisotropies of the stacking ApA molecules. When ApA is intramolecularly stacked

with each of the bases preferentially oriented in the anti conformation, the H_2 protons are relatively far-removed from the sugar-phosphate backbone and are easily influenced by ring-current effects of the other stacking molecules. However, the H_8 protons of ApA are somewhat sterically shielded from the bases of the other molecules as intermolecular stacks are formed; and the concentration shifts of the H_8(A) protons are smaller than the H_2(A) shifts.

The net concentration shift of H_8(A) of ApG is also much smaller than the H_2(A) shift (Table VI). This smaller shift may also be attributed to the steric shielding of the H_8(A) proton by the ribose-phosphate backbone. In addition the net concentration shift of H_8(G) of ApG is significantly smaller than the H_8(A) or H_2(A) shift of this dinucleotide. A similar observation was made in the case of ApA where the concentration shift of the H_8(5') proton is smaller than those of the other adenine protons. This smaller concentration shift is the result of the conformation of intramolecularly stacked ApA. When the dinucleotide assumes a 3'-anti, 5'-anti conformation, the H_8(5') proton is situated in the cleft formed by the adenine rings and the molecular backbone. In this site, H_8(5') is more extensively shielded from the bases of other molecules as intermolecular stacks are formed. The smaller concentration shift of H_8(G) of ApG can also be attributed to similar steric effects since it is also an H_8(5') proton. When the guanine base of ApG is oriented in the anti conformation, H_8(G) is sterically shielded from the externally stacking molecules. Accordingly, the small concentration shift of the H_8(G) proton of ApG provides good evidence that the guanine base of ApG is preferentially oriented in the

anti conformation. The concentration shift of H_8(G), however, appears to be unusually small. For example, at 0.11 M the H_8(G) resonance is shifted only 0.03 ppm upfield from its extrapolated infinite dilution value compared to an upfield shift of 0.115 ppm for H_8(5') of ApA and 0.064 ppm for H_8(5') of UpA. This extremely small shift of H_8(G) of ApG is somewhat puzzling, especially in view of the comparatively large concentration shift of the H_8(5') proton of ApA. The much smaller shift of the guanine proton can be attributed to several effects, and it is difficult to determine the relative importance of these different possibilities.

The purine-binding experiments reported in Section F of this chapter demonstrate that purine-adenine base-stacking interactions are somewhat stronger than purine-guanine interactions. On the basis of these experiments, one might expect adenine-adenine base-stacking to be more stable than adenine-guanine base-stacking. Accordingly, the smaller concentration shift of H_8(5') of ApG relative to H_8(5') of ApA or UpA may be a consequence of the smaller tendency of the guanine base to form intermolecular base stacks.

Another factor which could contribute to the extremely small concentration shift of the H_8(5') proton of ApG is the possible greater stability of the anti conformation of the guanine base of ApG compared to the 5'-anti base conformation of ApA or UpA. The infinite dilution chemical shifts of the adenine protons of ApA have shown that although the 3'-anti, 5'-anti conformation of this dinucleotide is the energetically most favorable conformation at 30°C, other conformations in which one or both bases assume the syn conformation are also significantly populated. CPK molecular models of ApG demonstrate that

steric hindrance between the 2-amino group of the guanine base and the protons and the oxygen atoms of the ribose-phosphate backbone could conceivably destabilize the syn conformation of guanine in an intramolecularly stacked ApG molecule. This would result in a greater fraction of the dinucleotides assuming a configuration in which the guanine base is oriented in the anti conformation and a corresponding increased shielding for the H_8(G) proton.

The data of Table VI also suggest another possibility. If relatively few adenine-guanine base stacks are formed, but intermolecular association of folded ApG molecules occurs primarily by the formation of adenine-adenine base-stacks and guanine-guanine base-stacks, the concentration shifts summarized in Table VI could result. The relatively large adenine proton shifts would be the natural consequence of the A-A base-stacks and the very small H_8(G) shift could be attributed to specific guanine base orientations in the G-G base-stacks in which very little ring-current anisotropy of the opposing guanine base is experienced by H_8(G). Although this possibility is suggested by the data, there is very little supporting evidence to substantiate this hypothesis, and the other rationales of the small H_8(G) concentration shift of ApG appear to be more reasonable.

Although our concentration data on GpA are limited, it is apparent from Figure 33 that H_8(G) of GpA is shifted more with variation of the dinucleotide concentration than H_8(G) of ApG. This is a direct result of the more limited intramolecular steric shielding effects for the H_8(G) proton of GpA.

3. Concentration Shifts of the Ribose $H_{1'}$ Protons. The total concentration shifts of the two ribose $H_{1'}$ proton resonances of ApG are appreciably different (Figure 34). At 0.2 M, the downfield or $H_{1'}$(A) doublet is 0.09 ppm upfield from its value at infinite dilution while the $H_{1'}$(G) resonance shifts only 0.04 ppm over the same concentration range. We feel that the adenosine proton experiences a greater upfield shift than $H_{1'}$(G) because ApG has a greater tendency to form intermolecular adenine-adenine base-stacks than adenine-guanine or guanine-guanine base stacks. The limited observations made for GpA also indicate that the adenosine ribose $H_{1'}$ resonance shifts more with variations in concentration than $H_{1'}$ of the guanosine nucleoside. These observations are consistent with the results obtained for ApU and UpA. In each case the adenosine $H_{1'}$ resonance is more concentration-dependent. The $H_{1'}$ ribose concentration shifts of ApG corroborate the conclusion that adenine-adenine base-stacking interactions are somewhat stronger than those of adenine-guanine and that the adenine-guanine interactions are significantly larger than adenine-uracil base-stacking interactions.

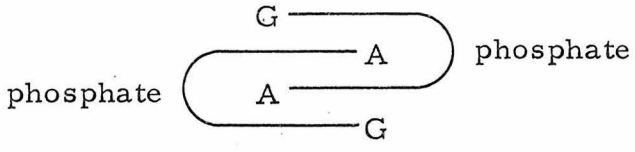
4. Resonance Linewidths of the Base Protons. The linewidths of the base proton resonances at various ApG concentrations are given in Table VII. Although the linewidths of each of the adenine resonances are somewhat greater at 0.186 M, all of the base proton resonances are relatively sharp over the entire concentration range studied. The observation of very little linebroadening with increasing concentration is good evidence that few self-intercalated complexes are formed by ApG. A self-intercalated complex results when one of the bases of a

TABLE VII. Linewidths[a] of the Base Proton Resonances of ApG at Various Concentrations and 29°C.

Concentration (Molar)	Resonance Linewidth[a]		
	H_2(A)	H_8(A)	H_8(G)
0.186	1.6	1.8	2.4
0.135	1.3	1.7	2.6
0.097	1.5	1.6	2.3
0.069	1.0	1.5	2.3
0.048	1.2	1.5	2.5
0.030	0.8	1.3	2.5
0.018	1.0	1.4	2.9
0.009	1.0	1.3	2.0
0.005	1.0	1.5	2.8

[a]In cps at half signal intensity.

dinucleoside monophosphate inserts itself between the two bases of another molecule while stacking vertically with these bases. In this complex, the molecules may, for example, be coiled about each other in the following manner:



Evidence for the formation of such complexes comes from the observation of increased linewidths for some of the base proton resonances

with increasing concentration. The linebroadening is caused by nuclear dipole-dipole interactions between the protons of the intercalated base of one molecule and ribose ring protons of the other. Such linebroadening has been observed for the adenine protons of UpA at high concentrations and for purine complexes of several dinucleotides. The limited linewidths of the ApG base proton resonances at high concentrations is reasonable evidence that this molecule forms relatively stable intramolecular stacks.

5. Coupling Constants $|J_{H_1'-H_2'}|$ of the Ribose Protons. It is noteworthy that the coupling constants, $|J_{H_1'-H_2'}|$, of the H_1' protons of ApG are independent of the concentration. At 29°C the coupling constant of each of the ribose H_1' protons is approximately 4 cps over the entire concentration range studied. This indicates that the conformations of the ribose rings do not change with dinucleotide concentration and is also good evidence that self-intercalated complexes are not formed. Since these coupling constants and their relationship to the ribose ring conformations are discussed in detail in following sections of this chapter, we shall not elaborate on them here.

6. Summary. ApG self-associates extensively in aqueous solution, and the intermolecular association proceeds primarily by vertical stacking of the intramolecularly folded molecules. Adenine-adenine base-stacking interactions are somewhat more favorable than adenine-guanine interactions in the self-association process of ApG. Due to the relatively strong intramolecular base-stacking, few intercalation-type intermolecular complexes are formed. At higher concentrations (~0.1 M) polymerization of ApG via base-stacking proceeds beyond the formation of dimers.

These self-association studies have also demonstrated that the guanine base of ApG is preferentially oriented in the anti conformation in the stable intramolecular base stacks at 30°C.

E. Temperature Studies

1. Introduction. In order to obtain some quantitative information concerning the strength of the intramolecular stacking interaction between the adenine and guanine bases along polynucleotide chains and in an effort to elucidate the conformations of these bases with respect to their ribose rings, we have studied the proton magnetic resonance spectra of ApG and GpA as a function of temperature. As in the concentration studies, it is of interest to compare the results reported here with those obtained for ApA.

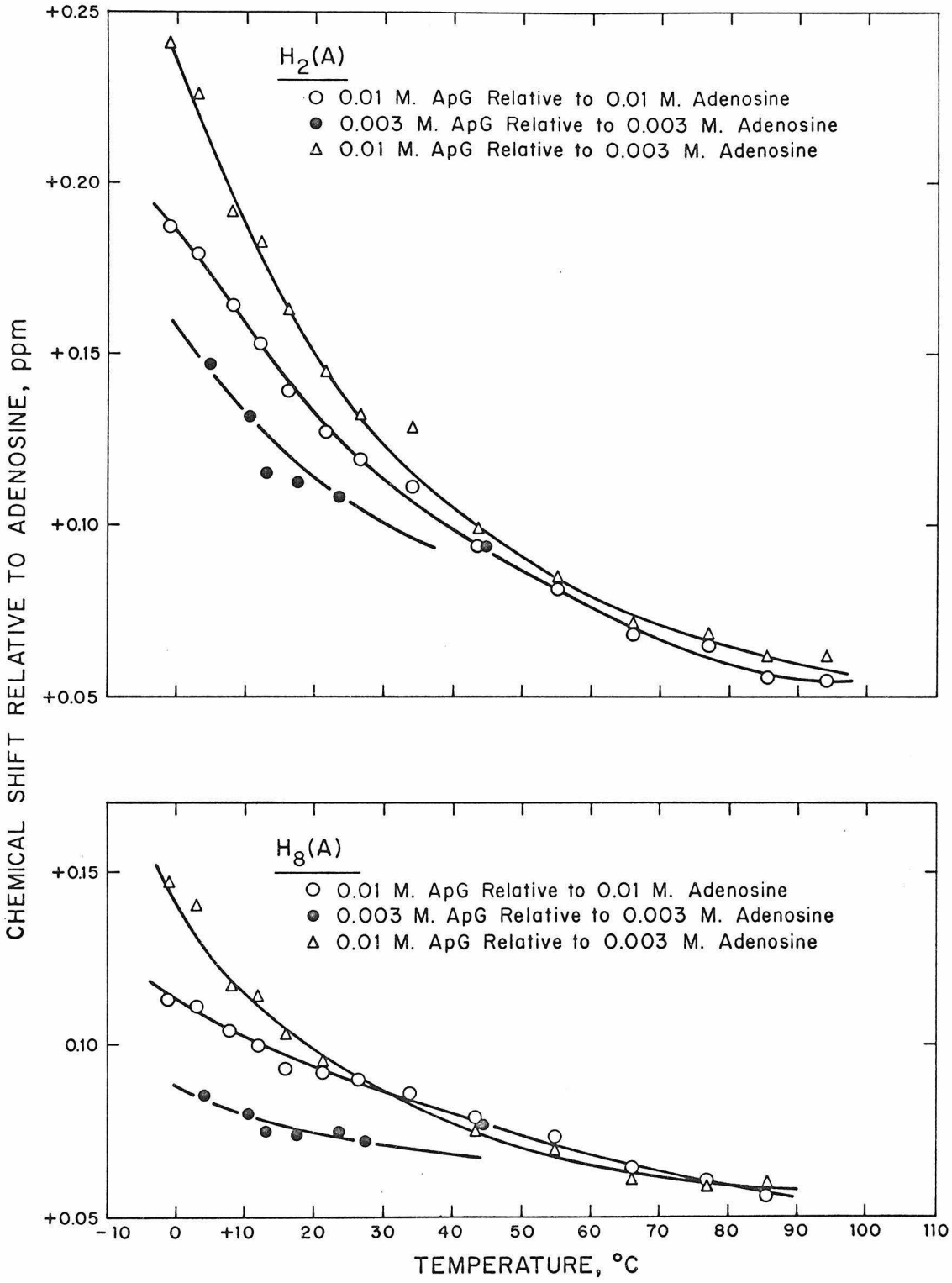
In this section we discuss the temperature dependence of the chemical shifts of several protons and also the effect of temperature on the $H_{1'}$-$H_{2'}$ coupling constants of 0.01 M ApG and 0.01 M GpA. Since the concentration employed in the temperature studies of ApA was only 0.003 M, we have also investigated the temperature dependence of the ApG proton resonances at this concentration.

2. Reference Compounds and Reference Solutions. As in the case of ApA, we have found that the profiles of chemical shift vs temperature curves vary significantly, depending on the reference compound used to monitor the chemical shifts. Changes in the bulk susceptibility of the solution with temperature can be compensated by utilizing an inert reference compound in the sample. However, the proton chemical shifts of both the reference compound and the dinucleoside

monophosphate are subject to solvation effects and since the temperature shifts, therefore, reflect changes in the solvent-solute interactions and the solvent structure with temperature in addition to intramolecular changes in the dinucleoside monophosphate, we have used the simple nucleoside molecules as reference compounds in order to minimize effects arising from differences in solvation. We have accordingly, monitored the base proton chemical shifts of ApG and GpA relative to the corresponding proton chemical shifts of adenosine and guanosine. These reference molecules were studied under approximately the same experimental conditions as the dinucleotides.

In Figure 35, the adenine proton chemical shifts of 0.01 M ApG are plotted as a function of temperature utilizing two different concentrations of adenosine (0.01 M and 0.003 M) as reference solutions. In addition, the adenine chemical shifts of 0.003 M ApG have been monitored with respect to the corresponding adenine resonances of 0.003 M adenosine as a function of temperature. The resulting curves illustrate the importance of using low solution concentrations in the application of p.m.r. spectroscopy to the study of intramolecular base-stacking interactions in dinucleotides and oligonucleotides and demonstrate the necessity of choosing proper experimental conditions for the reference molecules. For example, when 0.003 M adenosine is employed as a reference compound, the resonance of the H_2(A) proton of 0.01 M ApG is approximately 0.06 ppm upfield from the same proton resonance of 0.003 M ApG at 5°C. At temperatures above 60°C, however, there is very little difference in the adenine proton chemical shifts of the two ApG solutions.

Figure 35. The effect of the concentration of the reference solution on the temperature variation of the chemical shifts of the adenine protons of ApG. The $H_2(A)$ [$H_8(A)$] protons of ApG are referenced with respect to $H_2(A)$ [$H_8(A)$] of adenosine.


H2(A)
O 0.01 M. ApG Relative to 0.01 M. Adenosine
● 0.003 M. ApG Relative to 0.003 M. Adenosine
Δ 0.01 M. ApG Relative to 0.003 M. Adenosine
H8(A)
O 0.01 M. ApG Relative to 0.01 M. Adenosine
● 0.003 M. ApG Relative to 0.003 M. Adenosine
Δ 0.01 M. ApG Relative to 0.003 M. Adenosine
CHEMICAL SHIFT RELATIVE TO ADENOSINE, ppm
+0.25
+0.20
+0.15
+0.10
+0.05
0.10
-10
0
+10
20
30
40
50
60
70
80
90
100
110
TEMPERATURE, °C

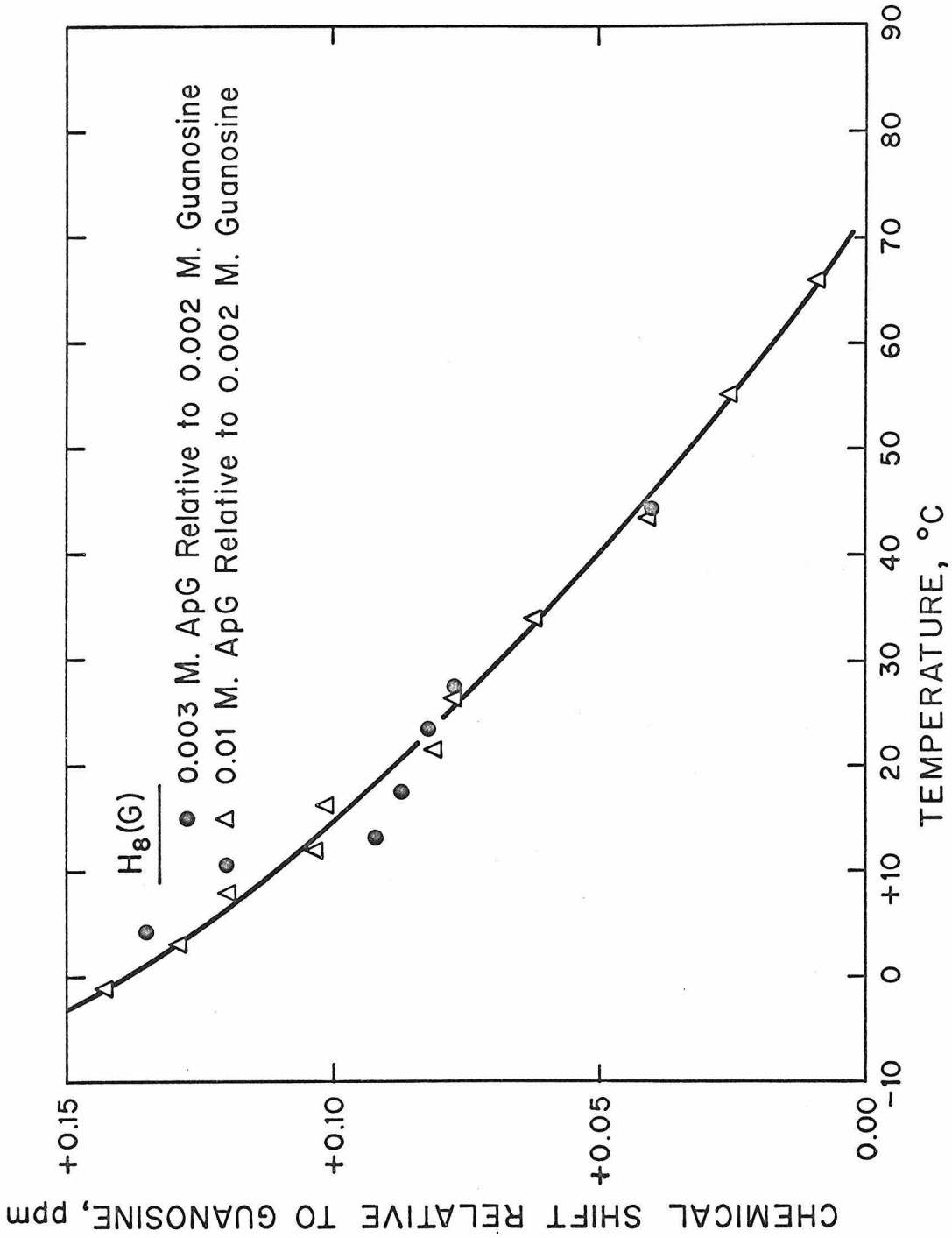
The significantly higher field shift observed at lower temperatures for the more concentrated solution is the direct result of increased intermolecular base-stacking.

A similar effect was observed for the H_8(A) proton of ApG. In this case, the resonance of 0.01 M ApG is only 0.04 ppm upfield from that of 0.003 M ApG. As shown in Figure 33, the concentration shift of H_8(A) of ApG is considerably smaller than the H_2(A) shift. This explains the smaller differences observed in the H_8(A) resonances of the two different ApG solutions at low temperatures.

In contrast to the results obtained for the adenine protons of ApG, the chemical shifts of the H_8(G) proton resonances of 0.01 M and 0.003 M ApG are very similar over the entire temperature range studied when referenced relative to H_8(G) of 0.002 M guanosine (Figure 36). In view of the very small concentration shift of the H_8(G) proton of ApG noted in Figure 33, these results are not unexpected.

In order to minimize intermolecular effects in our temperature studies, we have employed a 0.01 M adenosine solution to reference the adenosine chemical shifts of 0.01 M ApG and 0.01 M GpA. Under these conditions and at temperatures less than 40°C, each of the adenine resonances of ApG is ~0.02–0.03 ppm further upfield from the respective reference proton than the corresponding resonance of 0.003 M ApG referenced relative to 0.003 M adenosine. These differences are due to intermolecular effects, but they are sufficiently small to justify the use of 0.01 M solutions of the dinucleotides in the temperature studies of intramolecular base-stacking provided the adenosine reference solutions are also 0.01 M. These differences are also

Figure 36. The temperature variation of the $H_8(G)$ proton chemical shifts of ApG at two different concentrations. The chemical shifts are referenced to $H_8(G)$ of 0.002 M guanosine.


CHEMICAL SHIFT RELATIVE TO GUANOSINE, ppm
+0.15
+0.10
+0.05
0.00
−10
0
+10
20
30
40
50
60
70
80
90
TEMPERATURE, °C
$\frac{H_8(G)}{}$
● 0.003 M. ApG Relative to 0.002 M. Guanosine
△ 0.01 M. ApG Relative to 0.002 M. Guanosine

sufficiently small to permit a realistic comparison of the results obtained for 0.01 M solutions of ApG and GpA with the results of the temperature studies of 0.003 M ApA.

Due to the limited solubility of guanosine in D_2O, we have employed a 0.002 M guanosine solution to reference the temperature shifts of the guanosine resonances of the dinucleotides. We feel that the use of this relatively dilute solution is justified because the chemical shifts of the $H_8(G)$ and $H_{1'}(G)$ protons of guanosine-5'-monophosphate are essentially independent of concentration. (The concentration shifts of these protons are illustrated in Figure 37.) Accordingly, intermolecular effects in the temperature studies would not be expected to alter significantly the chemical shifts of the reference guanosine protons.

3. Temperature Shifts of the Base Protons. The temperature shifts of the adenine and guanine protons of 0.01 M ApG and 0.01 M GpA are shown in Figure 38. These measurements were made over a temperature range of 0° to $95^{\circ}C$. Increasing the temperature results in downfield shifts for all of the base proton resonances. The magnitudes of the various proton temperature shifts differ considerably. For example, the total shift of the $H_2(A)$ resonance of ApG is ~ 0.13 ppm, whereas the corresponding change for $H_2(A)$ of GpA is only ~ 0.03 ppm (Table VIII). The shifts of $H_2(3')$ and $H_2(5')$ of ApA over a comparable temperature range are 0.18 ppm and 0.04 ppm, respectively. As discussed in Chapter II, it is reasonable to assume that these temperature shifts arise from changes in the intermolecular base-stacking

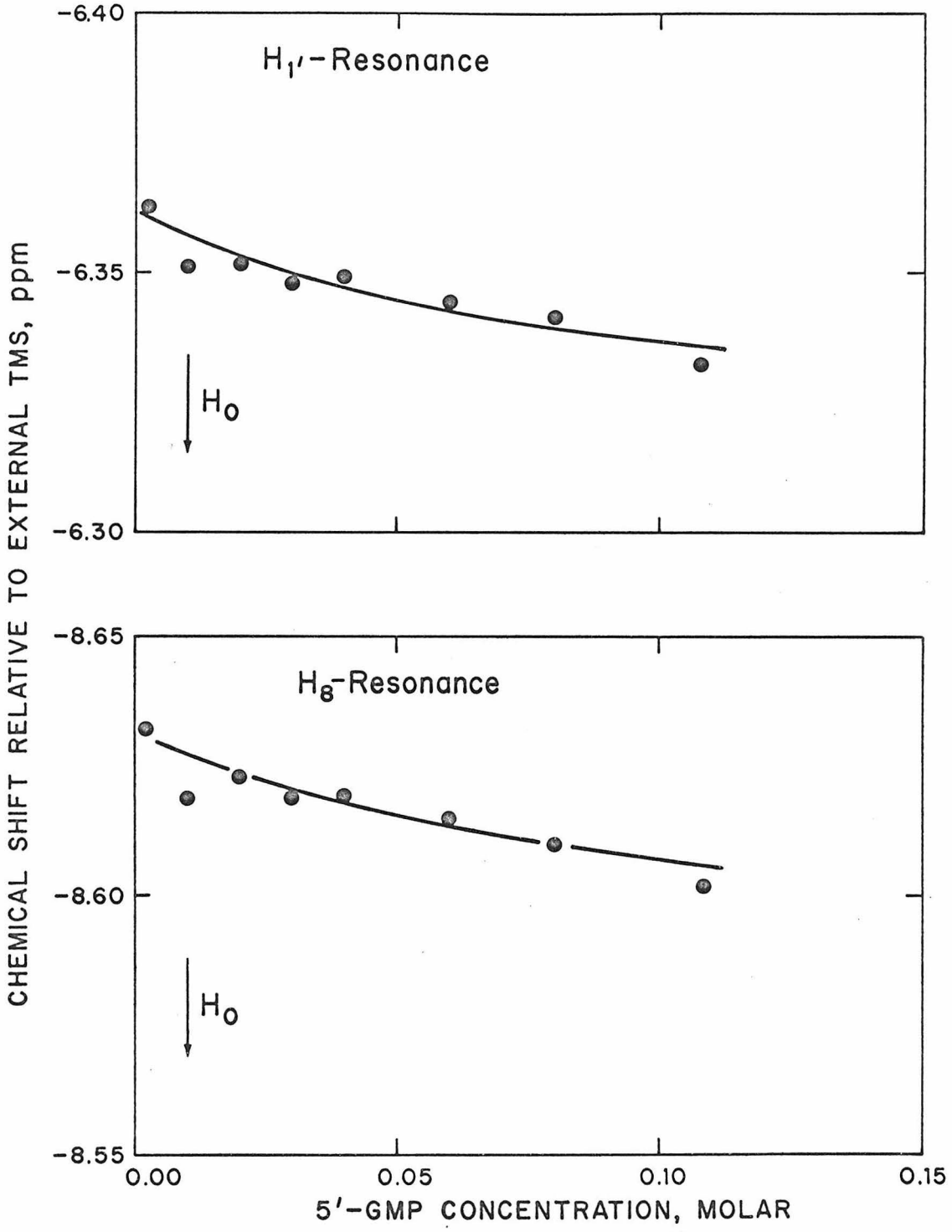


Figure 37. Chemical shifts of the $H_{1'}(G)$ and $H_8(G)$ protons of guanosine-5'-monophosphate as a function of concentration. The chemical shifts are referenced to external TMS and have not been corrected for bulk susceptibility.

Figure 38. The temperature dependence of the base protons of 0.01 M ApG and 0.01 M GpA. The adenosine shifts are referenced to the corresponding protons of 0.01 M adenosine under similar experimental conditions. Similarly, the guanosine protons are referenced relative to 0.002 M guanosine.

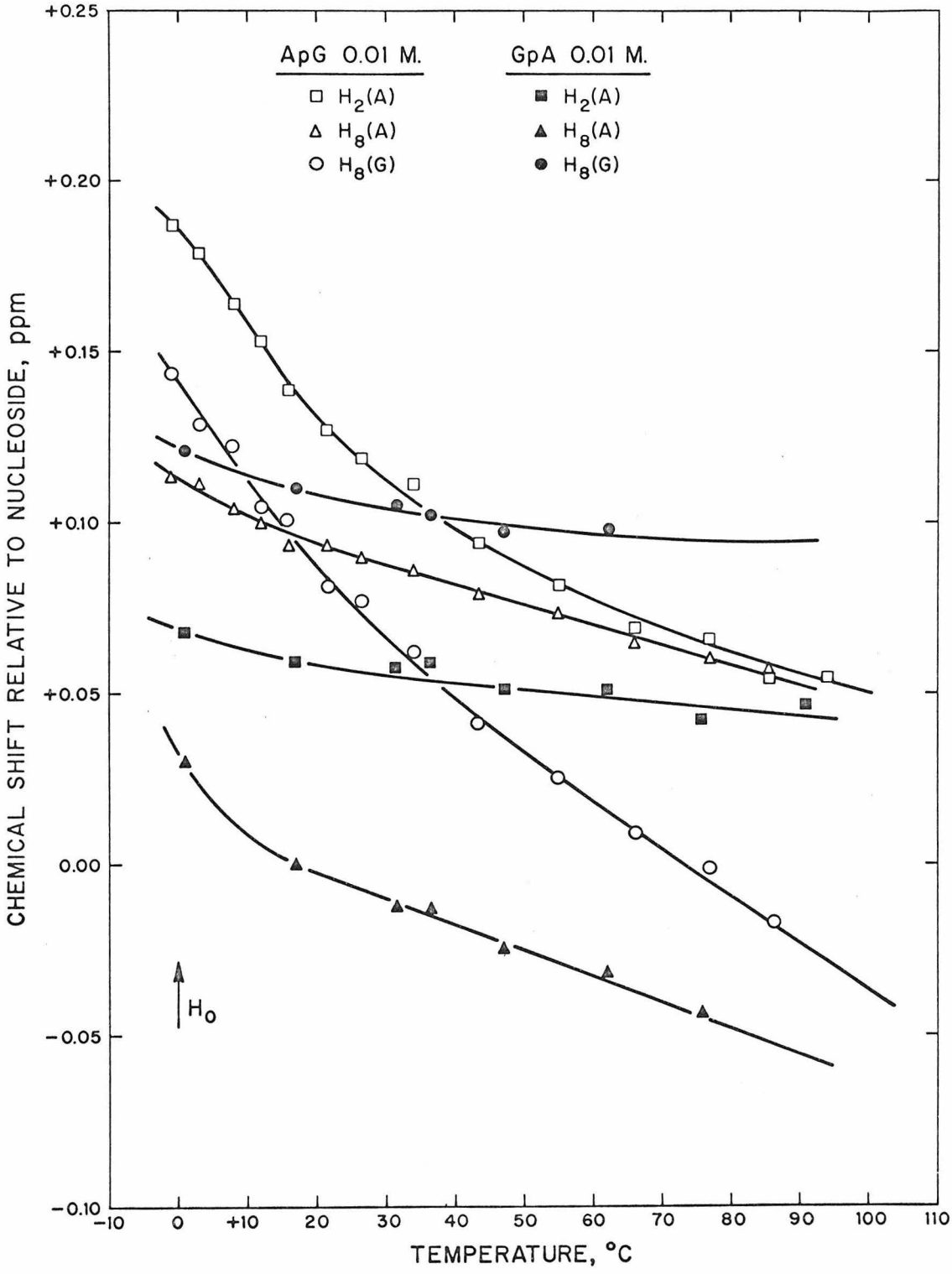

ApG 0.01 M.
H_2(A)
H_8(A)
H_8(G)
GpA 0.01 M.
H_2(A)
H_8(A)
H_8(G)
+0.25
+0.20
+0.15
+0.10
+0.05
0.00
−0.05
−0.10
CHEMICAL SHIFT RELATIVE TO NUCLEOSIDE, ppm
H_o
−10
0
+10
20
30
40
50
60
70
80
90
100
110
TEMPERATURE, °C

TABLE VIII. Total Variation of Proton Chemical Shifts of ApG, GpA, and ApA in the Temperature Range 0°–95°C.

Molecule	Total Shift,[a] ppm					
	$H_8(5')$	$H_8(3')$	$H_2(5')$	$H_2(3')$	$H_{1'}(5')$	$H_{1'}(3')$
ApG	0.17	0.05	- -	0.13	0.132	0.136
GpA	0.09	0.03	0.03	- -	0.039	0.149
ApA	0.16	0.025	0.04	0.18	. . .	. . .

[a]Total downfield shift in increasing the temperature from 0° to 95°C.

interactions and also from changes in the conformations of the bases relative to their ribose rings. Since the H_2 protons are far removed from the phosphate group and the ribose sugars, the temperature shifts of these protons must arise almost entirely from changes in the ring-current magnetic anisotropy of the neighboring base of the dinucleoside monophosphate. At 5°C, $H_2(A)$ of ApG is approximately 0.17 ppm upfield from the H_2 resonances of adenosine whereas the $H_2(3')$ resonance of ApA is ~0.28 ppm upfield from the adenosine H_2 signal. These shifts are tabulated in Table IX along with similar shifts of the other base protons of ApG and GpA. Since the reasonably large upfield shift of the $H_2(A)$ proton of ApG at low temperatures may be attributed only to the magnetic effects of the neighboring guanine base, it may be concluded that the guanine ring supports a significant ring-current magnetic anisotropy and that ApG is intramolecularly stacked at 5°C with the adenine base preferentially oriented in the anti conformation with respect to its furanose ring. If this base were preferentially stacked

TABLE IX. Chemical Shifts[a] of the Base Protons of ApA, ApG, and GpA in D_2O at 5°C.

Molecule	Concentration (Molar)	Chemical Shift,[a] ppm			
		$H_2(5')$	$H_2(3')$	$H_8(5')$	$H_8(3')$
ApA	0.003	0.10	0.28	0.11	0.10
ApG	0.010	...	0.17	0.13	0.11
GpA	0.010	0.07	...	0.02	0.12

[a]Upfield from the corresponding proton of the nucleoside at roughly the same concentration and 5°C.

in the syn conformation, $H_2(A)$ would be oriented away from the guanine base and would not experience a large upfield shift. As noted in Chapter II, the anti conformation is also energetically more favorable for the 3'-adenine base of ApA. However, at 5°C the upfield shift (due to intramolecular base-stacking) of $H_2(A)$ of ApG is ~ 0.10 ppm smaller than that observed for $H_2(3')$ of ApA. The smaller shift of the ApG proton could be attributed to two different effects: (1) the smaller ring-current anisotropy of the guanine ring; and (2) weaker adenine-guanine base-stacking interactions in comparison to adenine-adenine interactions. The relative importance of these two effects is not obvious from the data presented here. However, since the $H_8(5')$ resonance of ApG is at slightly higher field with respect to the corresponding nucleoside resonance than $H_8(5')$ of ApA, the second alternative presented above does not appear to account for the large difference in the shifts of the respective $H_2(3')$ protons of these two dinucleotides at 5°C. With regard to the first possibility, it is noteworthy that some

ring-current magnetic anisotropy may be associated with the guanine ring because protons adjacent to the ring in the intramolecularly stacked molecule experience an upfield shift (Table IX). This observation is in agreement with chemical shift measurements in deoxyguanylyl-(3'→5')-thymidine (18) and with theoretical calculations (99). In view of this fact, it is interesting that extremely small concentration shifts were observed for H_8(G) and $H_{1'}$(G) of 5'-GMP in the concentration range from 0.1 M to infinite dilution (Figure 37). Since guanine evidently has a significant ring-current anisotropy, some concentration shifts would be expected on the basis of other physical evidence which indicates that guanine stacks extensively at higher concentrations (98, 100, 101). We suggest that the ring-current magnetic anisotropy of guanine is localized in the 5-membered imidazole ring and that 5'-GMP forms intermolecular complexes with the 6-membered pyrimidine rings partially overlapped and the imidazole rings oriented away from each other so that very small concentration dependent shifts are observed for the H_8(G) and $H_{1'}$(G) protons. This model for intermolecular guanine base-stacking in 5'-GMP is especially attractive because the negatively charged phosphate groups are oriented away from each other.

In ApG, if the bases are intramolecularly stacked and the adenine base is in the anti conformation, H_2(A) would be situated directly above the imidazole ring of the neighboring guanine base; and, therefore, it would be influenced by the diamagnetic ring-current anisotropy associated with this ring. However, H_2(A) of ApG would not be expected to shift as far upfield in the stacked dinucleotide as H_2(A) of ApA due to the smaller average ring-current effect of the guanine base.

Increasing the temperature of ApG would be expected to result in a corresponding increase in the average distance of H_2(A) from the adjacent guanine ring and a relatively large downfield shift would be anticipated. On the other hand, if the adenine base is preferentially oriented in the syn conformation, H_2(A) would not be exposed to the magnetic effects of the guanine base and a relatively small temperature shift would be observed. A progressive downfield shift of 0.13 ppm is observed for H_2(A) of ApG as the temperature is raised from 0° to 95°C. This temperature shift provides excellent evidence that the adenine base of ApG preferentially assumes the anti conformation.

Temperature studies of the H_2(A) resonance of GpA have also enabled us to determine the most favorable conformation of the adenine base in this dinucleotide. At 5°C, H_2(A) is only 0.07 ppm upfield from the adenosine reference proton compared to 0.17 ppm for H_2(A) of ApG. The lower field resonance frequency of the GpA proton could be the result of either a weaker stacking tendency or base conformations in which H_2(A) experiences less exposure to the opposite guanine ring. As noted in Table VIII, the total temperature shift of H_8(A) of GpA is sufficiently large to indicate reasonably strong guanine-adenine stacking. Therefore, we are led to conclude that the smaller upfield shift of the H_2(A) resonance of GpA at low temperatures is the result of the preferential anti conformation of the adenine base. (In this conformation H_2(A) is far removed from the guanine ring-current anisotropy.) As in the case of the H_2(5') proton of ApA, the higher field resonance frequency of H_2(A) of GpA relative to the H_2 nucleoside proton is due to the formation of some intramolecular stacks in which the adenine base

assumes the syn conformation.

The similarity of the chemical shifts of H_2(A) of GpA and H_2(5') of ApA substantiates the conclusion that the anti conformation of the adenine base of GpA is more favorable. The GpA resonance is only 0.03 ppm downfield from that of ApA. It has been shown in Chapter II that the 5'-adenine base preferentially assumes the anti conformation, and in this conformation, H_2(5') experiences little upfield shift from the neighboring adenine base. Therefore, if the adenine base of GpA also assumes the anti conformation, similar chemical shifts would be expected for the H_2(5') protons of GpA and ApA.

In contrast to the relatively large temperature shifts of H_2(3') of ApG and ApA, the H_2(5') resonance frequency of both GpA and ApA changes very little with temperature variation. This provides conclusive evidence that the anti conformation is energetically more favorable for the 5'-adenine base of GpA as well as ApA.

The interpretation of the temperature dependence of the chemical shifts of the H_8 protons is not quite as straightforward. The resonance frequency of these protons may be influenced by the ribose ring. Accordingly, the conformation of the base with respect to the ribose ring determines to some extent the H_8 proton chemical shifts; and it is not obvious to what extent the intramolecular base-stacking in the dinucleoside phosphates stabilizes the base conformations with respect to the conformations assumed in the reference nucleosides. The chemical shift of the H_8 proton of the 5'-nucleosides is further complicated by phosphate deshielding effects.

At 5°C, the H_8(G) proton of ApG is ~0.13 ppm upfield from H_8

of guanosine (Table IX), and it shifts ~0.17 ppm over the temperature range 0° to 95°C. In comparison, the H_8(G) resonance of GpA is 0.12 ppm upfield from the reference resonance at 5°C, and shifts only ~0.03 ppm over the same temperature range. This very small temperature shift of H_8(3') of GpA is almost equivalent to that observed for H_8(3') of ApA (~0.025 ppm). The small shift of the H_8-adenine proton of GpA can be accounted for if the guanine base is more stable in the anti conformation. With the base in this conformation, the H_8(3') resonance is free from the ring-current effects of the 5'-adenine base. The H_8(3') proton chemical shift of ApG also shows relatively little temperature dependence, it shifts only 0.05 ppm over the entire temperature range studied. This is, again, due to the anti orientation of the 3'-base. It is noteworthy that the chemical shifts of the protons of the 3'-nucleoside are not complicated by phosphate deshielding effects.

The H_8(3') resonances of ApG, GpA, and ApA are all about 0.11 ppm upfield from the adenosine H_8 resonance at 5°C. Since the 3'-base of each of these dinucleotides preferentially stacks in the anti conformation, this relatively large upfield shift is somewhat puzzling. However, part of this residual low temperature upfield shift may be attributed to significant population of 3'-syn conformations in these dinucleotides. If there is a significant fraction of 3'-bases oriented in the syn conformation, a downfield shift would be expected for H_8(3') with increasing temperature. This effect would be compensated, however, by an upfield shift resulting from thermal population of the syn conformation as the temperature increases. This accounts for the relatively small temperature shifts of the H_8(3') protons of ApA, ApG,

and GpA even though a significant fraction of the 3'-bases assume the syn conformation as is evidenced by the low temperature chemical shifts.

The H_8(G) protons of ApG and GpA have almost equivalent upfield shifts (relative to H_8 of guanosine) at 5°C. If the guanine base of ApG, like that of GpA, preferentially assumes the anti conformation, the ring-current magnetic anisotropy of the adenine base would be expected to shift the H_8(G) resonance of ApG to a much higher field. However, this resonance would also experience a downfield shift from the phosphate deshielding effect because the H_8(5') protons of stacked dinucleotides are in close proximity to the phosphate when the base assumes the anti conformation. The deshielding effect of the phosphate group can be quite important. For example, the H_8 resonance of 5'-GMP (pD $\approx$ 8.3) is 0.175 ppm downfield from the guanosine H_8 proton resonance (neutral pD) at 30°C and a comparable concentration. Therefore, the H_8 proton of an anti oriented guanine base in ApG would experience two opposing magnetic effects, the adenine ring-current diamagnetic anisotropy and the phosphate deshielding. Neither of these effects is important for H_8 of anti guanine in GpA. This could explain why the H_8(G) resonance of ApG is not at a significantly higher field than the corresponding H_8(G) of GpA at low temperatures. An alternative explanation is that the guanine base of ApG preferentially stacks in the syn conformation. However, as we have already noted, a sizable downfield shift (0.13 ppm) was observed for the H_8(G) proton of ApG in increasing the temperature from 5° to 95°C. Since the phosphate deshielding effect is not significantly temperature dependent,

this large shift is due to the attenuation of the effect of the adenine ring-current anisotropy as destacking of the bases of the dinucleotide occurs. On the basis of the geometry of the molecule, a large temperature shift would be expected for H_8(G) of ApG only if the guanine base preferentially stacks in the anti conformation (Figures 9-12). Accordingly, we conclude that the anti conformation is also the energetically more favorable one for the guanine base of ApG.

The H_8(5') proton of GpA is only ~0.02 ppm upfield from the nucleoside resonance at 5°C. (The upfield shift due to the guanine base is compensated by the deshielding effect of the phosphate.) This resonance shifts only ~0.09 ppm over the temperature range studied. As noted in Tables VIII and IX, these shifts are much smaller than those observed for H_8(5') of ApG and ApA. We feel that this reflects the smaller ring-current anisotropy of the neighboring guanine base in comparison to adenine.

Finally, the temperature shift of the H_8 resonance of the 5'-esterified nucleoside of each of the purine-purine dinucleotides studied is greater than that of the H_8(3') resonance in agreement with the conclusion that each of the bases is preferentially stacked in the anti conformation in these molecules.

4. Temperature Shifts of the Ribose $H_{1'}$ Protons. We have also studied the temperature dependence of the p.m.r. spectra of the ribose $H_{1'}$ protons of GpA and ApG. To facilitate the discussion which follows, we have summarized the infinite dilution chemical shifts and the $H_{1'}$-$H_{2'}$ coupling constants of these protons in several nucleosides, nucleotides, and dinucleoside monophosphates in Table X. The $H_{1'}$

TABLE X. Infinite Dilution Chemical Shifts and Coupling Constants, $|J_{H_1'-H_2'}|$, of the H_1' Ribose Protons of Several Molecules at ~30°C and Approximately Neutral pH.

Molecule	Chemical Shift,[a] ppm	Coupling Constant, $\|J_{H_1'-H_2'}\|$, cps
Nucleosides and Nucleotides		
Adenosine	6.53	6.1
3'-AMP	6.54	5.9
5'-AMP	6.58	5.6
Guanosine	6.36	6.0
5'-GMP	6.37	6.2
Uridine[b]	6.35	4.5
3'-UMP[b]	6.38	4.3
5'-UMP[b]	6.44	4.8
Cytidine[c]	6.34	3.5
3'-CMP[c]	6.38	...
5'-CMP[c]	6.44	3.3

Dinucleoside Monophosphates				
	H_1'(A)	H_1'(G, U, or C)	H_1'(A)	H_1'(G, U, or C)
ApG	6.39	6.25	4.1	4.4
GpA	6.53	6.14	4.6	4.2
ApU	6.51	6.20	4.3	2.4
UpA	6.56	6.17	4.6	4.4
ApC[c]	6.50	6.20	3.9	2.0
CpA[c]	6.55	6.16	3.8	3.2
	H_1'(5')	H_1'(3')	H_1'(5')	H_1'(3')
ApA	6.42	6.30	3.4	3.5
CpC[c]	6.33	6.24	2.2	2.3
UpU[c]	6.38	6.33	...	4.7

[a]Downfield from external TMS; not corrected for bulk susceptibility.

[b]Data of J. H. Prestegard, private communication.

[c]Data of B. W. Bangerter, private communication.

resonance of guanosine is 0.17 ppm upfield from $H_{1'}$ of adenosine, and the $H_{1'}$ resonance of 5'-GMP is 0.21 ppm upfield from the corresponding resonance of 5'-AMP. On the basis of these observations, we have assigned the higher field resonance of the two $H_{1'}$ protons to the guanosine nucleoside proton in both ApG and GpA. The $H_{1'}$ infinite dilution chemical shifts of these two isomers and of ApA, ApU, UpA, UpU, ApC, CpA, and CpC are summarized in Table X.

In the monocleotides of adenosine, cytidine, and uridine, the chemical shifts of the $H_{1'}$ protons are dependent on the site of the phosphate esterification. In each of these cases, the $H_{1'}$ proton resonance of the 3'-mononucleotide is approximately 0.05 ppm upfield from the $H_{1'}$ resonance of the 5'-mononucleotide. Since the $H_{1'}$-$H_{2'}$ coupling constants of the two isomers in each set are quite similar, the chemical shift difference is not due to conformational differences in the two ribose rings. (We shall discuss the relationship between the coupling constant and the ribose ring conformation below.) Therefore, the differences observed for the $H_{1'}$ chemical shifts of the isomeric mononucleotides may be directly attributed to the phosphate group.

The infinite dilution chemical shifts of the $H_{1'}$ dinucleotide protons are referenced relative to the corresponding $H_{1'}$ chemical shifts of the respective mononucleotides in Table XI. With the exception of $H_{1'}$(5') of UpA, each of the $H_{1'}$ protons of the dinucleotides resonates at a higher field than the corresponding proton of the mononucleotide. We feel that the higher field shifts of the dinucleotide $H_{1'}$ protons are due to the different conformations of the ribose rings of the dinucleotides in comparison to those of the mononucleotides. Many factors could contribute to such conformational differences, and the

Table XI. Chemical Shifts and Coupling Constants, $|J_{H_1'-H_2'}|$, of the H_1' Ribose Protons of Several Dinucleoside Monophosphates Relative to the Respective Values of the Constituent Nucleotides.[a]

Molecule	Proton	Reference Molecule	Chemical Shift,[b] ppm	ΔJ[c], cps
ApG	$H_1'(3')$	3'-AMP	0.15	1.8
	$H_1'(5')$	5'-GMP	0.12	2.2
GpA	$H_1'(3')$	(5'-GMP)[d]	(0.23)[d]	(2.0)[d]
	$H_1'(5')$	5'-AMP	0.05	1.0
ApA	$H_1'(3')$	3'-AMP	0.24	2.4
	$H_1'(5')$	5'-AMP	0.16	2.2
ApU	$H_1'(3')$	3'-AMP	0.03	1.6
	$H_1'(5')$	5'-UMP	0.24	2.4
UpA	$H_1'(3')$	3'-UMP	0.21	0.1
	$H_1'(5')$	5'-AMP	-0.02	1.0
UpU[e]	$H_1'(3')$	3'-UMP	0.06	0.4
	$H_1'(5')$	5'-UMP	0.05	1.4
ApC[e]	$H_1'(3')$	3'-AMP	0.04	2.0
	$H_1'(5')$	5'-CMP	0.24	1.3
CpA[e]	$H_1'(3')$	3'-CMP	0.22	...
	$H_1'(5')$	5'-AMP	0.03	1.8
CpC[e]	$H_1'(3')$	3'-CMP	0.14	...
	$H_1'(5')$	5'-CMP	0.11	1.1

[a]All values taken from infinite dilution extrapolations of solutions at ~30°C and neutral pH.

[b]Upfield chemical shift from the reference molecule.

[c]$\Delta J = |J_{H_1'-H_2'}|$ (reference molecule) - $|J_{H_1'-H_2'}|$ (dinucleoside monophosphate).

[d]The chemical shifts and coupling constants of 3'-GMP were not available; 5'-GMP was used to reference the guanosine resonances of GpA.

[e]Data of B. W. Bangerter, private communication.

intramolecular stacking of the planar bases of the dinucleotides is expected to be one of the most important. Evidence for this has been found in temperature variation of the $H_{1'}$ chemical shifts in several different dinucleotides. The temperature shifts of the following dinucleoside monophosphates are summarized in Tables XII: ApG, GpA, ApA, ApC, CpA, CpC, and UpU.

With the exception of the adenosine resonance of CpA, all of the $H_{1'}$ resonances of the dinucleotides are shifted downfield with increasing temperature. These pronounced downfield shifts are not observed for the $H_{1'}$ resonances of the nucleosides. The specific temperature shifts of the $H_{1'}$ protons of ApG and GpA are illustrated in Figure 39. Since the intramolecular base-stacking interaction between the bases of these molecules decreases with increasing temperature, the downfield shifts may be associated with destacking of the bases and the resulting conformational changes in the molecule.

It is significant that in each of the dinucleotides listed in Table XII, the $H_{1'}$ proton resonances which experience the larger (smaller) temperature shifts also experience a larger (smaller) upfield shift when the respective mononucleotide is incorporated as one of the constituents of a dinucleotide. A comparison of the data of Tables XI and XII reveals that the temperature shifts and the upfield dinucleotide shifts are similar for almost all of the $H_{1'}$ resonances. For example, the $H_{1'}$(A) resonance of GpA is only 0.05 ppm upfield from $H_{1'}$ of 5'-AMP; and, accordingly, this resonance shifts only 0.04 ppm in the temperature range 0^{o}-95^{o}C. On the other hand, the large upfield shifts (~0.2 ppm) of the cytidine $H_{1'}$ protons of ApC and CpA are

TABLE XII. Temperature Variation of the Chemical Shifts and Coupling Constants, $|J_{H_1'-H_2'}|$, of the H_1' Protons of Several Dinucleoside Monophosphates.[a]

Molecule	Proton	Temperature Range	Chemical Shift,[b] ppm	Temperature Range	ΔJ^c, cps
ApG	$H_1'(3')$	0°–95°C	0.14	8°–86°C	1.6
	$H_1'(5')$		0.13		1.8
GpA	$H_1'(3')$	0°–95°C	0.15	1°–62°C	1.5
	$H_1'(5')$		0.04		1.2
ApA[d]	$H_1'(3')$	4°–71°C	0.13	4°–71°C	2.4
	$H_1'(5')$		0.12		2.4
ApC[e]	$H_1'(3')$	6°–86°C	0.04	6°–86°C	2.3
	$H_1'(5')$		0.24		1.7
CpA[e]	$H_1'(3')$	6°–86°C	0.17	6°–86°C	2.3
	$H_1'(5')$		-0.02		2.0
CpC[e]	$H_1'(3')$	14°–63°C	0.10	14°–63°C	2.2
	$H_1'(5')$		0.06		...
UpU[e]	$H_1'(3')$	14°–63°C	0.02	14°–63°C	0.7
	$H_1'(5')$		0.01		0.5

[a] ~0.01 M solutions at approximately neutral pH.

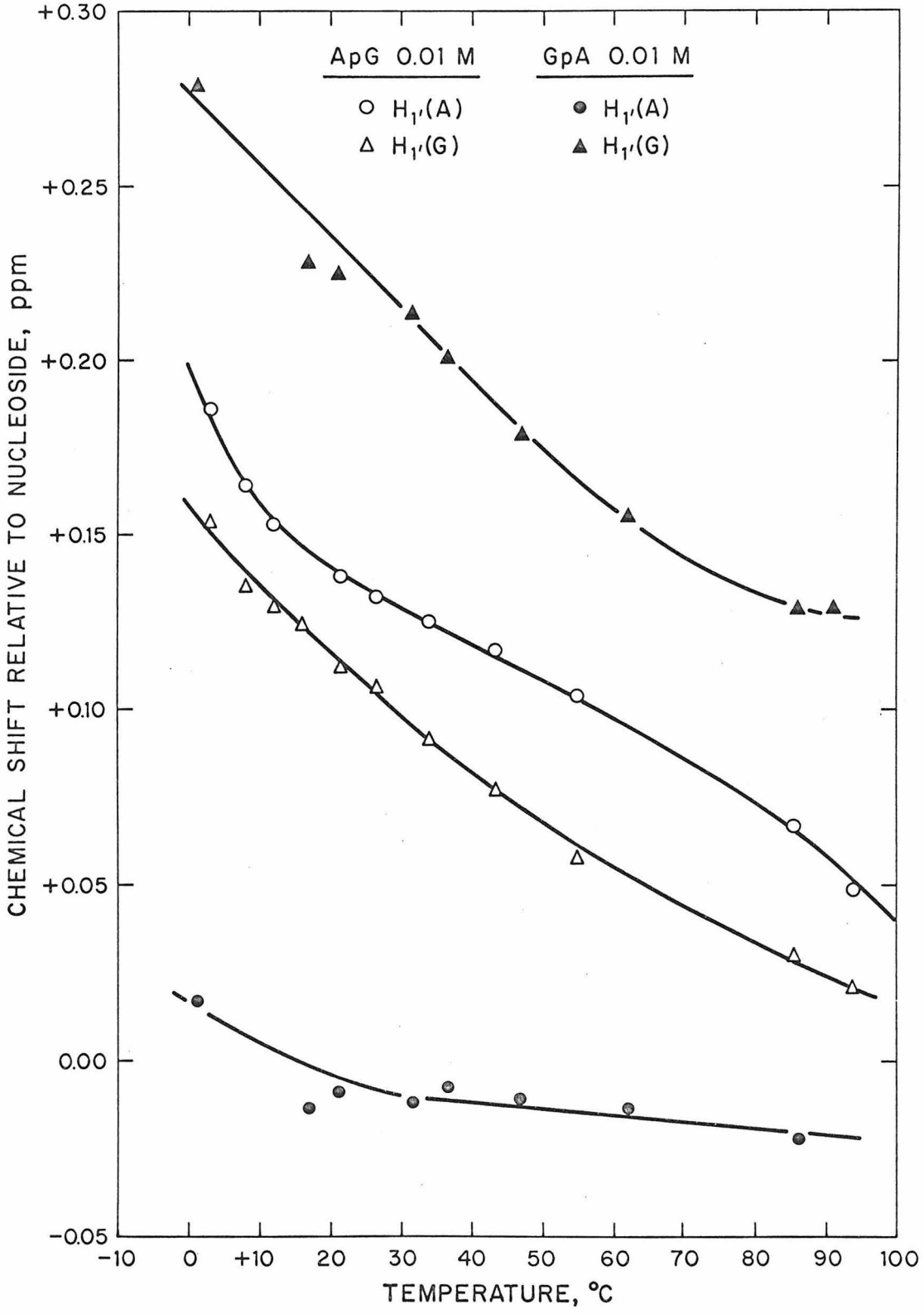
[b] Total downfield shift observed in the temperature range proceeding from lower to higher temperature. Shifts are referenced to the corresponding nucleoside protons at the same temperature.

[c] Increase in $|J_{H_1'-H_2'}|$ with increasing temperature.

[d] Data of F. E. Hruska and S. S. Danyluk, reference (78).

[e] Data of B. W. Bangerter, private communication.

Figure 39. Temperature variation of the $H_{1'}$ ribose resonances of ApG and GpA. The dinucleotide concentrations are 0.01 M, and the chemical shifts are referenced to the corresponding protons of the nucleosides under similar experimental conditions.


ApG 0.01 M
GpA 0.01 M
H1'(A)
H1'(G)
H1'(A)
H1'(G)
+0.30
+0.25
+0.20
+0.15
+0.10
+0.05
0.00
-0.05
CHEMICAL SHIFT RELATIVE TO NUCLEOSIDE, ppm
-10
0
+10
20
30
40
50
60
70
80
90
100
TEMPERATURE, °C

reflected in the greater temperature shifts (~0.2 ppm) of these protons. This provides good evidence that the conformational changes which accompany increased intramolecular base-stacking result in upfield shifts for the $H_{1'}$ ribose protons.

It is useful to consider the origin of these upfield shifts. In the isomer set, ApG–GpA, the ribose $H_{1'}$ proton of the 3'-adenosine is shifted further upfield with respect to the corresponding chemical shift of the mononucleotide than the $H_{1'}$ proton of 5'-adenosine. Of the two guanosine $H_{1'}$ resonances in this set, the proton of the 3'-nucleoside also experiences a greater upfield shift. Finally, in each of the homodinucleotides, ApA, CpC, and UpU, the $H_{1'}(3')$ resonance is shifted further upfield than $H_{1'}(5')$. The experimental evidence thus far presented for ApA, ApG, and GpA has shown that the bases of each of these molecules form intramolecular stacks in aqueous solution with each of the bases preferentially oriented in the anti conformation and with the sugar-phosphate backbone in a similar configuration as the molecular backbone in double helical DNA. In this configuration, the backbone of the dinucleotide represents a small segment of a right-handed helix. If the dinucleotide is stacked in this configuration, the $H_{1'}(3')$ proton is far removed from the 5'-base and, therefore, the greater upfield shift observed for this proton in these dinucleotides cannot be attributed to the magnetic anisotropy of the 5'-base. However, if the bases of the molecule are intramolecularly stacked in a configuration in which the sense of the partial helix of the sugar-phosphate backbone is left-handed rather than right-handed, the $H_{1'}(3')$ proton could conceivably be strongly influenced by the ring-current

magnetic anisotropy of the 5'-base and would be expected to shift more upfield with intramolecular base-stacking than the $H_{1'}(5')$ proton. The concentration shifts and the purine-induced shifts (discussed in the next section of this chapter) of the $H_8(5')$ protons of ApG, GpA, and ApA provide excellent evidence that the right-handed helix is preferred in these molecules. Optical studies of the dinucleotides have also shown that the right-handed helix is generally more favorable for the stacked dinucleotides (59). Furthermore, a consideration of molecular models of these compounds indicates that the right-handed helix is energetically more favorable from the standpoint of steric effects. We conclude, then, that the stacked dinucleotides preferentially assume a right-handed helical structure in solution and that the upfield shifts of the $H_{1'}(3')$ resonances of the dinucleotides relative to the respective 3'-mononucleotide protons are not due to ring-current effects of the 5'-base. Since the $H_{1'}(3')$ protons are shifted upfield to a greater extent than the $H_{1'}(5')$ protons (which in the most favorable molecular configuration could be affected by the magnetic anisotropy of the 3'-base), it is also apparent that the ring-current contribution to the upfield shift of the $H_{1'}(5')$ protons is not extensive. It is not surprising, then, that the magnitudes of the temperature shifts of the various $H_{1'}$ protons of ApG and GpA do not lend themselves to an interpretive argument involving the ring-current magnetic anisotropy of the neighboring base. In GpA the total temperature shift is much larger for $H_{1'}(3')$ ($\sim$0.15 ppm) than for $H_{1'}(5')$ ($\sim$0.04 ppm) while the two $H_{1'}$ ribose temperature shifts in ApG are approximately equal ($\sim$0.13 ppm). Since the $H_{1'}(3')$ proton of each of these dinucleotides is not exposed to the ring-current

magnetic anisotropy of the 5'-base when the molecule assumes the preferable DNA-like configuration, the effect of the ring-current of the neighboring base is not the principal cause of the dinucleotide $H_{1'}$ temperature shifts. This conclusion is supported by the observation that both $H_{1'}$ resonances of CpC have sizable temperature shifts (Table XI), although the cytosine ring has very little ring-current magnetic anisotropy (51, 79). In addition, the $H_{1'}$ infinite dilution shifts of CpC are considerably further upfield than the respective cytidine monophosphate $H_{1'}$ resonances (Table XI). These shifts evidently originate from sources other than ring-current magnetic anisotropy.

Changes in the conformation of the ribose ring and in the orientation of the base with respect to rotation about the glycosidic bond are likely responsible for the temperature shifts of the $H_{1'}$ dinucleotide protons as well as the higher field shifts of these protons relative to those of the mononucleotides. Evidence for this is discussed in the following sections.

5. Coupling Constants, $|J_{H_{1'}-H_{2'}}|$, of the Ribose Protons. Variations in the magnitude of the coupling constants, $|J_{H_{1'}-H_{2'}}|$, of ApG and GpA with temperature provide convincing evidence that the ribose rings of the dinucleotides change conformation as the intramolecular stacking interaction is modified by temperature perturbations.

Utilizing the Karplus relationship (102) between vicinal coupling constants and the dihedral angle, Jardetzky (91) has discussed the relationship of $|J_{H_{1'}-H_{2'}}|$ to the conformation of the ribose ring of several nucleotides in aqueous solution and has concluded that values

of $|J_{H_1'-H_2'}|$ of approximately 4.5 cps or greater correspond to a ribose ring conformation which is primarily 2'-endo and that smaller coupling constants reflect the existence of conformations which are more 3'-endo in character. Chan and Nelson (82, Chapter II, this thesis) and also Hruska and Danyluk (78) have independently attributed the increase of $|J_{H_1'-H_2'}|$ in ApA with increasing temperature to the destacking of the dinucleotide accompanied by increasing 2'-endo character for the ribose ring conformations.

The magnitudes of $J_{H_1'-H_2'}$ observed for each of the H_1' ribose protons of ApG and of GpA are plotted as a function of temperature in Figure 40 and Figure 41, respectively. There is some scattering in the data of these figures due to the intrinsic difficulty of accurately determining the coupling constants in solutions of low concentration. However, these data clearly show that the magnitude of each of the $H_1'-H_2'$ coupling constants of ApG and GpA increases from a value of $\sim$3 cps at 0°C to $\sim$5 cps at 90°C. Therefore, it appears that the conformations of the ribose rings of these molecules change considerably as the temperature increases and that destacking of the bases results in more 2'-endo character for each of the ribose rings. Also illustrated in Figures 40 and 41 are the magnitudes of the $H_1'-H_2'$ coupling constants of guanosine and adenosine. In contrast to the dinucleotide coupling constants, these coupling constants do not increase with increasing temperature. There is, conversely, a small decrease in $|J_{H_1'-H_2'}|$ of each of these nucleosides as the temperature increases from 0° to 95°C. At the higher temperatures, the values of $|J_{H_1'-H_2'}|$ of the dinucleoside monophosphates closely approach those of the

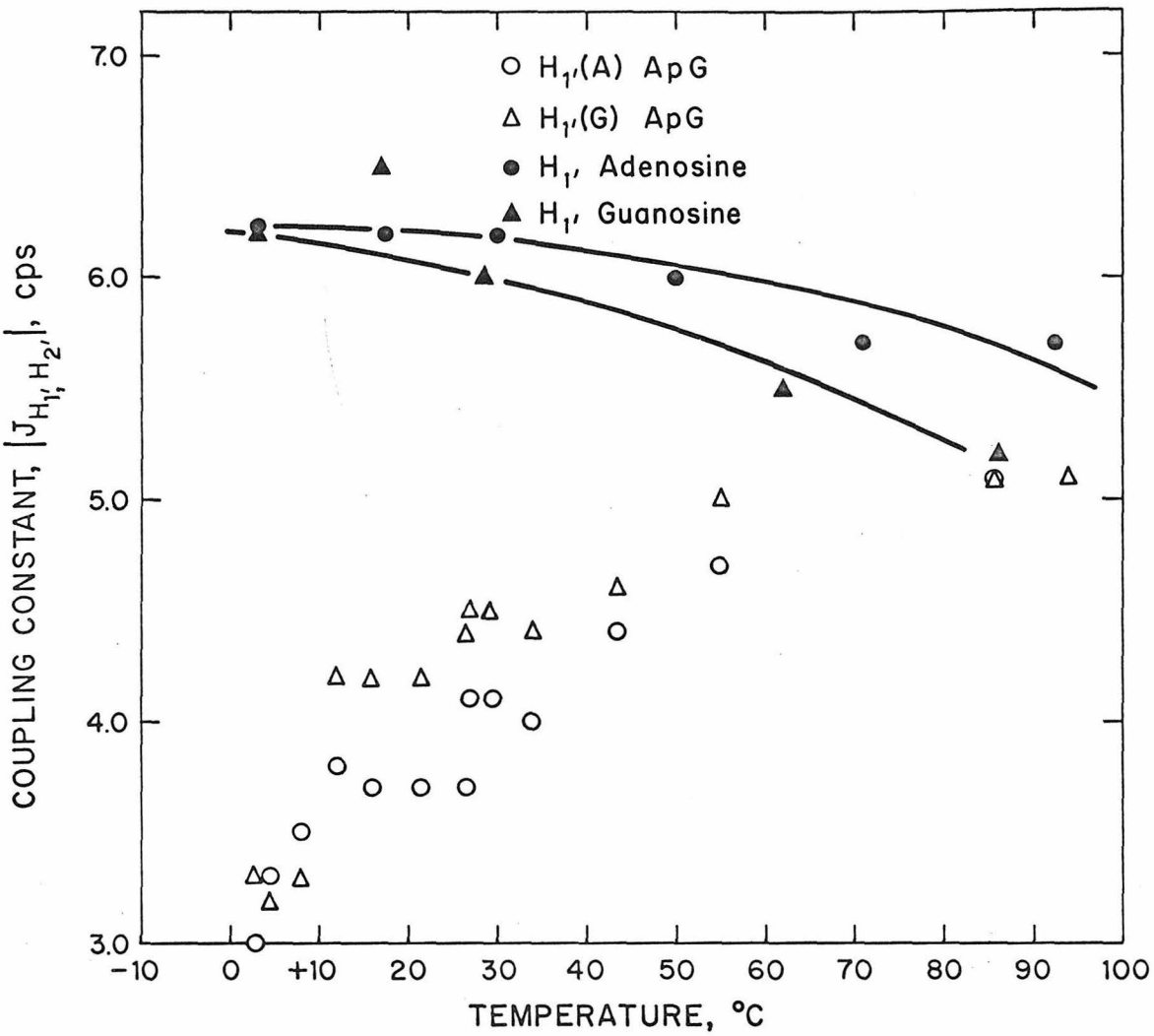


Figure 40. Temperature variation of the coupling constants, $|J_{H_1'-H_2'}|$, of ApG, adenosine, and guanosine. Solution concentrations: 0.01 M.

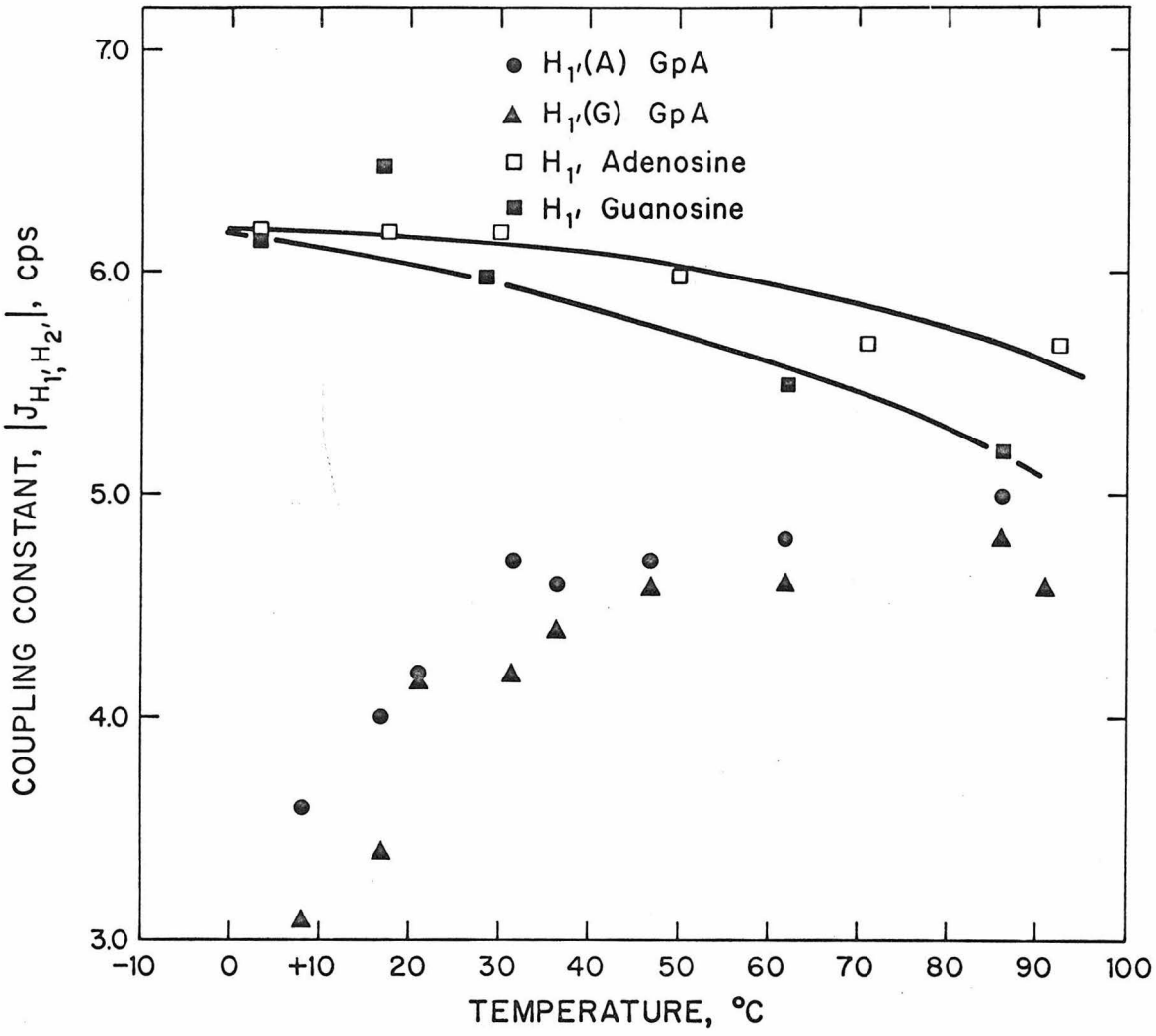


Figure 41. Temperature variation of the coupling constants, $|J_{H_1'-H_2'}|$, of GpA, adenosine, and guanosine. Solution concentrations: 0.01 M.

nucleosides as would be expected if the dinucleotides are significantly destacked.

It is noteworthy that the magnitudes of the coupling constants of ApA are consistently smaller than those of ApG and GpA in the temperature range 0°-50°C, and that the total change observed in $|J_{H_1'-H_2'}|$ is greater in ApA than in either ApG or GpA. These observations indicate that ApA is more strongly intramolecularly stacked at lower temperatures than these other dinucleotides.

The total change of $|J_{H_1'-H_2'}|$ of several different dinucleoside monophosphates over large temperature ranges is also shown in Table XII. As in the case of the H_1' chemical shifts, the temperature-induced change in the H_1'-H_2' coupling constant of the adenosine nucleosides in the isomer sets, ApG - GpA and ApC - CpA, is larger for the 3'-nucleoside. In nearly all of the dinucleotides listed in Table XII, there is some correlation between the temperature shifts of the H_1' resonances and the temperature-induced changes in the H_1'-H_2' coupling constants. For example, both the temperature shifts and the temperature-induced changes in the coupling constants are large for the ApA protons and very small for the UpU protons. In addition, the H_1'(A) proton of GpA has a much smaller temperature shift and a significantly smaller coupling constant change than the other H_1' protons of GpA and ApG. Apparently, the effect of the base-stacking interaction and the attending conformational changes is somewhat less pronounced for the 5'-nucleoside of GpA.

The large temperature shifts of the cytidine H_1' resonances of ApC and CpA are accompanied by large changes in the coupling

constants. However, the adenosine $H_{1'}$ protons of ApC and CpA have surprisingly small temperature shifts in view of the large changes which occur in the coupling constants. With the exception of these two $H_{1'}$ protons, modification of the intramolecular base-stacking interactions and the attending conformational changes apparently alter both the $H_{1'}$ chemical shifts and the $H_{1'}$-$H_{2'}$ coupling constants in the dinucleotides, with the p. m. r. spectra of the more strongly stacked molecules such as ApA experiencing the greatest changes, and the more weakly intramolecularly stacked molecules such as UpU showing small temperature-induced effects.

From Table X it is evident that the $H_{1'}$-$H_{2'}$ coupling constants of the dinucleoside monophosphates are generally smaller than those of the constituent mononucleotides at $\sim 30^{\circ}C$. As might be expected, the magnitude of the difference in the coupling constant values of the nucleosides and dinucleotides depends on the nature of the bases and agrees reasonably well with the magnitude of the changes in the coupling constants of the dinucleotides over large temperature ranges. Again, the greatest effects are generally observed for the more strongly intramolecularly stacked molecules.

It is pertinent to discuss factors which might be responsible for changes in the ribose ring conformations as a result of the stacking interactions in the dinucleoside monophosphates. The experimental observations indicate that the 2'-endo conformation is more favorable in the simple 3'- and 5'-mononucleotides, but in all of the dinucleoside monophosphates we have studied, the intramolecular stacking interaction between the two bases favors the 3'-endo conformation.

Examination of CPK molecular models of the dinucleotides indicates that resistance to intramolecular base-stacking through steric hindrance is less pronounced when the two ribose ring conformations of these molecules are 3'-endo. If the ribose conformation of the 3'-nucleoside is 2'-endo, the two bases of the dinucleoside monophosphate cannot overlap because of non-bonded repulsion of the 2'—CHOH group of the 3'-nucleoside by the base and the ether oxygen of the 5'-nucleoside. This steric repulsion is relieved when the ribose conformation of the 3'-nucleoside assumes more 3'-endo character. Similar non-bonded repulsions 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 in this case. From the models it appears that there is less steric interaction between the 5'-base and the 5'-CH_2 group in the stacked dinucleotide when the ribose ring of the 5'-nucleoside is more 3'-endo in character. In the 2'-endo conformation, the ribose ring of the 3'-nucleoside offers more resistance to the formation of intramolecular base stacks than the 2'-endo conformation of the 5'-ribose ring; and in most of the dinucleotides, intramolecular base-stacking appears to effect greater changes in the conformation of the ribose ring of the 3'-nucleoside.

These observations indicate that the stacking energy of many of the dinucleotides is sufficiently great to cause significant changes in the ribose ring conformations.

6. The Effect of Molecular Conformational Changes on the $H_{1'}$ Chemical Shifts of the Dinucleotides. Let us consider the effect of changes in the ribose ring conformations of the dinucleotides on the

chemical shifts of the $H_{1'}$ protons.

At infinite dilution, the $H_{1'}$ chemical shift of 2'-deoxyadenosine is approximately 0.4 ppm downfield from the $H_{1'}$ resonance of both 3'-deoxyadenosine and adenosine (35). The values of $|J_{H_{1'}-H_{2'}}|$ of 2'-deoxyadenosine, 3'-deoxyadenosine, and adenosine are 6.5 cps, 2.2 cps, and 6.1 cps, respectively. Although the conformations of the ribose rings of 3'-deoxyadenosine and adenosine are very dissimilar (as indicated by the difference in the respective $H_{1'}$-$H_{2'}$ coupling constants), the $H_{1'}$ chemical shifts of these two molecules are about the same. Therefore, different ribose ring conformations are not intrinsically associated with different $H_{1'}$ chemical shifts. Since the $H_{1'}$-$H_{2'}$ coupling constants of 2'-deoxyadenosine and adenosine are similar, the ribose ring conformations in these two molecules are also presumably quite similar. Apparently, the much higher field chemical shift of the $H_{1'}$ proton of adenosine is not the direct result of conformational differences in the ribose rings of adenosine and 2'-deoxyadenosine, but, rather, may be attributed to the effects of the 2'-oxygen atom of adenosine.

The 2'-oxygen atom can influence the $H_{1'}$ resonance frequency of adenosine primarily in two ways. (1) It is possible for the oxygen atom to alter the electron density at the $H_{1'}$ proton through inductive effects. As a result of the large electronegativity of the oxygen atom, decreased electronic shielding of the $H_{1'}$ nucleus would be expected. (2) The magnetic anisotropy of the oxygen atom can also affect the magnetic field at the site of the $H_{1'}$ proton. In contrast to the inductive effects, this phenomenon operates directly through space; and it is,

therefore, dependent upon the orientation of the oxygen atom with respect to the $H_{1'}$ proton and upon the distance between the two atoms. NMR studies of model compounds (103) have shown that both the inductive effect and the effect of the magnetic anisotropy of the 2'-oxygen atom could make significant contributions in determining the chemical shift of the neighboring $H_{1'}$ proton. It is difficult to estimate accurately the relative importance of these two effects. However, since the spectral position of the $H_{1'}$ proton of adenosine is upfield rather than downfield from that of the $H_{1'}$ 2'-deoxyadenosine resonance, it would appear that the inductive effect is relatively unimportant in accounting for the difference in the chemical shifts of the $H_{1'}$ protons of these two molecules.

If the magnetic anisotropy of the 2'-oxygen atom of adenosine makes the principal contribution to the upfield shift of the adenosine $H_{1'}$ proton with respect to the corresponding 2'-deoxyadenosine resonance, variations in the ribose ring conformation would be expected to change the $H_{1'}$ resonance frequency because such variations would alter both the $H_{1'}$-$O_{2'}$ distance and the orientation of the oxygen atom with respect to the $H_{1'}$ proton. For example, the 2'-oxygen atom of a 3'-endo ribose ring is much closer to the neighboring $H_{1'}$ proton than the $O_{2'}$ atom of a ribose ring in the 2'-endo conformation. If the effect of the magnetic anisotropy of $O_{2'}$ is to shield the $H_{1'}$ proton, an upfield shift would be expected for $H_{1'}$ as the ring conformation changes from 2'-endo to 3'-endo. We feel that this effect makes a major contribution in determining the temperature shifts of the $H_{1'}$ ribose protons of many of the dinucleotides. Decreasing the temperature results in increased

intramolecular base-stacking. The formation of base stacks is accompanied by increased 3'-endo conformational character for the ribose rings; this manifests itself in smaller $H_{1'}$-$H_{2'}$ coupling constants and higher field $H_{1'}$ chemical shifts.

Additional molecular conformational changes in the dinucleotides also influence the $H_{1'}$ chemical shifts. Changes in the average orientation of the respective bases about the glycosidic linkage can alter the spectral position of the $H_{1'}$ protons because the distance between these protons and centers of diamagnetic anisotropy, such as keto-oxygens and nitrogen lone pair electrons, of the planar bases may vary considerably depending on the orientation of the base about the glycosidic bond.

The large upfield dinucleotide shifts of the $H_{1'}$ pyrimidine protons of ApC, CpA, ApU, and UpA (Table XI) may be attributed to the formation of intramolecular base-stacks in which the pyrimidine bases are preferentially oriented in the anti conformation so that the 2-keto-oxygen of the pyrimidine base is in close proximity to the $H_{1'}$ proton of the pyrimidine nucleoside. The intramolecular base-stacking would be expected to decrease the average angle of rotation of the base about the glycosidic bond relative to the rotation in the simple nucleotide or nucleoside; and the average O_2-$H_{1'}$ distance would be smaller in the pyrimidine nucleoside of the dinucleotides than the corresponding distance in the simpler pyrimidine derivatives.

The relatively large temperature shifts of the $H_{1'}$ cytidine resonances of ApC and CpA (Table XII) may also be accounted for by temperature-induced changes in the rotation of the cytosine bases about the N_1-$C_{1'}$ bond. Previous studies (79) have shown that the cytosine bases

of ApC and CpA preferentially assume the anti conformation and that these two dinucleoside monophosphates form relatively stable intramolecular base-stacks at low temperatures. As indicated above, in intramolecular base-stacks of ApC and CpA in which the cytosine base is anti oriented, the 2'-keto-oxygen atom is in relatively close proximity to the $H_{1'}$ proton of the pyrimidine nucleoside; and diamagnetic shielding of the ribose proton by the lone pair electrons of the keto oxygen could account for the low temperature upfield shifts of the cytosine protons. As the temperature is increased, the intramolecular base-stacking interaction decreases and larger average angles of rotation of the bases about the glycosidic bond would be expected. In addition, the syn conformations of the cytosine bases would become more populated. Each of these effects increase the average O_2-$H_{1'}$ distance in the dinucleotides, and the net result would be downfield shifts of the $H_{1'}$ resonances of the dinucleotides as observed.

The shielding effect of the 2-keto-oxygen atom would also contribute to the large temperature shifts and the high field dinucleotide shifts of the $H_{1'}$ protons of CpC. The absence of large shifts in UpU is additional evidence that the uracil bases of this molecule are weakly stacked.

In a similar manner, it is possible for the magnetic anisotropy of the lone pair electrons of N_3 of an adenine or a guanine base to influence the $H_{1'}$ chemical shifts of purine nucleosides in dinucleotide molecules. It is difficult to estimate the effect of these nitrogen lone pairs. However, when the bases of the dinucleotide are somewhat destacked and the rotation of the bases about the glycosidic bond is less

hindered, the average distance between $H_{1'}$ and N_3 decreases. It is possible that increased diamagnetic shielding and an upfield shift could result from the effect of the N_3 lone pair electrons as the dinucleotide uncoils from a stacked conformation.

The ring-current magnetic anisotropy of bases such as adenine which are able to accommodate large ring currents can also affect the chemical shift of the $H_{1'}$ proton of the ribose residue of the same nucleoside in the dinucleotide molecules. [As a possible example of this effect, the infinite dilution chemical shift of $H_{1'}$ of adenosine is ~0.18 ppm downfield from each of the corresponding shifts of uridine, cytidine, and guanosine (Table X).] Changes in the orientation of the base about the glycosidic bond can alter the effect of the ring-current magnetic anisotropy at the $H_{1'}$ site and, therefore, change the chemical shift. Destacking of the bases of a dinucleotide results in less hindered rotations of bases about the glycosidic bond; and it appears that the position of the $H_{1'}$ proton is more likely to be in the plane of the base of its nucleoside as intramolecular destacking occurs. This change can cause either an upfield or a downfield shift in the $H_{1'}$ resonance, depending on the dimensions of the ring current and the average location of the ribose proton with respect to the base. An examination of CPK molecular models and the Johnson-Bovey Tables (45, 46) permits a qualitative estimate of the nature of the adenine ring-current effect on the adenosine $H_{1'}$ protons of dinucleotides, and it appears that an upfield shift would accompany the destacking process as a result of changes in the effect of the ring-current anisotropy.

In ApA, ApG, and GpA, relatively large downfield shifts (with increasing temperature) were observed for all of the adenosine $H_{1'}$ protons except that of GpA. (The change in the $H_{1'}$-$H_{2'}$ coupling constant of the adenosine nucleoside of GpA with temperature is also considerably smaller.) We may conclude that there is not a significant upfield shift due to the ring-current effect or the N_3 lone pair electrons of the adenine base as these molecules become less strongly stacked. In comparison, the downfield temperature shifts of the adenosine $H_{1'}$ protons of ApC and GpA are very small even though the temperature-induced changes in the $H_{1'}$-$H_{2'}$ coupling constants are large in these adenine-cytosine dinucleotides (Table XII). In these molecules it is possible that the downfield shift which results from decreased stacking and the consequential changes in the ribose ring conformations is compensated by upfield shifts caused by changes in the rotation of the adenine base about the glycosidic bond. In ApC and CpA, the rotation of the adenine base is possibly less restricted than in ApA, ApG, and GpA because the neighboring base of the dinucleoside monophosphate is smaller and the intramolecular base-stacking interactions are somewhat less stable.

7. Summary. The temperature shifts of the base protons of ApG and GpA demonstrate that the intramolecular stacking interaction in each of these dinucleotides is relatively strong in aqueous solution. Furthermore, the geometrically analogous base protons of ApA, ApG, and GpA have similar temperature shifts which are modified primarily by the different ring-current magnetic anisotropies of the adenine and

guanine bases. These results provide conclusive evidence that, as in ApA, each of the bases of GpA and ApG preferentially stacks in the anti conformation.

In comparison to the nucleosides and mononucleotides, the dinucleotide molecules have smaller $H_{1'}$-$H_{2'}$ coupling constants. These smaller coupling constants are due to the conformational changes effected in the ribose rings of the dinucleotides by intramolecular base-stacking. The magnitudes of the dinucleotide coupling constants increase with increasing temperature. As the molecules unfold, the ribose ring conformations change from 3'-endo to 2'-endo and larger values of $|J_{H_{1'}\text{-}H_{2'}}|$ are observed. The larger temperature-induced changes in the ApA coupling constants provide good evidence that the intramolecular base-stacking is stronger in this molecule than in either ApG or GpA.

The temperature shifts of the $H_{1'}$ ribose protons of the dinucleotides are determined by many different intramolecular effects, and it is not meaningful to compare indiscriminately the $H_{1'}$ shifts of all dinucleotides. However, the bases and the molecular configurations of ApA, ApG, and GpA are sufficiently similar to permit a cursory comparison. Over comparable temperature ranges, the shifts of the adenosine $H_{1'}$ proton resonances of ApA are larger than the corresponding shifts of ApG and GpA (Table XII). This also demonstrates the stronger intramolecular stacking in ApA. In view of the relatively large temperature shifts of the $H_{1'}$ ribose protons of GpA and ApG, the small temperature shift of the GpA adenosine proton (Figure 39) is somewhat anomalous. This smaller shift may be indicative of less stable base-stacking in GpA.

F. Dinucleotide-Purine Interactions

1. Introduction. Previous studies (75, 79, Chapter II, this thesis) have demonstrated that purine binds to the bases of dinucleoside monophosphates via vertical base-stacking. Purine can, therefore, serve as a useful probe molecule in the investigation of the intramolecular base-stacking interactions in these larger molecules. In this section we report the results of purine-binding studies of ApG and GpA and compare these results with those obtained for ApA.

In order to minimize effects arising from intermolecular association of the dinucleotides, 0.01 M solutions of ApG and of GpA were utilized in these purine-binding experiments.

2. Purine-induced Chemical Shifts. The base proton resonances and the $H_{1'}$ resonances of the dinucleotides shift upfield with the addition of purine due to the formation of base stacks between the dinucleotide bases and purine. The purine-induced shifts of the adenine, guanine, and $H_{1'}$ ribose protons of ApG and GpA are plotted as a function of purine concentration in Figure 42 and Figure 43, respectively. In addition, the total purine-induced shifts of these proton resonances at a purine concentration of 1.0 M are summarized in Table XIII.

At 1.0 M purine, the upfield purine-induced shift of the H_2 proton of ApG is ~0.06 ppm greater than the shift of the H_2 proton of GpA. Thus, the H_2 resonance of the 3'-base is shifted more by the addition of purine than the corresponding resonance of the 5'-base in the ApG-GpA isomer set. This is in agreement with the results of purine-binding

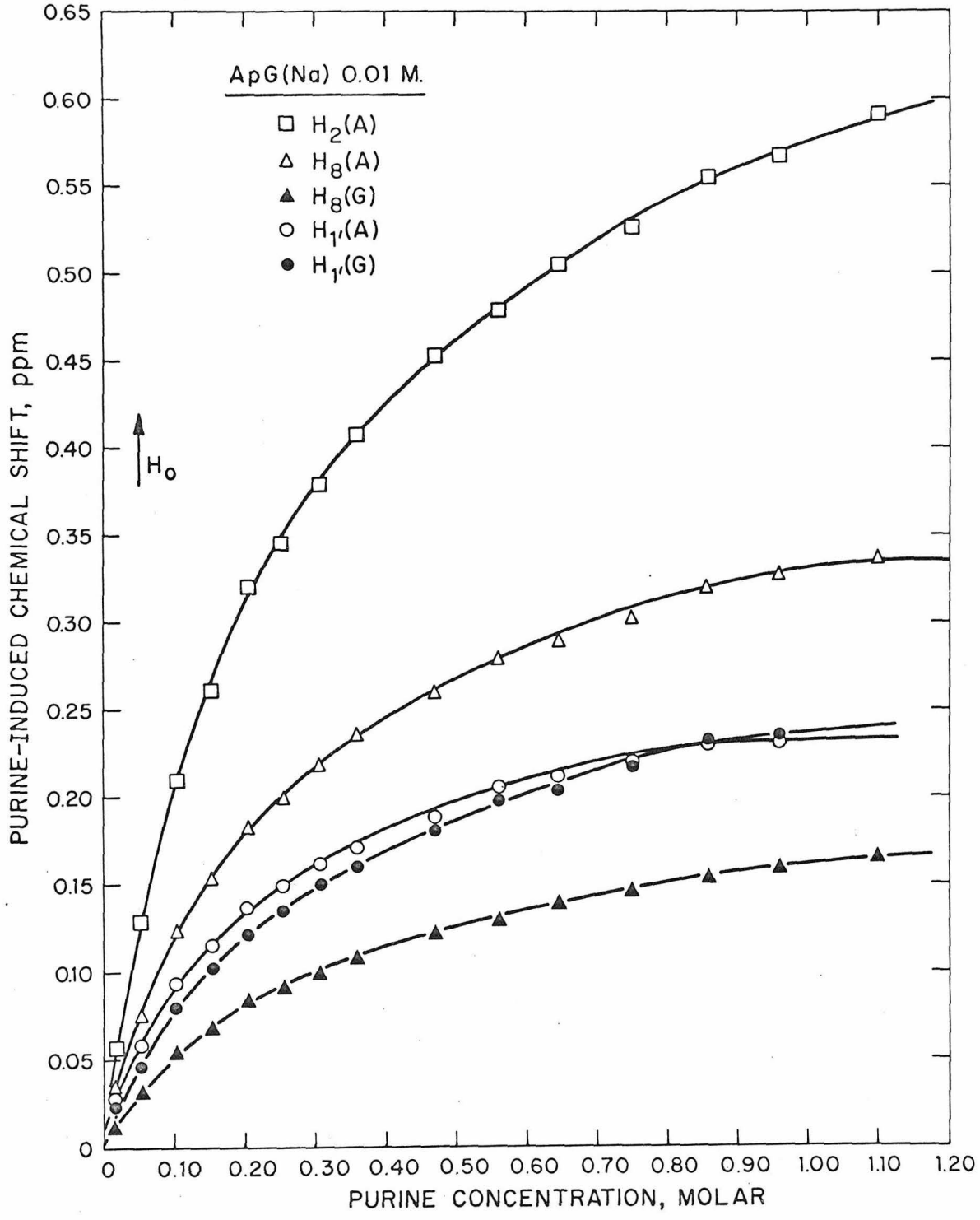


Figure 42. Purine-induced chemical shifts (100 Mcps) of the $H_2(A)$, $H_8(A)$, $H_8(G)$, $H_{1'}(A)$, and $H_{1'}(G)$ protons of ApG (sodium salt). ApG concentration = 0.01 M.

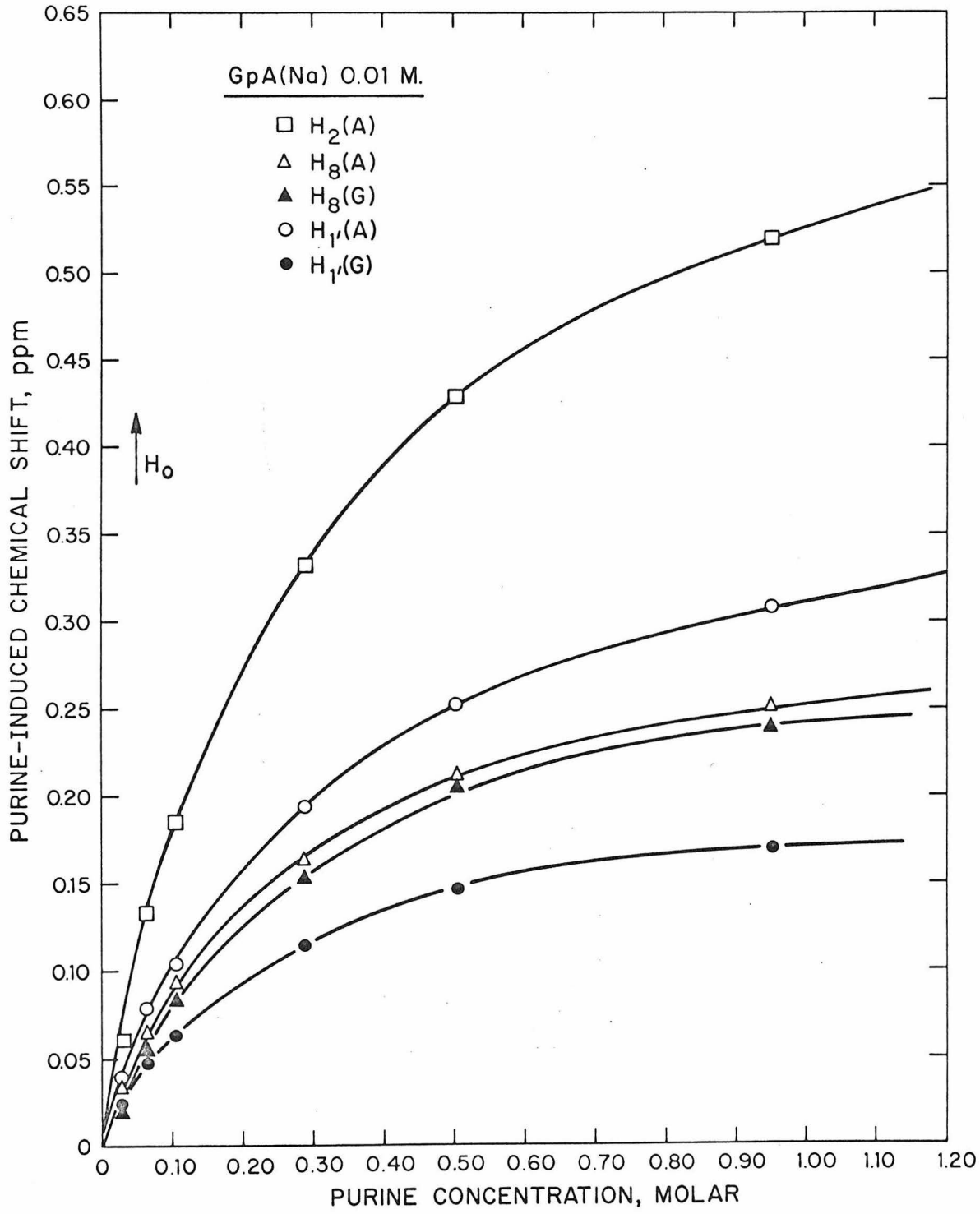


Figure 43. Purine-induced chemical shifts (100 Mcps) of the $H_2(A)$, $H_8(A)$, $H_8(G)$, $H_{1'}(A)$, and $H_{1'}(G)$ protons of GpA (sodium salt). GpA concentration = 0.01 M.

TABLE XIII. Total Purine-Induced Chemical Shifts of the H_2, H_8, and $H_{1'}$ Protons of ApA, ApG, and GpA at 1.0 M Purine.

Molecule	Chemical Shift,[a] ppm					
	$H_2(5')$	$H_2(3')$	$H_8(5')$	$H_8(3')$	$H_{1'}(5')$	$H_{1'}(3')$
ApA	0.52	0.48	0.20	0.31	0.25	0.17
ApG	- -	0.58	0.16	0.33	0.24	0.23
GpA	0.52	- -	0.25	0.24	0.31	0.17

[a]Upfield shift relative to solutions with no purine.

experiments involving several other dinucleoside monophosphates in which it was demonstrated that the protons of the 3'-base generally experience greater upfield shifts with purine addition (75, 79). However, under conditions of neutral pH, an opposite effect was observed for the H_2 resonances of ApA (Table XIII) due to the unusually strong base-stacking in ApA and the relatively large ring-current magnetic anisotropy of the adenine base (Chapter II, this thesis). (In ApA solutions which are sufficiently acidic (pD - 3.7) to cause destacking of the dinucleotide, the addition of purine also results in a larger upfield shift for the protons of the 3'-adenine base.) Since the purine-induced shifts of the $H_2(5')$ protons of ApA and GpA are almost identical (Table XIII), the contrasting behavior of the $H_2(A)$ proton resonances of ApA and the corresponding $H_2(A)$ resonances of the ApG-GpA isomer set with purine addition is due to the greater purine-induced shift of the $H_2(3')$ resonance of ApG relative to $H_2(3')$ of ApA (Table XIII). This greater shift is primarily the result of the smaller ring-current anisotropy of the guanine base in comparison to adenine. In ApA the

purine-induced shift of $H_2(3')$ is compensated by a reduction in the upfield shift of this proton due to the 5'-adenine ring-current magnetic anisotropy as purine intercalation occurs. Since the corresponding upfield shift of $H_2(3')$ of intramolecularly stacked ApG is caused by the guanine ring current, it is significantly smaller, and the reduction of the purine-induced shift by intramolecular destacking is less severe in this case. Accordingly, the purine-induced shift of $H_2(3')$ of ApG is greater than the analogous shift in ApA. The respective infinite dilution shifts of $H_2(3')$ of ApA (- 8.48 ppm relative to external TMS; Table V) and $H_2(3')$ of ApG (- 8.61 ppm) support the above conclusion. In addition we note that a smaller intramolecular base-stacking interaction in ApG relative to that of ApA is also consistent with the purine-binding experiments and with the infinite dilution chemical shifts of the base protons listed in Table V.

A comparison of the purine-induced chemical shifts listed in Table XIII indicates that purine-adenine base-stacking interactions are stronger than purine-guanine interactions. The purine-induced shift of the $H_8(5')$ resonance of ApG is significantly less than the $H_8(5')$ purine-induced shifts of ApA and GpA; and, similarly, the shift of $H_8(3')$ of GpA is much smaller than those of ApA and ApG. In each of these cases the proton of the guanine base experiences a smaller purine-induced shift than the analogous adenine base protons. In addition, the guanosine nucleoside proton in each of the two $H_{1'}$ ribose resonance pairs [the $H_{1'}(3')$ pair and the $H_{1'}(5')$ pair] of ApG and GpA is shifted upfield less with the addition of purine than the counterpart ribose proton of adenosine. Evidently, in the adenine-guanine

dinucleotides the purine base has a greater affinity to form intermolecular base stacks with adenine.

Perhaps the most interesting result pertaining to the purine-induced shifts of the H_8 resonances of ApG and GpA is that the induced shifts of the two H_8 GpA absorption signals are almost identical (Figure 43), while the H_8 adenine resonance of ApG is shifted to a much greater extent than H_8 of guanine (Figure 42).

In the previous discussions we have shown that the $H_8(5')$ proton of purine-purine dinucleotides is sterically shielded from the ring-current magnetic anisotropy of planar bases which are capable of forming stable base-stacks with the dinucleotide 5'-base. All other effects being equal, then, $H_8(3')$ would be expected to experience a greater upfield shift with the binding of purine than $H_8(5')$ in ApA, ApG, and GpA. In accordance with this observation, the purine-induced shift of the H_8 proton of the 3'-nucleoside of ApA is considerably greater than the $H_8(5')$ shift (Figure 18). However, in GpA the steric effect is compensated by the greater affinity of the purine probe molecule to bind to the adenine base, and the net result is very similar purine-induced shifts for the two H_8 resonances of GpA (Figure 43). Conversely, the H_8 adenine proton of the ApG is sterically less shielded from the magnetic anisotropy of an externally binding purine molecule than $H_8(G)$. This effect coupled with the intrinsically greater affinity of the adenine base to form stable base-stacks with purine results in a much larger purine-induced shift for the adenine H_8 proton in ApG (Figure 42).

As discussed previously, the $H_8(A)$ resonance of ApG is shifted further upfield ($\sim$0.09 ppm) than the $H_8(G)$ resonance of GpA at a purine

concentration of 1.0 M even though the adenosine nucleoside of ApG and the guanosine nucleoside of GpA are geometrically and conformationally quite similar and the site of the H_8 proton is essentially the same in the respective adenine and guanine bases. The greater purine-induced shift of the adenine proton reflects the greater affinity of the adenine base to form intermolecular base-stacks with purine. For this reason, the purine-induced shift of the H_8(A) proton of GpA is also larger (~0.09 ppm at 1.0 M purine) than the corresponding shift of the H_8(G) resonance of ApG.

The two H_8(A) resonances of ApG and GpA experience larger purine-induced shifts at a purine concentration of 1.0 M than the corresponding H_8(A) resonances of ApA. This reflects the greater stability of the intramolecular base-stacking in ApA. In a dinucleoside monophosphate which is more strongly intramolecularly stacked, the H_8 protons [especially H_8(5')] are more efficiently shielded from the ring-current effects of the externally binding purine; and, in addition, there is greater resistance to purine intercalation. Each of these effects results in smaller purine-induced shifts for the dinucleotide base protons.

The purine-induced shift of the $H_{1'}$(5') ribose proton of ApA is larger than the corresponding $H_{1'}$(3') shift (Table XIII). A comparison of the purine-induced shifts of the adenosine $H_{1'}$ protons of ApG and GpA shows that the proton of the 5'-nucleoside experiences a greater upfield shift as a result of purine binding. Similarly, of the two $H_{1'}$ guanosine protons of ApG and GpA, the purine-induced chemical shift is greater for the proton resonance of ApG. In fact, results of

purine-binding studies of several other dinucleoside monophosphates (79) have also shown that the $H_{1'}(5')$ ribose proton experiences higher field induced shifts when purine-dinucleotide complexes are formed than $H_{1'}(3')$. As noted previously, experimental evidence indicates that the sense of the partial helix formed by the sugar-phosphate backbone of the folded dinucleotides is right-handed. Accordingly, on the basis of the configuration of the dinucleotides, it appears that purine intercalation is responsible for the larger purine-induced shifts of the $H_{1'}(5')$ protons. The 5' ribose proton of a folded dinucleotide would be readily exposed to the magnetic anisotropy of an intercalating purine molecule while the 3' proton would not. On the other hand, $H_{1'}(3')$ would be expected to experience a greater upfield shift from externally stacking purine bases.

As in the case of the H_8 protons, two different effects determine the relative magnitudes of the purine-induced shifts of the two $H_{1'}$ protons in each of the adenine-guanine dinucleotides. First, the geometry of the stacked dinucleotides is such that the $H_{1'}(5')$ ribose proton resonance would be subject to a greater shift at a given purine concentration than the $H_{1'}(3')$ signal when other effects are neglected; and, second, the purine probe molecule forms more stable complexes with the base of the adenosine nucleoside. In GpA, where the $H_{1'}(5')$ proton is also an adenosine nucleoside proton, the purine-induced shift of the resonance of this proton is much greater than the $H_{1'}(3')$ shift (Figure 43). However, in ApG the purine-induced shifts of the two $H_{1'}$ resonances are approximately equal (Figure 42) because the 5'-nucleoside in this case is guanosine and purine has a smaller tendency to stack with the guanine base.

3. The Effect of Purine-Binding on the $H_{1'}$-$H_{2'}$ Coupling Constants. It is interesting to note the variation of the $H_{1'}$-$H_{2'}$ coupling constants with the addition of purine. In the temperature studies summarized in section E of this chapter, we reported that the magnitude of each of the $H_{1'}$-$H_{2'}$ coupling constants of ApG and GpA increases with increasing temperature; and we attributed this change to the intramolecular conformational changes which accompany the destacking of the bases of these dinucleotides. The $|J_{H_{1'}\text{-}H_{2'}}|$ values in ApG and GpA also increase with increasing purine concentration.

The variation of the $H_{1'}$-$H_{2'}$ coupling constants of ApG is illustrated in Figure 44. In the absence of purine, the value of $|J_{H_{1'}\text{-}H_{2'}}|$ of the adenosine nucleoside is ~4.0 cps. With the addition of purine, the magnitude of this coupling constant gradually increases until it reaches a value of ~5.6 cps at 1.1 M purine. The change observed in the $H_{1'}$-$H_{2'}$ coupling constant of guanosine is much less pronounced. The magnitude of this coupling constant increases from ~4.5 cps in the absence of purine to ~5.0 cps at 1.1 M purine.

The purine-induced changes in the $H_{1'}$-$H_{2'}$ coupling constants of GpA are shown in Figure 45. In this dinucleotide, $|J_{H_{1'}\text{-}H_{2'}}|$ of guanosine increases from ~4.0 cps in the absence of purine to ~5.4 cps at 1.0 M purine. Similarly, the magnitude of the adenosine coupling constant varies from ~4.3 cps to ~4.8 cps over the same concentration range.

The conformation of the two ribose rings of each of these dinucleotides changes as purine-binding occurs. We believe the origin of the conformational changes is the formation of purine-intercalated

Figure 44. Variation of the $|J_{H_1'-H_2'}|$ coupling constants of ApG with concentration of added purine. ApG concentration = 0.01 M.

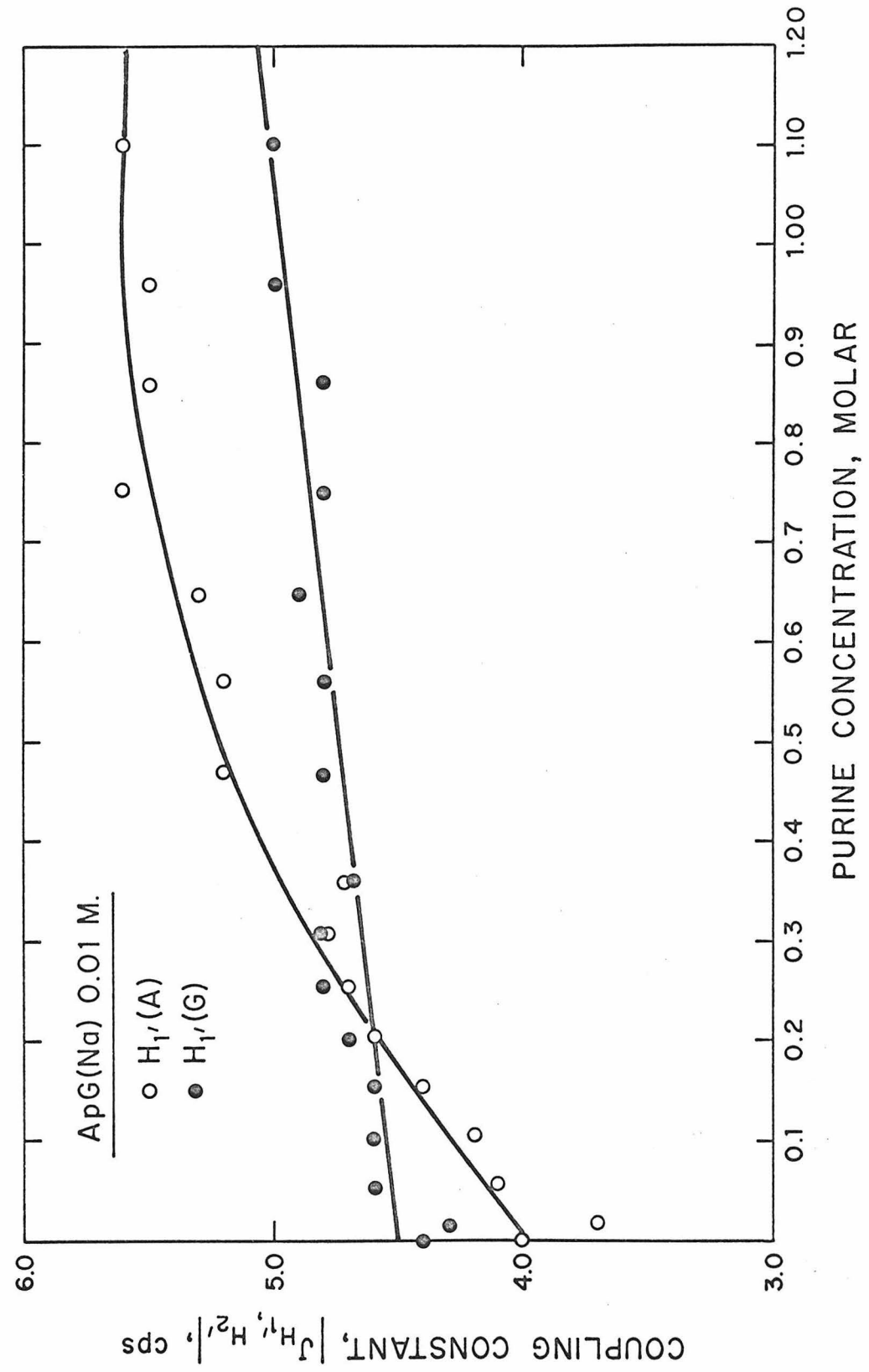
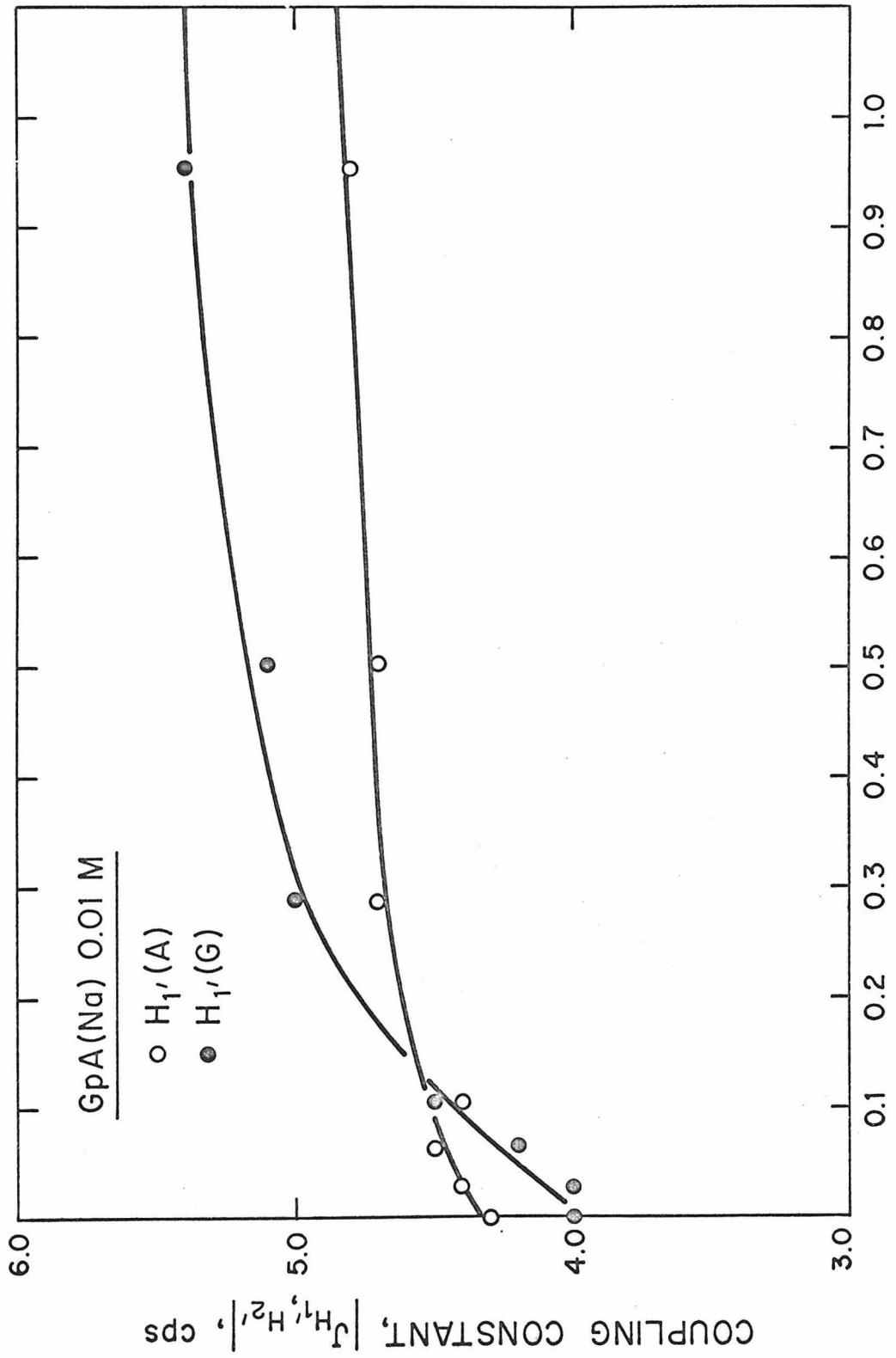

ApG(Na) 0.01 M.
o $H_{1'}$(A)
● $H_{1'}$(G)
COUPLING CONSTANT, $|J_{H_{1'}, H_{2'}}|$, cps
6.0
5.0
4.0
3.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.00
1.10
1.20
PURINE CONCENTRATION, MOLAR

Figure 45. Variation of the $|J_{H_1'-H_2'}|$ coupling constants of GpA with concentration of added purine. GpA concentration = 0.01 M.


GpA(Na) 0.01 M
o $H_{1'}$(A)
● $H_{1'}$(G)
COUPLING CONSTANT, $|J_{H_{1'},H_{2'}}|$, cps
6.0
5.0
4.0
3.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
PURINE CONCENTRATION, MOLAR

complexes. When the bases of the dinucleotide move apart to incorporate a purine molecule, there is, apparently, a tendency for the ribose conformations to be restored to that of the mononucleotide. Accordingly, the dinucleotide ribose rings assume more 2'-endo conformational character as the purine concentration increases, and this is manifested in increasing values of $|J_{H_{1'}-H_{2'}}|$. Increasing the temperature also has the effect of restoring the dinucleotide ribose conformations to that of the mononucleotide (2'-endo) as would be expected if the molecule is unfolded by the temperature increase.

It is interesting to compare the purine-induced changes in the $H_{1'}$-$H_{2'}$ coupling constants of ApG (Figure 44) and GpA (Figure 45) with those of ApA which are depicted in Figure 20. In the absence of purine, the value of $|J_{H_{1'}-H_{2'}}|$ of the 3'-nucleoside of each of these dinucleotides is smaller than the 5'-nucleoside coupling constant. The addition of purine effects greater changes in the 3'-nucleoside coupling constant in each case so that at approximately 1.0 M purine the magnitude of this coupling constant is significantly greater than the 5'-nucleoside coupling constant. Evidently, the purine-intercalation process causes greater changes in the 3'-ribose ring conformation in each of these purine-purine dinucleotides regardless of the base composition or base-sequence.

It is significant that no change was observed in the $H_{1'}$-$H_{2'}$ coupling constants of ApG as the concentration of this dinuelcotide was increased from 0 to 0.2 M, although intermolecular base stacking occurs as is evidenced by the concentration shifts of the ApG base protons. The intramolecular base-stacking in ApG is, evidently,

sufficiently strong to prevent significant perturbations of the ribose ring conformations as intermolecular complexes are formed.

On the other hand, the value of $|J_{H_{1'}\text{-}H_{2'}}|$ of 5'-GMP is somewhat concentration dependent. It decreases from ~6.3 cps at infinite dilution to ~5.6 cps at 0.11 M. Although only very small changes occur in the chemical shifts of the $H_{1'}$ and H_8 protons of 5'-GMP over the same concentration range (Figure 37), the variation of the $H_{1'}\text{-}H_{2'}$ coupling constant indicates that intermolecular complexes are formed; and that, as a result, some small changes occur in the ribose ring conformation. Just as the intramolecular base-stacking in the dinucleotides causes the ribose rings to assume more 3'-endo character, the intermolecular base-stacking in 5'-GMP also results in more 3'-endo character for the ribose ring. Similar concentration-induced changes in the $H_{1'}\text{-}H_{2'}$ coupling constants have been noted for both 3'-AMP and 5'-AMP; in these nucleotides, the coupling constant changes are accompanied by large concentration shifts due to the adenine ring-current magnetic anisotropy.

4. Purine Resonance Linewidths. Evidence for purine intercalation in dinucleotides 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 purine molecules intercalated in the dinucleotide is relatively high. The purine resonances were not extensively broadened in our purine-binding studies of ApG and GpA

because even at the lowest purine concentrations investigated, the proportion of unbound purine is relatively high. However, some purine line-broadening was observed for ApG and GpA complexes at the lower purine concentrations. As in the case of all other dinucleotides we have studied, the linebroadening effect is greatest for the purine H_6 resonance and least for the H_2 resonance. Under comparable conditions, the linewidths of the purine resonances are somewhat greater for the GpA complexes than for those of ApG. Since the purine line-broadening is presumably due to the effect of the nuclear magnetic dipoles of the 2'-, 3'-, and 5'- ribose protons of the 3'-nucleoside, the larger purine line-broadening in the case of GpA cannot be attributed to the greater stability of the adenine-purine base stacks relative to guanine-purine base stacks. We feel that the greater purine line resonances in GpA are caused by the formation of more intercalated complexes with this molecule. Evidently, the intramolecular base-stacking is somewhat less in GpA than in ApG so that purine intercalation is more probable. In agreement with these observations, we note that the purine resonances in ApA-purine mixtures are sharper than those of either ApG or GpA under comparable conditions.

G. Summary

1. Introduction. The p. m. r. spectra of adenylyl-(3'→5')-guanosine and guanylyl-(3'→5')-adenosine in D_2O have been studied as a function of dinucleotide concentration, temperature, and concentration of added purine. The chemical shifts and resonance linewidths of the

adenine and guanine ring protons and the $H_{1'}$ ribose protons of each dinucleotide have been monitored under various experimental conditions. In addition, the $H_{1'}$-$H_{2'}$ coupling constants and the details of the purine spectra have also been investigated.

2. Conclusions. The results of the present p. m. r. study of the conformational properties of ApG and GpA in aqueous solution indicate that the adenine and guanine bases in each of these molecules are strongly intramolecularly stacked at room temperature and neutral pH. A comparison of the results of this study with those previously reported in this thesis for ApA suggests that the intramolecular stacking interactions in each of these sequence isomers is somewhat weaker than the A-A stacking interactions in ApA. Consistent with this observation, the results of the purine-binding experiments demonstrated that the purine-adenine base-stacking interactions are energetically more favorable than the corresponding purine-guanine interactions under the conditions of our experiments. The present study also provided information concerning the rotational conformation of the bases about the glycosidic bond in the intramolecular stacks and the conformational changes in the ribose rings which accompany changes in intramolecular base-stacking. The data obtained for both ApG and GpA are consistent with stacked conformations in which both bases of the respective dinucleoside monophosphates are preferentially oriented in the anti conformation as in a similar dApdG or dGpdA (dA = deoxyadenosine; dG = deoxyguanosine) segment along a single strand of double helical DNA. Both ribose rings of each isomer assume more 3'-endo (versus 2'-endo) average conformational character as intramolecular base-stacking interactions

increase; the conformational change is more pronounced for the ribose ring of the 3'-nucleoside in each case. Finally, these dinucleotides have been shown to undergo extensive intermolecular association with polymerization proceeding beyond dimerization at concentrations greater than 0.1 M, emphasizing the necessity of working at low dinucleotide concentrations in experiments concerned with intramolecular effects.

IV. THE NATURE OF BASE-STACKING INTERACTIONS IN ADENYLYL-(3'→5')-URIDINE AND URIDYLYL-(3'→5')-ADENOSINE

A. Introduction

In the preceding chapters, we have discussed the base-stacking tendencies and the conformational properties of ApA, ApG, and GpA in aqueous solution as elucidated by proton magnetic resonance spectroscopy. In these studies it was shown that the base-stacking interaction between the two purine bases of each of these dinucleotides is relatively strong and that the purine rings are preferentially oriented in the anti conformation. In an effort to compare the conformational properties of these strongly stacked dinucleotides with the properties of dinucleotides consisting of at least one weakly stacking base, we have also studied the proton magnetic resonance spectra of ApU and UpA under various experimental conditions.

The concentration-dependent properties of these two sequence isomers are summarized in this chapter. In addition, the interaction of adenylyl-(3'→5')-uridine with 6-methylpurine and with ribosylpurine is reported; and the results of these studies are compared with similar studies of the binding of unsubstituted purine to ApU and UpA (79). The temperature dependence of the p.m.r. spectra of these two dinucleoside monophosphates has been studied by other workers (84) in this laboratory, and these data are not treated in the present discussion.

ApU differs from UpA only in the esterification of the phosphate group to the ribose moieties of the two nucleosides (Figure 46); and this structural difference may result in significant sequence-dependent effects. It is therefore of interest to compare the results obtained for these two sequence isomers.

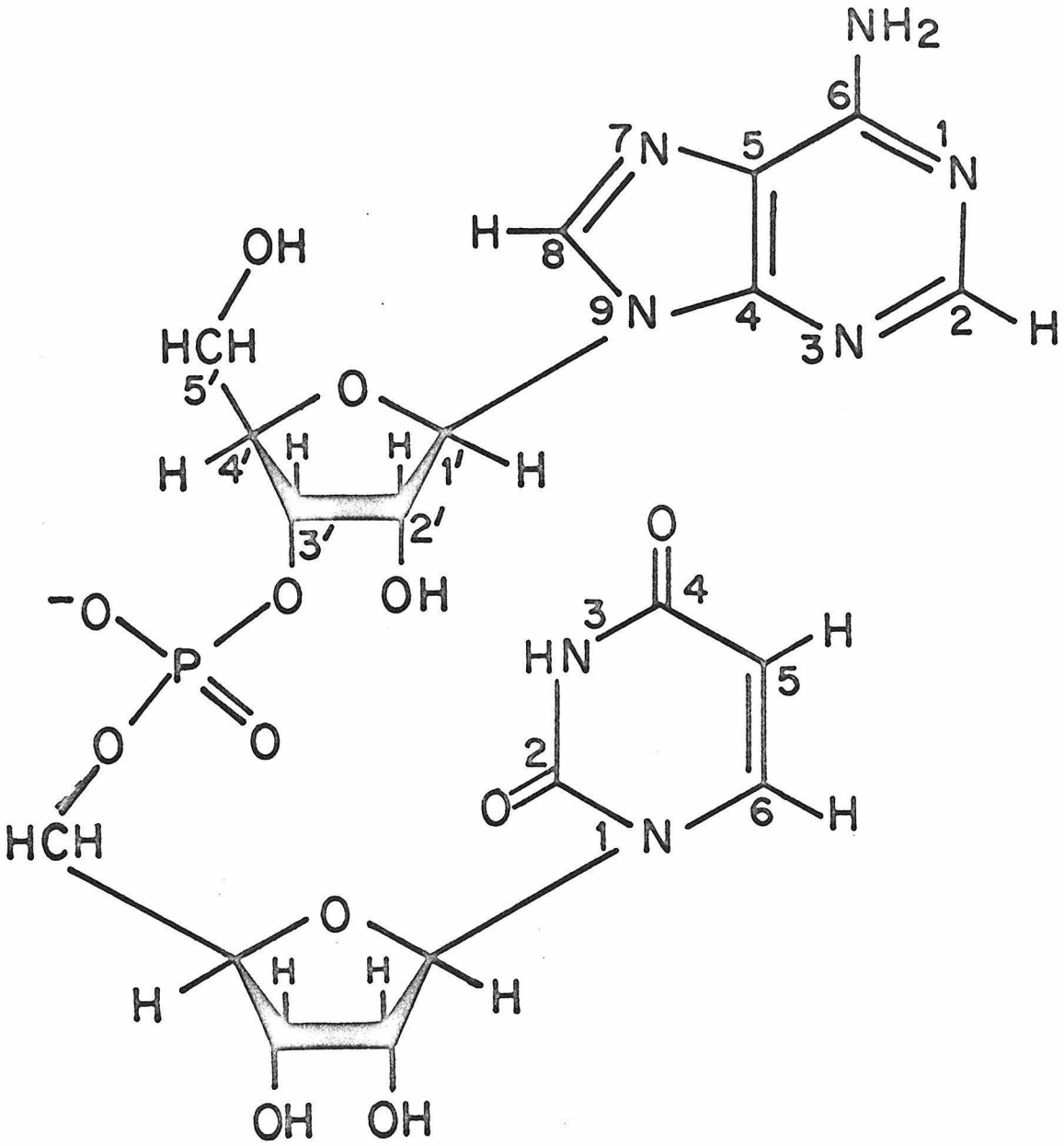
B. Experimental

A-grade adenosine, uridine, ApU, and UpA were obtained from Calbiochem, Los Angeles, California. Ribosylpurine and 6-methylpurine were purchased from K and K Laboratories, Inc., Hollywood, California. The 6-methylpurine was sublimed *in vacuo* before use, and the other materials were used without further purification. A column of Dowex 50W-X8 cation exchange resin was used to convert the dinucleoside monophosphates from the acid forms to the sodium salts. All samples were prepared in D_2O (99.7 mole %) supplied by Columbia Organic Chemicals, Columbia, South Carolina.

The spectra were recorded on a Varian HA-100 spectrometer in the usual manner (see Chapter II, this thesis). When necessary, weak signals were enhanced by time-averaging with a Varian C-1024 time-averaging computer.

C. Spectra and Assignment of ApU and UpA Proton Resonances

The 100 Mcps p.m.r. spectra in the region of the $H_2(A)$, $H_8(A)$, and $H_6(U)$ protons of the sodium salts of UpA (0.011 M) and ApU (0.015 M) at 30°C are shown in Figure 47. For the purpose of comparison, the same spectral regions of 0.01 M solutions of the component



ApU

Figure 46. Adenylyl-(3'→5')-Uridine.

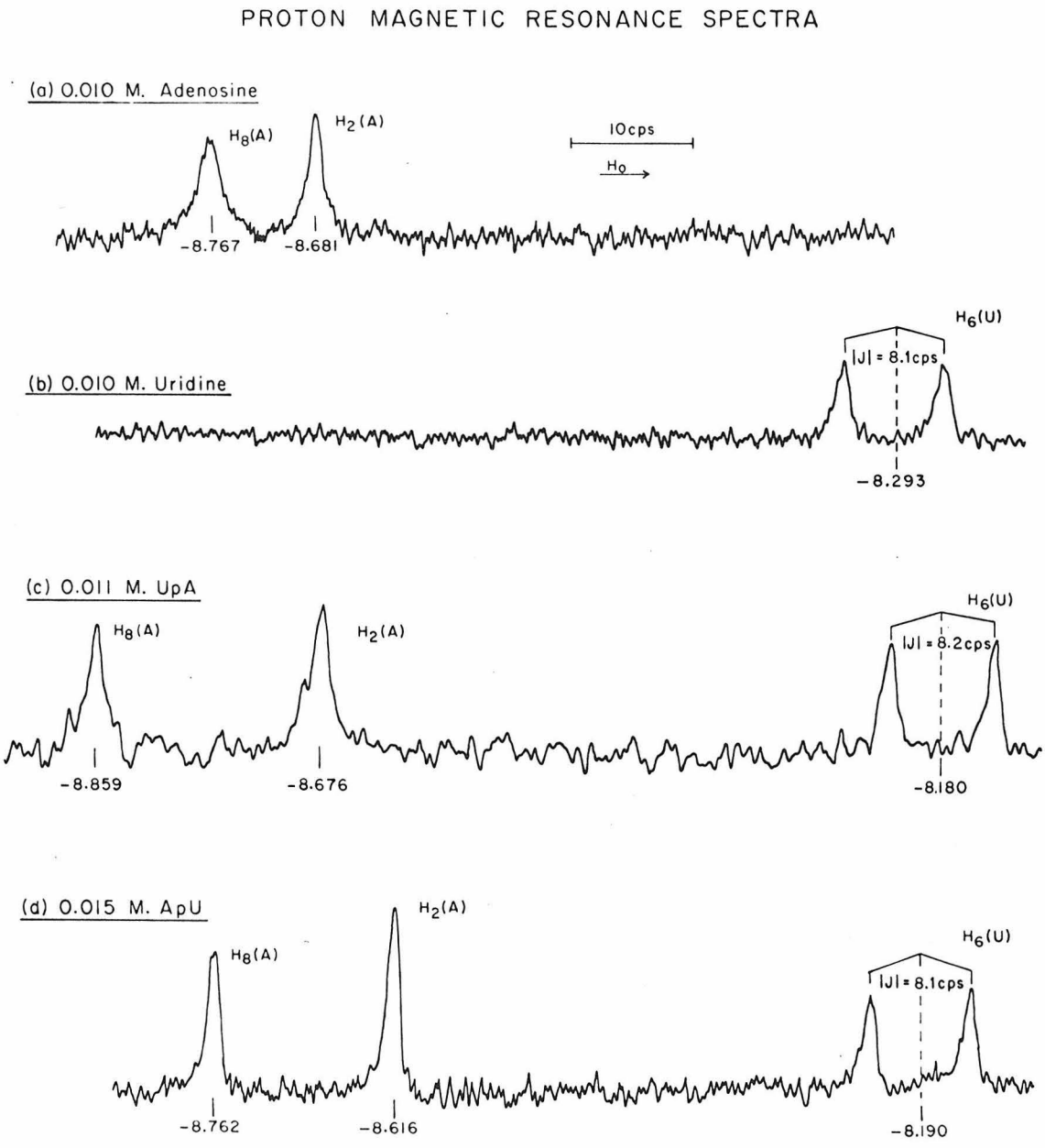


Figure 47. Proton magnetic resonance spectra of (a) 0.010 M adenosine, (b) 0.010 M uridine (data of J. H. Prestegard), (c) 0.011 M UpA, and (d) 0.015 M ApU in the $H_2(A)$, $H_8(A)$, $H_6(U)$ spectral region at 30°C and approximately neutral pD.

nucleosides, adenosine and uridine, are also shown. The assignment of the base proton resonances of each of the dinucleotides is straightforward. As in the case of ApA, ApG, and GpA, the resonances of the adenine H_8 protons were identified by deuterium exchange at elevated temperatures, and these are the lowest field signals in the p. m. r. spectra of both ApU and UpA. The adenine H_2 resonance of each of these dinucleotides is readily distinguished from the uracil H_6 proton by a comparison with the nucleoside spectra. The H_6(U) proton is spin-spin coupled to H_5(U), and each of these resonances appears as a doublet in the dinucleotide and nucleoside spectra ($|J_{H_5-H_6}| \cong 8.2$ cps in both ApU and UpA). For both of these dinucleoside monophosphates the spectral assignment of the protons in the lowest field spectral region is, in the order of increasing field: H_8(A), H_2(A), and H_6(U).

The p. m. r. spectra of the $H_{1'}$ ribose protons and the H_5(U) protons of ApU and UpA are shown in Figure 48. Again, the same spectral region is shown for uridine and adenosine under comparable experimental conditions. The assignments of the dinucleotide resonances depicted in this figure were also made by a comparison with the nucleoside spectra. The adenosine $H_{1'}$ resonances appear at significantly lower fields than the $H_{1'}$ resonances of uridine due to the ring-current magnetic anisotropy of the adenine base. The spin-spin coupling of the $H_{1'}$ and $H_{2'}$ protons of each ribose ring gives rise to $H_{1'}$ resonance doublets in each of the dinucleotide and nucleoside spectra. The magnitudes of the respective $H_{1'}$-$H_{2'}$ and H_5-H_6 coupling constants of ApU, UpA, and the constituent nucleosides are listed in Table XIV. In each of the molecules possessing a uridine nucleoside, the magnitude of the $H_{1'}$-$H_{2'}$ coupling constant

PROTON MAGNETIC RESONANCE SPECTRA

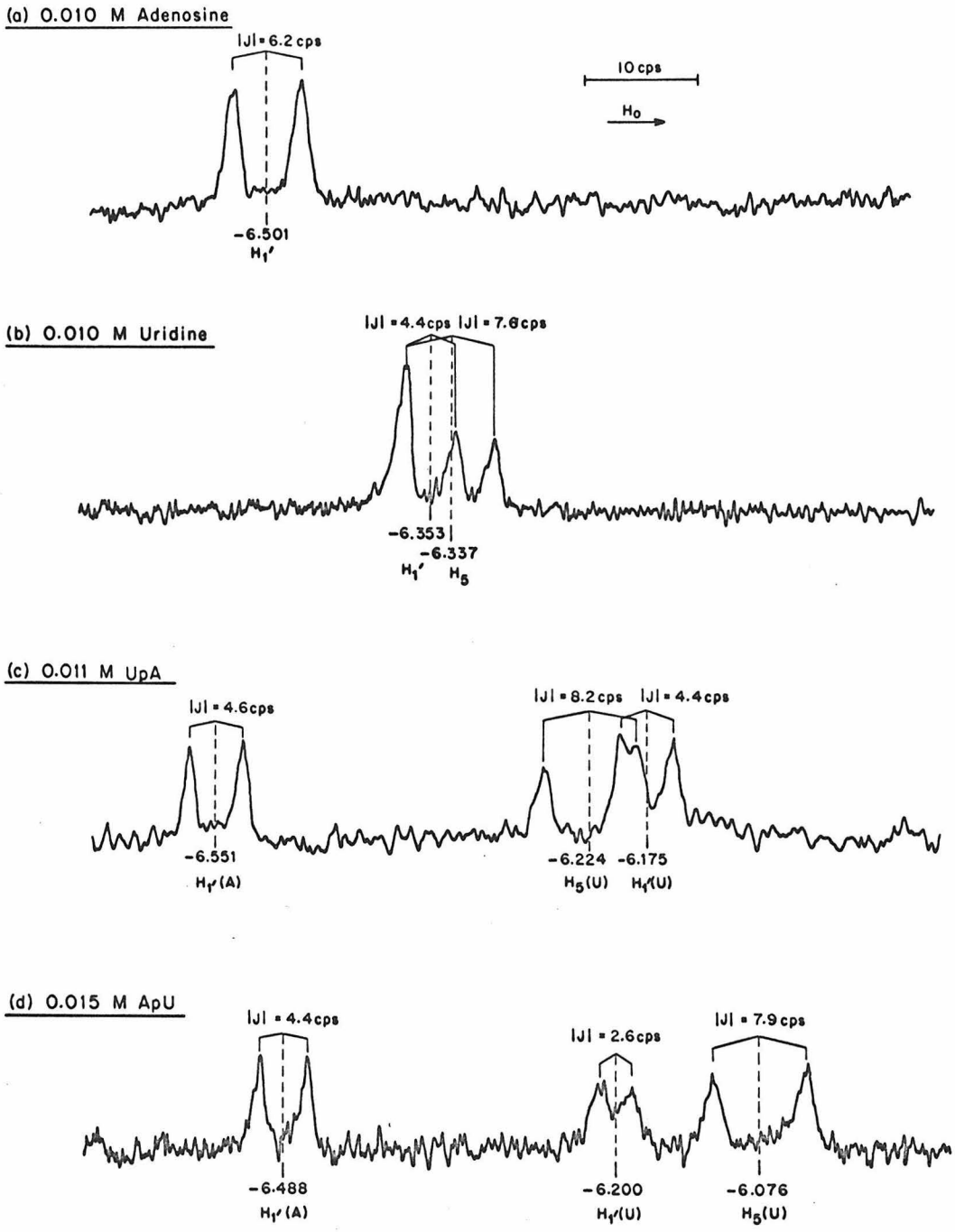


Figure 48. Proton magnetic resonance spectra of (a) 0.01 M adenosine, (b) 0.010 M uridine (data of J. H. Prestegard), (c) 0.011 M UpA, and (d) 0.015 M ApU in the $H_{1'}$, $H_5(U)$ spectral region at 30°C and approximately neutral pD.

is smaller than $|J_{H_5\text{-}H_6}|$. Accordingly, the H_5(U) resonance may be easily distinguished from the $H_{1'}$ ribose resonances. The coupling constants, $|J_{H_{1'}\text{-}H_{2'}}|$, of the dinucleotides are smaller than those of the respective nucleosides due to conformational changes which occur in the dinucleotide ribose rings as a result of intramolecular interactions. These conformational changes have been discussed in detail in Chapter III, and further elaboration is not necessary here.

TABLE XIV. $H_{1'}$-$H_{2'}$ and H_5-H_6 Coupling Constants[a] of Uridine, Adenosine, ApU and UpA

Molecule	Coupling Constant, $\|J\|$, cps		
	$H_{1'}$-$H_{2'}$(A)	$H_{1'}$-$H_{2'}$(U)	H_5-H_6(U)
Uridine	- -	4.5	8.1
Adenosine	6.1	- -	- -
ApU	4.3	2.4	8.2
UpA	4.6	4.4	8.2

[a]Concentration $\simeq$ 0.01 M.

In addition to the resonances shown in Figures 47 and 48, the ribose $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, and $H_{5''}$ protons give rise to resonances in the spectral region 4.0 to 5.3 ppm downfield from external TMS. There is extensive spin-spin coupling among these protons in each ribose ring; and, in addition, the analogous resonances of the 3'- and 5'-esterified nucleosides do not coincide. Due to these complications, this spectral region was not extensively studied. Finally, the hydroxyl, amino, and N-H protons do not give distinct resonances because of the rapid chemical exchange of these protons with the solvent D_2O.

D. Discussion of Infinite Dilution Proton Chemical Shifts

The extrapolated infinite dilution chemical shifts of various protons of uridine, adenosine, ApU, and UpA are presented in Table XV. With the exception of the H_8(A) proton of ApU and H_2(A) of UpA, all of the dinucleotide resonances monitored are shifted from their spectral positions in the component nucleosides. These shifts are due in part to the influence of the neighboring base of the dinucleotide. For example, the adenine base in both ApU and UpA possesses a large ring-current magnetic anisotropy which can effect large changes in the chemical shifts of the protons of the uracil base. As a consequence of its negative charge and the resulting electric field, the phosphate group can also produce changes in the resonance frequencies of protons of the dinucleotides. The resonances of analogous protons of the 3'- and 5'-nucleosides are influenced to a different extent by the phosphate effect due to the difference in the phosphate attachment. In addition, it has recently been demonstrated (104) that the chemical shifts of the adenine H_8 proton and the uracil H_6 proton are sensitive to the rotational conformation of the base about the glycosidic bond. In the dinucleotides, intramolecular interactions can affect both the rotational conformation of the bases and the ribose ring conformations. We note, therefore, that the chemical shifts of the H_6(U), H_8(A), and $H_{1'}$ ribose protons of ApU and UpA may be determined by several different intramolecular effects.

The influence of the phosphate group on the uridine proton chemical shifts of ApU and UpA can be somewhat compensated by comparing

TABLE XV. Extrapolated Infinite Dilution Chemical Shifts of Various Proton Resonances of Uridine, Adenosine, ApU, and UpA

Molecule	Chemical Shift[a] (ppm)					
	H_8(A)	H_2(A)	$H_{1'}$(A)	H_5(U)	H_6(U)	$H_{1'}$(U)
Uridine	- - -	- - -	- - -	6.35	8.29	6.32
Adenosine	8.78	8.69	6.53	- - -	- - -	- - -
ApU	8.784	8.651	6.507	6.074	8.197	6.200
UpA	8.865	8.696	6.558	6.232	8.180	6.175

[a]Downfield from external TMS.

the resonance frequencies of these protons with the corresponding protons of UpU. In Table XVI the H_6(U), H_5(U), and $H_{1'}$(U) infinite dilution chemical shifts of ApU, UpA, and UpU are summarized along with the upfield shift of the respective uridine proton resonances which results from the substitution of an adenine base for the neighboring uracil base in UpU. We note that the ring-current magnetic anisotropy of uracil is negligible so that the chemical shifts of neighboring protons are not influenced to a great extent by this base.

Since each of the H_5(U) protons of ApU and UpA is relatively far-removed from the molecular backbone of the dinucleotide, the higher field shifts of these protons relative to the analogous H_5 UpU protons are derived almost exclusively from the ring-current anisotropy of the respective neighboring adenine bases. These shifts indicate that although uracil is a relatively weakly stacking base, intramolecular base-stacking does occur in both ApU and UpA at $\sim 30^{\circ}$C. The much

TABLE XVI. Extrapolated Infinite Dilution Chemical Shifts of the Uridine Protons of ApU, UpA, and UpU; and the Shifts Resulting from the Substitution of Adenine for Uracil as the Adjacent Base in UpU

Molecule	Chemical Shift[a] (ppm)					
	H_6		H_5		$H_{1'}$	
	(3')	(5')	(3')	(5')	(3')	(5')
ApU	- - -	8.197	- - -	6.074	- - -	6.200
UpA	8.180	- - -	6.232	- - -	6.175	- - -
UpU[b]	8.332	8.342	6.308	6.333	6.332	6.380
Shift	+0.152	+0.145	+0.076	+0.259	+0.157	+0.180

[a]Downfield from external TMS.

[b]Data of B. W. Bangerter; Reference (79).

larger higher field shift of the ApU resonance is the result of the conformational properties of the uracil bases in the dinucleoside monophosphates. Prestegard and Chan have shown that the uracil base of the uridine monophosphates preferentially assumes the anti conformation about the glycosidic bond (104). Accordingly, the uracil bases of the dinucleotides might also be expected to be energetically more stable in the anti conformation. The H_5(U) proton of intramolecularly stacked ApU is considerably more exposed to the ring-current anisotropy of the neighboring adenine base than H_5(U) of UpA when each of the uracil bases of these molecules is anti oriented. Therefore, the larger upfield shift of the ApU H_5 proton may be readily understood in terms of the base conformations of the dinucleoside monophosphates. Differences

in the stability of the intramolecular base-stacking in the two isomers could also contribute to the different chemical shifts of the two H_5(U) protons of this isomer set.

Factors determining the extent of the higher field shifts of the H_6(U) protons of ApU and UpA are considerably more complicated. Prestegard and Chan (104) have also shown that the H_6(U) protons of these dinucleotides would be expected to shift downfield relative to the corresponding nucleoside or mononucleotide resonances due to the formation of intramolecular base-stacks and the consequential smaller average distance between H_6(U) and the furanose ether oxygen. (The magnetic anisotropy of the ether oxygen apparently deshields the H_6(U) proton.) The intramolecular base-stacking interaction in either ApU or UpA is expected to be stronger than the U-U base-stacking interaction in UpU. The H_6(U) resonances of the adenine-uracil dinucleotides would, therefore, be expected to appear at lower fields (if only the magnetic effect of the furanose ether oxygen is considered) than the corresponding UpU resonances. As indicated in Table XVI, however, the H_6(U) resonances are at appreciably higher fields in ApU and UpA. It is evident that the uracil H_6 protons of both of these dinucleotides are also exposed to the neighboring adenine ring-current anisotropy. On the basis of the large difference in the upfield shifts of the H_5(U) protons, we might also expect a large differential shift for the H_6(U) resonance pair. The H_6(U) shifts are, however, very similar. Apparently, the compensating downfield shifts due to the furanose ether oxygen atoms and to other magnetic effects are different in the two dinucleotides. In any event, the reasonably large ring-current shifts

observed for the H_5 and H_6 resonances of both ApU and UpA indicate that each of these molecules is somewhat intramolecularly stacked at 30°C.

Several different factors also determine the chemical shifts of the $H_{1'}$(U) protons of ApU and UpA. As discussed in Chapter III, the $H_{1'}$ ribose protons are sensitive to the respective conformations of their ribose rings. The dependence of the $H_{1'}$ chemical shift on the ribose ring conformation is largely due to the shielding effect of the 2'-oxygen atom. When the ribose ring assumes the 3'-endo conformation as opposed to 2'-endo, the $H_{1'}$ proton is somewhat closer to $O_{2'}$, and the $H_{1'}$ resonance frequency is shifted to higher field. In the dinucleoside monophosphates, strong intramolecular base-stacking results in more 3'-endo character for the ribose rings. Accordingly, the larger upfield shifts of the $H_{1'}$ protons in the ApU-UpA isomer set relative to the corresponding $H_{1'}$ UpU chemical shifts reflect the stronger base-stacking interactions in the former molecules.

The $H_{1'}$ uridine chemical shifts may also be sensitive to the rotational conformation of the uracil base about the glycosidic bond due to the magnetic anisotropy of the 2-keto-oxygens. This effect in relationship to dinucleotides has been discussed in detail in Section E of Chapter III, and we shall not elaborate further here.

Finally, it is reasonable to assume that the ring-current anisotropy of the neighboring base has only a small effect on the chemical shift of the dinucleotide $H_{1'}$ protons because the net upfield shift effected in each of the $H_{1'}$ resonances of UpU with adenine substitution in the opposing nucleoside is quite similar. Due to the geometry of the stacked

dinucleotides, a similar shift would not be expected for each of the $H_{1'}$ protons if ring-current effects are important in determining the chemical shifts of these protons.

The shifts resulting from the substitution of uracil for adenine as the adjacent base in ApA are tabulated in Table XVII for each of the adenine protons. Due to the negligible ring-current magnetic anisotropy of uracil and its smaller tendency to form base-stacks, each adenine resonance is shifted appreciably downfield. Consistent with the preferential 3'-anti, 5'-anti conformation of ApA, the extent of the downfield shifts is greatest for $H_8(5')$ and $H_2(3')$. However, the shifts of the other protons are sufficiently large to indicate that the syn conformations are also significantly populated in ApA at 30°C.

TABLE XVII. Extrapolated Infinite Dilution Chemical Shifts of the Adenine Protons of ApU, UpA, and ApA; and the Shifts Resulting from the Substitution of Uracil for Adenine as the Adjacent Base in ApA

Molecule	Chemical Shift[a] (ppm)			
	H_8		H_2	
	(3')	(5')	(3')	(5')
ApU	8.784	- - -	8.651	- - -
UpA	- - -	8.865	- - -	8.696
ApA	8.68	8.71	8.48	8.61
Shift	-0.10	-0.15	-0.17	-0.09

[a]Downfield from external TMS.

The data of Table XVII do not demonstrate the explicit effect of a neighboring uracil base on the chemical shifts of the adenosine protons of a dinucleotide. This can be examined by comparing the infinite dilution chemical shifts of these protons in ApU and UpA with the corresponding shifts of the 3'- and 5'-mononucleotides of adenosine. This comparison is made in Table XVIII. The data indicate that the esterification of uridine to 3'-AMP or to 5'-AMP results in only very small shifts for all of the protons except H_8 of the 5'-mononucleotide and H_2 of 3'-AMP. The relatively large shift of the $H_8(5')$ proton undoubtedly reflects the difference in the phosphate shielding effect in the mononucleotide and the dinucleotide. The sizable upfield shift observed for the $H_2(3')$ proton is surprising in view of the lack of sensitivity of the adenine H_2 chemical shift to the molecular backbone and the small

TABLE XVIII. Chemical Shifts[a] of Adenosine Proton Resonances Resulting from Esterification of Uridine to the Adenosine 3'- and 5'-Monophosphates

Molecule	Proton		
	H_8	H_2	$H_{1'}$
ApU	8.784	8.651	6.507
3'-AMP-Na^{+b}	8.805	8.697	6.530
Shift	+0.021	+0.046	+0.023
UpA	8.865	8.696	6.558
5'-AMP-Na^{+b}	8.957	8.705	6.578
Shift	+0.092	+0.009	+0.020

[a] In ppm.

[b] pD = 5.9; data of Schweizer et al., Reference (52).

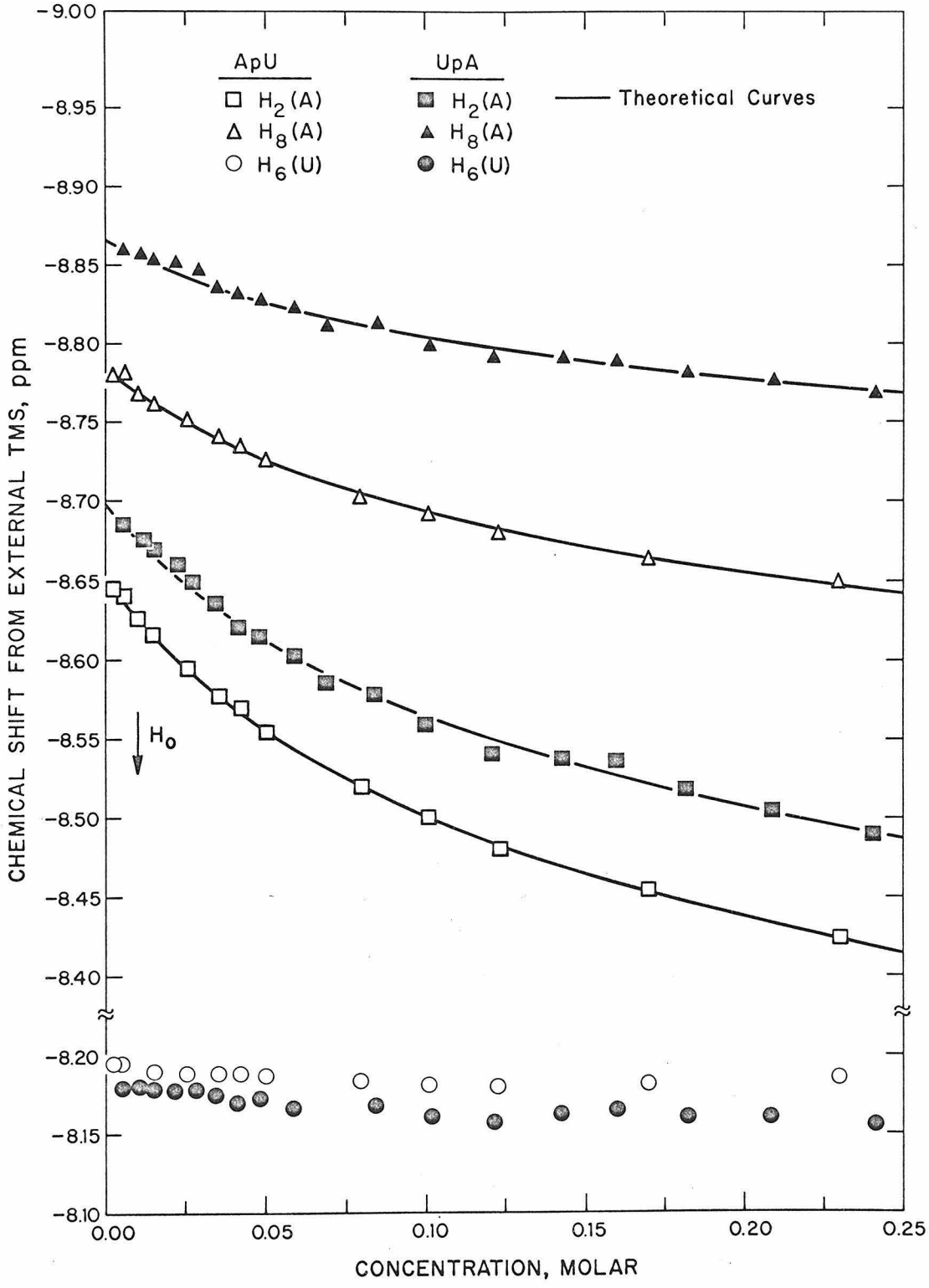
ring-current magnetic anisotropy of the uracil base. The present observations would seem to indicate that the uracil base has a small influence on the resonance frequency of a proton located in its immediate vicinity.

E. Self-Association of the Dinucleoside Monophosphates

1. Introduction. The effect of concentration on the p.m.r. spectra of ApU and UpA was studied to determine the extent of the intermolecular association of these dinucleotides, and to ascertain the effects of self-association on the chemical shifts and resonance linewidths of the various protons. The self-association of monomeric bases, nucleosides, and nucleotides as well as ApA, ApG, and GpA via vertical base-stacking has been noted previously in this thesis; and both ApU and UpA would be expected to exhibit similar behavior. As we have indicated in the preceding chapters, an understanding of the nature of the intermolecular association process is important in interpreting correctly the results of experiments designed to study the intramolecular base-stacking interactions in the dinucleotide molecules.

2. Concentration Dependence of the Dinucleotide Proton Chemical Shifts and Resonance Linewidths. The chemical shifts of the base protons and the $H_{1'}$ ribose protons of ApU and UpA were monitored over the concentration range 0.005 M to 0.24 M at $\sim 30^{\circ}C$, and the results are summarized in Figures 49 and 50. The extrapolated infinite dilution shifts have already been discussed in Section D of this chapter, and they are tabulated in Table XV. The net concentration shifts at 0.10 M and at 0.23 M are listed in Table XIX. Changes in the bulk magnetic susceptibility of the solution with concentration are small over these

Figure 49. Concentration dependence of the chemical shifts of $H_2(A)$, $H_8(A)$, and $H_6(U)$ protons of ApU and UpA (sodium salts) at $\sim 30^{\circ}C$. The solid lines represent calculated curves (see Section E, Part 3).


ApU
UpA
$H_2(A)$
$H_8(A)$
$H_6(U)$
$H_2(A)$
$H_8(A)$
$H_6(U)$
Theoretical Curves
H_o
CHEMICAL SHIFT FROM EXTERNAL TMS, ppm
CONCENTRATION, MOLAR
-9.00
-8.95
-8.90
-8.85
-8.80
-8.75
-8.70
-8.65
-8.60
-8.55
-8.50
-8.45
-8.40
-8.20
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-8.10
0.00
0.05
0.10
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0.20
0.25

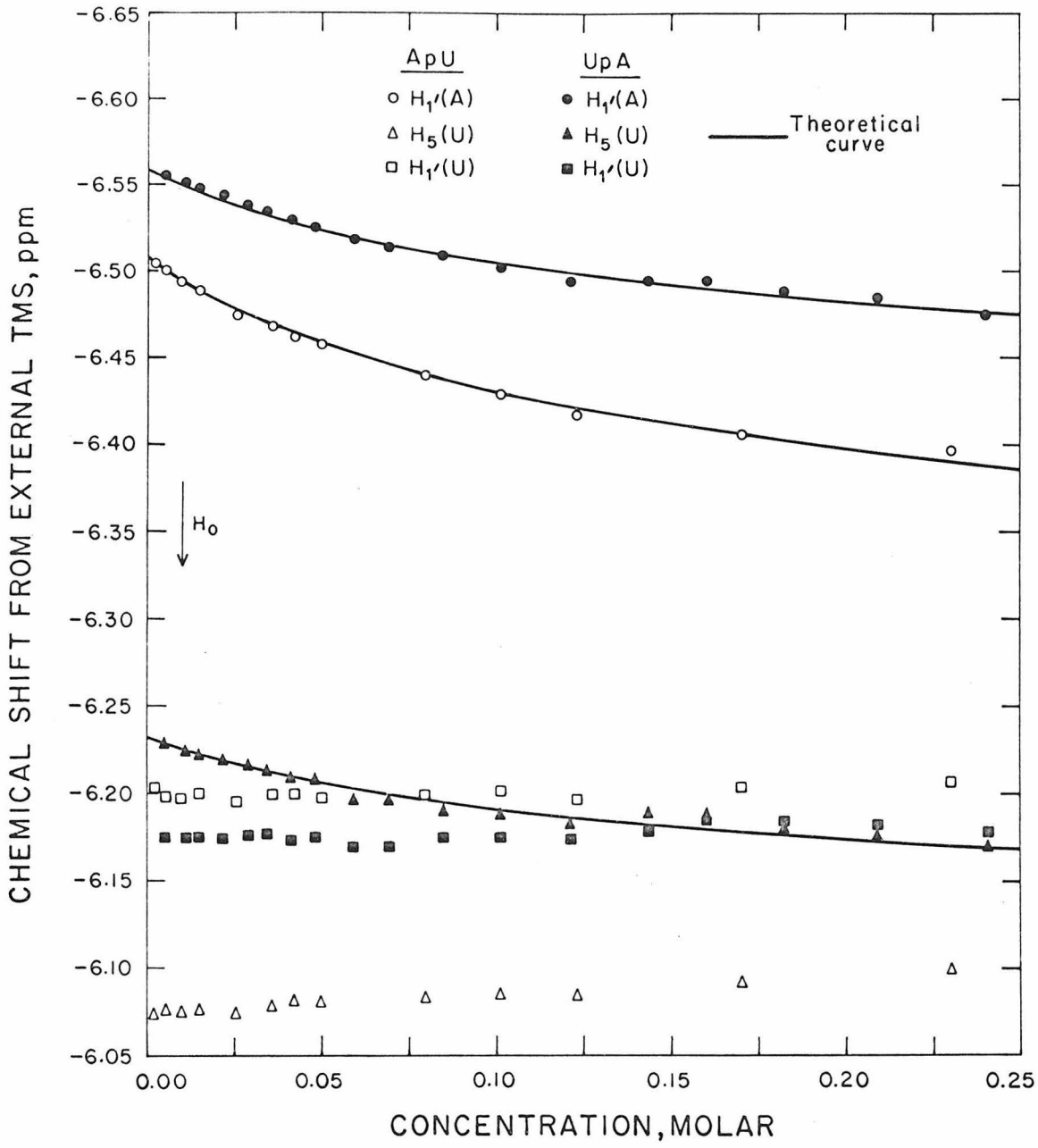


Figure 50. Concentration dependence of the chemical shifts of the $H_1'(A)$, $H_1'(U)$, and $H_5(U)$ protons of ApU and UpA (sodium salts) at ~ 30°C. The solid lines represent calculated curves (see Section E, Part 3).

TABLE XIX. Concentration Shifts[a] of the Proton Resonances of ApU and UpA at 30°C.

Proton	Concentration Shift at 0.10 M		Concentration Shift at 0.23 M	
	ApU	UpA	ApU	UpA
H_8(A)	0.091	0.065	0.134	0.093
H_2(A)	0.149	0.136	0.226	0.199
H_6(U)	0.015	0.019	0.010	0.023
$H_{1'}$(A)	0.078	0.056	0.110	0.080
H_5(U)	-0.011	0.044	-0.025	0.060
$H_{1'}$(U)	0.000	0.000	-0.005	-0.004

[a]Upfield shift in ppm from the extrapolated infinite dilution chemical shift values.

concentration ranges, and no corrections have been applied to the data of Table XIX. Each of the adenosine resonances of both dinucleotides is shifted to higher field with increasing concentration, and significant shifts are experienced by these resonances in the concentration range 0.10 M to 0.23 M. With the exception of H_5(U) of UpA, the uridine proton chemical shifts exhibit little concentration dependence.

The higher field shifts observed for many of the protons of ApU and UpA with increasing concentration indicate that these molecules self-associate intermolecularly by base-stacking. Since the uracil base has a negligible ring-current magnetic anisotropy, the concentration shifts in ApU and UpA must be a consequence of the adenine ring current. The large shifts of the adenine ring protons indicate considerable A-A base-stacking in the intermolecularly associated species of each

dinucleotide. In the case of ApU, none of the uridine protons is significantly affected by the intermolecular association, implying that relatively little A-U intermolecular base-stacking occurs in the ApU complexes. A similar observation has been made for the cytidine protons of ApC (79). However, each of these protons shifts farther upfield with increasing concentration than the corresponding uridine proton of ApU; this is an indication that the cytosine base is somewhat more involved in the formation of intermolecular complexes than uracil. In comparison to the corresponding uracil base proton resonances of ApU, the H_5 and H_6 resonances of UpA show larger concentration shifts; and the extent of A-U base-stacking would appear to be greater in the intermolecular association of UpA. The upfield shifts of the cytidine resonances of CpA are greater than the analogous UpA uridine concentration shifts as would be expected if the cytosine base forms more stable base stacks with adenine than does uracil.

In the ApU-UpA isomer set, the greater concentration shifts of the uracil protons of UpA is somewhat surprising. A consideration of the possible types of intermolecular complexes is helpful in understanding this sequence-dependent effect. If a dinucleotide is strongly intramolecularly stacked, self-association will proceed primarily via base-stacking of the external or exposed faces of the bases of the folded molecule. The differences in the concentration shifts of the uracil protons of ApU and UpA could be attributed to different A-U base-stacking interactions and different adenine-uracil base overlap as the respective folded dinucleotides associate intermolecularly. If this is the case, however, different concentration shifts might also be expected for the

$H_{1'}$(U) protons of ApU and UpA. Since neither of these protons experiences a significant concentration shift, it would appear that intermolecular association of folded dinucleotide molecules is not entirely responsible for the sequence-dependent uracil concentration shifts. It is not unreasonable to assume that the formation of self-intercalated intermolecular dimer complexes may account for the differential concentration shifts of the uracil protons of ApU and UpA. With the formation of dimer complexes in which the adenine base of one molecule is inserted between the two bases of the other, the H_5(U) proton (and to a small extent the H_6(U) proton) of both UpA and ApU would be expected to shift to higher fields due to the anisotropy of the intercalated adenine base. This shift is compensated, however, by a reduction in the intramolecular ring-current effect of the adjacent adenine base of the dinucleotide as intramolecular base-stacking is disrupted by intercalation. Since the uracil protons of ApU experience a larger upfield shift from the effects of the neighboring adenine base in the intramolecular stack than those of UpA (Table XVI) and since an incorporated adenine base is expected to shift the uracil proton resonances in both dinucleotides to a similar extent, the larger concentration shifts of the UpA uracil resonances are not unexpected.

The concentration dependence of the resonance linewidths of the protons of ApU and UpA provides additional evidence that the sequence-dependent properties of the uracil proton concentration shifts of this isomer set arise primarily from the formation of self-intercalated complexes. The resonance linewidths (at various concentrations) of the monitored protons of ApU and UpA are presented in Table XX. We have

TABLE XX. Concentration Dependence of the Linewidths of the Base Proton and $H_{1'}$ Ribose Proton Resonances of ApU and UpA

Concentration (Molar)	Resonance Linewidth at Half Signal Intensity, cps											
	H_8(A)		H_2(A)		$H_{1'}$(A)		H_5(U)		H_6(U)		$H_{1'}$(U)	
	ApU	UpA	ApU	UpA	ApU	UpA	ApU	UpA	ApU	UpA	ApU	UpA
0.24	- -	4.2	- -	5.0	2	1.8	- -	>7[a]	- -	1.6	- -	2.5[a]
0.23	2.1	- -	1.5	- -	- -	- -	1.8	- -	1.8	- -	2.5	- -
0.21	- -	5.2	- -	4.4	- -	2.5	- -	a	- -	2.2	- -	a
0.18	- -	5	- -	6	- -	2	- -	a	- -	2.2	- -	a
0.17	1.7	- -	1.1	- -	2	- -	1.8	- -	1.2	- -	2.5	- -
0.16	- -	4.2	- -	4.8	- -	1.7	- -	6[a]	- -	1.8	- -	2[a]
0.14	- -	4.5	- -	4.5	- -	2.6	- -	>5[a]	- -	2.5	- -	a
0.12	2.0	3.8	1.6	3.8	2	2	2	>4[a]	1.8	1.2	2.5	2[a]
0.10	1.5	4.4	1.1	4.0	2	2.0	2	4.4	1.2	2.0	2.2	2.0
0.08	1.6	3.4	- -	3.3	2	1.8	2	3.2	1.2	1.4	2.2	2.0
0.07	- -	2.4	1.0	2.6	- -	1.2	- -	2.0	- -	1.2	- -	1.8
0.06	- -	3.0	- -	2.6	- -	1.5	- -	3.6	- -	2.0	- -	2.6
0.05	1.2	2.8	0.9	2.7	1.4	1.3	1.4	2.0	1.1	1.2	2	1.8
0.04[b]	1.4	3.0	1.0	2.9	1.3	1.9	1.5	2.8	1.0	1.6	2.0	2.4
0.035[b]	1.2	- -	0.7	- -	1.5	- -	1.7	- -	1.2	- -	2.4	- -
0.034[b]	- -	2.2	- -	2.4	- -	1.6	- -	2.0	- -	1.4	- -	1.8
0.025[b]	1.3	- -	0.9	- -	1.4	- -	1.5	- -	1.2	- -	2.0	- -

TABLE XX.--Continued

Concentration (Molar)	Resonance Linewidth at Half Signal Intensity, cps											
	H_8(A)		H_2(A)		$H_{1'}$(A)		H_5(U)		H_6(U)		$H_{1'}$(U)	
	ApU	UpA	ApU	UpA	ApU	UpA	ApU	UpA	ApU	UpA	ApU	UpA
0.021[b]	- -	3.0	- -	2.8	- -	1.6	- -	2.5	- -	1.8	- -	2.0
0.015[b]	1.1	2.6	1.0	2.4	1.2	2.0	1.8	2.3	1.0	1.8	2.2	2.4
0.011[b]	- -	1.6	- -	1.6	- -	1.4	- -	1.6	- -	1.0	- -	1.4
0.010[b]	1.4	- -	1.2	- -	1.2	- -	1.0	- -	1.0	- -	2	- -
0.005[b]	1.4	2	0.8	2	1.6	1.2	1.4	1.2	1.0	1.8	2.0	2
0.002[b]	1.4	- -	1.0	- -	1.4	- -	1.3	- -	1.5	- -	2	- -

[a]At higher concentrations, the H_5(U) and $H_{1'}$(U) doublets overlap and resonance linewidths are difficult to determine.

[b]Time-averaged spectra.

noted previously that base intercalation frequently results in increased linewidths for the resonances of the incorporated base due to magnetic dipolar coupling between the protons of the incorporated base and the ribose protons of the dinucleotide molecule which provides the "shell" for the inserted base. Therefore, we might expect to observe some linebroadening for the ApU or UpA resonances at higher concentrations if a significant number of intercalated dimers are formed. Only a very small increase in the resonance linewidths is observed for the ApU proton resonances at high concentrations. Hence it would appear that this dinucleotide has only a small tendency to form intercalated complexes. On the other hand, the H_2(A), H_8(A), and H_5(U) resonances of UpA are appreciably broadened at concentrations greater than 0.15 M, while the two ribose $H_{1'}$ and the H_6(U) resonance linewidths of this molecule are relatively narrow and show very little concentration dependence. The linebroadening of the adenine resonances of UpA at high concentration is illustrated in Figure 51. When adenine intercalated complexes are formed, the specific sites of each of the protons of the inserted adenine base render them vulnerable to the relaxation effects of the fluctuating magnetic fields which originate from the magnetic dipoles of the ribose protons of the second dinucleotide molecule comprising the dimer complex. Likewise, the H_5 proton of an intercalated uracil base is also subject to these effects. Therefore, these proton resonances are significantly broadened by the magnetic dipolar field fluctuations when complexation of this nature occurs. On the other hand, the magnetic dipolar relaxation mechanism would not be expected to alter the resonance linewidths of the $H_{1'}$(A), $H_{1'}$(U), or H_6(U) protons as readily

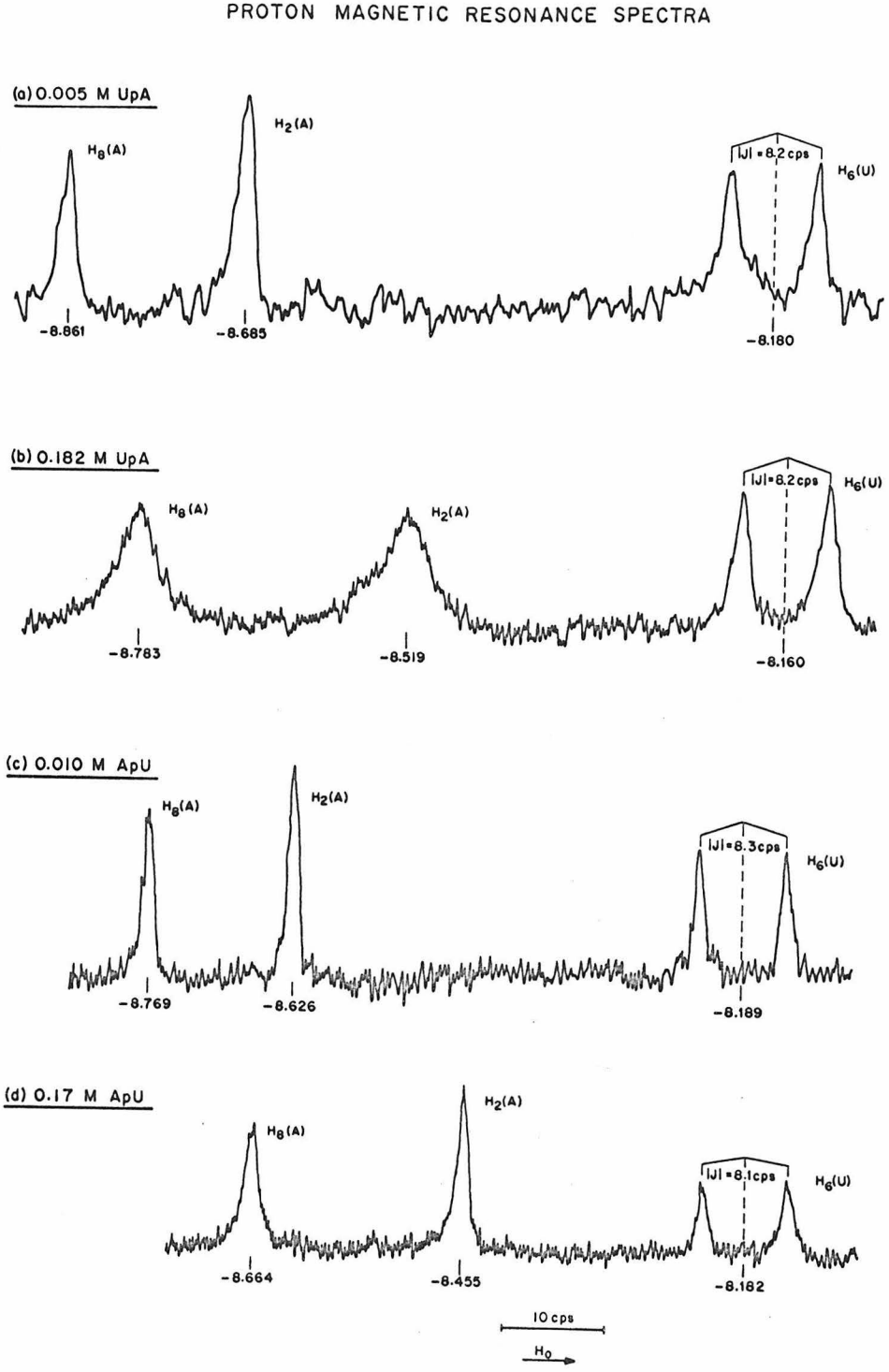


Figure 51. Proton magnetic resonance spectra of (a) 0.005 M UpA, (b) 0.182 M UpA, (c) 0.010 M ApU, and (d) 0.17 M ApU in the region of the $H_8(A)$, $H_2(A)$, and $H_6(U)$ resonances. These spectra of the sodium salts of UpA and ApU (in D_2O) were recorded at ~30°C, and the chemical shifts were monitored relative to external TMS.

because, in the intercalated dimer, these protons are relatively far-removed from the ribose protons responsible for the relaxation mechanism. Apparently, the linebroadening observed for the adenine protons and the H_5(U) proton of UpA at higher concentrations results from the formation of intercalated complexes. Under the conditions of our experiments, the formation of such complexes is obviously more favorable for UpA than for ApU, suggesting that the intramolecular base-stacking interaction in ApU is somewhat stronger. This observation is in agreement with the results of other studies which have indicated that, in isomer sets in which one of the dinucleotide bases is adenine, the ApX isomer appears to be more strongly stacked than XpA (X = G, U, or C), and with the results of purine-binding experiments in which it has been shown that UpA solutions cause a greater increase in purine proton resonance linewidths than comparable solutions of ApU (79).

The formation of more self-intercalated dimers in the case of UpA would be expected to result in larger concentration shifts for the uracil protons of this molecule. Because A-A stacking interactions are stronger, it is likely that the largest fraction of the intercalated dimers involve adenine insertion. The resulting stable A-U base-stacking would shift the uracil protons of UpA to higher field. The linewidth of the H_5(U) resonance at higher concentrations indicates that uracil insertion is also a favorable process in the intermolecular association of UpA. The stable A-U base-stacking interactions in complexes of this nature may also contribute to the uracil concentration shifts of this molecule.

The concentration shifts of the adenine H_2 protons of UpA and ApU are reasonably similar as is expected, and the smaller shift of the UpA H_8 resonance in comparison to the corresponding resonance of ApU is a direct result of the steric shielding of this H_8 proton by the adenine base and the molecular backbone as explained previously in Chapters II and III.

On the basis of the concentration data, it is not possible to determine the relative importance of the various intermolecular complexes which contribute to the proton concentration shifts, but it is probable that both types of complexes considered above are present in the solutions of ApU and UpA. However, the formation of intercalated dimers is apparently more favorable for UpA.

Finally, we note that no direct evidence was found in these concentration studies for the formation of intermolecular complexes via hydrogen bonding between the complementary adenine and uracil bases although base-pairing interactions between externally base-stacked complexes of folded dinucleotides are not inconsistent with the results of these experiments. The formation of intercalated complexes, however, indicates that the vertical base-stacking interactions are more important than the horizontal hydrogen-bonding interactions under the experimental conditions at which intercalation occurs.

3. Calculations of Dimerization Equilibrium Constants.

Although the dinucleoside monophosphates, like the simpler bases and nucleosides, may associate intermolecularly to form dimers, trimers, tetramers, and more highly associated species in aqueous solution, it is reasonable to assume that only the dimer is of significance in the

case of ApU or UpA at dinucleotide concentrations below 0.25 M. This is evidenced by the relatively small tendency of the uracil base to participate directly in the intermolecular base-stacking interactions of ApU and UpA as discussed in the previous section. Accordingly, we have interpreted the concentration shifts of the protons of these isomers in terms of a dimer equilibrium.

Let us define a system in which the dimerization of dinucleoside monophosphates may proceed via n different equilibria to produce n different types of dimers, each characterized by a specific equilibrium constant. For example, we have

$$
\begin{aligned}
M + M &\longrightarrow C_1 \qquad (K_1) \ ; \\
M + M &\longrightarrow C_2 \qquad (K_2) \ ; \\
&\ \ \vdots \\
M + M &\longrightarrow C_i \qquad (K_i) \ ; \\
&\ \ \vdots \\
M + M &\longrightarrow C_n \qquad (K_n) \ ;
\end{aligned}
$$

where K_i is the dimerization equilibrium constant for the formation of dimer, C_i, from the association of two monomers, M. In the limit of rapid chemical exchange where only an average chemical shift is observed for each proton of the dinucleoside monophosphate, the concentration shifts of a particular proton are given by

$$
\begin{aligned}
&\delta_k - \delta_M \\
&\quad = f_1(\delta_1 - \delta_M) + f_2(\delta_2 - \delta_M) + \ldots + f_i(\delta_i - \delta_M) + \ldots + f_n(\delta_n - \delta_M) \qquad \text{(IV-1)}
\end{aligned}
$$

The average chemical shift of the proton at the specific dinucleotide concentration designated by the index k is δ_k, while δ_M is the proton chemical shift in the monomer (infinite dilution value); and δ_i is the proton chemical shift in dimer C_i. The value of f_i, the mole fraction of the dinucleoside monophosphate in C_i, is given by twice the dimer concentration, c_i, divided by the total dinucleotide concentration, c_o. Since $c_i = K_i[M]^2$, we can rewrite equation (IV-1) as

$$\delta_k - \delta_M = \left\{ 2[M] \left[\sum_{i=1}^{n} (K_i D_i) \right] \right\} \Big/ c_o \qquad \text{(IV-2)}$$

where D_i is the dimer shift $\delta_i - \delta_M$. To calculate the equilibrium monomer concentration, we write:

$$\sum_{i=1}^{n} f_i = 1 \qquad \text{(IV-3)}$$

Substituting the appropriate terms for the f_i's into this equation and rearranging, we obtain

$$f_M = [M]/c_o = 1 - \left\{ 2[M]^2 \left(\sum_{i=1}^{n} K_i \right) \right\} \Big/ c_o \ . \qquad \text{(IV-4)}$$

We can now solve equation (IV-4) for [M] and substitute this expression into (IV-2). The result is

$$\delta_k - \delta_M = \left\{ 1+4 c_o \left(\sum_{i=1}^{n} K_i \right) - \left[1+8 c_o \left(\sum_{i=1}^{n} K_i \right) \right]^{\frac{1}{2}} \right\} \Big/ 4 c_o \left(\sum_{i=1}^{n} K_i \right) \cdot$$

$$\left\{ \sum_{i=1}^{n} (K_i D_i) \right\} \Big/ \left\{ \sum_{i=1}^{n} K_i \right\} \qquad \text{(IV-5)}$$

As is well known, it is not possible to distinguish between the formation of one versus several dimer species in the analysis of the concentration shifts of ApU and UpA due to rapid chemical exchange. Where n different dimer species are involved as is probably the case here, the overall association constant K is the sum of the formation constants of all the n different dimer species and the average dimer shift D represents a weighted mean over all these species; i.e.,

$$K = \sum_{i=1}^{n} K_i \qquad \text{(IV-6)}$$

and

$$D = \left\{ \sum_{i=1}^{n} (K_i D_i) \right\} \Big/ \left\{ \sum_{i=1}^{n} K_i \right\} \qquad \text{(IV-7)}$$

Substituting expressions (IV-6) and (IV-7) into (IV-5), we obtain

$$\delta_k - \delta_M = \left\{ D \left[(1+4 c_o K) - (1+8 c_o K)^{\frac{1}{2}} \right] \right\} \Big/ 4 c_o K \qquad \text{(IV-8)}$$

A computer least squares fit of the concentration data of Figures 49 and 50 to equation (IV-8) was used to determine the dimerization constant K and the dimer shift D. In this analysis K was varied from 1.0 to 6.5 molar^{-1} in increments of 0.1 molar^{-1}. The best fit to the experimental data for a given value of K was determined by treating D as an adjustable parameter in the following manner. We define a square deviation E as

$$E = \sum_{k=1}^{N} (\delta_k - \Delta_k)^2 \qquad \text{(IV-9)}$$

where δ_k is the chemical shift calculated from equation (IV-8) and Δ_k is the experimentally observed chemical shift. Each of these shifts is associated with a specific total dinucleotide concentration designated by the index k, and the summation in equation (IV-9) runs over the N different concentrations at which the proton chemical shifts were measured. By minimizing E with respect to the dimer shift D, the value of D which yields the best fit to the experimental data may be determined for each arbitrary dimerization constant. This dimer shift is given by

$$D = \left[\sum_{k=1}^{N} \Delta_k f_D(k)\right] \Big/ \left[\sum_{k=1}^{N} f_D^2(k)\right] \qquad \text{(IV-10)}$$

where $f_D(k)$ is the mole fraction of dinucleotide in dimers at the concentration characterized by k. Finally, the best value of the dimerization constant is that which corresponds to the smallest square deviation, E.

Only the concentration shifts of the adenosine protons of ApU were analyzed since the other resonances were not shifted appreciably with concentration. The data of the $H_5(U)$ proton of UpA were analyzed in addition to the adenosine proton data of this dinucleotide. The value of K yielding the smallest square deviation for the data of each proton analyzed and the corresponding dimer shift are listed in Table XXI. In addition, the value of K which yields the smallest total square deviation for the collective data of specific sets of protons was also determined, and these results along with the corresponding dimer shifts are also summarized in Table XXI.

TABLE XXI. Dimerization Constants K Giving the Best Fit to the Concentration Data of Various Protons of ApU and UpA, and the Dimer Shifts[a] Calculated from these Dimerization Constants

Proton	ApU		UpA	
	Best K (Molar^{-1})	Dimer Shift[a] (ppm)	Best K (Molar^{-1})	Dimer Shift[a] (ppm)
A. Square Deviation[b] Minimized Separately for Data of Each Proton				
H_8(A)	2.5	0.338	1.9	0.268
H_2(A)	2.4	0.568	2.3	0.508
$H_{1'}$(A)	3.1	0.254	2.6	0.193
H_5(U)	- -	- - -	3.7	0.129
B. Square Deviation Minimized for Collective Data of H_2(A) and H_8(A)				
H_8(A)	2.4	0.342	2.2	0.243
H_2(A)		0.566		0.524
$H_{1'}$(A)		0.291		0.210
H_5(U)		- - -		0.162
C. Square Deviation Minimized for Collective Data of All Protons Analyzed				
H_8(A)	2.5	0.556	2.4	0.235
H_2(A)		0.337		0.506
$H_{1'}$(A)		0.286		0.203
H_5(U)		- - -		0.157

[a]The difference in the dimer-complex chemical shift of a given proton and its monomer chemical shift.

[b]See text for definition and discussion of square deviation.

It is obvious from these results that the value determined for the dimerization equilibrium constants of ApU and UpA by this method is dependent on the proton chosen for analysis. For example, in the case of UpA, the value of the equilibrium constant determined from the data

of H_8(A) is 1.9 molar^{-1}, while the data of H_2(A), $H_{1'}$(A), and H_5(U) yielded values of 2.3, 2.6, and 3.7 molar^{-1}, respectively. Similarly, the computer least squares fit of the concentration shifts of the various ApU protons also gave different dimerization constants: 2.5, 2.4, and 3.1 molar^{-1} respectively for the data of the adenosine H_2, H_8, and $H_{1'}$ protons. These differences reflect the intrinsic uncertainty in our empirical method. We note, however, that the square deviation calculated from the $H_{1'}$(A) data of ApU and UpA and the H_5(U) data of UpA is somewhat insensitive over a relatively wide range of equilibrium constants. Several different values of K (within a range of ± 0.5 molar^{-1} of the best K for the $H_{1'}$ protons and a range of ± 1.0 molar^{-1} for the H_5(U) proton of UpA) yielded calculated chemical shift curves which closely fit the experimental data. The deviation of the calculated curves from the experimental curves is considerably more sensitive to the value of the equilibrium constant in the analysis of the data of H_2(A) and H_8(A) for both ApU and UpA. In each of these cases, only a small range of equilibrium constants (± 0.2 molar^{-1}) gives satisfactory results. Accordingly, we have chosen as the best equilibrium constant for the dimerization of ApU and UpA the value of K for which the total square deviation is smallest in the treatment of the concentration shift data of the H_2(A) and H_8(A) protons. As indicated in Table XXI, these values are 2.4 molar^{-1} for ApU and 2.2 molar^{-1} for UpA. A similar procedure which determined the dimerization constant by minimizing the total square deviation for the data of <u>all</u> protons analyzed yielded values of 2.5 and 2.4 molar^{-1} for ApU and UpA respectively.

The solid lines depicted in Figures 49 and 50 represent the chemical shift versus concentration curves calculated from the best K and the dimerization shift values listed in Part B of Table XXI. Each of these theoretical curves fits the experimental data very well over the entire concentration range studied.

The dimerization constants of 2.4 molar^{-1} for ApU and 2.2 molar^{-1} for UpA show that the intermolecular association tendencies of these two dinucleoside monophosphates are quite similar. In comparison the self-association constants of adenosine and uridine as determined previously by osmotic studies are 4.5 molal^{-1} (35) and 0.6 molal^{-1} (33), respectively. The esterification of uridine monophosphate to adenosine reduces its dimerization constant by a factor of ~ 2. This likely results from the reduced accessibility of the adenine base and from the effects of the negatively charged phosphate group.

The dimer shifts obtained in these studies are very reasonable. For example, the larger shifts of the H_2(A) protons of ApU and UpA reflect their greater exposure to the magnetic anisotropy of the base of the adjacent molecule in the dimer complex. The H_8(A) protons are located nearer the ribose-phosphate backbone in both molecules and would be expected to have a smaller dimer shift. The site of the $H_{1'}$(A) protons is relatively far-removed from the bases in the intermolecular base-stacks accounting for their smaller dimer shifts. Finally, it is interesting that the steric shielding of the H_8(5') proton in this isomer set is reflected in the calculated dimer shifts. The dimer shifts we have calculated for the adenine proton resonances of ApU and UpA are comparable to those reported earlier by Chan et al. (48): H_6, 0.59 ppm;

H_2, 0.68 ppm; H_8, 0.47 ppm. In addition, Johnson and Bovey (45, 46) have calculated a ring-current diamagnetic anisotropy shielding of 0.58 ppm at the locus of points $z = 3.0$ Å, $\rho = 2.0$ Å in a system of cylindrical co-ordinates with the origin at the center of a benzene ring and the z-axis perpendicular to the molecular plane. These observations indicate that the preceding treatment of the intermolecular association of ApU and UpA is reasonable.

4. Summary. The concentration dependence of the p.m.r. spectra of ApU and UpA demonstrate that each of these dinucleotides has an appreciable tendency to associate intermolecularly. The self-association proceeds primarily via vertical stacking of the adenine bases with polymerization beyond dimerization somewhat limited at concentrations smaller than 0.25 M. The chemical shift data in the concentration range 0–0.25 M are consistent with a dimer equilibrium model in which the respective equilibrium constants of ApU and UpA are 2.4 and 2.2 molar^{-1}.

F. The Binding of 6-Methylpurine to ApU

Purine has proved to be a useful probe molecule in the study of base-stacking properties of several dinucleoside monophosphates. The binding of purine to the bases of ApA and the adenine-guanine dinucleotides has been discussed in the preceding chapters of this thesis, and similar purine-binding experiments have been reported for ApU and UpA (79). In each of these dinucleotides and several pyrimidine-pyrimidine dinucleotides (79), the formation of purine-dinucleotide

complexes results in higher field chemical shifts for the dinucleotide protons due to the intermolecular base-stacking and the magnetic anisotropy of the purine ring. These upfield shifts provide a convenient means of studying the nature of intramolecular base-stacking in the dinucleotides as well as the characteristics of the intermolecular association. Of equal interest in the purine-binding studies is the effect of the dinucleotide molecules on the magnetic resonance spectra of the purine protons when complex formation occurs. In the purine-binding experiments of each of the dinucleotides we have studied, the resonance linewidths of the purine protons are enhanced by the formation of intermolecular complexes. Since this effect is not observed in purine-nucleoside stacking interactions, it is not unreasonable to assume that intercalation of the purine molecule between the two dinucleotide bases is responsible for the purine resonance linebroadening phenomenon. There are several factors which could account for the increased linewidths of the purine resonances in the intercalated complexes. Most of these can be readily eliminated from consideration.

Since only an average chemical shift is observed for each purine resonance, the rate of exchange of purine between bound and unbound sites is relatively rapid. If, however, the exchange rate is comparable to the difference in the chemical shifts of a purine proton in the bound state and the free state, some linebroadening would be expected. If this were the case, a similar linebroadening effect would also be observed for the dinucleotide proton resonances. Since none of the base proton resonances of each dinucleotide we have studied is significantly broadened when intercalated complexes are formed, we feel that

the exchange mechanism cannot account for the linewidth enhancement of the purine absorption signals. Moreover, this rationale would require an unreasonably long lifetime of approximately 10^{-2} seconds for a purine molecule in a given state (free or bound).

A longer rotational tumbling time as a result of complex formation could also conceivably account for the purine linebroadening. This appears to be inconsistent with the results of other NMR experiments however. Nitrogen-14 NMR studies of purine (105) have shown that the formation of intermolecular purine complexes causes an increase in the molecular rotational correlation time of the purine molecules. However, no corresponding increase is observed in the linewidths of the proton resonances under equivalent experimental conditions. This observation does not entirely rule out the possibility that changes in the rotational correlation time of the purine molecule effected by intermolecular association with a dinucleotide could be responsible for the increased purine resonance linewidths.

It is significant that the linebroadening pattern of the purine proton resonances of the purine-dinucleotide intercalated complexes is identical for every dinucleotide we have studied. The H_6 proton resonance experiences the largest increase in linewidth, the H_2 resonance is broadened least, and H_8 exhibits intermediate behavior. This suggests that some phenomenon intrinsic in the structure of the purine molecule itself might be partially responsible for the resonance linebroadening. Specific intramolecular effects which could result in increased linewidths for the purine resonances when intermolecular complexes are formed with the dinucleotides include (1) nuclear spin-

spin coupling between the purine protons of interest and the ^{14}N electric quadrupoles of the purine molecule and (2) nuclear magnetic dipole interaction between each of the purine protons and the remaining nuclei of the molecule.

Let us consider the first possibility. Nitrogen-14 has a spin number I = 1 and therefore possesses a quadrupole moment which can interact with fluctuating electric field gradients produced at the ^{14}N nucleus by other molecular degrees of freedom. This interaction can result in rapid spin-lattice relaxation for the ^{14}N nuclear quadrupole. Under conditions of rapid molecular tumbling the spin-lattice relaxation time, T_1, of the ^{14}N quadrupole is given by

$$\frac{1}{T_1} = \frac{3}{8} \hbar^{-2} (eq)^2 (eQ)^2 \tau_c \qquad \text{(IV-11)}$$

where eQ is the electric quadrupole moment, eq is the electric field gradient, and τ_c is the correlation time determined by molecular rotation (106). Hence changes in the molecular tumbling time of the purine molecule can alter the spin-lattice relaxation time of the ^{14}N quadrupole moment. It is well known that the absorption bands of nuclei coupled to ^{14}N nuclei are frequently broadened due to quadrupole relaxation of the ^{14}N nuclear spin and the linewidths are often a function of the relaxation rate of the ^{14}N quadrupole (107). Therefore, if the purine protons were coupled to the ^{14}N nuclei, and if intermolecular association of the purine molecule with a dinucleotide resulted in a smaller spin-lattice relaxation time for the ^{14}N nuclei, the purine proton resonance linewidths could be increased by intramolecular ^{14}N

quadrupole effects. Different relaxation effects for the respective ^{14}N nuclei and dissimilar nuclear spin-spin coupling could account for the specific linebroadening pattern observed for the purine proton resonance spectra. This linebroadening mechanism does not appear to be operative in the case of the purine protons, however, because the resonances of these protons are narrow in the absence of the dinucleotides, and no ^{1}H-^{14}N coupling is observed (the proton resonances are narrow singlets) in the proton magnetic resonance spectrum of purine. Under conditions of rapid molecular tumbling and relatively inefficient ^{14}N quadrupole relaxation, the purine resonances would be expected to appear as multiplets if the protons were coupled to the ^{14}N muclei. In connection with this observation, it is noteworthy that long range ^{1}H-^{14}N coupling constants have not been observed in the resonance spectra of any nitrogen-containing heterocyclic molecules (108), and the linebroadening effect of the ^{14}N quadrupole relaxation appears to be important only for protons attached directly to the nitrogen atom.

We now consider the possibility of the relaxation (and the accompanying linebroadening effect) of the purine protons in the dinucleotide complex by intramolecular nuclear magnetic dipolar interaction. This mechanism could account for the specific purine proton resonance linebroadening pattern if the intramolecular effects were different for each of the purine protons. Within the limits of reasonable assumptions, the contribution of intramolecular dipolar interactions to the relaxation rate $(1/T_1)$ of nucleus i is given by

$$\frac{1}{T_{1i}} = \frac{\hbar^2 \tau_c \gamma_i}{2}\left(3\gamma_i^2 \sum_j r_{ij}^{-6} + 2\sum_f^{*} \gamma_f^2 r_{if}^{-6}\right) \qquad \text{(IV-12)}$$

In this formula, summations $\sum_j$ are over nuclei of the same species (protons) and $\sum_f^{*}$ over all other nuclei; τ_c is the correlation time for the rotational motion of the molecule. The magnetogyric ratios of the protons and of the other nuclei are designated as γ_i and γ_f, respectively. The distance between the i^{th} proton and the j^{th} proton of the purine molecule is r_{ij} and the corresponding distance between proton i and nucleus f is r_{if} (109). An increase in the correlation time, τ_c, of purine could be effected by purine intercalation, and according to equation (IV-12) this could result in an increased relaxation rate for the proton nuclei due to intramolecular dipolar interactions. The increased relaxation rate would be manifested in the resonance spectrum of the protons through greater resonance linewidths. In an effort to determine if this mechanism is responsible for the purine-linebroadening pattern when purine-intercalated complexes are formed with the dinucleotides, we have measured the proton resonance linewidths of purine in D_2O, glycerol, and various mixtures of these two solvents. The molecular tumbling time would be expected to increase as the fraction of glycerol in the solvent is increased. If changes in the rotational correlation time of the purine molecule and the accompanying intramolecular relaxation effects as outlined above are entirely responsible for the purine linebroadening pattern, a similar pattern might also be expected for the purine resonances in solvent systems of high glycerol content. The results of these experiments are summarized in Table XXII.

TABLE XXII. Resonance Linewidths[a] of the H_6, H_2, and H_8 Protons of Purine in D_2O, Glycerol, and Various D_2O-Glycerol Mixtures

Purine Concentration (Molal)	Solvent System	Purine Resonance Linewidth[a] (cps)		
		H_6	H_2	H_8
0.050	D_2O	0.8	1.0	0.9
0.044	11.4%[b] Glycerol in D_2O	0.8	0.9	0.8
0.036	28.1%[b] Glycerol in D_2O	0.8	1.1	0.9
0.012	55.3%[b] Glycerol in D_2O	0.9	1.0	0.9
0.050	Glycerol	6.	6.	6.

[a]Full linewidth at half signal intensity.

[b]By weight.

There is no noticeable change in the purine proton resonance linewidths in the solvent systems ranging from pure D_2O to 55% (by weight) glycerol; and in neat glycerol each of the resonance linewidths is only ~ 6 cps. The very small linebroadening effect observed in these glycerol-D_2O solvents strongly suggests that the intercalation-related linebroadening is not the direct result of a simple change in the purine molecular tumbling time and increased proton nuclear relaxation rates resulting from intramolecular dipole interactions. Perhaps an even more interesting aspect of these experiments is the fact that in each of the solvent systems presented in Table XXII, the H_6, H_2, and H_8 resonance linewidths are all about equal. There is no selective linebroadening with an increase in the molecular tumbling time. This is illustrated in Figure 52 which depicts the proton magnetic resonance spectra of

PURINE PROTON RESONANCE SPECTRA

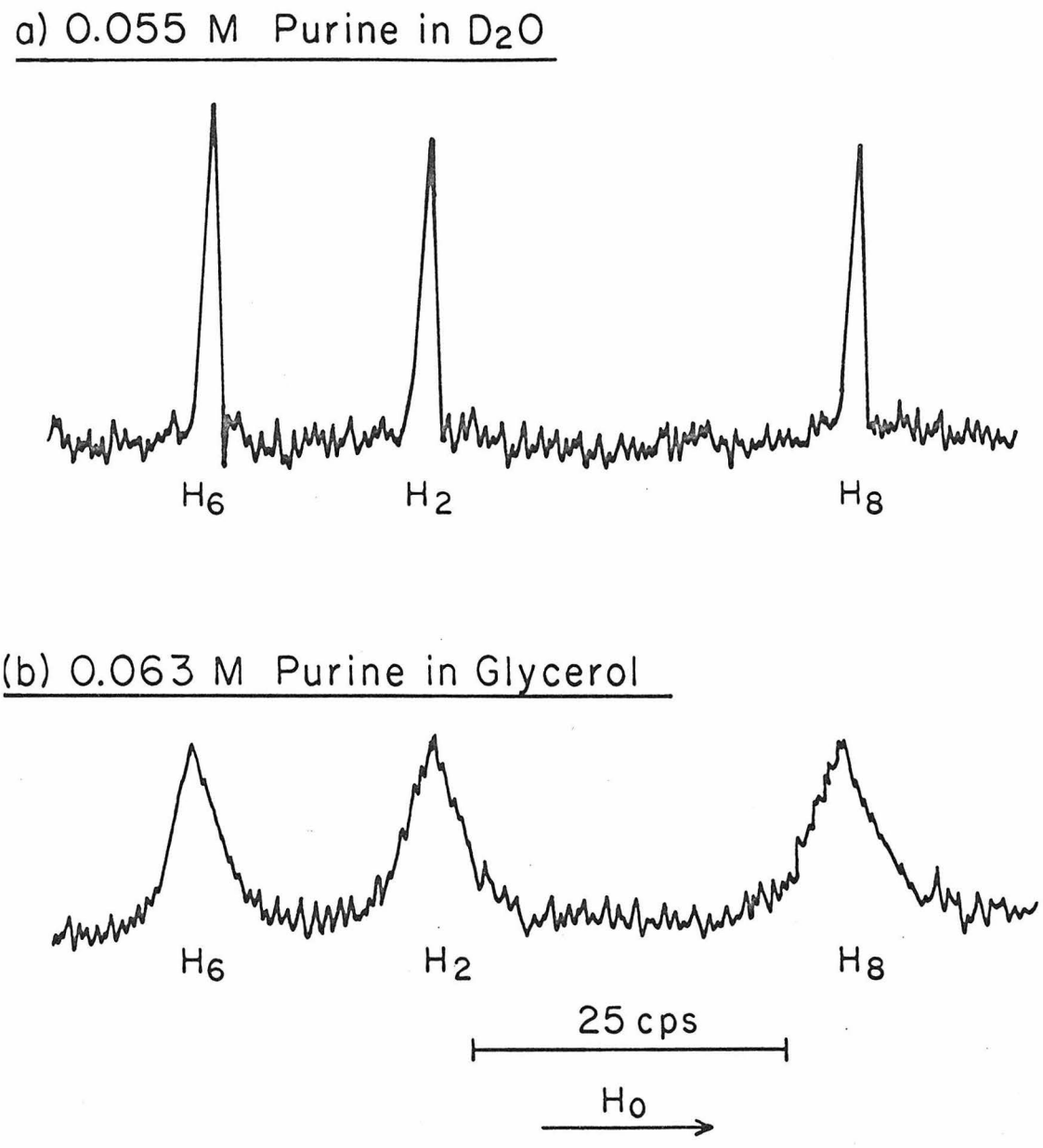


Figure 52. Proton magnetic resonance spectra (100 Mcps) of (a) 0.055 M purine in D_2O and (b) 0.063 M purine in glycerol.

0.055 M purine in D_2O and 0.063 M purine in glycerol. Although the longer rotational tumbling time of purine in the glycerol solution increases the resonance linewidths, no selective linebroadening pattern is observed. This implies that intramolecular effects are not solely responsible for the increased linewidths of the purine proton absorption bands and the specific linebroadening pattern observed with the formation of purine-intercalated complexes.

As discussed in Chapters II and III, purine intercalation can result in the exposure of the purine protons to strong fluctuating magnetic dipolar fields produced by the protons of the dinucleotide ribose-phosphate backbone. The magnetic dipolar coupling between the purine protons and the dinucleotide protons presumably provides an effective relaxation mechanism for the purine protons. The preferential orientation of the purine molecule in each of the purine-dinucleotide intercalated complexes evidently accounts for the specific linebroadening pattern of the purine resonances. The average location of the H_6 proton is apparently much closer to the source of the relaxation mechanism of each dinucleotide than either H_2 or H_8; hence, this proton experiences the greatest linewidth enhancement in each case. In view of the observations of the preceding paragraphs, this rationale of the purine linebroadening appears most reasonable; additional support for this mechanism has been provided by the binding of 6-methylpurine to adenylyl-(3'→5')-uridine.

Due to steric interaction between the methyl group of 6-methylpurine and the dinucleotide (ApU) atoms, the average orientation

of an ApU-intercalated 6-methylpurine molecule would be expected to be appreciably different from that of a similarly intercalated purine molecule. Accordingly, the respective linebroadening patterns of purine and 6-methylpurine would also be different provided the intermolecular dipolar relaxation mechanism is responsible for the linewidth enhancement of the proton resonances of the intercalating molecule. The changes in the resonance linewidths of the purine and 6-methylpurine protons due to the formation of an intercalated complex with ApU are summarized in Table XXIII; and the effect of ApU (at two different concentrations) on the proton magnetic resonance spectrum of purine is illustrated in Figures 53 and 54. The resonances of ~ 0.05 M solutions of both purine and 6-methylpurine are narrow in the absence of the dinucleotide. The addition of ApU to the purine solution results in the familiar linebroadening pattern: H_6 linewidth $> H_8 > H_2$. (A higher dinucleotide concentration results in greater purine resonance linewidths due to the incorporation of a greater fraction of purine into the intercalated environment.) The spectra in Figures 53 and 54 show that the effect of ApU on the p. m. r. spectrum of 6-methylpurine is much different. For example, the H_8 resonance is significantly more broadened than the other two resonances (H_2 and CH_3) of 6-methylpurine and considerably more broadened than the H_8 resonance of purine under similar experimental conditions. As illustrated in Figure 53, the resonance of the methyl protons at the 6-position of the purine ring are only slightly broadened at ApU and purine concentrations of 0.099 M and 0.055 M, respectively. The linebroadening pattern of the 6-methylpurine spectrum in the presence of ApU is: H_8 linewidth $> H_2 >$ 6-CH_3. It is obvious that the

TABLE XXIII. Proton Resonance Linewidths[a] of Purine, 6-Methylpurine (6-MP), Ribosylpurine (RP), and Adenylyl-(3'→5')-Uridine (ApU) under Various Experimental Conditions

Solution[b]	Resonance Linewidth[a]						
	H_8(A)	H_2(A)	H_8(P)	H_2(P)	H_6(P)	6-CH_3	$H_{1'}$(RP)
0.063 M ApU	1.2	0.8	- -	- -	- -	- -	- -
0.05 M Purine	- -	- -	0.9	1.0	0.8	- -	- -
0.057 M 6-MP	- -	- -	1.2	1.2	- -	1.0	- -
0.06 M RP	- -	- -	1.2	0.9	0.9	- -	1.4
0.063 M ApU 0.055 M 6-MP	1.3	1.0	2.1	1.3	- -	1.2	- -
0.099 M ApU 0.055 M 6-MP	2.3	1.8	6.0	2.8	- -	2.1	- -
0.08 M ApU 0.08 M Purine[c]	1.2	0.8	~ 4	1.4	4.3	- -	- -
0.08 M ApU 0.04 M Purine[c]	1.2	0.8	3.2	2.8	5.6	- -	- -
0.022 M RP 0.05 M ApU	1.2	0.9	1.6	1.4	2.2	- -	1.4

[a]Full linewidth in cps at half signal intensity.

[b]All solutions in D_2O.

[c]Data of B. W. Bangerter, Reference (79).

substitution of the larger methyl group for the H_6 proton of purine changes the average orientation of the purine molecule in the ApU-purine intercalated complex, thus altering the effect of the fluctuating magnetic fields which are produced at the dinucleotide backbone on the respective purine protons. For the purpose of comparison, the changes effected in the p. m. r. spectrum of ribosylpurine by the formation of intercalated

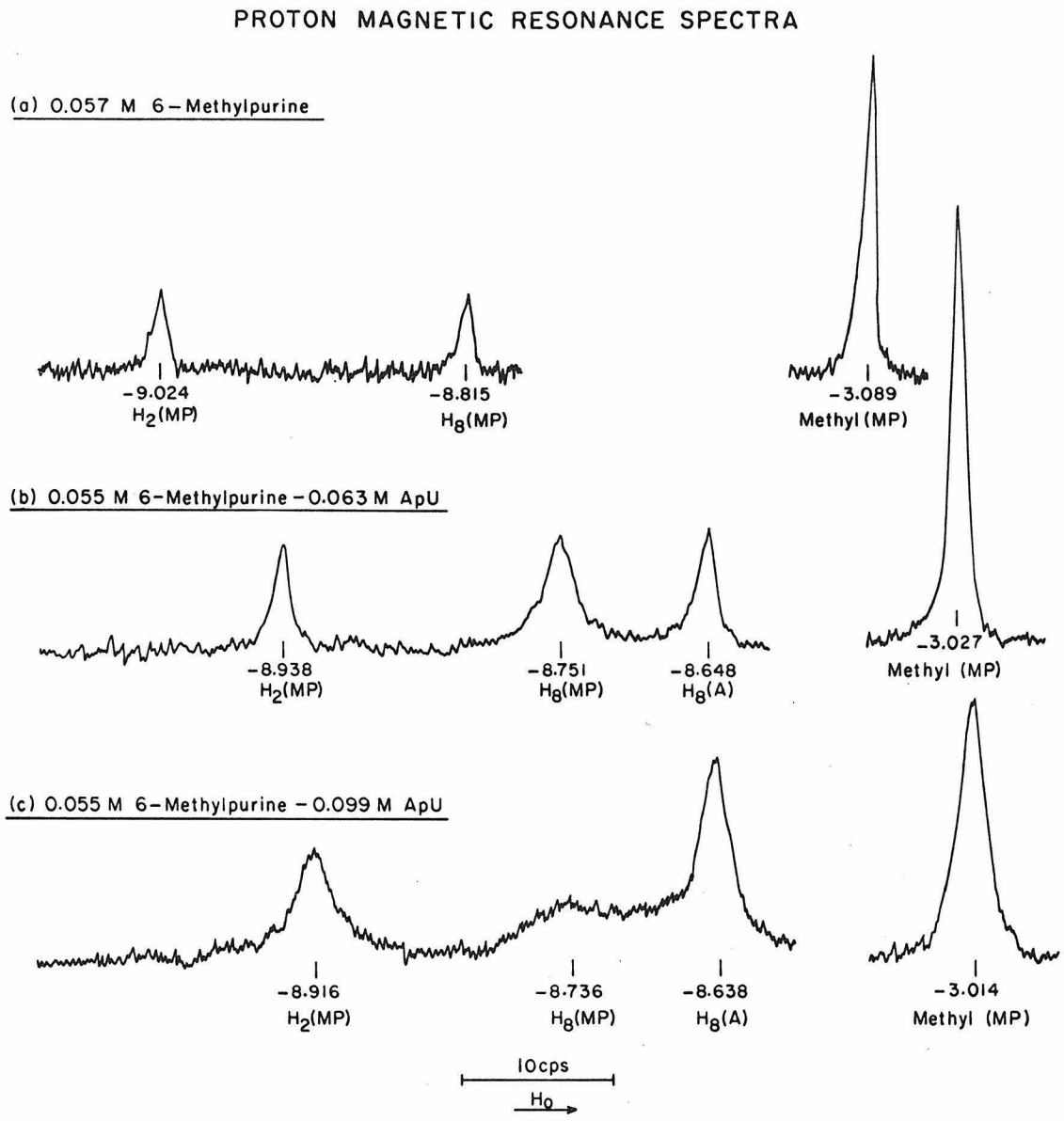


Figure 53. Proton magnetic resonance spectra (100 Mcps) of (a) 0.057 M 6-methylpurine, (b) 0.055 M 6-methylpurine—0.063 M ApU, and (c) 0.055 M 6-methylpurine—0.099 M ApU. The chemical shifts are referenced to external TMS.

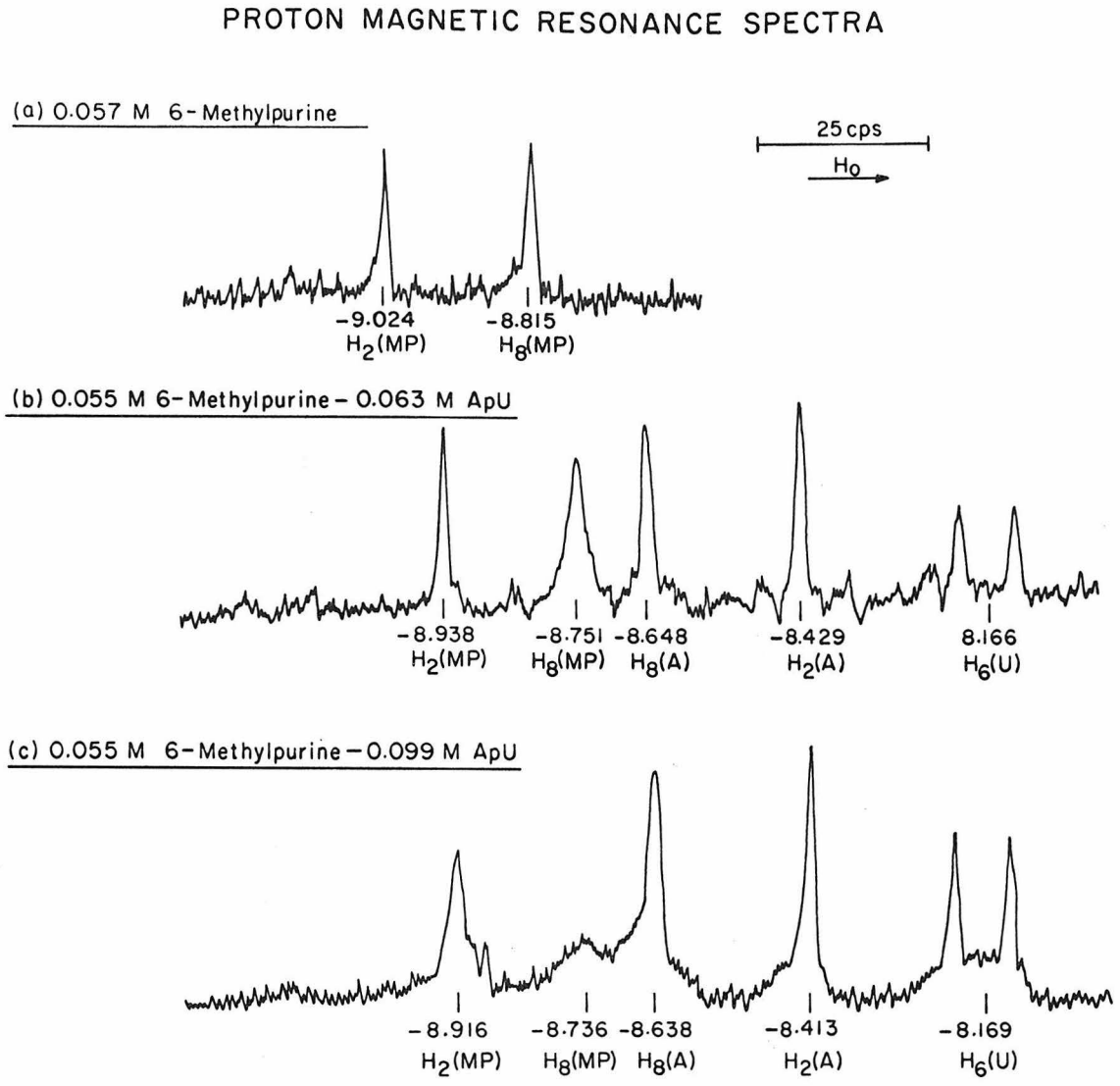


Figure 54. Proton magnetic resonance spectra (100 Mcps) of (a) 0.057 M 6-methylpurine, (b) 0.055 M 6-methylpurine—0.063 M ApU, and (c) 0.055 M 6-methylpurine—0.099 M ApU. The chemical shifts are referenced to external TMS.

complexes with ApU are also presented in Table XXIII and depicted in Figure 55. Due to the steric effects of the relatively bulky ribosyl group, the intercalation process is appreciably hindered. Evidence for this is provided by the much narrower linewidths of the ribosylpurine proton resonances (in comparison to the resonances of purine and 6-methylpurine) in the presence of ApU. However, some linebroadening is observed and the H_6 resonance linewidth is greater than that of either H_8 or H_2 as is expected from steric considerations. It is noteworthy that no change is effected in the linewidth of the H_1, proton resonance of ribosyl purine by ApU, emphasizing that this proton is far-removed from the dinucleotide-phosphate backbone when the intercalated complex is formed.

G. Summary

Although the base-stacking tendency of uracil is relatively small, the infinite dilution chemical shifts of ApU and UpA show that there is appreciable intramolecular base-stacking in each of these dinucleotides at 30°C. However, the intramolecular interaction in these dinucleotides is not as strong as the adenine-adenine base-stacking in ApA.

Both of the adenine-uracil dinucleotides associate intermolecularly by vertical stacking of the adenine bases to form dimers in the concentration range 0—0.25 M. The data of these studies are consistent with a dimerization constant of approximately 2.4 molar^{-1} for each of these isomers.

The binding of purine, 6-methylpurine, and ribosylpurine to ApU has provided excellent evidence that the purine resonance linebroadening

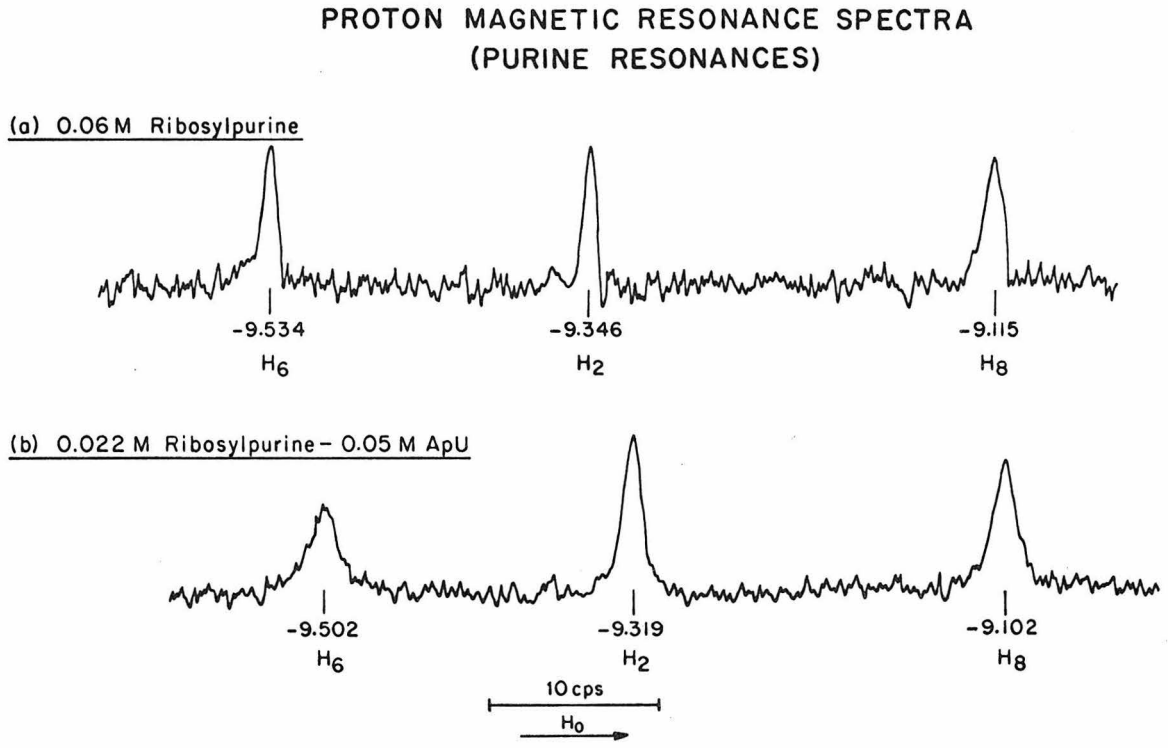


Figure 55. Proton magnetic resonance spectra (100 Mcps) of the purine protons of (a) 0.06 M ribosylpurine and (b) 0.022 M ribosylpurine—0.05 M ApU. The chemical shifts are referenced to external TMS.

which accompanies the formation of purine-dinucleotide complexes is caused by purine intercalation and that the relaxation mechanism is the magnetic dipolar interaction between the purine protons and the protons of the ribose-phosphate backbone. The intercalated purine base is preferentially oriented with the H_6 proton relatively close to the molecular backbone of the dinucleotide and H_2 reasonably far-removed. The steric interaction of the methyl hydrogens of 6-methylpurine with the dinucleotide atoms causes this molecule to be oriented much differently in the intercalated complex; the H_8 proton is in closer proximity to the dinucleotide backbone with the bulkier methyl group far-removed. The specific base-purine interactions do not appear to be an important factor in determining the energetically most favorable orientation of the purine base in the purine-dinucleotide intercalated complexes because very similar purine base orientations are observed for a wide variety of dinucleoside monophosphates, e.g., ApU, UpA, ApA, UpU, TpT (T = thymidine), CpC, ApG, GpA, ApC, CpA, dUpT (dU = deoxyuridine), and TpdU.

V. BASE-STACKING INTERACTIONS IN SMALL OLIGORIBONUCLEOTIDES OF ADENINE

A. Introduction

A natural extension of the proton magnetic resonance studies of the conformational properties of dinucleoside monophosphates in aqueous solution (Chapters II, III, IV; this thesis) is the study of similar conformational properties of oligonucleotides. The information accumulated in the earlier studies involving the simpler dimer molecules would be expected to be helpful in the interpretation of the results of the oligonucleotide proton magnetic resonance experiments. Although the dinucleoside monophosphates provide a convenient model for studying the interaction between two specific bases along a polynucleotide chain, the base-stacking interactions in the oligonucleotides would be expected to be slightly more representative of the interactions which are of primary importance in determining the structural and conformational properties of native nucleic acids and polynucleotides. The two bases of the dinucleotides are analogous to the two terminal bases of a polynucleotide chain. Therefore, the properties of these bases may be somewhat different from those of the interior bases of a polynucleotide. The oligonucleotides can serve as useful models for the study of the base-stacking tendencies and the conformational properties of the interior polynucleotide bases in aqueous solution. Accordingly, our proton magnetic resonance studies have been extended to the investigation of the base-stacking properties and conformational properties of

several oligonucleotides.

We have chosen the adenine (3'→5')-oligoribonucleotides as the initial system for our p. m. r. studies of the oligomers because the p. m. r. spectral properties of adenylyl-(3'→5')-adenosine (ApA) have been studied under several different experimental conditions, and sufficient data are available to make meaningful comparisons between the results of the experiments involving this dinucleotide and the adenine oligonucleotides. In addition, the results of various optical studies of oligoadenylic acids of varying chainlength are also available for comparison. These studies have demonstrated that the oligoadenylate molecules possess a considerable degree of ordered structure in aqueous solution at neutral pH and $\sim 25^{\circ}C$; this secondary structure may be attributed to the formation of intramolecular adenine-adenine base-stacks (57, 63, 64). The ordered structure of the adenine oligonucleotides is consistent with the strong intramolecular base-stacking interaction observed for ApA (Chapter II, this thesis). The optical studies of the oligomers provide very little information pertaining to the specific conformations of these molecules in solution. In view of the successful investigations of the conformational properties of the dinucleoside monophosphates by proton magnetic resonance, similar techniques would also be expected to yield more detailed information concerning the conformation of the respective oligonucleotides than the optical experiments. Proton magnetic resonance spectroscopy has recently been applied to the study of the properties of ApGp, CpGp, and UpGp (76) and to deoxytrinucleotides containing thymine, adenine, and guanine (77). However, the interpretation of the results of each of these

studies solely in terms of intramolecular processes is not feasible because the experiments were carried out at relatively high concentrations where the effects of intermolecular association are undoubtedly important in determining the spectral properties of these molecules.

Several proton magnetic resonance spectra of polyadenylic acid have also been reported (26, 27, 49). Due to inadequate resolution and the relatively slow rotational correlation time of the polymer chain and the accompanying dipolar relaxation mechanism (the proton resonances are appreciably broadened at room temperature), all of the H_8(A) protons give rise to only one broadened resonance absorption signal; the same observation is true for the H_2(A) and $H_{1'}$(A) protons in the poly A spectra. The rotational correlation time of the oligoribonucleotides of adenine is expected to be considerably shorter than that of poly A. Resolution of the various H_8 (H_2 or $H_{1'}$) protons would, therefore, be anticipated at least for the smaller oligomers. It is of interest to determine if such resolution is possible, and at what chain length the H_8 (H_2 or $H_{1'}$) resonances can no longer be satisfactorily distinguished. The observation of distinct resonances makes possible the accumulation of important information concerning the conformations of the bases of the oligonucleotides, provided the respective absorption bands can be correctly assigned.

In an effort to determine the extent of the intramolecular base-stacking in the oligoadenylates and to elucidate the most favorable conformations of each of the adenine bases in these molecules, we have investigated the effect of temperature variation and purine-binding on the p. m. r. spectra of these molecules at low oligomer concentrations

($< 10^{-2}$ M). In this chapter, we report the results of preliminary temperature studies of adenylyl-(3'→5')-adenylyl-(3'→5')-adenosine (ApApA) and the (3'→5')-tetranucleoside triphosphate of adenine (ApApApA) as well as purine-binding studies of the trimer. The proton magnetic resonance spectra of the H_2, H_8, and $H_{1'}$ protons of ApApA and of the H_2 and H_8 protons of ApApApA have been studied under various conditions. Due to spectral complications arising from complex coupling patterns and overlapping resonances, the magnetic resonance spectra of the other protons of these oligonucleotides have not been extensively studied.

B. Experimental

The adenylate oligomers, ApApA and ApApApA, were purchased from Miles Laboratories, Inc., Elkhart, Indiana, and were used without further purification. A column containing Dowex 50W-X8 cation exchange resin was used to convert the oligoribonucleotides from the acid forms to the respective sodium salts, and the sodium salts were employed in each of the experiments reported in this chapter. Purine, supplied by Calbiochem, Los Angeles, California, was sublimed *in vacuo* before use. All samples were prepared in deuterium oxide (99.7 mole %) obtained from Columbia Organic Chemicals, Columbia, South Carolina.

The proton magnetic resonance spectra were recorded with a Varian HA-100 spectrometer operating in the frequency-sweep mode. Neat TMS sealed in capillary tubing was used to provide the field/frequency lock signal, and the chemical shifts were measured in the usual manner (see Chapter II, this thesis). A Varian C-1024 time-

averaging computer was used to enhance the weak absorption signals. Unless specified otherwise, all measurements were made at 29 ± 1°C.

C. Proton Magnetic Resonance Studies of ApApA

1. Assignment of the H_2, H_8, and $H_{1'}$ Proton Resonances. The p.m.r. spectrum of the adenine ring protons of ApApA at neutral pH, ~30°C, and an oligomer concentration of 0.0074 M is depicted in Figure 56. The resonances of the ApApA protons in this spectral region are well-resolved under these experimental conditions. As observed previously with molecules of a similar nature, the adenine H_8 resonances are readily distinguished from the H_2 absorption bands by deuterium exchange of the H_8 protons with the solvent, D_2O, at elevated temperatures; and at ~30°C and neutral pH, the three H_8 resonances are at lower field than the H_2 signals.

We have assigned the individual H_2 resonances of ApApA by comparison with the spectrum of ApA under similar experimental conditions. The H_2 chemical shifts of each of these molecules at neutral pH and room temperature are summarized in Table XXIV. The assignment of the H_2 trimer resonances as presented in the table is discussed in the following paragraphs.

The resonance frequency of one of the H_2 protons of ApApA is at considerably lower field than that of the remaining two H_2 resonances and is very similar to the $H_2(5')$ resonance frequency of ApA. In accordance with this observation we have assigned the lowest field H_2 resonance of ApApA to the $H_2(5')$ proton. ($H_2(5')$ designates the H_2 proton of the trimer nucleoside which possesses a free 3'-hydroxyl group and is esterified to the phosphate through its 5'-hydroxyl group.)

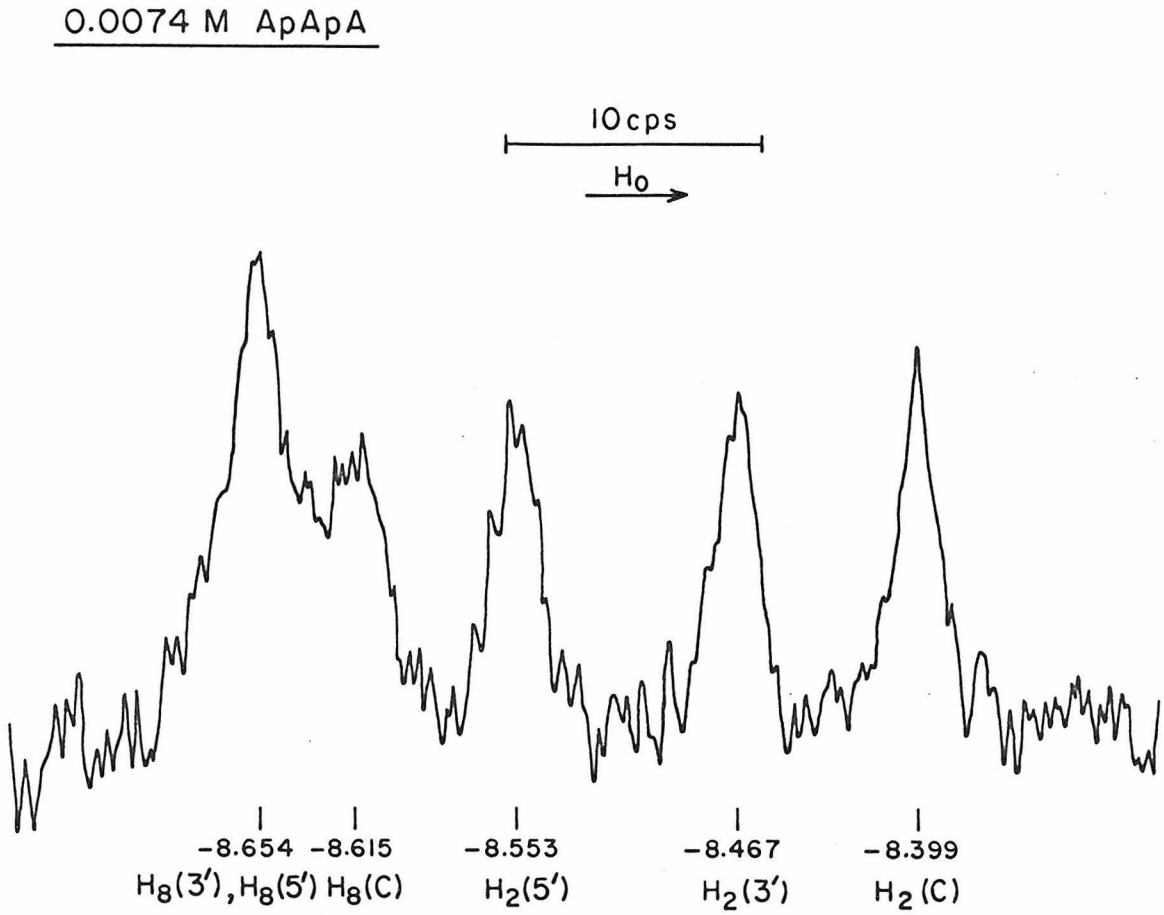


Figure 56. Proton magnetic resonance spectrum (100 Mcps) of the H_2 and H_8 adenine protons of ApApA (0.0074 M) at neutral pH and ~ 30°C. The chemical shifts are monitored relative to external TMS.

TABLE XXIV. Chemical Shifts[a] of the H_2 Protons of ApA, ApApA, and ApApApA

Molecule	Conc. (Molar)	Temp. (°C)	pD	Chemical Shift (ppm)		
				$H_2(5')$[b]	$H_2(C)$[c]	$H_2(3')$[d]
ApA[e]	0.01	29	7-8	8.57	- -	8.43
ApApA[e]	0.007	28	7-8	8.531	8.366	8.445
ApApApA[e]	0.007	29	7-8	8.516	[8.360, 8.382][f]	8.393

[a]Downfield shift relative to external TMS.

[b]$H_2(5')$ designates the H_2 proton of the nucleoside (with a free 3'-hydroxyl group) esterified to the phosphate through the 5'-hydroxyl group.

[c]$H_2(C)$ designates the H_2 proton of the central nucleoside along the ribose phosphate backbone of the trimer.

[d]$H_2(3')$ designates the H_2 proton of the nucleoside with a free 5'-hydroxyl group.

[e]Sodium salt.

[f]The specific assignment of the H_2 resonances of the two interior nucleosides of the tetramer has not been determined; the chemical shift of both of these resonances is listed in the $H_2(C)$ column.

Similarly, the chemical shifts of the $H_2(3')$ proton of ApA and the central resonance of the H_2 spectral region of ApApA are almost equivalent; and the central ApApA signal has been assigned to $H_2(3')$. ($H_2(3')$ designates the H_2 proton of the trimer nucleoside possessing a free 5'-hydroxyl group.) The remaining H_2 resonance (the highest field resonance in the H_2 spectral pattern of ApApA) has been assigned to the base proton of the central nucleoside along the molecular backbone of the trimer, $H_2(C)$. On the basis of the ring-current magnetic anisotropy

effects, the H_2(C) proton of the trimer would be expected to have a higher field chemical shift than either of the other H_2 protons if relatively strong intramolecular base-stacks are formed, and the chemical shift values tabulated in Table XXIV indicate that there is strong intramolecular base-stacking in ApApA at ~30°C and neutral pH. The spectral assignments discussed above are not rigorous, but they are reasonable in view of the experimental results summarized in the preceding chapters of this thesis. In fact, a different assignment appears to be untenable on the basis of previous experiments and the experimental evidence reported in the following sections.

Additional support for the H_2 assignment outlined above is provided by a comparison of the chemical shifts of the H_2 protons of ApA and ApApA at low temperatures where intramolecular base-stacking interactions are stronger. The low temperature chemical shifts of these H_2 protons, referenced with respect to the H_2 proton chemical shift of adenosine under approximately the same experimental conditions, are presented in Table XXV. The H_2(5') resonances of both ApA and ApApA are shifted approximately 0.11 ppm upfield relative to H_2 of adenosine, and the higher field shifts of the respective H_2(3') resonances are also quite similar (~0.27 ppm) at low temperature. Finally, the H_2(C) proton resonance experiences the most pronounced upfield shift at low temperature. These data substantiate the spectral assignment of the H_2 trimer resonances.

An assignment of the adenine H_8 resonances of ApApA by a comparison with the H_8 chemical shifts of ApA at normal probe temperature is not as straightforward because the resonance frequencies of

TABLE XXV. Low Temperature Chemical Shifts of the H_2 Protons of ApA, ApApA, and ApApApA

Molecule	Conc. M	Temp. °C	Chemical Shift[a] (ppm)		
			$H_2(5')$	$H_2(C)$	$H_2(3')$
ApA	0.003	5	0.10	- -	0.28
ApApA	0.007	3	0.120	0.384	0.264
ApApApA	0.007	1	0.142	[0.362, 0.388][b]	0.302

[a]Upfield shift from the H_2 resonance of adenosine under similar experimental conditions.

[b]The specific assignment of the H_2 resonances of the two interior nucleosides of the tetramer has not been determined.

the H_8 trimer protons are quite similar to 30°C. We shall discuss the assignment of the H_8 resonances in more detail in the temperature studies reported in Part 2 of Section C. A tentative assignment of the H_8 spectral region of ApApA is possible, however, by comparing the dimer and trimer H_8 resonance frequencies at high and low temperatures. This comparison is presented in Table XXVI. In this table, the resonances are labelled as they were assigned from the results of the detailed temperature studies discussed in Section C, Part 2.

At higher temperatures, the difference in the chemical shifts of the respective adenine ring protons caused by the ring-current magnetic anisotropy of the planar bases becomes less pronounced. For example, at 95°C the resonance frequencies of the three H_2 proton resonances of ApApA are practically equivalent. A similar situation would be expected for the H_8 protons of ApApA if the phosphate

TABLE XXVI. Chemical Shifts[a] of the H_8 Protons of ApA, ApApA, and ApApApA at High and Low Temperatures

Molecule	Conc. M	Temp. °C	Chemical Shift[a] (ppm)		
			$H_8(5')$	$H_8(C)$	$H_8(3')$
ApA	0.01	5	0.11	- -	0.10
ApApA	0.007	3	0.199	0.256	0.108
ApApApA	0.007	1	0.230	[0.282, 0.374][b]	0.120
ApA	0.01	80	-0.02	- -	0.08
ApApA	0.007	78	0.005	0.021	0.113
ApApApA	0.007	80	0.008	[0.042, 0.052][b]	0.123

[a]Upfield shift from the H_8 resonance of adenosine under similar experimental conditions.

[b]The specific assignment of the H_8 resonances of the two interior nucleosides of the tetramer has not been determined.

deshielding effect (Chapter II, this thesis) were neglected. Since the deshielding of the adenine H_8 protons by the phosphate moiety is not noticably sensitive to temperature variation in molecules of this nature, however, the resonance signals of the H_8 protons of the two ApApA nucleosides which have a 5'-esterified phosphate group would be expected to be at lower field than the remaining H_8 resonance at high temperature. The data listed in Table XXVI show that one of the H_8 resonances of the trimer is approximately 0.1 ppm upfield from the other two H_8 absorption signals at 78°C; this resonance has, therefore, been assigned to the $H_8(3')$ proton of ApApA. Of the two remaining resonances in the H_8 spectral pattern of the trimer, the resonance

frequency of the proton of the central nucleoside would be expected to be at higher field than that of $H_8(5')$ (particularly at lower temperatures where base-stacking interactions are stronger) because the ring protons of the central adenine base may be subject to ring-current anisotropy effects of both neighboring bases. For example, the chemical shifts listed in Table XXV show that the $H_2(C)$ proton of ApApA experiences a much greater upfield shift at temperatures less than 5°C than either $H_2(3')$ or $H_2(5')$. Accordingly, the highest field H_8 resonance of ApApA under low temperature conditions may be assigned to the $H_8(C)$ proton.

The chemical shifts of the ribose $H_{1'}$ protons of ApA and ApApA at normal probe temperature are listed in Table XXVII. The $H_{1'}(5')$ resonance of ApA is 0.12 ppm downfield from $H_{1'}(3')$. Since one of the ApApA $H_{1'}$ proton resonances is considerably downfield from the other two and has a chemical shift quite similar to the $H_{1'}(5')$ ApA proton, we have assigned the lowest field $H_{1'}$ resonance of the trimer to $H_{1'}(5')$. On the basis of a similar comparison, it appears that the highest field ribose $H_{1'}$ resonance signal should be assigned to $H_{1'}(3')$ although this assignment is not as straightforward because the chemical shifts of $H_{1'}(C)$ and $H_{1'}(3')$ differ by only 0.039 ppm at 28°C. At temperatures below 10°C, the $H_{1'}(3')$ proton resonance of ApA is ~0.15 ppm upfield from the $H_{1'}(5')$ absorption band and approximately 0.3 ppm upfield from the $H_{1'}$ doublet of adenosine under similar conditions of temperature, solution pH, and concentration. Of the two resonances of ApApA having chemical shifts similar to $H_{1'}(3')$ of ApA at ~28°C (Table XXVII), one (the central resonance of the $H_{1'}$ spectral pattern at 28°) is ~0.2 ppm upfield from the corresponding $H_{1'}$ resonance of adenosine

TABLE XXVII. Chemical Shifts[a] of the $H_{1'}$ Protons of ApA and ApApA

Molecule	Conc. M	Temp. °C	Chemical Shift (ppm)		
			$H_{1'}(5')$	$H_{1'}(C)$	$H_{1'}(3')$
ApA	0.01	29	6.42	- -	6.30
ApApA	0.007	28	6.380	6.319	6.280

[a]Downfield shift from external TMS.

while the other (the highest field $H_{1'}$ doublet at 28°C) is ~0.3 ppm (the same as $H_{1'}(3')$ of ApA) upfield from the $H_{1'}$ adenosine resonance. Therefore, the assignment of the highest field $H_{1'}$ ribose resonance signal of ApApA at normal probe temperature to the $H_{1'}(3')$ proton of the trimer as indicated in Table XXVII appears to be reasonable.

2. Temperature Variation of the ApApA Proton Resonances.

The temperature dependence of the H_2, H_8, and $H_{1'}$ chemical shifts of ApApA has been determined in an effort to study the intramolecular base-stacking interactions in this trinucleoside diphosphate. The importance of minimizing intermolecular effects by working at a low trinucleotide concentration has been noted previously. Accordingly, in the temperature studies of ApApA, we have employed a 0.0074 M solution. Our earlier studies of the dinucleoside monophosphates (Chapters II, III, and IV, this thesis) have shown that at this concentration the effect of the intermolecular association on the spectral properties of molecules of this nature is small. At such low concentrations, however, computer time averaging is necessary to obtain a suitable signal-to-noise ratio in the p.m.r. spectra. An additional

experimental consideration is the choice of an appropriate reference compound for the chemical shift measurements. This problem has been discussed in detail in Chapter III (Section E; Part 2) of this thesis. In the discussion presented there, it was noted that the proton chemical shifts of various possible reference compounds exhibit different temperature dependence in D_2O with respect to the proton chemical shift of the external TMS sample, suggesting that changes in the solute-solvent interaction with temperature variation may influence the resonance frequency of the solute molecule. Since the primary objective of the ApApA temperature studies is to examine changes effected in the intramolecular interactions in the trinucleoside diphosphate by temperature variations, it is important to choose a reference compound which interacts with the D_2O solvent in a manner similar to ApApA. It is also critically important to monitor the proton chemical shifts of the reference compound at a solution concentration comparable to the molecule of interest. This minimizes differences arising from intermolecular association effects of the two molecules. For these reasons, the H_2, H_8, and H_1, resonances of the 0.0074 M ApApA solution have been monitored with respect to the corresponding proton resonances of 0.0099 M adenosine in D_2O. Both the adenosine reference resonances and the trimer chemical shifts were monitored relative to the proton resonance of internal $N(CH_3)_4^+$ at various temperatures ranging from 1^o-98^oC. The use of a very small concentration of $N(CH_3)_4Cl$ in each solution made possible a direct comparison of the chemical shifts of the adenosine protons and the corresponding ApApA protons at each temperature at which measurements were made with the $N(CH_3)_4^+$ resonance serving as an intermediate reference.

The detailed temperature dependence of the H_2 chemical shifts of ApApA is illustrated in Figure 57, and the total shifts of the H_2 resonances as well as the H_8 resonances of ApA, ApApA, and ApApApA over comparable temperature ranges are summarized in Table XXVIII. The chemical shifts of the ApApA H_2 protons were measured over a temperature range of 3° to 95°C.

Each of the H_2 trimer resonances is shifted downfield relative to the corresponding adenosine nucleoside resonance as the temperature is increased. Since the H_2 protons of ApApA are relatively far-removed from the ribose-phosphate backbone and the spectral positions of these protons do not appear to be particularly sensitive to the nature of the ribose-phosphate backbone, it is reasonable to assume that the temperature induced changes in the H_2 chemical shifts arise primarily from changes in the intramolecular base-stacking interactions in the ApApA molecule and the accompanying changes in the effect of the ring-current magnetic anisotropy of the respective adenine bases on the protons of neighboring bases. For example, as the base-stacking interactions decrease with increasing temperature, downfield shifts would be expected for protons exposed to the anisotropy of an adjacent base. Therefore, important information concerning the intramolecular interaction between the bases of the trinucleotide may be inferred from the temperature shifts of the various proton resonances, particularly the H_2 resonances because they are influenced only to a small extent by the magnetic effects associated with the atoms of the molecular backbone.

The temperature shift of the $H_2(C)$ proton resonances of ApApA is significantly larger than that of the $H_2(3')$ or $H_2(5')$ proton resonance

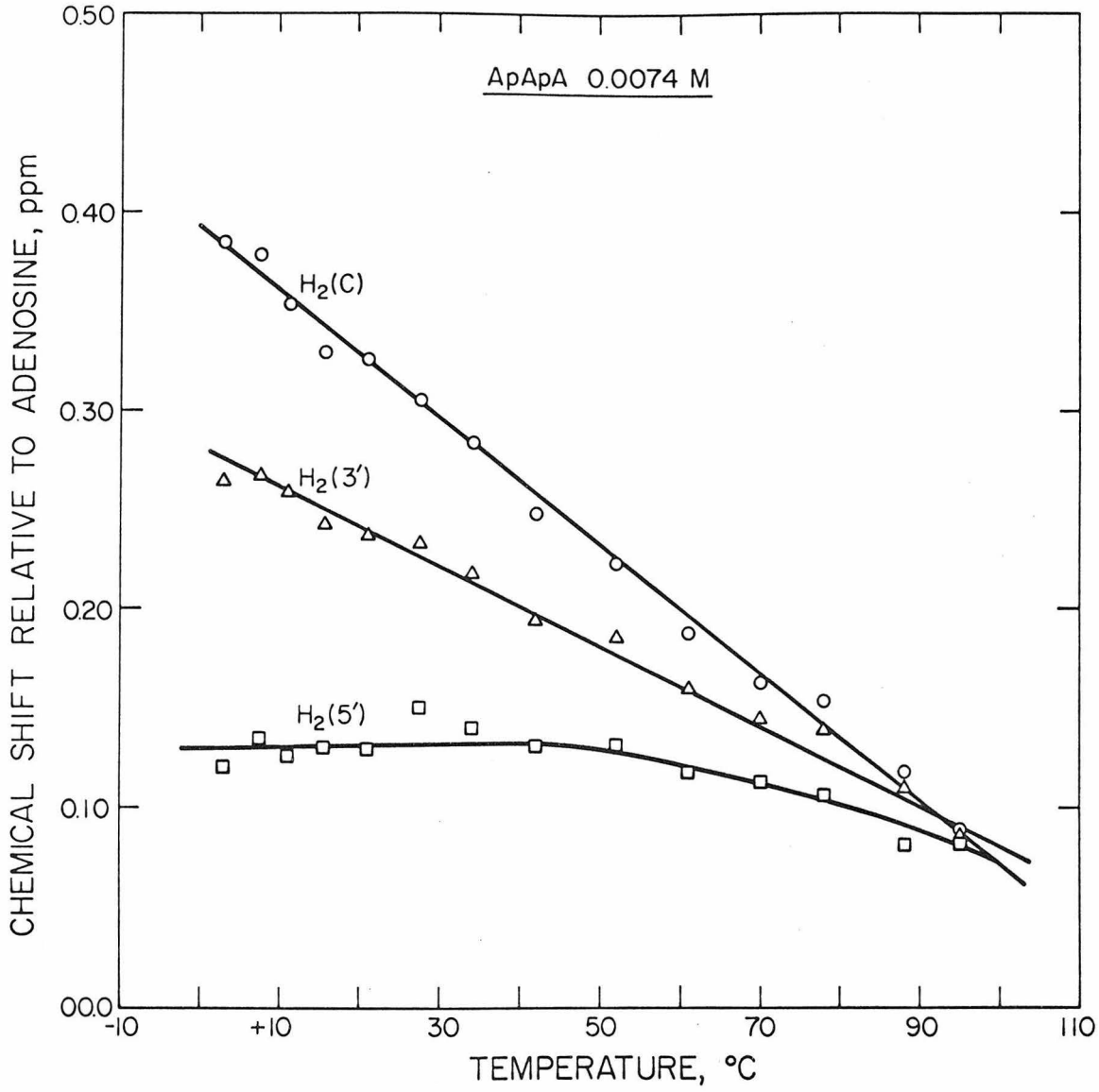


Figure 57. Temperature variation of the H_2 chemical shifts of ApApA (0.0074 M). The chemical shifts are monitored relative to the H_2 proton resonance of adenosine under similar experimental conditions.

TABLE XVIII. Temperature Shifts of the H_2 and H_8 Proton Resonances of ApA, ApApA, and ApApApA

Molecule	Temp. Range °C	Conc. M	Total Shift[a]					
			$H_2(5')$	$H_2(C)$	$H_2(3')$	$H_8(5')$	$H_8(C)$	$H_8(3')$
ApA	5-95	0.003	0.045	- -	0.191			
ApApA	3-95	0.007	0.037	0.295	0.175			
ApApApA	1-98	0.007	0.041	[0.194, 0.258][b]	0.145			
ApA	5-80	0.003				0.140	- -	0.015
ApApA	3-78	0.007				0.194	0.235	0.005
ApApApA	1-80	0.007				0.222	[0.240, 0.322][b]	-0.003

[a]Total downfield shift in ppm with increasing temperature.

[b]The specific assignment of the two H_8 and of the two H_2 resonances of the two interior nucleosides of the tetramer has not been determined.

of either ApApA or ApA. Since the $H_2(C)$ resonance is 0.384 ppm upfield from the H_2 proton resonance of adenosine at 3°C (Table XXV), the large downfield shift of this resonance with increasing temperature is not unexpected. It may be concluded from this large shift that ApApA is strongly intramolecularly stacked at low temperatures and that the stacking interaction decreases with increasing temperature. Since the temperature shifts of the two remaining H_2 resonances of the trinucleotide are much smaller, it is also reasonable to assume that the $H_2(C)$ proton experiences an upfield shift from the ring-current anisotropy of both neighboring adenine bases. Apparently, both the <u>syn</u> conformation and the <u>anti</u> conformation of the central adenine base of ApApA are

significantly populated at low temperatures. Otherwise, the much larger temperature shift of $H_2(C)$ would be unexpected.

The data of Table XXVIII also show that the $H_2(3')$ resonances of ApA and ApApA experience a similar downfield shift with increasing temperature. Accordingly, it would appear that the chemical shift of each of these protons is influenced to a comparable extent by the ring-current magnetic anisotropy of the respective neighboring base. This implies that the strength of the intramolecular base-stacking interaction is approximately the same in both molecules (the somewhat smaller temperature shift in ApApA may reflect slightly weaker base stacking in the trinucleotide) and that the average conformation of the 3'-adenine base of both molecules is similar. Since the results of earlier studies have shown that the 3'-base of ApA preferentially assumes the anti conformation with respect to rotation about the glycosidic bond, we may conclude that the 3'-base of ApApA is also preferentially oriented in the anti conformation. With the 3'-base in this conformation, the $H_2(3')$ proton is located directly below the adenine ring of the neighboring nucleoside. Therefore, at low temperatures where strong intramolecular base-stacks are formed, this proton experiences a sizable shift to higher field due to the effect of the ring-current anisotropy of the adjacent base. Increased temperature results in decreased base-stacking and a downfield shift for $H_2(3')$.

As was observed for the $H_2(3')$ temperature shifts of ApA and ApApA, the temperature shifts of the $H_2(5')$ protons of the adenine dinucleotide and the adenine trinucleotide are also very similar over comparable temperature ranges. For these proton resonances, however, the shifts are relatively small (~ 0.04 ppm over

the temperature range 3° - 95°C). If the 5'-base of each of the molecules under consideration preferentially assumes the anti conformation, the $H_2(5')$ proton of each molecule would be relatively far-removed from the ring-current anisotropy of the neighboring base and would not be expected to experience a large shift to higher field with the formation of strong intramolecular base-stacks. Consequently, a small temperature shift would also be expected for each $H_2(5')$ proton. Accordingly, the small temperature variation of the $H_2(5')$ proton chemical shift of ApA has been attributed to the preferential anti conformation of the 5'-base; and it is reasonable to assume that the similarly small temperature shift of the adenine trinucleotide $H_2(5')$ resonance also arises from the preferential anti conformation of the 5'-base of this molecule.

Finally, at approximately 95°C the chemical shifts of all three ApApA H_2 protons are almost equivalent and ~0.09 ppm upfield from the H_2 resonance of adenosine. This residual higher field shift at 95° indicates that the trimer molecule is not completely unstacked at this temperature. Since the chemical shifts of all the H_2 protons are equal, however, sufficient destacking of the bases has occurred to result in similar intramolecular ring-current shifts for all of the H_2 protons.

The H_2 proton temperature shifts of ApApA provide excellent experimental evidence that both the 3'- and 5'-bases are preferentially oriented in the anti conformation. The preferential conformation of the base of the central nucleoside cannot be determined from the temperature shifts of the H_2 protons. However, on the basis of other experiments, it would appear that this base is also preferentially oriented in the anti conformation.

The temperature shifts of the trinucleotide H_8 protons are illustrated in Figure 58 and summarized in Table XXVIII. Since the temperature variation of the H_2 chemical shifts has demonstrated that the 3'- and 5'-bases of ApApA preferentially assume the anti conformation, the nature of the various H_8 proton temperature shifts may be used to assign the H_8 proton resonance spectrum of ApApA. Due to the preferential anti conformation of the 3'-base, a small temperature shift would be expected for the $H_8(3')$ proton. The shifts depicted in Figure 58 demonstrate that the chemical shift of only one of the H_8 protons of ApApA is essentially independent of temperature when monitored relative to the H_8 resonance of adenosine. Therefore this resonance signal has been assigned to $H_8(3')$; this assignment is in agreement with the spectral assignment discussed previously. Of the two remaining H_8 resonances of ApApA, that of the central nucleoside would be expected to experience a greater temperature shift. [In the case of the H_2 resonances of ApApA, the temperature shift of $H_2(C)$ is considerably greater than that of either $H_2(3')$ or $H_2(5')$ (Table XXVIII)]. Since the higher field resonance of the remaining pair of H_8 absorption signals is shifted further with temperature variation, this resonance has been assigned to $H_8(C)$. This observation is also in agreement with our earlier assignment of the H_8 resonances.

The spectral position of the $H_8(C)$ proton resonance of ApApA at 80°C is particularly significant. It is similar to that of $H_8(5')$ and at considerably lower field than that of $H_8(3')$. This would be expected only if the $H_8(C)$ proton chemical shift is appreciably influenced by the 5'-esterified phosphate group of the central nucleoside. Apparently the

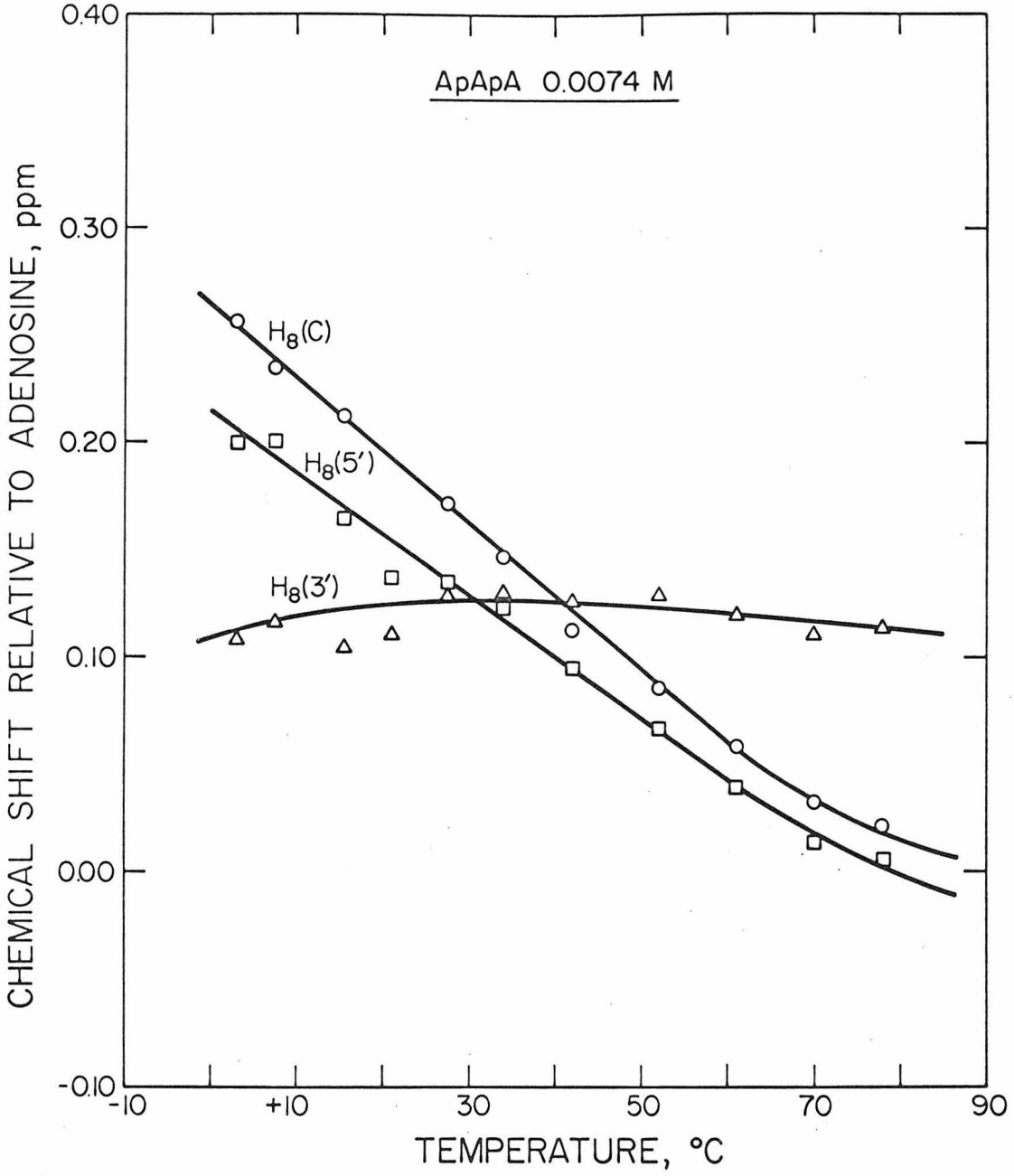


Figure 58. Temperature variation of the H_8 chemical shifts of ApApA (0.0074 M). The chemical shifts are monitored relative to the H_8 proton resonance of adenosine under similar experimental conditions.

average location of the H_8(C) proton of ApApA is relatively close to the 5'-esterified phosphate of the interior adenosine nucleoside. Since H_8(C) can be in close proximity to this phosphate group only when the base of the interior nucleoside assumes the anti conformation, it appears that the central adenine base of ApApA is also preferentially oriented in the anti conformation.

The similar temperature behavior of the two H_8(5') resonances and of the two H_8(3') resonances of ApA and ApApA is evident from the results summarized in Table XXVIII. Again, these similarities emphasize the conformational similarity of these two molecules in aqueous solution.

The temperature variation of each of the chemical shifts of the $H_{1'}$ ribose protons of ApApA is depicted in Figure 59; and Table XXIX summarizes the total change in the $H_{1'}$ chemical shifts and the total change in the magnitudes of the respective $H_{1'}$-$H_{2'}$ coupling constants of the trinucleotide over the temperature range 9°-85°C. The temperature shifts of the $H_{1'}$ ribose protons of ApApA are somewhat difficult to interpret because several different factors including the ribose ring conformation and the rotational conformation of the base about the $C_{1'}$-N_9 glycosidic bond may influence the resonance frequency of these protons. Since these factors have been discussed in detail in Chapter III, Section E(4), we shall not discuss them extensively here.

All of the $H_{1'}$ resonances of ApApA shift downfield with increasing temperature although the extent of the downfield shift is markedly different for each proton. Similarly, downfield shifts have been observed for the $H_{1'}$ ribose protons of ApA (78, Chapter II, this thesis) and of

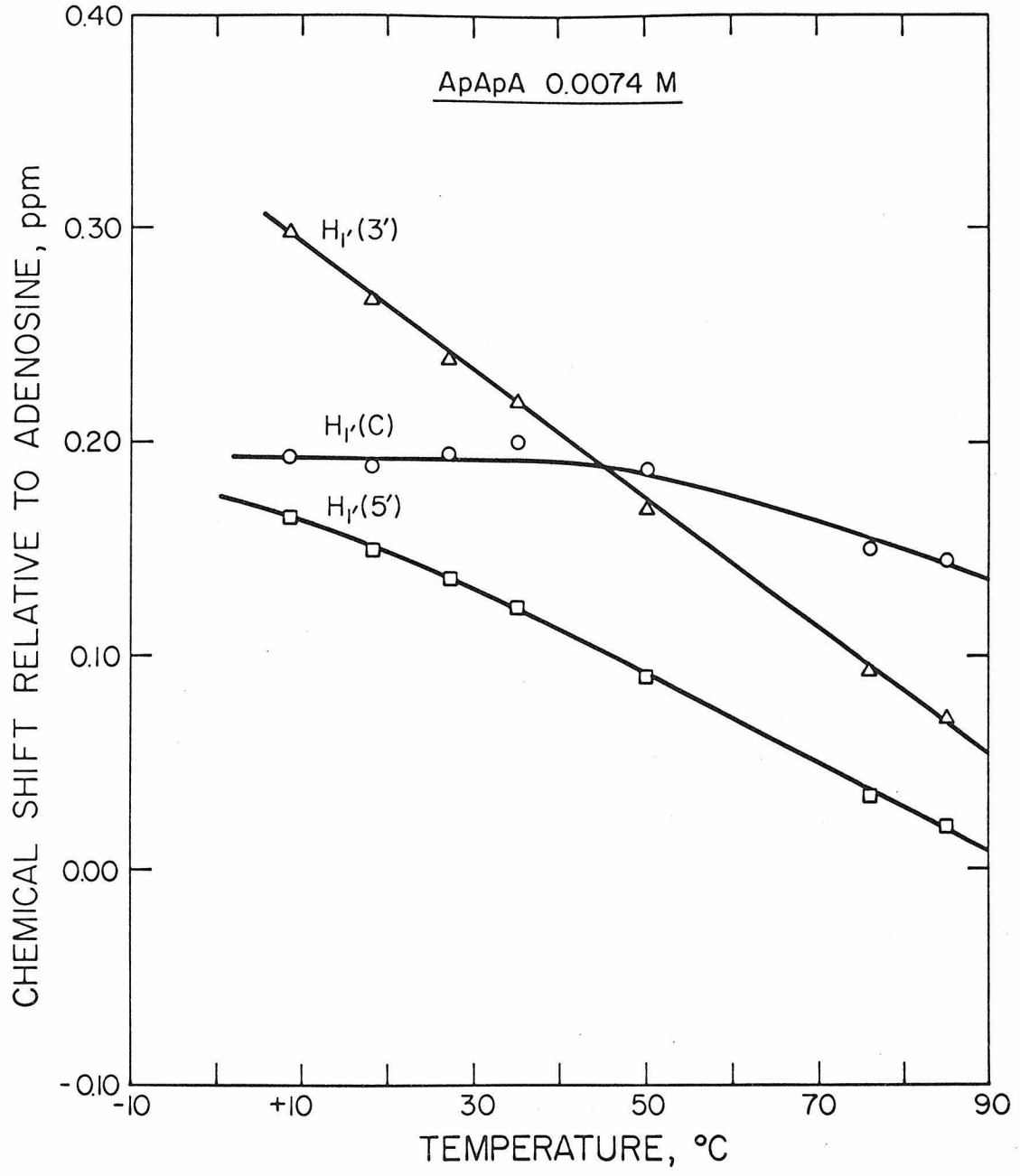


Figure 59. Temperature variation of the H_1' chemical shifts of ApApA (0.0074 M). The chemical shifts are monitored relative to the H_1' proton resonance of adenosine under similar experimental conditions.

TABLE XXIX. Temperature Variation of the $H_{1'}$ Chemical Shifts and $H_{1'}$-$H_{2'}$ Coupling Constants of ApApA (0.007 M)

Temp. °C	$H_{1'}$(3')		$H_{1'}$(C)		$H_{1'}$(5')	
	Chem. Shift[a]	|J|[b]	Chem. Shift	|J|	Chem. Shift	|J|
9	0.298	2.0	0.193	2.6	0.164	2.7
18	0.266	2.4	0.188	3.2	0.149	3.2
27	0.239	2.9	0.194	3.8	0.135	3.3
50	0.169	3.4	0.187	3.4	0.090	3.5
85	0.071	5.1	0.145	4.7	0.020	5.0
Total Change (9-85°C)	0.227	3.1	0.048	2.1	0.144	2.3

[a]Upfield shift in ppm from the $H_{1'}$ resonance of adenosine under similar experimental conditions.

[b]In cps.

several other dinucleoside monophosphates (Chapter III, this thesis). These temperature shifts are caused by a decrease in the intramolecular base-stacking interactions and the accompanying conformational changes which occur within the molecule. Although the shifts are complicated by various factors, it has been noted in Chapters II and III that the ribose rings of the dinucleotides assume more 2'-endo (vs. 3'-endo) conformational character with increasing temperature (decreasing intramolecular base-stacking); and more 2'-endo conformational character results in downfield shifts for the $H_{1'}$ ribose resonances. A similar effect would be expected for ApApA, and from the data tabulated in Table XXIX it would appear that greater changes are effected in the ribose ring conformation of the 3'-esterified nucleoside of ApApA by variations in

temperature than in the conformation of either of the other ribose rings. The total temperature shift of the $H_{1'}(3')$ proton of ApApA is significantly greater than that of either $H_{1'}(C)$ or $H_{1'}(5')$ of the trinucleotide and also greater than the temperature shifts of the $H_{1'}$ protons of ApA. In addition, a greater change is produced in the $H_{1'}$-$H_{2'}$ coupling constant of the 3'-nucleoside of ApApA. A smaller value of $|J_{H_{1'}\text{-}H_{2'}}|$ is indicative of more 3'-endo conformational character for the furanose ring. The value of $|J_{H_{1'}\text{-}H_{2'}}|$ of the 3'-nucleoside of ApApA changes from 2.0 cps at 9°C to 5.1 cps at 85°C, an increase of 3.1 cps over the entire temperature range. In comparison, the magnitudes of the $H_{1'}$-$H_{2'}$ coupling constants of the 5'-nucleoside and the interior nucleoside increase 2.3 and 2.1 cps, respectively, over the same range of temperatures. These changes are comparable to those observed for ApA. Apparently the destacking process in ApApA is accompanied by increased 2'-endo conformational character for each of the ribose rings with the ribose conformation of the 3'-nucleoside experiencing the greatest change. The greater conformational change induced in the ribose ring of the 3'-nucleoside partially accounts for the unusually large temperature shift of the $H_{1'}(3')$ proton. It is noteworthy that the ribose ring of the 3'-nucleoside of several dinucleoside monophosphates has also been observed to undergo greater conformational changes with destacking of the planar bases than the 5'-ribose ring. In the dinucleotides, it appears that greater changes must be effected in the ribose conformation of the 3'-nucleoside to obtain effective intramolecular base-stacking. (For a more detailed discussion see Section E (4) of Chapter III.) Evidently, greater changes are also effected in the 3'-nucleoside of

ApApA by the intramolecular base-stacking interactions.

In summary, the temperature dependence of the p. m. r. spectrum of ApApA has shown that the intramolecular base-stacking interactions in this oligonucleotide are relatively strong and comparable to those in ApA. Each of the adenine bases of ApApA preferentially assumes the anti conformation in the stacked configuration of the molecule, and the 3'-endo conformation of each of the ribose rings becomes more favorable with increased base-stacking.

3. Purine-ApApA Interactions. The study of the intermolecular interaction between purine and several dinucleoside monophosphates has proven to be useful in the elucidation of the conformational properties and the base-stacking properties of the dinucleotides. Similar purine-binding studies have also been applied to the study of the base-stacking interactions in ApApA and the results of these studies are reported in this section.

The purine-induced shifts of the adenine protons of the trinucleotide at 30°C are illustrated in Figure 60. These shifts are compared with similar purine-induced shifts for the corresponding protons of ApA in Table XXX. The results of purine-binding studies of ApApA at 6°C are also summarized in this table.

The purine-induced shifts of the $H_{1'}$ ribose protons were not monitored at several different purine concentrations due to complications in the spectra arising from overlapping of the resonance absorption bands; and, therefore, the effect of purine-binding on these resonances is not reported here.

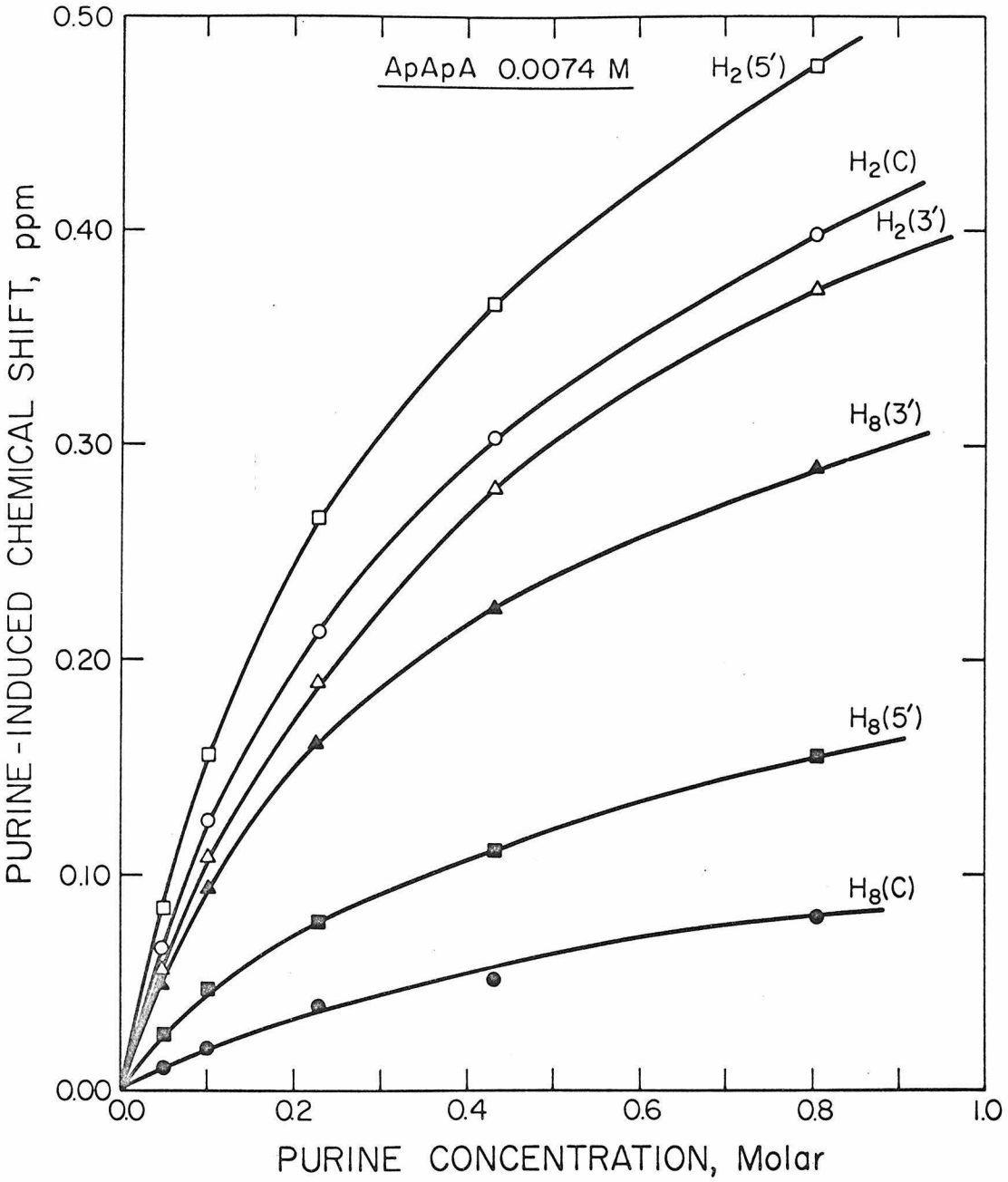


Figure 60. Purine-induced shifts of the H_2 and H_8 adenine resonances of ApApA (0.0074 M) at 30°C.

TABLE XXX. Purine-Induced Shifts of the Adenine Protons of ApA (0.01 M) and ApApA (0.007 M) in 0.8 M Purine

Molecule	Temp. °C	Purine-Induced Shift[a] (ppm)					
		$H_8(5')$	$H_8(C)$	$H_8(3')$	$H_2(5')$	$H_2(C)$	$H_2(3')$
ApA	30	0.190	- -	0.295	0.475	- -	0.450
ApApA	30	0.155	0.080	0.290	0.476	0.398	0.373
ApApA	6	0.123	0.032	[b]	0.546	0.402	0.436

[a]Upfield shift with purine addition.

[b]$H_8(3')$ resonance overlapped by intense purine band at 6°C.

All of the adenine proton resonances of the trinucleotide are shifted upfield with the addition of purine. This higher field shift is the result of the formation of intermolecular base-stacks between purine and the planar adenine rings of the trimer. As was observed in the case of ApA, the H_8 resonances experience smaller shifts than the H_2 resonances because the H_8 protons are in closer proximity to the ribose-phosphate backbone of the molecule, and are, therefore, less exposed to the ring-current effects of the purine bases as intermolecular complexes are formed.

The purine-induced shift of the $H_8(C)$ proton resonance is much smaller than that of either $H_8(3')$ or $H_8(5')$ and considerably less pronounced than the purine-induced shifts observed for the $H_8(A)$ proton resonances of dinucleoside monophosphates composed of adenosine nucleosides. This is the result of the severe steric shielding of the $H_8(C)$ proton of ApApA. When the central adenine base assumes the anti conformation, $H_8(C)$ is extensively shielded from the ring-current

effects of complexing purine molecules by the ribose-phosphate backbone and the neighboring adenine rings. However, intercalated purine molecules would be expected to cause some upfield shift for both the $H_8(C)$ and the $H_2(C)$ proton resonances. Since a much larger purine-induced shift is observed for the $H_2(C)$ resonance, it is reasonable to assume that the central adenine base of the trinucleotide is preferentially oriented in the anti conformation. In this conformation, the $H_8(C)$ proton is not readily influenced by the ring-current magnetic anisotropy of either a purine base stacked on the external faces of the terminal ApApA bases or an intercalated purine molecule.

The induced chemical shift of $H_8(3')$ is 0.135 ppm greater than that of $H_8(5')$ at a purine concentration of 0.8 M. The significantly larger shift of the $H_8(3')$ proton is the direct result of the preferential anti conformations of both the 3'-base and the 5'-base of ApApA. Two different effects contribute to the larger upfield shift of the $H_8(3')$ proton with the addition of purine. First, the $H_8(5')$ proton is shielded by the molecular backbone of ApApA when the 5'-base assumes the anti conformation. This reduces the effectiveness of the ring-current magnetic anisotropy of a complexing purine base stacked on the external face of the 5'-adenine base in shifting the $H_8(5')$ resonance to higher field. Due to the geometry of the ApApA molecule, the $H_8(3')$ proton is not similarly shielded, and a larger shift would be expected for this proton. Second, when the 5'-adenine base assumes the anti conformation, $H_8(5')$ is located directly above the adenine base of the central nucleoside of ApApA. Therefore, when purine intercalation disrupts the adenine-adenine base-stacking interaction between the 5'-base and the interior

base of the trinucleotide, the upfield shift effected in the $H_8(5')$ resonance by the ring-current magnetic anisotropy of the intercalated purine molecule would be partially compensated by a reduction in the upfield ring-current effect of the interior adenine base on the $H_8(5')$ resonance. Since the $H_8(3')$ proton of ApApA is not significantly influenced by the magnetic anisotropy of the central adenine base when the 3'-base assumes the more favorable anti conformation, a similar attenuation of the purine-induced shift would not be expected for $H_8(3')$ when purine intercalation occurs. Hence, as in the case of ApA, the nature of the differential purine-induced shift of the $H_8(3')$ and $H_8(5')$ protons of ApApA is a direct consequence of the preferential anti conformations of both the 3'-base and the 5'-base.

The resonance frequency of the $H_2(5')$ proton is shifted more extensively to higher field with the addition of purine than that of either $H_2(C)$ or $H_2(3')$. The energetically more favorable anti conformations of the adenine bases are again responsible for the differential purine-induced shifts. Due to the preferential anti conformation of each of the bases, the upfield purine-induced shift of the $H_2(5')$ proton is not reduced by changes which occur in the intramolecular base-stacking interactions as a result of purine intercalation, while the purine-induced higher field shifts of both $H_2(3')$ and $H_2(C)$ are less pronounced because of decreased intramolecular adenine-adenine base-stacking.

It is somewhat surprising that the H_2 resonance of the central nucleoside of ApApA shifts further upfield with the addition of purine than $H_2(3')$. On the basis of the compensating effect of reduced intramolecular base-stacking on the upfield purine-induced shifts, $H_2(C)$

might be expected to experience a much smaller shift than either $H_2(3')$ or $H_2(5')$. Apparently, the formation of purine-intercalated complexes, especially those in which a purine base is inserted between the 3'-base and the central base of the trinucleotide (a very small compensating downfield shift of $H_2(C)$ results from the decreased adenine-adenine base-stacking), is sufficiently favorable to cause the $H_2(C)$ resonance to experience a greater upfield with the addition of purine than $H_2(3')$. Increasing the intramolecular base-stacking interactions would, therefore, be expected to result in a smaller purine-induced shift for $H_2(C)$ relative to that of either $H_2(3')$ or $H_2(5')$. A smaller relative shift would be expected for the $H_2(C)$ proton from two different viewpoints. First, the purine intercalation process would be less favorable; and, second, the reduction of the purine-induced chemical shifts by intramolecular ring-current effects would be more pronounced, and this would be expected to influence the net shift of $H_2(C)$ to a much greater extent than that of either $H_2(5')$ or $H_2(3')$.

Since the intramolecular base-stacking interactions in ApApA are considerably stronger at low temperatures, we have studied the binding of purine to this trinucleotide at 6°C. Although the binding affinity of purine for the adenine bases is increased at lower temperatures, the relative tendency of purine to form intercalated complexes is reduced by increased intramolecular adenine-adenine base-stacking and increased purine intermolecular association. The results of the purine-binding experiments at 6°C are shown in Table XXX. The purine-induced shift of each of the H_2 resonances is increased. However, the increase in shift of the $H_2(5')$ proton is greatest while that of

H_2(C) is least, as expected from the observations of the previous discussion. In fact, under these experimental conditions, H_2(C) experiences a smaller purine-induced shift than either of the other H_2 resonances.

It is interesting that the H_8(C) proton resonance of ApApA is shifted upfield only 0.032 ppm at 0.8 M purine and 6°C. This upfield shift is approximately 0.05 ppm smaller than the corresponding purine-induced shift observed for the same resonance at 30°C. The extremely small purine-induced shift at the lower temperature is, of course, a reflection of the effective steric shielding of the H_8(C) proton under conditions which favor strong intramolecular base-stacking interactions.

Finally, it is of interest to compare the purine-induced shifts of the analogous protons of ApA and ApApA at normal probe temperature (Table XXX). Both of the H_2(5') protons experience a very similar shift (~0.29 ppm) at a purine concentration of 0.8 M; the purine-induced shifts of the H_8(3') protons are also equivalent (0.475 ppm) at this purine concentration. On the other hand, the shift of H_2(3') of ApApA is only 83% as great as the corresponding shift of the dinucleotide in a solution of 0.8 M purine. Similarly, the purine-induced shift of the H_8(5') resonance of the trimer is only 82% as great as that of the analogous dimer shift. The chemical shift of each of the trinucleotide protons which exhibits a smaller purine-induced shift than the analogous proton of ApA [H_2(3') or H_8(5')] is significantly influenced by the ring-current magnetic anisotropy of the adjacent adenine base in the intramolecular stack, whereas the resonance frequency of each of the

trinucleotide protons having a purine-induced shift similar to the corresponding dinucleotide proton [$H_2(5')$ or $H_8(3')$] is affected only to a small extent by the neighboring base. Thus, it would appear that the smaller shifts of the $H_8(5')$ and $H_2(3')$ protons of ApApA with the addition of purine arise primarily from subtle differences in intramolecular base-stacking effects in ApApA and ApA.

As noted previously, the formation of purine-intercalated complexes with the dinucleoside monophosphates results in linebroadening for the purine resonances. The purine resonances linewidths are also increased in the presence of ApApA. In Table XXXI the linewidths of the H_6, H_2, and H_8 resonances of purine in the presence of ApA and of ApApAp are summarized at various purine concentrations. The linebroadening pattern of the purine resonances is the same for each molecule: H_6 linewidth > H_8 > H_2. Therefore, the orientation of the intercalated purine molecule appears to be approximately the same for both ApA and ApApA. There is one significant difference in the effect of these two molecules on the purine proton magnetic resonance spectrum, however. The trinucleotide appears to broaden each of the purine resonances to a greater extent than the dinucleotide under similar conditions of concentration. This could be attributed to the formation of more stable intercalated complexes in the case of the trinucleotide molecule or to differences in the kinetics of the two systems.

The results of these purine-binding experiments show that the formation of intermolecular complexes between purine and ApApA involves vertical base-stacking interactions and that a significant number of purine-intercalated complexes are formed. More important, however,

these results demonstrate that there is relatively strong intramolecular base-stacking in ApApA at 30°C and that each of the adenine bases preferentially assumes the anti conformation in the intramolecular base-stacks.

TABLE XXXI. Purine Resonance Linewidths in the Presence of ApA and of ApApA

Molecule	Purine Conc. (M)	Purine Resonance Linewidth[a] (cps)		
		H_6	H_2	H_8
ApA (0.01 M)	0.02	2.0	1.2	2.0
	0.57	0.8	0.8	0.8
	0.7	1.0	0.9	1.0
	0.8	0.9	1.0	0.8
ApApA (0.007 M)	0.048	~20	4.5	~20
	0.101	10	2.8	9.2
	0.228	6.8	2.1	5.0
	0.804	3.0	1.5	2.4

[a]Full linewidth at half signal intensity.

D. Proton Magnetic Resonance Studies of ApApApA

1. Assignment of the H_2 and H_8 Resonances. The resonances of the adenine ring protons of 0.0074 M ApApApA are sufficiently narrow in the temperature range 1°-98°C to be generally well-resolved although some overlapping of the spectral peaks is observed at various temperatures as might be expected. The H_8 resonances were distinguished from the H_2 absorption signals by deuterium exchange of the

H_8 protons at elevated temperatures in the usual manner; and at temperatures greater than 40°C, each of the H_8 proton resonances is at lower field than the H_2 resonance signals.

The specific spectral assignment of the H_2 resonances of the tetranucleotide have been made on the basis of a comparison of the resonance frequencies of the H_2 protons of this molecule with those of ApA and ApApA, particularly at low temperatures. The low temperature chemical shifts of the H_2 protons are summarized in Table XXV. The $H_2(5')$ resonance of both ApA and ApApA is at a much lower field than the other H_2 resonances at 3°-5°C. Correspondingly, one of the H_2 resonances of the adenine tetranucleotide is also considerably downfield from the other resonances in the H_2 spectrum of this oligonucleotide, and this resonance has been assigned to the $H_2(5')$ proton. As discussed previously in the assignment of the adenine trinucleotide spectrum, the H_2 protons of the central adenine bases of an oligonucleotide would be expected to resonate at higher field than either of the H_2 resonances of the terminal bases at low temperatures. The resonance frequencies of the H_2 protons of the interior bases of ApApA and ApApApA would also be expected to be quite similar when experimental conditions favor strong intramolecular base-stacking. According to the chemical shifts listed in Table XXV, the highest field H_2 absorption signal in the p. m. r. spectrum of ApApA and the two highest field H_2 resonances of ApApApA have similar resonance frequencies at temperatures lower than 3°C. It is reasonable, then, to assign these two high field resonances of the H_2 proton spectrum of the tetranucleotide to the two interior bases. Although the H_2 protons of the two interior

adenine bases are not chemically equivalent, no distinguishing assignment has been made, and each of these protons is listed as $H_2(C)$ in the tables of this chapter. As indicated in Table XXV, the remaining proton resonance is assigned to $H_2(3')$, and at 1°C the spectral peak of this proton is 0.06-0.09 ppm downfield from the two $H_2(C)$ resonances.

The assignment of the H_8 resonances in the proton magnetic resonance spectrum of ApApApA has been made in a similar manner. Due to the effects of phosphate deshielding, the $H_8(3')$ proton, which is not deshielded by a phosphate group, would be expected to resonate at a higher field than the other H_8 resonances at higher temperatures where the effects of the adenine ring-currents are more limited. The H_8 chemical shifts of ApApApA, ApApA, and ApA at 80°C are summarized in Table XXVI. In the tetranucleotide spectrum, one of the H_8 resonances is 0.07-0.12 ppm upfield from the remaining H_8 resonances, and this resonance signal has been assigned to the $H_8(3')$ proton. It is noteworthy that the chemical shift of this proton is very similar to that of the corresponding proton of ApApA as would be expected.

The low temperature shifts of the H_8 protons provide a convenient method for assigning the remaining H_8 resonances of ApApApA. Again, the absorption signals of the two interior adenine bases would be expected to be at higher fields than that of $H_8(5')$ when strong intramolecular base-stacks are formed. Therefore, at 1°C, it is reasonable to assign the two highest field resonances in the H_8 spectral pattern of ApApApA to the H_8 protons of the interior adenine bases. At 1°C, the assignment of the H_8 resonances of ApApApA is, in the order of increasing field: $H_8(3')$, $H_8(5')$, and the $H_2(C)$'s. The data of

Table XXVI show that the expected similarity in the $H_8(5')$ chemical shifts of ApApA and ApApApA at both high and low temperatures is observed.

Finally, for reference purposes we have summarized the chemical shifts of the adenine ring protons of ApApA and ApApApA relative to external TMS at normal probe temperature in Table XXXII.

2. Temperature Variation of the ApApApA Proton Resonances. The temperature dependence of the chemical shifts of the adenine protons of ApApApA has been studied in a manner similar to that described previously for the adenine protons of the trinucleotide.

The temperature shifts of the H_2 resonances of ApApApA are shown in Figure 61, and the total shift of these resonances as well as the H_2 resonances of ApApA and ApA over large temperature ranges are summarized in Table XXVIII. The tetranucleotide H_2 chemical shifts were measured over a temperature range of 1^{o}-$95^{o}C$, and each of the H_2 resonances is shifted downfield at $95^{o}C$ relative to its spectral position at $1^{o}C$. Since the H_2 protons are far-removed from the ribose-phosphate backbone and the resonance frequency of each of these protons does not appear to be particularly sensitive to the properties of the molecular backbone, it is reasonable to assume that the temperature shifts of the H_2 protons of ApApApA arise from changes in the intramolecular base-stacking. As indicated in Table XXVIII, the total variation of each of the chemical shifts of the adenine tetranucleotide over the temperature range 1^{o}-80^{o} is comparable to that of the corresponding shift of ApApA. It may be concluded from this

TABLE XXXII. Chemical Shifts of the Adenine Protons of ApApA and ApApApA Relative to External TMS at Normal Probe Temperature

Molecule	Conc. M	Temp. °C	Chemical Shift (ppm)							
			$H_8(5')$	$H_8(C)^a$	$H_8(C)^a$	$H_8(3')$	$H_2(5')$	$H_2(C)^a$	$H_2(C)^a$	$H_2(3')$
ApApA	0.0074	28	8.626	8.589		8.632	8.531	8.366		8.445
ApApApA	0.0074	29	8.619	8.590	8.511	8.613	8.516	8.382	8.360	8.393

[a]The specific assignment of the resonances of the two interior nucleosides of the tetranucleotide has not been determined.

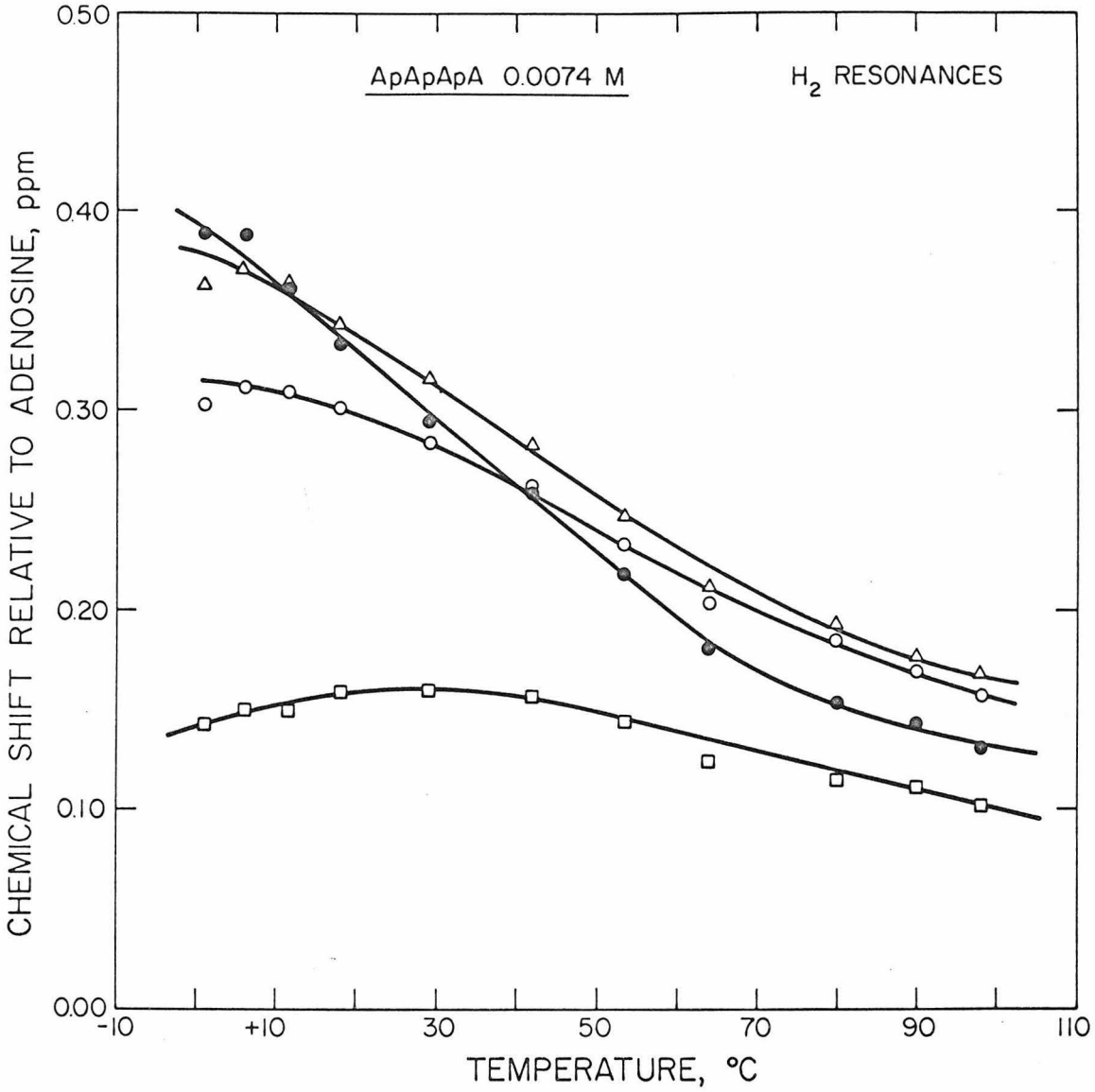


Figure 61. Temperature variation of the H_2 chemical shifts of ApApApA (0.0074 M). The chemical shifts are monitored relative to the H_2 proton resonance of adenosine under similar experimental conditions. $H_2(5')$, (□); $H_2(3')$, (○); $H_2(C)$, (△); $H_2(C)$, (●). Each of the H_2 resonances of the two interior adenine bases is designated $H_2(C)$. A distinguishing assignment has not been determined.

observation that the strength of the intramolecular base-stacking interactions in these molecules is similar.

The most notable feature of the data illustrated in Figure 61 is the extremely small temperature shift of the $H_2(5')$ proton. A similarly small shift was observed for $H_2(5')$ of ApApA. As explained previously, the small $H_2(5')$ temperature shift is the result of the preferential anti conformation of the adenine base of the 5'-esterified nucleoside. Since the 3'-adenine base of the trinucleotide preferentially assumes the anti conformation, the similar temperature dependence of the $H_2(3')$ protons of ApApA and ApApApA would seem to indicate that the anti conformation is also energetically more favorable for the base of the 3'-nucleoside of the tetramer.

Similar temperature shifts were observed for the $H_2(5')$ protons of the adenine dimer, trimer and tetramer. Although the agreement is not quite as good in the case of the $H_2(3')$ proton, the temperature shift of this proton is also similar in each of the three adenine oligomers. This would be expected if the terminal base conformations are similar in each molecule.

The temperature shifts of the two H_2 protons of the interior bases of ApApApA are greater than those of the other two H_2 protons. Therefore, it is reasonable to assume that the resonance frequency of these protons is influenced by both neighboring bases in the intramolecular stacks.

As in the case of ApApA, the chemical shifts of the H_2 protons of ApApApA become increasingly similar with increasing temperature due to weaker intramolecular base-stacking interactions. At 95°C, however, there appears to be more intramolecular base-stacking in

the tetranucleotide because the shifts are at higher fields and are more dissimilar than the H_2 chemical shifts of ApApA.

The temperature dependence of the H_8 protons of ApApApA is shown in Figure 62; and the total shifts of these protons in the temperature range 1°-80°C are listed in Table XXVIII. The preferential anti conformation of the base of the 3'-esterified nucleoside is reflected in the small shift observed for $H_8(3')$, and the similar shifts of the $H_8(5')$ protons of ApApApA and ApApA provide additional evidence that the anti conformation of the 5'-base of the tetranucleotide is energetically more favorable.

Although one of the $H_2(C)$ resonances of the tetranucleotide exhibits approximately the same temperature dependence as $H_2(C)$ of ApApA, the other $H_2(C)$ proton is shifted unusually far upfield at low temperatures. The explicit reason for this large upfield shift is not obvious, but it probably has its origin in subtle conformational changes in the molecule, and it demonstrates the sensitivity of proton magnetic resonance spectroscopy to such conformational changes. The larger upfield shift of one of the two $H_2(C)$ protons of ApApApA with decreasing temperature could arise from a more favorable orientation of the proton with respect to the ring-current anisotropy of the neighboring bases, an increase in the average distance between the proton and the phosphate group, or a different rotational conformation of the adenine base about the glycosidic bond.

The chemical shifts of the two $H_8(C)$ protons and $H_8(5')$ are approximately equivalent at 95°C while $H_8(3')$ is ~0.10 ppm upfield. The protons of each of the three lower field resonances are evidently

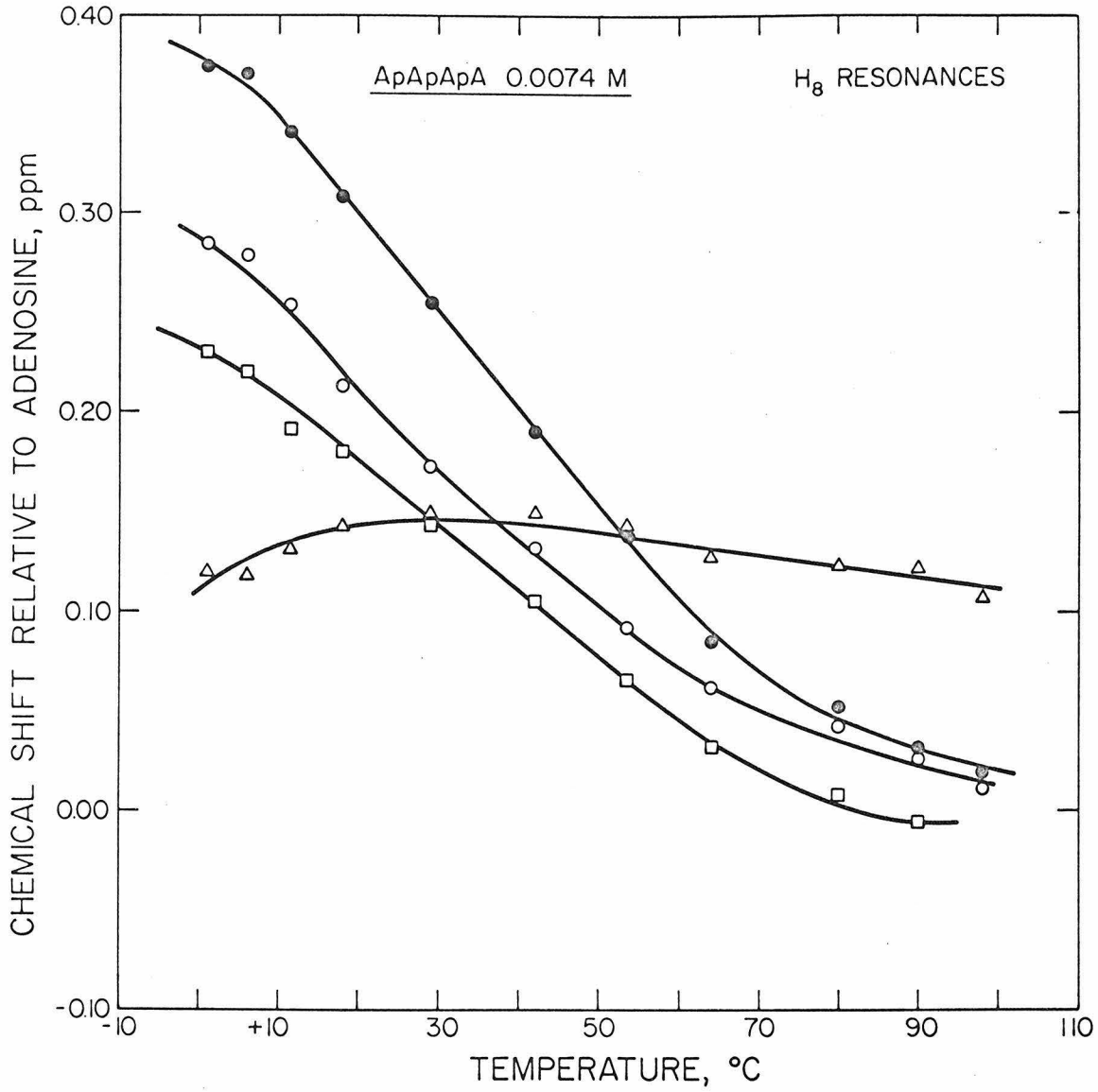


Figure 62. Temperature variation of the H_8 chemical shifts of ApApApA (0.0074 M). The chemical shifts are monitored relative to the H_8 proton resonance of adenosine under similar experimental conditions. $H_8(3')$, (△); $H_8(5')$, (□); $H_8(C)$, (○); $H_8(C)$, (◉). Each of the H_8 resonances of the two interior adenine bases is designated as $H_8(C)$. A distinguishing assignment has not been determined.

deshielded by the 5'-esterified phosphate group of the respective nucleosides, and the deshielding effect is similar for all of these protons. Since the 5'-nucleoside of ApApApA is oriented in the anti conformation, it appears from the nature of the high temperature H_8 shifts, that the two interior bases of the tetranucleotide also preferentially assume the anti conformation.

These proton magnetic resonance studies of ApApApA have shown that the resonances of the H_2 and H_8 adenine ring protons are relative narrow and reasonably well-resolved in the temperature range 1^{o}-98^{o}C. The temperature shifts of these protons demonstrate that the tetranucleotide is strongly intramolecularly stacked in aqueous solution at room temperature, and that the intramolecular stacking interactions appear to be comparable to those in ApApA and ApA. Apparently, the base-stacking interactions observed for many of the dinucleoside monophosphates are equally important in determining the conformational properties of the larger nucleic acids. Each of the adenine proton resonances of ApApApA is shifted differently with temperature variation, but the temperature shifts of the analogous protons of ApA, ApApA, and ApApApA are remarkably similar. From these shifts we have concluded that the adenine bases of each molecule are preferentially stacked in the anti conformation, as in a similarly stacked segment along one strand of double helical DNA, and that the stacking properties of the interior bases of the adenine oligonucleotides are similar to those of the terminal bases.

E. Conclusion

Through the application of NMR spectroscopy to the study of the base-stacking interactions in oligonucleotides and dinucleotides, we have been able to make some definite statements concerning the conformational properties of these molecules in solution and the relative energetics of the stacking interactions between various bases. In addition we have discussed the importance of these observations in relation to structural properties of the biologically significant nucleic acids. The vertical base-stacking interactions between two purine bases are stronger than the analogous purine-pyrimidine or pyrimidine-pyrimidine interactions along a polynucleotide chain, and adenine-adenine interactions appear to be important in determining the structural properties of native nucleic acids and polynucleotides in aqueous solution.

It is interesting that the adenine bases of ApA and of the oligoribonucleotides, ApApA and ApApApA, are stacked with the bases preferentially oriented in the anti conformation as in a similarly stacked dApdApdApdA (dA = deoxyadenosine) segment along one strand of double helical DNA. The free energy differences between various stacked conformers probably do not arise from differences in the details of the base-stacking interaction between the adenine bases. (The anti conformation is also energetically more favorable than the syn conformation for guanine, cytosine and uracil in the stacked configuration of the dinucleoside monophosphates we have studied.) However, the structural properties of the native nucleic acids and their derivatives are intimately related to the origin of the base-stacking

interactions. The hydrophobic stacking interactions between bases, nucleosides, and nucleotides in aqueous solution have generally been attributed to van der Waals and London dispersion forces (16), and with few exceptions (110), the special role of the solvent has not received sufficient recognition. We suggest that water plays a significant role in determining these base-stacking interactions. These bases are intrinsically unstable in aqueous solution, and were it not for certain polar side groups which stabilize them in solution, they might not even be soluble in water. The instability of the bases in aqueous solution is inherent in the strongly hydrogen-bonded water structure and the resultant strong self-cohesion energy of the solvent. Considerable free energy is therefore required to create a cavity in aqueous solution to accommodate the base and to form the interface between the planar bases and the solvent. There is, thus, a driving force for bases to form stacks in aqueous solution to attain the minimum interface contact surface between the solute and the solvent. If this were the only important driving force, the base-stacking tendencies should increase with the size of the base. The experimental facts are not inconsistent with this picture. We have already noted that purine bases self-associate more strongly than pyrimidine bases, and it is well known that the larger acridine dyes (111) associate extensively in aqueous solution even at extremely low concentrations. The total free energy of stacking must, naturally, also include any direct electrostatic interaction between the bases themselves. This electrostatic interaction can be either attractive or repulsive, depending upon the relative orientation of the bases, the extent of base-overlap, etc. Rough estimates of these interaction

energies have been made by DeVoe and Tinoco (16), by Claverie, Pullman, and Caillet (72), and by Sinanoglu and Abdulnur (110); and the magnitudes of these interaction free energies are typically quite large. It is therefore surprising that the free energy differences between the various stacked conformers of ApA, ApApA, or ApApApA do not reflect any dependence upon those details which are expected to influence the direct electrostatic energy between the two bases.

We believe that conformation energies of the adenine base relative to the ribose moiety about the glycosidic bond play a prominent role in determining the preferred conformation of the adenine rings in the intramolecular stack. Of the many possible base conformations relative to the ribose moiety, it is clear that the strong intramolecular stacking interaction between the two adenine bases would stabilize only the DNA-like anti conformation and the syn conformation in which the adenine base is rotated 180° about the glycosidic bond from its DNA-like orientation. Hence we need only to concern ourselves with the difference in the intrinsic conformation energy between these two base orientations about the glycosidic linkage. Ts'o and co-workers (30, 35) have proposed that the anti base conformation is favored in the ribose purine nucleosides because of the formation of an intramolecular hydrogen bond between the 2'-hydroxyl group of the ribose ring and N_3 of the purine base. We feel, however, that this cannot be the primary explanation, since we expect the anti base conformation to be of comparable energetic favorability in the deoxyribose purine nucleosides, in which the 2'-hydroxyl would not be present. We suggest that the DNA-like (anti) base conformation is energetically more favorable in the purine

nucleosides and, therefore, in the adenine oligoribonucleotides because of repulsive interactions between the N_3 nitrogen lone pairs and the lone pairs of the ether oxygen of the furanose ring when the adenine base is oriented in the syn conformation. A similar repulsive interaction presumably exists in the pyrimidine nucleosides between the 2-keto oxygen and the same ether oxygen of the furanose ring when the conformation of the pyrimidine base is syn.

Although these studies demonstrate that vertical base-stacking interactions are instrumental in determining the conformational properties of nucleic acids and their derivatives in aqueous solution, it is also evident that several other factors make important contributions. The vital biological functions of the nucleic acids are therefore dependent on a delicate balance and interplay of subtle intramolecular forces and continued scrutiny into the nature of these fundamental forces will hopefully bring us closer to an understanding of life itself.

REFERENCES

1. F. Miescher, Hoppe-Seyler's Med. Chem. Unters., 441 (1871).

2. O. Avery, C. MacLeod, and M. McCarty, J. Exptl. Med. 79, 137 (1944).

3. J. Watson and F. Crick, Nature 171, 737 (1953).

4. H. R. Mahler and E. H. Cordes, Biological Chemistry (Harper and Row, New York, 1966), p. 787.

5. F. H. C. Crick, Biochem. Soc. Symp. (Cambridge, England) No. 14, 25 (1957).

6. J. R. Fresco, B. M. Alberts, and P. Doty, Nature 188, 98 (1960).

7. P. Doty, H. Boedtker, J. R. Fresco, R. Haselkorn, and M. Litt, Proc. Natl. Acad. Sci. U. S. 45, 482 (1959).

8. A. S. Spirin, J. Mol. Biol. 2, 436 (1960).

9. J. R. Fresco, A. Adams, R. Ascione, D. Henley, and T. Lindahl, Cold Spring Harbor Symp. Quant. Biol. 31, 527 (1966).

10. S. W. Englander and J. J. Englander, Proc. Natl. Acad. Sci. U. S. 53, 370 (1965).

11. T. Lindahl, A. Adams, and J. R. Fresco, Proc. Natl. Acad. Sci. U. S. 55, 940 (1966).

12. B. Pullman, J. Chem. Phys. 43, S233 (1965).

13. T. T. Herskovits, S. J. Singer, and E. P. Geiduschek, Arch. Biochem. Biophys. 94, 99 (1961).

14. E. P. Geiduschek and T. T. Herskovits, Arch. Biochem. Biophys. 95, 114 (1961).

15. P. O. P. Ts'o, G. K. Helmkamp, and C. Sander, Proc. Natl. Acad. Sci. U. S. 48, 686 (1962).

16. H. DeVoe and I. Tinoco, Jr., J. Mol. Biol. 4, 500 (1962).

17. D. M. Crothers and B. H. Zimm, J. Mol. Biol. 15, 455 (1966).

18. C. C. McDonald, W. D. Phillips, and J. Lazar, J. Am. Chem. Soc. 89, 4166 (1967).

19. C. C. McDonald, W. D. Phillips, and J. Penswick, Biopolymers 3, 609 (1965).

20. G. Felsenfeld and H. T. Miles, Ann. Rev. Biochem. 36, 407 (1967).

21. R. F. Steiner and R. F. Beers, Jr., Polynucleotides (Elsevier, Amsterdam, 1961), Chap. 7.

22. J. Applequist and V. Damle, J. Am. Chem. Soc. 88, 3895 (1966).

23. D. N. Holcomb and I. Tinoco, Jr., Biopolymers 3, 121 (1965).

24. M. Leng and G. Felsenfeld, J. Mol. Biol. 15, 455 (1966).

25. J. Brahms, A. M. Michelson, and K. E. Van Holde, J. Mol. Biol. 15, 467 (1966).

26. J. P. McTagne, V. Ross, and J. H. Gibbs, Biopolymers 2, 163 (1964).

27. C. C. McDonald, W. D. Phillips, and S. Penman, Science 144, 1234 (1964).

28. G. D. Fasman, C. Lindblow, and L. Grossman, Biochemistry 3, 1015 (1964).

29. E. G. Richards, C. P. Flessel, and J. R. Fresco, Biopolymers 1, 431 (1963).

30. P. O. P. Ts'o, S. A. Rapaport, and F. J. Bollum, Biochemistry 5, 4153 (1966).

31. P. K. Sarkar and J. T. Yang, J. Biol. Chem. 240, 2088 (1965).

32. B. W. Bangerter and S. I. Chan, Biopolymers 6, 983 (1968).

33. P. O. P. Ts'o, I. S. Melvin, and A. C. Olson, J. Am. Chem. Soc. 85, 1289 (1963).

34. P. O. P. Ts'o and S. I. Chan, J. Am. Chem. Soc. 86, 4176 (1964).

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

36. P. O. P. Ts'o, "The Physicochemical Basis of Interactions of Nucleic Acid," in Molecular Associations in Biology, ed. by B. Pullman (Academic Press, New York, 1968), pp. 40-44.

37. K. E. Van Holde and G. P. Rossetti, Biochemistry 6, 2189 (1967).

38. G. P. Rossetti and K. E. Van Holde, Biochem. Biophys. Res. Commun. 26, 717 (1967).

39. S. J. Gill, M. Downing, and G. F. Sheats, Biochemistry 6, 272 (1967).

40. P. R. Stoesser and S. J. Gill, J. Phys. Chem. 71, 564 (1967).

41. J. A. Schellman, Compt, Rend. Trav. Lab. Carlsberg, Ser. Chim. 29, 223 (1956).

42. J. A. Pople, W. G. Schneider, and H. J. Bernstein, High-resolution Nuclear Magnetic Resonance (McGraw-Hill, New York, 1959), p. 400.

43. R. R. Shoup, H. T. Miles, and E. D. Becker, Biochem. Biophys. Res. Commun. 23, 194 (1966).

44. L. Katz and S. Penman, J. Mol. Biol. 15, 220 (1966).

45. J. W. Emsley, J. Feeney, and L. H. Sutcliffe, High Resolution Nuclear Magnetic Resonance Spectroscopy (Pergamon Press, Oxford, 1965), Vol. 1, pp. 140, 595-604.

46. C. E. Johnson and F. A. Bovey, J. Chem. Phys. 29, 1012 (1958).

47. G. K. Helmkamp, S. I. Chan, M. P. Schweizer, and P. O. P. Ts'o, "Physical Chemical Studies on the Secondary Structures of Nucleic Acid Polymers and on the Interactions of Free Bases and Nucleosides," in Physikalische Chemie biogener Makromolekule (Akademie, Berlin, 1964), pp. 273-285.

48. S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, J. Am. Chem. Soc. 86, 4182 (1964).

49. O. Jardetzky, "Proton Magnetic Resonance of Purine and Pyrimidine Derivatives," in Biopolymers Symposia No. 1, ed. by M. Weissbluth (Interscience, New York, 1964), p. 501.

50. G. K. Helmkamp and N. S. Kondo, Biochim, Biophys. Acta 145, 27 (1967).

51. M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, J. Am. Chem. Soc. 87, 5241 (1965).

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

53. A. M. Michelson, J. Chem. Soc., 3655 (1959).

54. M. F. Singer, L. A. Heppel, G. W. Rushizky, and H. A. Sober, Biochim. Biophys. Acta 61, 474 (1962).

55. A. M. Michelson, Chemistry of Nucleosides and Nucleotides (Academic Press, London, 1963), pp. 445-452.

56. K. E. Van Holde, J. Brahms, and A. M. Michelson, J. Mol. Biol. 12, 726 (1965).

57. J. Brahms, A. M. Michelson, and K. E. Van Holde, J. Mol. Biol. 15, 467 (1966).

58. J. Brahms, J. C. Maurizot, and A. M. Michelson, J. Mol. Biol. 25, 465 (1967).

59. G. B. Zavil'gel'skii and L. Li, Molekulyarnaya Biologiga 1, 323 (1967).

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

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

62. M. M. Warshaw, C. A. Bush, and I. Tinoco, Jr., Biochem. Biophys. Res. Commun. 18, 633 (1965).

63. A. M. Michelson, T. L. V. Ulbricht, R. T. Emerson, and R. J. Swan, Nature 209, 873 (1966).

64. D. Poland, J. N. Vournakis, and H. A. Scheraga, Biopolymers 4, 223 (1966).

65. J. N. Vournakis, H. A. Scheraga, G. W. Rushizky, and H. A. Sober, Biopolymers 4, 33 (1966).

66. Y. Inoue, S. Aoyagi, and K, Nakaniski, J. Am. Chem. Soc. 89, 5701 (1967).

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

68. M. M. Warshaw and I. Tinoco, Jr., J. Mol. Biol. 13, 54 (1965).

69. M. M. Warshaw and I. Tinoco, Jr., J. Mol. Biol. 20, 29 (1966).

70. C. R. Cantor and I. Tinoco, Jr., Biopolymers 5, 821 (1967).

71. C. R. Cantor, S. R. Jaskunas, and I. Tinoco, Jr., J. Mol. Biol. 20, 39 (1966).

72. P. Claverie, B. Pullman, and J. Caillet, J. Theoret. Biol. 12, 419 (1966).

73. E. Shefter, M. Barlow, R. Sparks, and K. Trueblood, J. Am. Chem. Soc. 86, 1872 (1964).

74. F. E. Hruska and S. S. Danyluk, Biochim. Biophys. Acta 157, 238 (1968).

75. S. I. Chan, B. W. Bangerter, and H. H. Peter, Proc. Natl. Acad. Sci. U. S. 55, 720 (1966).

76. Y. Inoue and S. Aoyagi, Biochem. Biophys. Res. Commun. 28, 973 (1967).

77. K. H. Scheit, F. Cramer, and A. Franke, Biochim, Biophys. Acta 145, 21 (1967).

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

79. B. W. Bangerter, Ph. D. Thesis, California Institute of Technology, Pasadena, California, 1969.

80. B. W. Bangerter and S. I. Chan, in preparation.

81. B. W. Bangerter and S. I. Chan, Proc. Natl. Acad. Sci. U. S. 60, 1144 (1968).

82. S. I. Chan, and J. H. Nelson, J. Am. . Chem. Soc., in press.

83. J. H. Nelson, B. W. Bangerter, J. H. Prestegard, and S. I. Chan, in preparation.

84. J. H. Prestegard, private communication.

85. P. K. Glasoe and F. A. Long, J. Phys. Chem. 64, 188 (1960).

86. S. S. Danyluk and F. E. Hruska, Biochemistry 7, 1038 (1968).

87. J. Donohue and K. N. Trueblood, J. Mol. Biol. 2, 363 (1960).

88. R. G. Shulman, H. Sternlicht, and B. J. Wyluda, J. Chem. Phys. 43, 3116 (1965).

89. E. Walaas, Acta Chem. Scand. 12, 528 (1958).

90. C. D. Jardetzky and O. Jardetzky, J. Am. Chem. Soc. 82, 222 (1960).

91. C. D. Jardetzky, J. Am. Chem. Soc. 84, 62 (1962).

92. C. D. Jardetzky, J. Am. Chem. Soc. 82, 229 (1960).

93. A. Albert and D. J. Brown, J. Chem. Soc., 2060 (1954).

94. D. O. Jordon, The Chemistry of Nucleic Acids (Butterworths, Washington, D.C., 1960), p. 137.

95. J. M. Read, Jr., and J. H. Goldstein, J. Am. Chem. Soc. 87, 3440 (1965).

96. H. Simpkins and E. G. Richards, Biochemistry 6, 2513 (1967).

97. H. T. Miles, F. B. Howard, and J. Frazier, Science 142, 1458 (1963).

98. H. T. Miles and J. Frazier, Biochim Biophys. Acta 79, 216 (1964).

99. C. Giessner-Prettre and B. Pullman, Compt. Rend. 261, 2521 (1965).

100. R. K. Ralph, W. J. Connors, and H. G. Khorana, J. Am. Chem. Soc. 84, 2265 (1962).

101. M. Gellert, M. N. Lipsett, and D. R. Davies, Proc. Natl. Acad. Sci. U. S. 48, 2013 (1962).

102. M. Karplus, J. Chem. Phys. 30, 11 (1959).

103. J. W. Emsley, J. Feeney, and L. H. Sutcliffe, High Resolution Nuclear Magnetic Resonance Spectroscopy (Pergamon Press, Oxford, 1966), Vol. 2, pp. 670-674.

104. J. H. Prestegard and S. I. Chan, in preparation.

105. B. W. Bangerter, private communication.

106. J. A. Pople, Mol. Phys. 1, 168 (1958).

107. A. Carrington and A. D. McLachlan, Introduction to Magnetic Resonance (Harper and Row, New York, 1967), pp. 210-211.

108. Ref. 103, p. 1040.

109. Ref. 42, Chapter 9.

110. O. Sinanoglu and S. Abdulnur, Photochem. Photobiol. 3, 333 (1964).

111. D. J. Blears and S. S. Danyluk, J. Am. Chem. Soc. 88, 1084 (1966).

PROPOSITIONS

PROPOSITION I

It is proposed that the binding sites and thermodynamic quantities of specific metal-ligand interactions be determined in systems of nucleosides and nucleotides by nuclear magnetic resonance spectroscopy, Raman spectroscopy, and calorimetry, and that the results of these studies be applied in the investigation of the role of metal ions in biological systems.

The interaction of metal ions with nucleic acids is biologically important from several points of view. Of particular interest is the biological role of several cations in the stabilization of nucleic acids and the related transmission of hereditary information. In addition, certain metal ions have important catalytic roles in metabolic pathways involving nucleotides. Previous studies indicate that binding sites on polynucleotides vary for different cations and different experimental conditions resulting in either stabilization or destabilization of the macromolecule. For example, Cu (II) and Cd (II) ions at higher concentrations reduce the stability of double helical DNA (1-3), whereas Mn (II), Co (II), Ni (II), and other metal ions stabilize the double helix (4). Moreover, it has been demonstrated (5) that Cu (II) ions affect the dissociation of the double helical complex formed by the interaction of poly A with poly U, and of poly I with poly C (A = adenylic acid, U = uridylic acid, C = cytidylic acid, I = inosinic acid). The Cu (II) ion also has the interesting property of effecting the cleavage of phosphodiester bonds in polynucleotides (6). It is likely that the binding site of

the cation determines its effect on the molecule with which it interacts. Therefore, a study of the interaction of simple nucleosides and nucleotides (which can serve as model compounds for the macromolecules) with characteristic cations is proposed in an effort to determine the nature of the interaction and the specific binding sites of the ions. Eventually, these preliminary studies could also be extended to include dinucleotides, oligonucleotides, and even the polynucleotides. Since the guanine-cytosine content of nucleic acids apparently influences the properties of these larger molecules when metal binding occurs (7), a comparison of the binding properties of the various nucleotide constituents of nucleic acids is pertinent.

The interaction of paramagnetic ions with nucleosides and nucleotides may be investigated by monitoring the resonance linewidths of the base protons, the sugar protons, and the phosphorus nuclei (in the case of the nucleotides) as a function of metal ion concentration. When a paramagnetic ion such as Cu (II) binds to a molecule, the linewidths of the resonances of neighboring nuclei are selectively increased relative to those of nuclei farther removed from the binding site. This line broadening is the result of the decreased relaxation time of the nuclei in close proximity to the magnetic moment of the bound paramagnetic ion as a result of nuclear spin-electron spin dipolar interaction. Accordingly, a comparison of the linewidths of the nuclear magnetic resonances of a given molecule at various metal ion concentrations may determine the preferential binding site. These experiments must be carried out at a metal ion concentration sufficiently low to prevent complete collapse of the resonance signals. These concentrations will

be sufficiently low that the chemical shifts of the nucleoside (or nucleotide) resonances will not be significantly altered. However, the binding site of diamagnetic ions such as Mg (II) may be investigated by monitoring the chemical shifts of the biological moieties as a function of the cation concentration. Nuclei which are in close proximity to the binding site will presumably be deshielded to a greater extent than those nuclei which are farther removed. It appears that nuclear magnetic resonance spectroscopy is well suited to the study of the binding of both paramagnetic and diamagnetic metal ions to nucleosides, nucleotides, and larger molecules of a similar nature.

It is noteworthy that interactions of metal ions with nucleic acid derivatives may also be investigated by Raman spectroscopy since the binding of the cation to an aromatic base would be expected to cause significant changes in the π-electron distribution in the molecule (8). This would have considerable effect on polarizability changes accompanying the vibrations of atoms involved in the ion-nucleoside (nucleotide) bond. It is proposed that this method be used to supplement the NMR data.

As an important application of the spectroscopic determination of the metal ion binding sites, it is proposed that the results of these experiments be utilized in the interpretation of thermodynamic K, ΔH, and ΔS data which may be obtained from thermometric titrations and conventional calorimetry.

A specific proposition including the application of each of the procedures and methods discussed in the previous paragraphs to the study of a particular system is outlined below.

We begin with a cursory summary of pertinent major research findings. Line broadening effects of paramagnetic ions on the base proton resonances of several purines and pyrimidines have been observed (4, 6). However, the concentrations of the biological molecules were 0.1 M or greater. Since many of these planar molecules interact to form intermolecular complexes at higher concentrations (9-11), it is likely that the cation-base complexes involve more than one base, nucleoside, or nucleotide. Therefore, interpretation of the observations of experiments at high concentrations solely on the basis of the formation of one-to-one complexes may not be valid. Similar studies involving chemical shift changes in nucleic acids and their constituents as a result of the binding of diamagnetic ions have also been carried out at high concentration. More meaningful results would be anticipated in experiments involving lower nucleoside (nucleotide) concentrations.

Raman spectroscopy has been applied in the investigation of the binding of Hg (II) to cytidine (8). The results of these studies indicate that the heavy metal ion binds to the available N_3 position of the cytosine ring.

Phillips (12) has recently summarized the spectroscopic and thermodynamic data available for proton and metal ion interactions with adenosine and the adenine nucleotides. This review clearly shows that metal ions exhibit marked differences in their respective affinities for chain and ring binding sites and indicates the desirability of coordinated spectroscopic-thermodynamic studies of these metal complexes.

The following procedures are proposed in the initiation of such studies:

(1) The nuclear magnetic resonance linewidths of the adenine and ribose protons and the phosphate nuclei (in the nucleotides) of adenosine, and its -5'-mono-, -5'-di-, and -5'-triphosphates would be monitored at a low adenine derivative concentration (~0.01 M) as a function of the concentration of Mn (II) and of Cu (II). The concentration range of the metal ions would be approximately 10^{-2} to 10^{-6} M. These studies would be carried out in D_2O to eliminate the large proton resonance of water. Due to the small concentrations involved, time-averaging methods would be utilized in obtaining the nuclear magnetic resonance spectra. A comparison of the linewidths of the various resonances would be instrumental in the determination of the binding sites of each of these paramagnetic cations.

(2) Similarly, Mg (II) would be added to 0.01 M solutions of the adenine derivatives and the chemical shifts of the resonances would be monitored as a function of the cation concentration. These studies would also be carried out in the D_2O solvent with the aid of computer time-averaging. A comparison of the resonance positions would be helpful in elucidating the binding site of Mg (II) on each molecule.

(3) The Raman spectra of complexes formed between Cu (II), Mn (II), and Mg (II) and each of the adenine derivatives in aqueous solution would be investigated and correlated with the NMR data.

(4) Each of these metal ion-adenine systems would then be studied by conventional calorimetry (13) and/or thermometric titration methods (14-16) to determine equilibrium constants for the specific metal ligand interactions.

It is proposed that the determination of the binding sites and the thermodynamic quantities for these metal ligand interactions would result in increased understanding of the role of metal ions in biological systems.

REFERENCES

1. G. L. Eichorn and P. Clark, Proc. Natl. Acad. Sci. U. S. 53, 586 (1965).

2. Y. T. Zakharenko and Y. S. Moshkovski, Biofizika 11, 945 (1966).

3. S. Hiai, J. Mol. Biol. 11, 672 (1965).

4. G. L. Eichorn, Advances in Chemistry, No. 62, 378 (1967).

5. G. L. Eichorn and E. Tarien, Biopolymers 5, 273 (1967).

6. G. L. Eichorn, P. Clark, and E. D. Becker, Biochemistry 5, 245 (1966).

7. H. Venner and C. Zimmer, Biopolymers 4, 321 (1965).

8. R. C. Lord and G. J. Thomas, Jr., Biochim. Biophys. Acta 142, 1 (1967).

9. M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, J. Am. Chem. Soc. 87, 5241 (1965).

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

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

12. R. Phillips, Chem. Revs. 66, 501 (1966).

13. J. D. Hale, R. M. Izatt, and J. J. Christensen, J. Phys. Chem. 67, 2605 (1963).

14. J. J. Christensen, R. M. Izatt, L. D. Hansen, and J. A. Partridge, J. Phys. Chem. 70, 2003 (1966).

15. R. M. Izatt, D. Eatough, J. J. Christensen, and R. L. Snow, J. Phys. Chem. 72, 1208 (1968).

16. J. J. Christensen, R. M. Izatt, and L. D. Hansen, Rev. Sci. Instr. 36, 779 (1965).

PROPOSITION II

Ultraviolet (UV)-irradation of DNA and similar polynucleotides produces significant physicochemical changes in the structure of these molecules (1). These UV-induced structural alterations in the DNA of living cells are particularly important because they frequently result in the death or mutation of the cell. Ultraviolet-radiation in similar doses does not have similar effects on all living systems, however. For example, the mutant B_{S-1} strain of E. coli is markedly more sensitive to UV-radiation than strain B/r (2). Experimental evidence indicates that strains of bacteria capable of surviving doses of radiation which are lethal to other strains are also capable of repairing the molecularly damaged DNA (3). Details of the UV-induced physicochemical changes of DNA and the subsequent biological repair of these changes are not yet fully understood. However, there is good evidence that the changes include base alterations such as pyrimidine dimer formation (4). Experimental conditions apparently determine the nature of the DNA structural variations. Some proposed molecular steps involved in the repair mechanism are (a) excision of fragments of base-altered DNA, (b) degradation of segments of single strands of DNA, (c) repair replication of degraded DNA, and (d) covalent bonding of newly-synthesized DNA to the original strand. That these steps are completely responsible for DNA repair has not been demonstrated, and the order in which they occur and the molecular mechanism by which they function is not known. Therefore it is proposed to study the repair-replication of UV-damaged DNA

in an attempt to elucidate the molecular mechanism involved in the repair of bacterial DNA.

As mentioned above, a specific change produced in DNA by UV-radiation is the formation of cyclobutane-type dimers between contiguous pyrimidine bases along one strand of a polynucleotide (4). The formation of these dimers is detrimental to the proper functioning of a living cell and they are apparently eliminated from DNA during repair replication. Since these dimers may easily be followed by radioactive label and are stable to drastic isolation procedures, such as acid hydrolysis, they provide a convenient model system for studying the DNA repair mechanism (5).

Although newly-synthesized DNA has been observed in the chromosomal DNA of UV-damaged E. coli cells (6), no definitive evidence that the newly incorporated material actually replaces excised regions has been accumulated. Experimental procedures which would provide such evidence are proposed.

The demonstration that observed aberrant DNA synthesis is repair synthesis would involve two steps. The first is a control experiment designed to demonstrate that tritium-labeled thymine is removed as a cyclobutane-type dimer from the DNA of UV-irradiated E. coli cells that continue to function properly following dimer removal. The labeling of the cellular DNA may be accomplished by growing the cells for several generations in either [^{14}C]thymine or [^{3}H]thymine (New England Nuclear Corporation, Boston, Mass.) (7). In addition, a series of experiments is proposed to determine the UV-radiation dose which results in the maximum yield of thymine dimers from cells that

continue to function properly after irradiation.

In the second step, it is proposed that cells with [^{14}C]thymine-labeled DNA be irradiated with the optimum radiation dose as determined in step 1 and that [^{3}H]thymine be incorporated during repair replication in a heavy medium characterized by ^{15}N-labeled nucleosides or similarly heavy atoms. Due to the incorporation of the heavier nucleosides, heavy and light strands of DNA would be formed. It is proposed that these components be isolated by centrifugation (8) and fractionation methods (9) and that the relative thymine-thymine sequences of each be determined. The latter could be done by irradiating with large fluxes at long wave length or in the presence of a triplet sensitizer. This would effect dimer formation between nearly all adjacent thymines. In each case the dimers could be collected and the relative T-T sequences determined by counting the radioactive labels. More T-T sequences in the heavier constituents would provide excellent evidence that the newly incorporated material actually replaced excised dimers during aberrant DNA-synthesis which follows UV-irradiation.

REFERENCES

1. R. B. Setlow, "The Repair of Molecular Damage to DNA," in Radiation Research, ed. by G. Silini (North-Holland Publishing Co., Amsterdam, 1967), pp. 525-537.

2. R. F. Hill and E. Simson, J. Gen. Microbiol. 24, 1 (1961).

3. R. B. Setlow, "Repair of DNA," in Regulation of Nucleic Acid and Protein Biosynthesis, ed. by V. V. Koningsberger and L. Bosch (Elsevier, Amsterdam, 1967), pp. 51-62.

4. R. B. Setlow, Science 153, 379 (1966).

5. R. B. Stelow, "Steps in Repair of DNA: Fact and Fancy," in Recovery and Repair Mechanisms in Radiobiology (U. S. Brookhaven National Laboratory, Upton, New York, 1967).

6. D. Pettijohn and P. Hanawatt, J. Mol. Biol. 9, 395 (1964).

7. R. Hewitt and D. Billen, J. Mol. Biol. 13, 40 (1965).

8. M. Meselson and F. W. Stahl, Proc. Natl. Acad. Sci. U. S. 44, 671 (1958).

9. R. B. Setlow and W. L. Carrier, "The Excision of Pyrimidine Dimers in vivo and in vitro," International Conference on Replication and Recombination of Genetic Material, Canberra, Australia, Aug. 28–Sept. 1, 1967.

PROPOSITION III

The self-association of planar bases, nucleosides, and nucleotides in aqueous solution via vertical base-stacking is well-established (1-3), and similar intramolecular base-stacking interactions have been shown to be important in determining the conformational properties of dinucleotides and oligonucleotides in solution (4-6). The nature of the specific forces involved in the hydrophobic stacking interactions is, however, not completely understood, and such forces are currently the subject of considerable speculation and research. With regard to this problem, it is of interest to determine whether intermolecular association of the planar monomer units proceeds with the bases assuming specific orientations with respect to each other or whether the monomers stack randomly in the intermolecular complexes.

As outlined in the following paragraphs, preliminary calculations involving purine as a model system have shown that it may be possible to investigate the specificity of the base-stacking interactions of purine in aqueous solution by measuring the proton spin-lattice relaxation times of purine and of specific deuterium labeled purine derivatives under various conditions.

Several mechanisms can lead to nuclear spin-lattice relaxation. Nuclei with spin $I = \frac{1}{2}$ have only a magnetic moment associated with the spin and therefore can interact only with local magnetic fields. Accordingly, thermal equilibration of spins can take place only through interaction with these fields. Molecular motion can give rise to fluctuating

local magnetic fields in several ways, and these fluctuating fields can, of course, result in nuclear spin relaxation. The most important sources of fluctuating magnetic fields at a specific nucleus include (1) magnetic moments of other nuclei, (2) spins of unpaired electrons, and (3) variable electronic screening of the static field H_0. In the purine system, fields due to the magnetic moments of other nuclei are the most important, and preliminary calculations of relaxation times from this source have been made to investigate the possibility of determining specificity in base-stacking interactions by proton spin-lattice relaxation experiments. The other sources of nuclear relaxation were neglected in these calculations.

In considering the magnetic fields produced at the site of a particular proton by the magnetic moments of other nuclei, one may divide these fields into two groups: those in the same molecule (intramolecular) and those in different molecules (intermolecular). Since the pertinent experiments would involve solutions which are not highly concentrated, intermolecular effects have been neglected in the calculations.

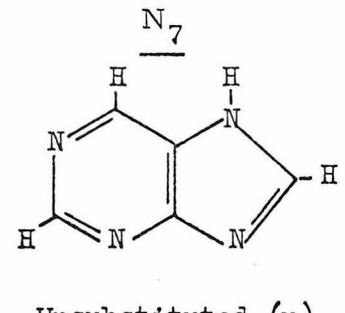
The formula presented below for the calculation of spin-lattice relaxation rates from magnetic fields of neighboring nuclei results from the treatment summarized in Pople, Schneider, and Bernstein (7) in which the behavior of the molecule is assumed to approach that of a sphere turning in a viscous fluid. The relaxation rate of nucleus i ($\frac{1}{T_{1i}}$) is given by

$$\frac{1}{T_{1i}} = \frac{2\pi\hbar^2 \gamma_i^2 \eta a^3}{3kT} \left\{ 3\gamma_i^2 \sum_j r_{ij}^{-6} + 2\sum_f^{*} \gamma_f^2 r_{if}^{-6} \right\} \qquad (1)$$

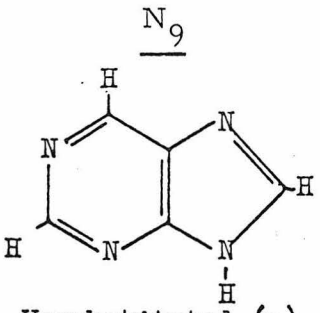
where η is the viscosity of the liquid solvent; r is a specific internuclear distance; and a is the radius of the model sphere. The summation $\sum_j$ is over nuclei of the same species as i and $\sum_f^*$ is over all other nuclei. Utilizing equation (1), the spin-lattice relaxation time of the H_6 proton of unsubstituted purine and of deuterium labeled purine (in which all purine protons except H_6 have been replaced by deuterium) has been estimated. Two sets of calculations have been made. One assumes a proton attached to N_7 of the imidazole ring and the other assumes this proton is at N_9 as shown in Table I. Bond distances were obtained from the crystal structure study of purine by Watson, Sweet, and Marsh (8). Dimers were considered to consist of two monomer units having the same orientation and being stacked directly above each other a distance of 4 Å apart. The results of preliminary calculations are summarized in Table I.

The relatively large values calculated for the ratio of $(1/T_1)_u$ to $(1/T_1)_s$ in the respective monomer units reflect the smaller nuclear magnetic moment of a deuterium atom in comparison to a hydrogen atom and the correspondingly more efficient relaxation mechanism provided by the proton system. Due to the larger magnetic moment of the proton, the spin-lattice relaxation rate of the H_6 proton of the unsubstituted dimer would be expected to be greater than that of the corresponding proton of the substituted dimer provided the H_6-H_6' distance is the same in both systems. (The prime designates a proton of a different monomer unit.) In the unsubstituted purine dimer, the H_6 proton magnetic dipole may interact with the magnetic dipoles of seven other protons, while in the deuterium labeled species the H_6 proton can

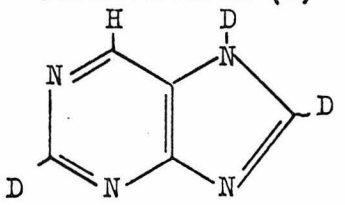
TABLE I



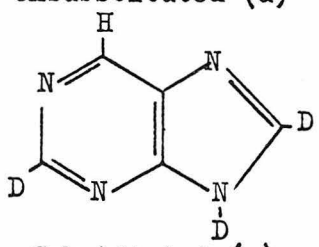
Unsubstituted (u)



Unsubstituted (u)



Substituted (s)

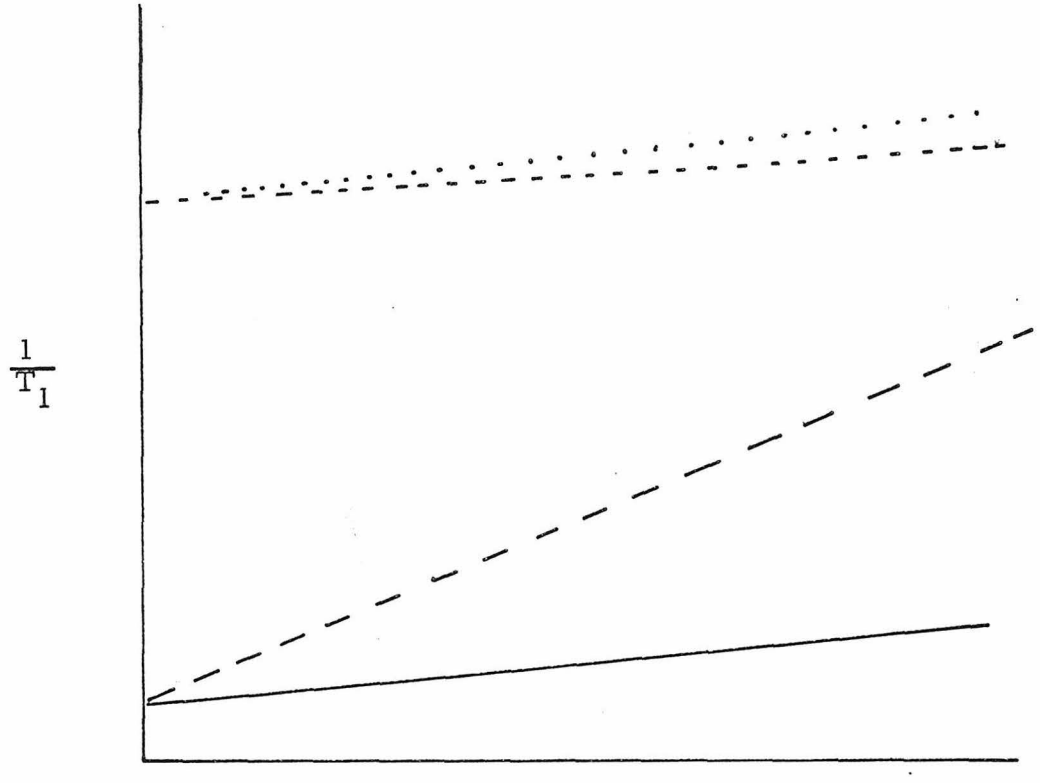


Substituted (s)

	N_7	N_9
$\frac{(1/T_1)_u \text{ (Monomer)}}{(1/T_1)_s \text{ (Monomer)}}$	20	6.0
$\frac{(1/T_1)_s \text{ (Dimer)}}{(1/T_1)_s \text{ (Monomer)}}$	6.4	7.5
$\frac{(1/T_1)_u \text{ (Dimer)}}{(1/T_1)_u \text{ (Monomer)}}$	2.1	3.0

interact with only one other proton dipole (H_6'). Due to similar effects, $(1/T_1)_s$ (Dimer)/$(1/T_1)_s$ (Monomer) would also be expected to be greater than $(1/T_1)_u$ (Dimer)/$(1/T_1)_u$ (Monomer) as indicated by the data in Table I. In connection with these observations, depicted in Figure 1 are some expected results involving $(1/T_1)$ as a function of purine concentration.

Figure 1



..... $(1/T_1)$ of H_6 of unsubstituted purine in a system in which specific base-stacks with a relatively small H_6-H_6' distance are formed.

------ $(1/T_1)$ of H_6 of unsubstituted purine in a system in which specific base-stacks with a large H_6-H_6' distance are formed.

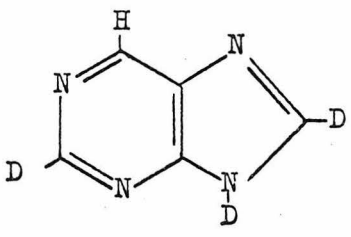
— — — $(1/T_1)$ of H_6 of 2,8,9-d_3-purine in a specific base-stacking system characterized by a relatively small H_6-H_6' distance.

——— $(1/T_s)$ of H_6 of 2,8,9-d_3-purine in a specific base-stacking system characterized by a large H_6-H_6' distance.

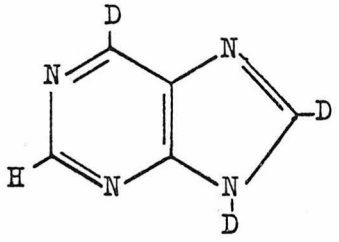
If the H_6-H_6' distance is increased in the unsubstituted dimer by rotation of the monomer units with respect to one another, $(1/T_1)$ would not be expected to change appreciably because the H_6 proton is always in relatively close proximity to another proton on the neighboring

monomer unit. Therefore, its environment [with respect to the environmental effect on $(1/T_1)$] does not change significantly. However, as the H_6-H_6' distance is increased by rotation in the substituted dimer $(1/T_1)$ would be expected to change appreciably because the interaction of the proton dipole moments is diminished as the distance between them increases.

This dependence of $(1/T_1)$ on the H_6-H_6' distance of the deuterium substituted dimer suggests the possibility of determining whether intermolecular vertical base-stacking occurs with preferred orientation of one monomer unit relative to another or whether the stacking occurs randomly. By studying at least two different deuterium-substituted purines, each having a single proton at different sites in the molecule as indicated below, one could measure the respective T_1 values for the protons in solutions of these molecules at various concentrations and particularly at a stoichiometric concentration where maximum dimer concentration would be expected.



I



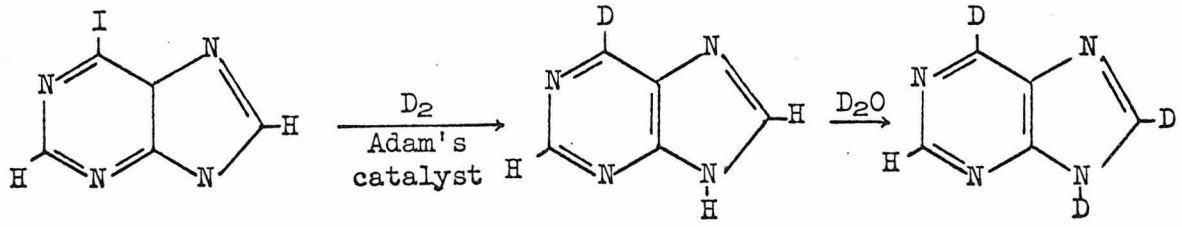
II

If the measured T_1 values of all the deuterium substituted compounds were the same under equivalent conditions, it may be assumed that random stacking occurs. This observation, of course, disregards the

unlikely possibility that the monomer units stack in a preferred orientation which results in equivalent T_1 values for the proton of each compound. However, the observation of different T_1 values for the protons of different sites would be indicative of specific base-stacking interactions.

Since the essential specifically deuterium labeled compounds are not readily available, methods are proposed for the synthesis of 2,8,9-d_3-purine (I) and 6,8,9-d_3-purine (II).

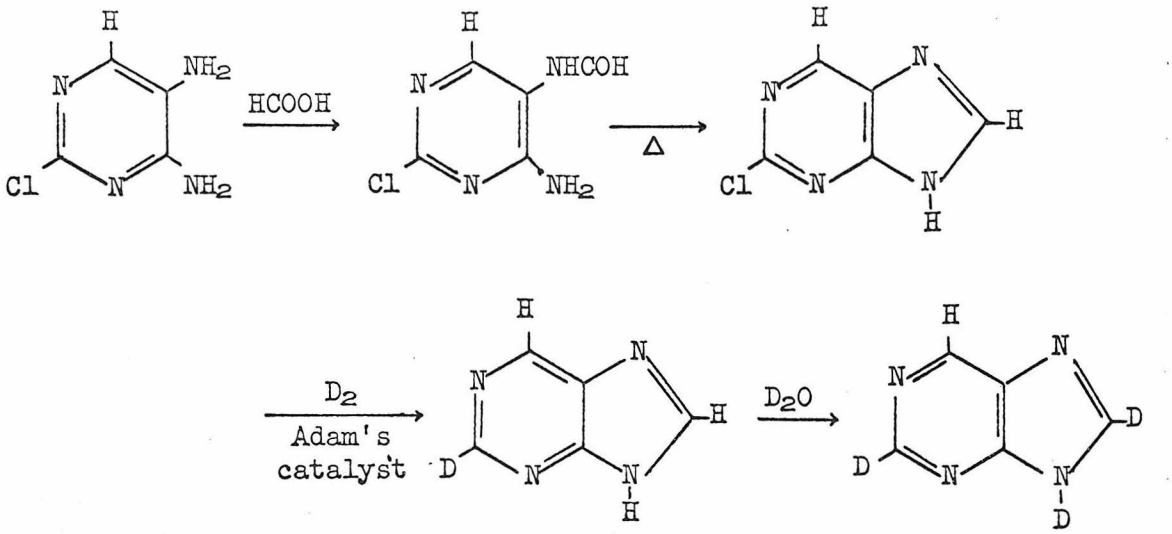
The proposed synthesis of 6,8,9-d_3-purine is summarized in the following scheme.



6,8,9-d_3-purine

6-Iodopurine which is available from K & K Laboratories, Los Angeles, California, may be reduced with deuterium in methanol-d in the presence of Adam's catalyst. Schweizer et al. (9) have shown that the amount of purine recovered is virtually quantitative. The H_8 and H_9 protons may be exchanged by boiling in D_2O for 4 hours at 105°C (9).

A scheme for the synthesis of 2,8,9-d_3-purine (I) is presented below:



2,8,9-d_3-purine (I)

The pyrimidine derivative, 4-amino-5-formamido-pyrimidine has been prepared by refluxing 4,5-diaminopyrimidine with formic acid for about one hour with an 80% yield (10). When the product was recrystallized from ethanol, 4-amino-5-formamidopyrimidine formed colorless needles which melted with the loss of water and resolidified to form purine. It is proposed to repeat this procedure with 2-chloro-4,5-diaminopyrimidine (commerically available from K & K Laboratories) and thereby obtain 2-chloropurine. The 2-chloropurine thus obtained may be reduced to 2-deuteropurine in a manner similar to the reduction of 6-iodopurine (9), and boiling 2-deuteropurine in D_2O will then yield the desired 2,8,9-d_3-purine.

REFERENCES

1. S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, J. Am. Chem. Soc. 86, 4182 (1964).

2. M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, J. Am. Chem. Soc. 87, 5241 (1965).

3. P. O. P. Ts'o, "The Physicochemical Basis of Interactions of Nucleic Acid," in Molecular Associations in Biology, ed. by B. Pullman (Academic Press, New York, 1968).

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

5. M. W. Warshaw and I. Tinoco, Jr., J. Mol. Biol. 20, 29 (1966).

6. S. I. Chan and J. H. Nelson, J. Am. Chem. Soc., in press.

7. J. A. Pople, W. G. Schneider, and H. J. Bernstein, High-resolution Nuclear Magnetic Resonance (McGraw-Hill Book Company, Inc., New York, 1959), Chapt. 9.

8. D. G. Watson, R. M. Sweet, and R. E. Marsh, Acta Cryst. 19, 573 (1965).

9. M. P. Schweizer, S. I. Chan, G. K. Helmkamp, and P. O. P. Ts'o, J. Am. Chem. Soc. 86, 696 (1963).

10. D. J. Brown, J. Appl. Chem. (London) 5, 358 (1955).

PROPOSITION IV

On the basis of the results of studies of several polyribonucleotides and polydeoxyribonucleotides by optical rotatory dispersion and supporting evidence provided by various investigations of the properties of purine and pyrimidine nucleosides and nucleotides, Ts'o and coworkers (1, 2) have proposed that intramolecular hydrogen bonding of the 2'-OH group to the 2-keto oxygen of pyrimidines and to the N_3 atoms of adenine may occur in the ribopolymers. Accordingly, the role of the 2'-OH group would be important in determining the structural properties of ribopolymers in solution, and could also partially account for the different properties of ribonucleic acids and deoxyribonucleic acids.

Zamecnik (3) has postulated that in the biosynthesis of proteins the formation of a similar hydrogen bond between the hydrogen atom of the 2'-OH group and N_3 of the terminal adenine base of transfer-RNA renders the oxygen atom of the 2'-hydroxyl position more nucleophilic than the corresponding oxygen of the 3'-position and that the 2'-oxygen atom therefore initiates a nucleophilic attack on the carbonyl carbon atom of an aminoacyl-AMP complex, resulting in the important linkage between transfer-RNA and amino acid residues. However, the studies of several other workers suggest that the 3'-OH group is equally important in the formation of the chemical bond between aminoacyl-AMP and transfer-RNA (4, 5).

It is well known (6) that DNA and RNA differ in their acid-base properties, DNA being more resistant to alkaline hydrolysis.

In view of these observations, the determination of the acidity of the sugar moieties of the ribonucleosides and deoxyribonucleosides of adenosine, guanosine, uridine, thymidine, and cytidine; the specific location of the acidic sight of the respective sugar groups; and a comparison of the results obtained for these molecules is of interest.

Spectrophotometric titrations by Fox and co-workers (7, 8) have shown that the ribose rings of pyrimidine nucleosides are somewhat more acidic than the ribose residues of the corresponding deoxynucleosides; and indirect evidence indicated that the acidic site of cytidine is the 2'-hydroxyl group. However, no quantitative pK_a values were determined in these studies and a direct comparison of the respective acidities of the various nucleosides was not possible.

Approximately forty years ago, Levene and co-workers (9-11) utilized electrometric titration methods in the determination of pK_a values for the ribose residues of cytidine, 5'-CMP, uridine, 5'-UMP and adenosine. Each of these pK_a values were in the pH range 12-13. These workers did not, however, determine the specific acidic site of the ribose rings.

More recently, Izatt et al. (10) have completed successful thermometric titration studies of adenosine and several adenosine derivatives. The results of these studies have enabled Izatt and co-workers to evaluate accurately the pK_a, ΔH^o, and ΔS^o values associated with the ribose moiety of adenosine ($pK_a = 12.35 \pm 0.03$; $\Delta H^o = 9.7 \pm 0.2$ kcal/mole; and $\Delta S^o = -24.0 \pm 0.7$ eu). Moreover, these studies indicate that the acidity in adenosine is associated with the 2'-position. This conclusion was confirmed by the fact that the heat change in the

alkaline titration of 2'-deoxadenosine is much less than that observed in the case of adenosine. However, the titration of 3'-deoxyadenosine yielded results similar to those of 2'-deoxyadenosine, suggesting that not one or the other, but both the 2'- and 3'-hydroxyl groups are required for the increased acidic character of adenosine. Corroborative evidence was obtained for this conclusion.

A systematic investigation of the nature of the acidity and the acidic site of the ribose rings of guanosine, cytosine, thymidine, uridine, and the respective deoxy-derivatives utilizing thermometric titrations methods (12-15) is proposed. A comparison of the results of these experiments will be helpful in elucidating the differences in the biological roles of the ribonucleosides and deoxyribonucleosides in DNA and RNA and in evaluating the importance of the 2'-OH group in determining the structural properties of polynucleotides.

REFERENCES

1. P. O. P. Ts'o, S. A. Rapaport, and F. J. Bollum, Biochemistry 5, 4153 (1966).

2. P. O. P. Ts'o, "The Physicochemical Basis of Interactions of Nucleic Acid," in Molecular Associations in Biology, ed. by B. Pullman (Academic Press, New York, 1968).

3. P. C. Zamecnik, Biochem. J. 85, 257 (1962).

4. H. Feldman and H. G. Zachau, Biochem. Biophys. Res. Commun. 15, 13 (1964).

5. C. S. McLaughlin and V. M. Ingram, Science 145, 942 (1964).

6. A. M. Michelson, The Chemistry of Nucleosides and Nucleotides (Academic Press, New York, 1963), p. 317.

7. J. J. Fox and D. Shugar, Biochim. Biophys. Acta 9, 369 (1952).

8. J. J. Fox, L. F. Cavalieri, and N. Chang, J. Am. Chem. Soc. 75, 4315 (1953).

9. P. A. Levene and H. S. Simms, J. Biol. Chem. 65, 519 (1925).

10. P. A. Levene, L. W. Bass, and H. S. Simms, J. Biol. Chem. 70, 229 (1926).

11. P. A. Levene, L. W. Bass, and H. S. Simms, J. Biol. Chem. 70, 243 (1926).

12. R. M. Izatt, J. H. Rytting, L. D. Hansen, and J. J. Christensen, J. Am. Chem. Soc. 88, 2641 (1966).

13. J. J. Christensen, R. M. Izatt, and L. D. Hansen, Rev. Sci. Instr. 36, 779 (1965).

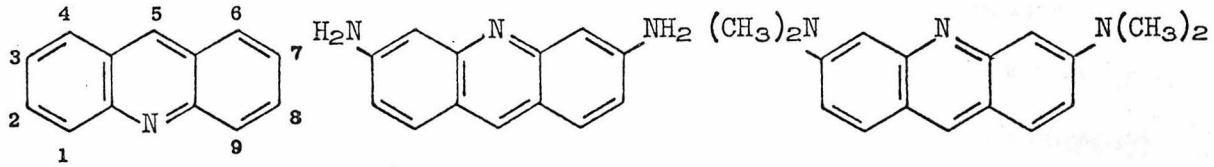
14. L. D. Hansen, J. J. Christensen, and R. M. Izatt, Chem. Commun. (London) No. 3, 36 (1965).

15. J. J. Christensen, R. M. Izatt, L. D. Hansen, and J. A. Partridge, J. Phys. Chem. 70, 2003 (1966).

PROPOSITION V

The effects of intermolecular interactions between the acridine dyes and nucleic acids are of considerable biological importance. For example, Orgel and Brenner (1) have shown that acridine, proflavin, and acridine orange (Figure 1) as well as other amino-substituted

Figure 1



Acridine Proflavin Acridine Orange

acridines, are strongly mutagenic in inducing reversion to wild type among a selected set of rII mutants of the bacteriophage T_4. They have also observed that the mutagenic activity of acridine is enhanced by the presence of amino groups on the rings as in proflavin. Methylation of the amino groups of proflavin results in diminution of the mutagenic effect. These differences may possibly be explained in terms of differences in the binding of the various acidines to DNA.

In addition to the mutagenic properties of the acridine dyes, these molecules also apparently act as inhibitors of the excision process in the repair replication of ultraviolet-damaged DNA (2).

In view of the preceding observations, an investigation of the binding of acridine dyes to nucleic acids and nucleic acid derivatives is of considerable interest, and numerous studies (3-14) employing a

variety of physicochemical techniques have been made in an effort to determine the structure of dye-nucleic acid complexes. The results of these studies indicate that two types of complexes are formed. Complex I, which exists at low dye/phosphate ratios, results from intercalation of the dye molecule between the planes of adjacent bases of the nucleic acid (6, 14, 15). Complex II, which is formed at high dye/phosphate ratios, presumably results from electrostatic interaction between the cationic dye molecules (neutral pH) and the negatively charged phosphate groups along the polymer chain (3). A study of the interaction of acridine, proflavine, and acridine orange to dinucleoside monophosphates and oligonucleotides by high-resolution nuclear magnetic resonance spectroscopy would be useful in obtaining additional information pertaining to the structure and relative stabilities of the intermolecular complexes formed between these dyes and nucleic acids. (The study of several different acridines is pertinent in that the difference in mutagenicity of the acridines may possibly be explained in light of their respective tendencies to bind to DNA.)

Previous studies have shown that interactions of this nature may be conveniently monitored by nuclear magnetic resonance (NMR) spectroscopy. For example, NMR studies have demonstrated that purine (16), purine nucleosides (17), and purine nucleotides (18) self-associate extensively in aqueous solution via vertical base-stacking interactions. These interactions result in upfield shifts for the ring protons and the $H_{1'}$ ribose protons as intermolecular association occurs. The higher field proton shifts arise from the ring-current magnetic anisotropy of the neighboring bases in the intermolecular stacks. Similar NMR

investigations have also shown that acridine orange (19, 20) forms stable base-stacks in aqueous solution, and that the stacking interactions in the self-associated complexes of this dye are much stronger than the corresponding interactions of the purine derivatives. In addition, nuclear magnetic resonance methods have been successfully extended to the study of the intermolecular stacking interaction between purine and several pyrimidine nucleosides (cytidine, thymidine, and uridine) (21); and more recently the intermolecular association of acridine orange with adenine, adenosine, the 2'-, 3'-, and 5'-mononucleotides of adenosine, and the 5'-monophosphates of guanosine, cytidine, uridine, and thymidine have also been investigated (22). From the results of the latter studies, it was concluded that (1) purines have a greater tendency to stack with acridine orange than do the pyrimidines, (2) the presence of the charged phosphate group on the nucleotides of adenosine increases the stability of the dye-adenine complex, and (3) the experimental data provide additional support for the intercalation mechanism of dye binding by nucleic acids and indicate that intercalation will be more favorable in purine-rich regions of the polynucleotides.

With regard to dye-polynucleotide interactions, it is proposed that more meaningful conclusions may be obtained from NMR studies of the interaction of the dyes with dinucleoside monophosphates and oligonucleotides since these molecules are more representative of the biologically significant polymers than the simple nucleosides and nucleotides. For example, the fact that the dyes interact more strongly with purines than with pyrimidines is not necessarily indicative that intercalation occurs more readily in purine-rich regions of polynucleotides

because the relatively strong intramolecular purine-purine interactions along the polymer chain may inhibit the intercalation process. Accordingly, a study of the binding of acridine dyes to specific purine-purine and pyrimidine-pyrimidine dinucleoside monophosphates is proposed. Similar studies of the binding of purine, 6-methylpurine, and ribosyl purine to dinucleoside monophosphates (23-26) and the trinucleoside diphosphate of adenine have shown that it is possible to observe experimentally the intercalation of purine between two bases of the nucleic acid derivatives and to determine the approximate orientation of purine in the intercalated complex. Therefore, it is likely that NMR studies of the binding of the acridine dyes to dinucleotides and oligonucleotides would also elucidate the existence of any intercalated complexes which are formed. Moreover, important information with respect to the orientation of the planar dye molecules in the intercalated complexes would likely be obtained in these studies.

A study of the binding of acridine, proflavin, and acridine orange to the homodinucleotides adenylyl-(3'→5')-adenosine (ApA), guanylyl-(3'→5')-guanosine (GpG), cytidylyl-(3'→5')-cytidine (CpC), and uridylyl-(3'→5')-uridine (UpU) at low concentrations is proposed. It is suggested that each of the dyes be added progressively to 0.01 M solutions of the dinucleotides in small quantities and that initial experiments be carried out at a dye concentration of ~0.001 - 0.002 M. In view of the high field high-resolution NMR spectrometers and computer time-averaging devices now available, experiments of this nature are certainly possible. By monitoring the proton chemical shifts and resonance linewidths of both the dye and the dinucleotide, the relative

binding tendencies and intercalation tendencies of the different systems, as well as the approximate orientation of intercalated dyes may be obtained. From the orientation of the dyes, information concerning the importance of the interaction between the negatively charged phosphate group and centers of positive charge on the dye molecules may be accumulated. In addition the effect of steric interactions of the methyl groups of acridine orange or the amino groups of proflavin may also be ascertained. The extension of these studies to heterodinucleotides and oligonucleotides may also prove useful.

Acridine orange has been found to self-associate more extensively in aqueous solution with the addition of sodium chloride (20). However, if electrostatic interactions between the phosphate group of a dinucleotide and the positive charge associated with the ring of the acridines (at neutral pH) are important in stabilizing the dinucleotide-dye complexes, the addition of salt would presumably result in weaker intermolecular interactions. Accordingly, the effect of salt (NaCl) concentration on the nature of acridine dye-dinucleotide complexes is also of considerable interest.

One final note is worthy of mention. Pullman and co-workers have made some interesting theoretical calculations concerning the electronic characteristics and dipole moments of various amino-acridines. Some of the results of these calculations are listed in Lerman's paper (15) on acridine mutagens. It would be interesting to compare the results of the proposed NMR studies with the electronic properties Pullman has investigated. A comparison of the relative

orientation of the stacked acridine dyes and nucleoside bases in the dinucleotide-dye complexes with the theoretically determined dipoles would also be of considerable interest.

REFERENCES

1. A. Orgel and S. Brenner, J. Mol. Biol. 3, 762 (1961).
2. R. B. Setlow, J. Cellular Comp. Physiol. 64, (Suppl. 1), 51 (1964).
3. D. F. Bradley and M. K. Wolf, Proc. Natl. Acad. Sci. U. S. 45, 944 (1959).
4. D. F. Bradley, and G. Felsenfeld, Nature 184, 1920 (1959).
5. A. L. Stone and D. F. Bradley, J. Am. Chem. Soc. 83, 3627 (1961).
6. L. S. Lerman, J. Mol. Biol. 3, 18 (1961).
7. S. Brenner, L. Barnett, F. H. C. Crick, and A. Orgel, J. Mol. Biol. 3, 121 (1961).
8. D. S. Drummond, V. F. W. Simpson-Gildemeister, and A. R. Peacocke 3, 135 (1965).
9. V. Luzzati, F. Masson, and L. S. Lerman, J. Mol. Biol. 3, 634 (1961).
10. J. C. Powers, Jr., and W. L. Peticolas, J. Phys. Chem. 71, 3191 (1967).
11. S. F. Mason, and A. J. McCaffery, Nature 204, 468 (1964).
12. G. Tomita, Biophysik. 4, 118 (1967).
13. D. J. Blears and S. S. Danyluk, Biopolymers. 5, 535 (1967).
14. L. S. Lerman, Proc. Natl. Acad. Sci. U. S. 49, 94 (1963).
15. L. S. Lerman, J. Cellular Comp. Physiol. 64, (Suppl. 1), 1 (1964).
16. S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, J. Am. Chem. Soc. 86, 4182 (1964).
17. A. D. Broom, M. P. Schweizer, and P. O. P. Ts'o, J. Am. Chem. Soc. 89, 3612 (1967).

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

19. D. J. Blears and S. S. Danyluk, J. Am. Chem. Soc. 88, 1084 (1966).

20. D. J. Blears and S. S. Danyluk, J. Am. Chem. Soc. 89, 21 (1967).

21. M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, J. Am. Chem. Soc. 87 (1965).

22. F. E. Hruska and S. S. Danyluk, Biochim. Biophys. Acta 161, 250 (1968).

23. S. I. Chan, B. W. Bangerter, and H. H. Peter, Proc. Natl. Acad. Sci. U. S. 55, 720 (1966).

24. S. I. Chan and J. H. Nelson, J. Am. Chem. Soc., in press.

25. B. W. Bangerter, Ph. D. Thesis, California Institute of Technology, Pasadena, California, 1969.

26. J. H. Nelson, Ph. D. Thesis, California Institute of Technology, Pasadena, California, 1969.