STUDIES OF DINUCLEOSIDE MONOPHOSPHATES AND MONOMER-POLYNUCLEOTIDE INTERACTIONS BY PROTON MAGNETIC RESONANCE

Thesis by

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ABSTRACT

The nature of the intra- and intermolecular base-stacking interactions involving several dinucleoside monophosphates in aqueous solution have been investigated by proton magnetic resonance spectrosocopy, and this method has been applied to a study of the interaction of polyuridylic acid with purine and adenosine monomers.

The pmr spectra of adenylyl (3′→ 5′) cytidine (ApC) and cytidylyl (3′→ 5′) adenosine (CpA) have been studied as a function of concentration and temperature. The results of these studies indicate that the intramolecular base-stacking interactions between the adenine and cytosine bases of these dinucleoside monophosphates are rather strong, and that the stacking tendencies are comparable for the two sequence isomers. The chemical shifts of the cytosine H_5 and adenine H_2 protons, and their variations with temperature, were shown to be consistent with stacked conformations in which both bases of the dinucleoside monophosphates are preferentially oriented in the anti conformation as in similar dApdC, and dCpdA (dA = deoxyadenosine; dC = deoxycytidine) segments in double helical DNA. The intramolecular stacking interaction was found to have a pronounced effect on the conformations of the ribose moieties, and these conformational changes are discussed. The concentration studies indicate extensive self-association of these dinucleoside monophosphates, and analysis of the concentration data facilitated determination of the dimerization constant for the association process as well as the nature

of the intermolecular complexes.

The dependence of the ribose conformation upon the extent of intramolecular base-stacking was used to demonstrate that the base-base interaction in cytidylyl (3′→5′) cytidine (CpC) is rather strong, while there appears to be little interaction between the two uracil bases of uridylyl (3′→5′) uridine (UpU).

Studies of the binding of purine to several ribose and deoxyribose dinucleoside monophosphates show that the mode of interaction is base-stacking, and evidence for the formation of a purine-dinucleoside monophosphate intercalated complex is presented. The purine proton resonances are markedly broadened in this complex, and estimates of the purine linewidths in the complex and the equilibrium constant for purine intercalation are obtained.

A study of the interaction of unsubstitued purine with poly-uridylic acid at 29°C by pmr indicated that purine binds to the uracil bases of the polymer by base-stacking. The severe broadening of the purine proton resonances observed provides strong evidence for the intercalation of purine between adjacent uracil bases of poly U. This interaction does not result in a more rigid or ordered structure for the polymer.

Investigation of the interaction between adenosine and poly-uridylic acid revealed two modes of interaction between the monomer and the polymer, depending on the temperature. At temperatures above 26°C or so, monomeric adenosine binds to poly U by non-cooperative A-U base stacking. Below this temperature, a rigid triple-stranded 1A:2U complex is formed, presumably via cooperative

hydrogen-bonding as has previously been reported.

These results clearly illustrate the importance of base-stacking in non-specific interactions between bases, nucleosides and nucleotides, and also reveal the important role of the base-stacking interactions in cooperatively formed structures involving specific base-pairing where both types of interaction are possible.

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I. INTRODUCTION

1. Structure and Conformation of Polynucleotides

The elucidation of the double helical secondary structure of deoxyribosenucleic acid (DNA) by Watson and Crick in 1953,[1] and the implications of this structure with regard to storage and transfer of genetic information, heralded the birth of molecular biology. In the past decade and a half, this has become one of the most active areas of scientific pursuit. Subjects of particular importance in the physical chemistry of nucleic acids are determination of conformation and secondary structure of DNA and RNA, particularly in aqueous solution, and investigation of the forces responsible for the stability of the secondary structure of these molecules. Determination of sequence by means other than chemical degradation of the polymers is also of considerable interest.

A brief description of the structure of nucleic acids is helpful to this discussion. A polynucleotide strand consists of a linear sequence of sugar (pentose) and phosphate moieties with each phosphate group esterified to the 3′-hydroxyl group of the preceding pentose and to the 5′-hydroxyl group of the following pentose. Attached to each sugar at the 1′-position is a heterocyclic base, derived from either purine or pyrimidine. In DNA, the sugar is 2′-deoxyribose and the bases are adenine and guanine (purines), and thymine and cytosine (pyrimidines). In RNA, the sugar is ribose and thymine is replaced by the base uracil. The combination of base and pentose is called a nucleoside, and the

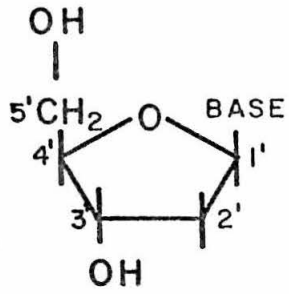
base-pentose-phosphate combination is termed a nucleotide. The structures of the nucleosides are shown in Figure 1. A polynucleotide strand has a unique direction, and the ordering of nucleotides along the strand, or sequence, is generally specified in the direction of 3′→ 5′-phosphate attachment. The sequence of a polynucleotide strand is termed its primary structure.

The presence of a number of chemical bonds about which complete or partial rotation can occur allows a polynucleotide strand to adopt many different conformations. These conformational properties, and the possible interaction between two (or more) strands, constitute the secondary structure of polynucleotides. The basis for secondary structure also resides in the base sequence of a strand. The possibility of base-pairing by hydrogen-bonding of complementary bases (adenine--thymine/uracil, guanine--cytosine) is an important aspect of the secondary structure of polynucleotides. The DNA double helix, in which two strands with complementary base sequences are bound together by A-T and G-C base pairs, is the secondary structure which characterizes most native DNA. Such a structure would also appear to be possible for complementary RNA strands, although most native RNA is not in this form. The existence of base paired regions in a single polynucleotide strand, as has been proposed for several transfer RNA molecules of known sequence,[2-4] seems quite likely, although such behavior has not been conclusively shown to occur.

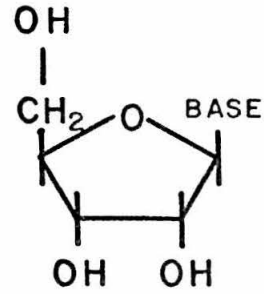
Until about 1962, attention had focused on the "horizontal" base pairing interactions by hydrogen-bonding as being the major source of

FIGURE 1

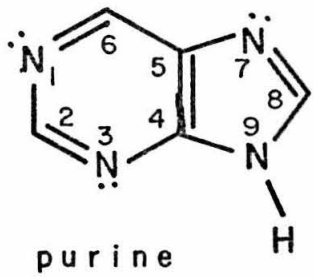
Structural diagrams of the pentose sugars ribose and 2′-deoxyribose, purine, pyrimidine, and the commonly occurring nucleosides adenosine, guanosine, thymidine, uridine and cytidine.



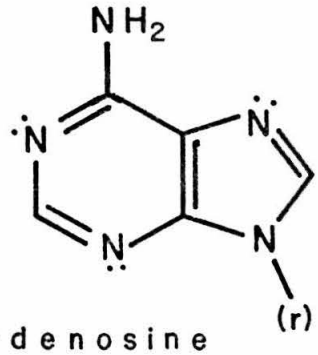
2'-deoxyribose (d)



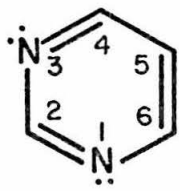
ribose (r)



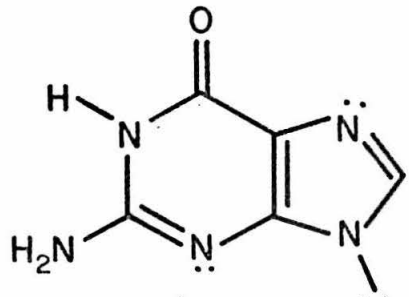
purine



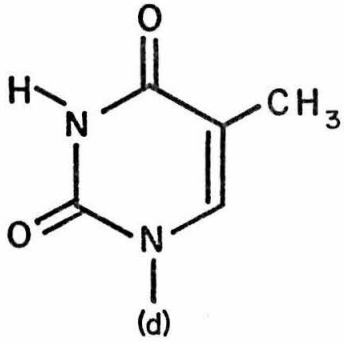
adenosine



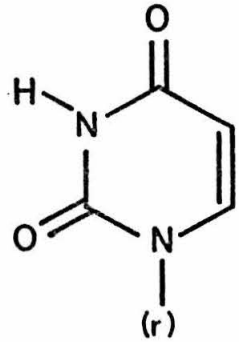
pyrimidine



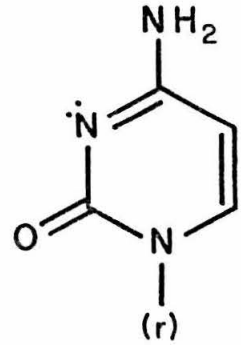
guanosine



thymidine



uridine



cytidine

stability of the DNA double helix.[5, 6] However the structure of the double helix is such as to involve a considerable degree of overlap of adjacent bases along a strand, and these "vertical" base-stacking interactions have more recently been recognized as making a sizable contribution to the stability of this structure.[7-9] These interactions are also responsible for the structure of ordered, non-base-paired regions of single stranded polynucleotides. Devoe and Tinoco[7] have considered the effects of direct interactions between bases in the double helix, including dipole-dipole, dipole-induced dipole and London dispersion forces, and have concluded that these forces make a large contribution to the stability of helical polynucleotides. It is well known that the nature of the solvent plays an important role in determining the secondary structure of polynucleotides in solution.[10-12] The unique role of water in regard to hydrophobic bonding (as base-stacking may be classified) has been considered by Sinanoğlu and Abdulnur,[13-15] and this phenomenon has been attributed to the surface free energy involved in forming a cavity in water to accommodate the solute. Némethy and Scheraga[16] have investigated hydrophobic bonding in proteins, with the conclusion that a negative entropy change for the solvent results from the addition of a non-polar molecule to water. Their treatment could conceivably be applied to polynucleotides as well as proteins.

The primary motivation for the work reported in this thesis has been the desire to study, in a detailed manner, the forces responsible for the interaction between adjacent bases in a polynucleotide strand, and the forces involved in the binding of small molecules, such as bases

and nucleosides, to polynucleotides. The role played by monomer-polymer interactions in the recognition process involving replication and information transfer in biological systems is of particular importance, and little detailed information regarding these interactions is presently available.

2. Properties of Monomeric Bases and Nucleosides

The obvious conclusion from studies of the primary and secondary structure of polynucleotides is that the forces responsible for the conformational stability of these molecules in solution must reside primarily in the interactions among the heterocyclic bases. It is therefore of interest to study, in a detailed manner, the interactions among various bases in model compounds, with the expectation that these results will give an insight into the nature of base-base interactions at the polymer level. The first phase in the investigation of the forces between bases was the study of monomeric bases and nucleosides in solution.

2. 1. Studies in Aqueous Solution

The molal osmotic coefficients of aqueous solutions of purine, 6-methylpurine, uridine, cytidine, 5-bromouridine, adenosine and a number of purine derivatives have been measured by Ts'o and co-workers[17-19] using vapor pressure osmometry. Activity coefficients determined from the data indicate that these solutes associate extensively in aqueous solution, and that the association process does not stop at the dimer stage; higher polymers are formed as well. Assuming

that the equilibrium constants for successive steps in the association process are equal, these workers determined association constants at 25°C for several bases and nucleosides. These are listed in Table I. Furthermore, the solubilities of the sparingly soluble bases adenine and thymine were shown to be greatly enhanced by the presence of more soluble bases and nucleosides, demonstrating the interaction of adenine and thymine with these compounds. It was found that interaction between two purine bases was stronger than that between a purine and a pyrimidine base, with the interaction between two pyrimidine bases weaker than either of these. These conclusions have been supported by the work of Solie and Schellman,[20] who also applied vapor pressure osmometry to the study of nucleosides in aqueous solution.

Van Holde and Rossetti have studied the association of purine in aqueous solution by equilibrium sedimentation,[21] and they agreed with Ts'o et al.[18] that a simple reversible polymerization is involved, with a constant free-energy increment for the addition of each successive purine molecule. The equilibrium constant at 25°C obtained in this work, 2.8 ℓ/mole, is somewhat larger than that reported by Ts'o et al. (2.1 molal^{-1}), and probably more accurate. Apparent equilibrium constants were determined at several temperatures by Van Holde and Rossetti, and a vant Hoff plot yielded an enthalpy of $\Delta H^\circ = -4.9$ kcal/mole for the binding of each purine molecule.

The mode of association of monomeric bases and nucleosides in aqueous solution was found to be stacking of the planar, heterocyclic bases. Proton magnetic resonance (pmr) spectroscopy was used by Chan and co-workers[22] to elucidate the mode of self-association of

TABLE I. Self-association constants of bases and nucleosides in aqueous solution at 25°C. Values reported by Ts'o and co-workers.[17-19]

Compound	K ($molal^{-1}$)
Purine	2.1
6-Methylpurine	6.7
Uridine	0.61
Cytidine	0.87
Adenosine	4.5

purine and 6-methylpurine. The proton resonances of these molecules are shifted to higher fields with increasing concentration over the range 0. 05 M to 1. 0 M. These shifts are a consequence of the ring-current magnetic anisotropy of the purine base. Since an understanding of this phenomenon is essential to the reading of this thesis, a brief description is presented here.

A molecule, when placed in a magnetic field, produces a small induced magnetic moment due to the circulation of electronic charge in the molecule. This is expressed in terms of the magnetic susceptibility tensor for the molecule, $\underset{\approx}{\chi}$[23]:

$$\vec{M}_{\text{induced}} = \underset{\approx}{\chi} \cdot \vec{H}_{\text{applied}}.$$

If the magnetic susceptibility tensor $\underset{\approx}{\chi}$ is anisotropic, there is a net contribution to the chemical shift of a proton bound to or in the vicinity of the molecule, after averaging over all molecular orientations with respect to the applied magnetic field. For a molecule with an axially symmetric $\underset{\approx}{\chi}$ tensor, the effect is that of an induced magnetic dipole at the center of the molecule, fixed with respect to the molecular axes. This induced moment produces a contribution to the shielding of a proton at a distance R from the dipole and an angle θ from the axis which is given by[24]:

$$\Delta\sigma = \frac{\chi_{\parallel} - \chi_{\perp}}{3R^3} (1 - 3\cos^2\theta).$$

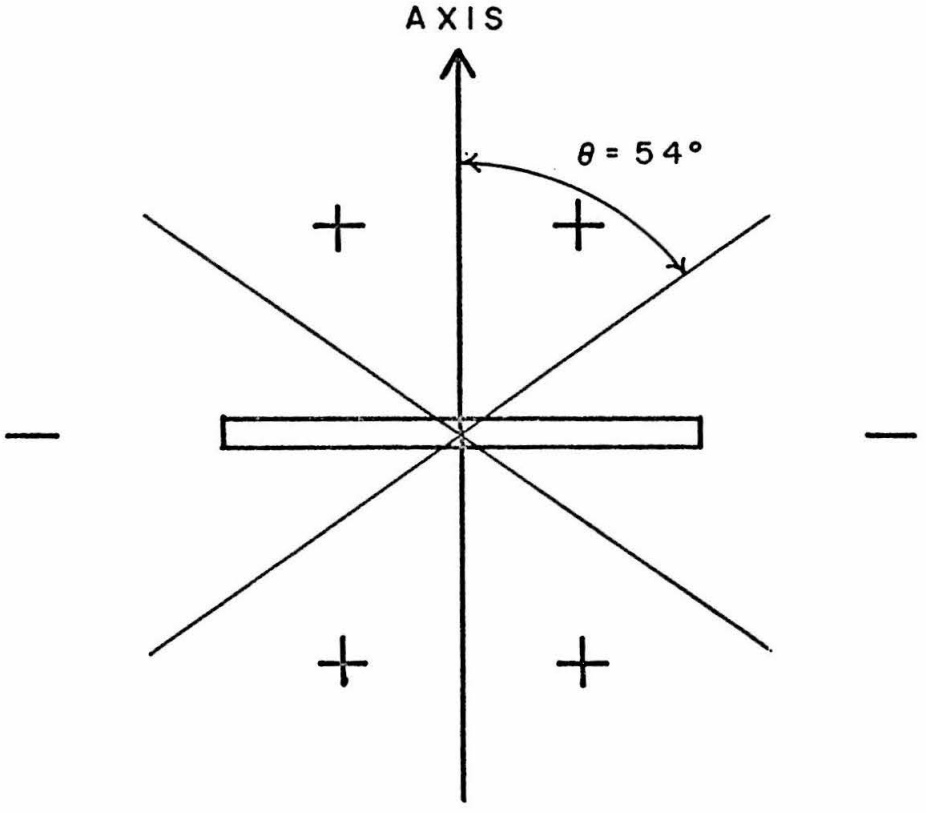
The principal components of the susceptibility tensor are $\chi_{||}$ and $\chi_{\perp}$, referring to fields applied parallel and perpendicular to the molecular symmetry axis, respectively.

For a planar aromatic molecule such as purine, $\underset{\approx}{\chi}$ is nearly of axial symmetry, with the unique axis perpendicular to the plane of the ring. Here, $|\chi_{||}| > |\chi_{\perp}|$ and both are of negative sign. This results in a shielding of protons lying within a cone of 54° from the axis perpendicular to the ring, and a deshielding of protons located outside of this region. Thus protons located above an aromatic ring system will experience a shift of their resonances to higher fields, and protons located toward the periphery of such a ring will experience a downfield shift of their resonances. This is depicted in Figure 2. The observation by Chan et al.[22] and by Jardetzky[25] that purine protons resonances shift upfield with increasing concentration indicates that the protons of a given purine molecule experience the shielding effect of an adjacent purine molecule, and thus on the average lie above the plane of the adjacent molecule. Intermolecular association of purine via horizontal hydrogen-bonding, on the other hand, would place the protons of one base in the deshielding region of the other base, resulting in shifts of the resonances to lower fields as such complexes are formed.

Shifts of proton resonances to higher fields with increasing concentration have also been observed for unsubstituted pyrimidine,[26] adenosine,[19] and various other purine derivatives[19, 27] as well, indicating that base-stacking interactions are prevalent with these compounds in aqueous solution. The chemical shifts of base protons

FIGURE 2

Edge-on view of a planar aromatic molecule. Protons located within the cone of revolution about this axis will be shielded by the ring current (resonances shifted upfield, +), while protons located outside of this region will be deshielded (resonances shifted downfield, -).


AXIS
θ = 54°

of uridine, cytidine, and thymidine have been found to be virtually independent of concentration,[28] although these molecules associate extensively in aqueous solution. This result has been taken to indicate that these bases have a negligibly small ring-current magnetic anisotropy. The existence of these bases in the keto tautomeric form renders them "nonaromatic", in that free circulation of electronic charge around the six-membered ring is no longer possible. The interaction of the pyrimidine nucleosides with purine[28] results in sizable upfield shifts of the pyrimidine base proton resonances, with smaller upfield shifts for the ribose protons, indicating that purine interacts with these nucleosides by base-stacking.

There is a great deal of evidence to indicate that the interaction of monomeric bases, nucleosides and nucleotides in aqueous solution involves base-stacking. The possibility of some solute-solute hydrogen-bonding cannot be discounted, although there is no evidence to indicate that this does indeed occur in aqueous solution. The base N-H protons which would be involved in hydrogen bond formation unfortunately undergo rapid chemical exchange with the solvent, and consequently do not give discrete resonances. This renders pmr useless in examining the possible involvement of these protons in hydrogen-bonding.

Knowledge of the relative tendencies of various bases to stack with one another is of course of considerable interest in assessing the probable strengths of interactions of bases along a polynucleotide strand. Use of monomers in studying interactions between two different bases or nucleosides is complicated by the fact that the cross inter-

action of interest, say between the A and C bases of adenosine and cytidine, occurs along with the self-interaction of the constituents of the system, in this case A-A and C-C base-stacking. This problem can be alleviated to some extent by proper choice of concentration ratios, but cannot be completely circumvented.

2. 2. Studies in Non-Aqueous Solution

The interactions among various base analogs and nucleosides in a variety of non-aqueous solvents have been investigated by several workers. The rationale for several of these studies has been the assertion that the environment of bases in non-hydroxylic solvents best represents the local environment of bases in a polynucleotide (compared to studies in aqueous solution) when working with bases and nucleosides at the monomer level. Hamlin, Lord and Rich[29] have demonstrated the association of adenine and uracil derivatives by hydrogen-bonding in chloroform, using infrared spectroscopy. Miller and Sobell[30] have made similar observations regarding several adenine and uracil derivatives, and Pitha, Jones, and Pithova[31] have shown the existence of hydrogen-bonded complexes of guanine and cytosine derivatives in chloroform, also by infrared spectroscopy. Association constants and thermodynamic parameters for the hydrogen-bonded complex of 9-ethyladenine and 1-cyclohexyluracil, as well as for the self-association of the components, have been determined calorimetrically[32] and by infrared spectroscopy.[33] The results of these two studies are in good agreement, yielding an association constant of ~ 100 ℓ/mole and an enthalpy for association of -6. 2 kcal/mole at 25°C for the A-U

mixed dimer.

Proton magnetic resonance studies by Katz and Penman[34] and by Shoup, Miles, and Becker[35] have shown that guanosine and cytidine hydrogen-bond strongly with one another in dimethyl-sulfoxide, this being the only pairwise base-base interaction found in this solvent. Interaction of adenine and uracil derivatives was, however, observed in chloroform solution.[34] These pmr studies utilized observation of the chemical shifts of the N-H and amino protons of the bases. These resonances show large shifts to lower fields with hydrogen bond formation.[36] It is interesting to note that no upfield shifts were observed for the adenine or guanine base protons with increasing concentration, or for cytosine or uracil protons when adenine or guanine derivatives were added, clearly showing that base-stacking does not occur to any appreciable extent in these solvents. It was also noted by Chan et al.[22] that purine self-association by base-stacking does not occur in dimethyl-sulfoxide.

The various hydrogen-bonded complexes of base analogs observed have been assumed to have a Watson-Crick type structure. The G-C pair involves both amino groups and the G-NH proton,[35] which is inconsistent with any other bonding schemes, although the possibility of the Hoogsteen bonding scheme[37] (which involves N_7 of adenine as an acceptor) must also be considered for the A-U complexes.

3. Properties of Dinucleotides

It is clear that the use of monomeric bases, nucleosides and nucleotides in modeling the interactions along a polynucleotide strand

is rather limited. To the extent that pairwise nearest-neighbor interactions between adjacent bases in a polynucleotide strand are of primary importance in determining the conformation of the molecule, dinucleoside monophosphates serve as the best objects for study. These compounds preserve the important feature of polynucleotides not found in the monomers: the ribose-phosphate backbone. The dinucleoside monophosphates consist of two nucleosides linked by a phosphate group esterified to the 3′-hydroxyl group of one nucleoside and to the 5′-hydroxyl group of the other nucleoside. (These molecules are also referred to as dinucleotides, though this is not a strictly accurate designation, and the two names are used interchangeably in the present discussion).

The dinucleotides have several clear advantages over the monomers as models for polynucleotide behavior. The base stacking interaction is intermolecular with monomers, so that a large negative contribution to the entropy of stacking due to translational diffusion of the molecules in solution must be taken into account. This translational entropy is not present when the interacting bases are joined by a ribose-phosphate backbone. Furthermore, the phosphate linkage in a dinucleotide restricts the geometry of the two bases relative to one another, as in the polymer, and this feature is missing with the monomers. Finally, it is possible to study the intramolecular interaction between two bases at very low concentrations of dinucleotide so that intermolecular effects are essentially eliminated. As mentioned previously, it is not possible to study, at the monomer level, the

interaction between two different bases in the absence of self-stacking of the components. Thus the intramolecular base-stacking interaction of dinucleoside monophosphates would appear to represent the simplest and best approximation to the interaction between adjacent bases in a polynucleotide strand.

There are two basic classes of dinucleotides: one involves the deoxyribose nucleosides of DNA, the other involves the ribose nucleosides of the RNA's. The conventional abbreviations used in naming dinucleoside monophosphates are specified by IUPAC-IUB rules.[38] These use capital letters to signify the nucleosides (A = adenosine, G = guanosine, C = cytidine, U = uridine, T = thymidine, dA = deoxyadenosine, etc.) and a small p to indicate phosphate, and are written in the order of 3′ to 5′-phosphate esterification. Considering the four ordinary nucleosides in either DNA or RNA, there are sixteen possible deoxyribose dinucleoside monophosphates and sixteen possible ribose dinucleoside monophosphates because of the asymmetric phosphate attachment to the two nucleosides. The molecules ApC and CpA, for example, obviously have different structures, and are termed sequence isomers. The conformational properties of sequence isomers may of course be quite different, leading to sequence-dependent interactions between a given pair of nucleotide residues in a polynucleotide strand. A number of unusual nucleosides (pseudouridine, dihydrouridine, inosine, etc.) also occur, particularly in the transfer RNA's, and these increase beyond sixteen the number of dinucleoside monophosphates which could be studied in a complete consideration of

pairwise base interactions.

3.1. Optical Studies

The optical properties of the dinucleoside monophosphates have been rather thoroughly investigated. Tinoco and co-workers[39-43] have used optical rotatory dispersion and ultraviolet absorption spectroscopy in studies of all sixteen ribose dinucleoside monophosphates derived from A, G, C and U. These measurements were made at 25°C and pH's of 1, 7 and 11.5, with an ionic strength of 0.1. The criteria of hypochromism and difference in optical rotation between dinucleotides and component monomers were used to classify the dinucleotides as either "stacked" or "unstacked" under the experimental conditions. The optical rotatory dispersion was highly sequence dependent, whereas the absorption measurements were not. The two classes of dinucleoside monophosphates at neutral pH are listed in Table II, in the order of decreasing tendency toward intramolecular base stacking. It can be noted from these results that purine-purine interactions are rather strong, on the average, and that pyrimidine-pyrimidine interactions are rather weak. Purine-cytosine interactions are quite a bit stronger than purine-uracil interactions; in fact, each of the five dinucleotides classified as "unstacked" has a uracil base. This pattern agrees rather well with base stacking tendencies at the monomer level, as seen in Table I.

Brahms, Maurizot and Michelson[44] have studied a variety of dinucleotides in concentrated salt solution, using circular dichroism and ultraviolet absorption spectroscopy. Investigation of the temperature

TABLE II. Classification of the sixteen ribose dinucleoside monophosphates as "stacked" or "unstacked" in neutral aqueous solution at 25°C. Based on optical studies by Warshaw and Tinoco.[41]

Stacked	Unstacked
GpG	ApU
CpG	UpA
GpC	UpC
ApA	GpU
ApC	UpU
CpA	
UpG	
CpC	
CpU	
GpA	
ApG	

dependence of these properties from -20°C to +80°C led these workers to determine thermodynamic parameters for the intramolecular stacking interaction, based on a two-state model. A number of discrepancies between these results and those of Tinoco and co-workers are evident. CpC was found to be the most highly stacked dinucleotide, followed by ApC, ApA and CpA. Enthalpies for stacking ranged from -6.0 to -8.0 kcal/mole, entropies for stacking were about -24 e.u., and ΔF° for stacking varied from -0.7 kcal/mole for CpC to -0.2 kcal/mole for GpA and CpU, the least stacked of the dinucleotides studied, at 0°C. The use of 4.7 M KF as the solvent in this study may have had a large effect on the base stacking interactions, and the extent to which these results are applicable to behavior in solutions of lower ionic strength is questionable.

Simpkins and Richards[45] have studied the UV absorptivity changes of ApA, ApU, UpA and UpU as the dinucleotides are titrated, and have applied the two-state model to a determination of the intramolecular stacking association constants. They concluded that, at 20°C, the fractions of the dinucleotides stacked were as follows: ApA, 84%; ApU, 29%; UpA, 5%; UpU, ~0%.

Several studies of oligomers, particularly trinucleotides, have also been reported.[46-50] These have involved use of optical rotatory dispersion or circular dichroism, and the results have agreed well with expectations based on examination of the dinucleotides.

3. 2. Pmr Studies

The first reported study of dinucleoside monophosphates by pmr[51] involved the deoxyribose species TpT, TpdU and dUpT, as discussed in Part III, Section 4, of this thesis. Recently, reports of several other studies have appeared in the literature. Inoue and Aoyagi[52] studied the dinucleotides ApGp, CpGp and UpGp, and Scheit, Cramer and Franke[53] have investigated trinucleotide species containing the base sequences TTT, TTA, TTG and TGG by pmr. These studies involved concentrations in excess of 0. 1 M, and the conclusions in both papers, based on discussion of intramolecular effects only, are highly doubtful in view of the extensive intermolecular association at these concentrations (see Part III, 2. 3). Hruska and Danyluk have investigated the effect of temperature on the base-stacking interaction in adenylyl (3′→ 5′) adenosine-2′, 3′-cyclic phosphate, ApA-c-p[54] and on the ribose conformation in ApA, ApA-c-p, and GpA.[55] This work indicated that ApA-c-p is appreciably stacked at room temperature, and that ribose conformation changes along with the degree of intramolecular base-stacking of a dinucleotide. Similar results concerning ApA[56] and several other dinucleoside monophosphates[57, 60] have been obtained by Chan and co-workers in this laboratory.

In addition to this published work, Schweizer, Hollis and Ts'o[58] have studied the pmr spectra of ten ribose dinucleoside monophosphates, and McDonald and co-workers[59] have examined several deoxyribose dinucleoside monophosphates, although these studies have

not yet appeared in the literature.

4. Studies of Polynucleotides by Pmr

A great many studies of native DNA, RNA and synthetic oligo- and polynucleotides by various types of optical spectroscopy have been reported,[61-76] and several reviews of this work are available.[77-79] A few proton magnetic resonance studies of the polymers in aqueous solution have been reported, although this spectroscopic method has been used much less extensively than the optical methods, in large part because of the inherent difficulties discussed in the next section. Pmr spectra of polyadenylic acid (poly A), polyuridylic acid (poly U), polyinosinic acid (poly I) and polycytidylic acid (poly C), as well as data on spectra of the poly (A + 2U) complex, were reported by McTague, Ross and Gibbs[80] and by McDonald, Phillips and Penman.[81] These studies showed poly A and poly I, as well as the poly (A + 2U) complex, to be rather highly ordered at room temperature. These systems became disordered at high temperatures, with poly (A + 2U) showing a sharp "melting" transition, in agreement with optical studies. The property of the pmr spectra which characterizes the degree of order in these molecules is the linewidth of the resonances. In a rigid structure, the magnetic dipole interactions among the magnetic nuclei of the molecule are not averaged out effectively, due to slow overall rotation of the molecule in solution. This results in a large resonance linewidth. When a polynucleotide is in a random coil form, rapid local motion of the nucleotide residues averages the magnetic dipolar couplings, and narrow resonances are

observed. This was found to be the case for poly U and poly C.

Studies of DNA[59, 81] and transfer RNA[82, 83] by pmr have also been reported recently. Of particular interest is the observation of two distinct resonances for the methyl protons of thymine in denatured DNA at 90°C. These resonances arise from different magnetic environments for thymine, depending on whether there is a purine or pyrimidine base in the 5′-neighbor position.[59]

5. Suitability and Limitations of Pmr for Studies of Biological Molecules

The use of proton magnetic resonance spectroscopy to probe the structural details of polynucleotides and polypeptides in solution has several advantages over the optical methods, as well as some obvious limitations. The pmr measurement is highly localized in space, reflecting what happens at a single proton (or group of equivalent protons), while optical spectroscopy involves measurement of properties of the entire electronic system. The very high resolution of pmr allows extremely small and subtle effects to be observed, and the fact that each chemically unique proton provides (in principal) a distinct resonance means that the information content of a pmr spectrum is much greater than that of an optical spectrum. The NMR measurement is affected by events occurring at a much slower timescale than those which ordinarily affect an optical measurement, such as rotational diffusion in solution and the rates of some chemical exchange processes. This can provide an added dimension to the interpretation of pmr data.

Unfortunately, pmr spectroscopy is about four orders of magnitude less sensitive than the optical methods. Consequently,

concentrations of macromolecules in solution must be considerably higher than their concentrations in biological systems in many cases. This leads to aggregation and other undesirable intermolecular effects. The sensitivity of pmr can be increased by a factor of twenty or more through the use of spectrum-enhancement devices, such as the time-averaging computer, to improve the signal-to-noise ratio. One of the problems inherent in studies of biological macromolecules is the occurrence of a large number of resonances, many of which overlap one another to a great extent. A more serious problem is the severe broadening of resonances resulting from magnetic dipolar interactions among the magnetic nuclei of the molecule. These interactions are effectively averaged out by rapid rotational diffusion for a small molecule in solution, so that they have little effect on the resonance linewidths. The rotational motion of a macromolecule in solution is quite slow, however, often approaching the Larmor period for a proton ($\sim 10^{-8}$ sec), and the magnetic dipolar couplings then make the dominant contribution to the observed linewidths. The theory of proton resonance linewidths has been extensively developed, and several good discussions are available.[84-86]

The advent of NMR spectrometers operating at increasingly higher frequencies has made pmr spectroscopy a more and more useful tool for studies of systems of biological interest. The recent development of superconducting solenoids of high field homogeneity has resulted in a spectrometer operating at 220 Mcps for proton work. This system has provided a dramatic improvement in resolution for the pmr spectra of several biological macromolecules,[87, 88] and has

afforded an increase in sensitivity as well. Further advances in the quality and availability of instrumentation for high resolution pmr spectroscopy promise to make this method increasingly useful to the molecular biologist.

II. EXPERIMENTAL

1. Materials

Dinucleoside Monophosphates. -- The deoxyribose dinucleoside monophosphates thymidylyl (3′→ 5′) thymidine (TpT), thymidylyl (3′→ 5′) 2′-deoxyuridine (TpdU), and 2′-deoxyuridylyl (3′→ 5′) thymidine (dUpT) were gifts from Dr. Heinrich H. Peter, Division of Biology, California Institute of Technology. These compounds were in the form of the ammonium salts in D_2O solution, and were synthesized by Dr. Peter. The following ribose dinucleoside monophosphates were obtained from Calbiochem, Los Angeles, and were all A grade materials in the acid form: adenylyl (3′→ 5′) cytidine (ApC), cytidylyl (3′→ 5′) adenosine (CpA), cytidylyl (3′→ 5′) cytidine (CpC), adenylyl (3′→ 5′) uridine (ApU), and uridylyl (3′→ 5′) adenosine (UpA). Uridylyl (3′→ 5′) uridine (UpU) was obtained from Calbiochem as B grade ammonium salt, and was reported by the supplier to contain 3% of the (2′→ 5′) isomer. The CpC used in the purine binding study at 0. 01 M CpC concentration was obtained from Sigma Chemical Company, St. Louis. These compounds were used without further purification.

All of the ribose dinucleoside monophosphates were converted

to the sodium or ammonium salts by passing solutions of these samples through columns of Dowex 50W-X8 cation-exchange resin in the appropriate form. The ion-exchange resin was washed alternately in 1 M HCl, 1 M NaOH and 1 M NaCl, and thoroughly rinsed with distilled water before use. The columns were regenerated by passing 1. 0 M solutions of reagent grade NaCl or NH_4Cl through them, followed by distilled water until the addition of $AgNO_3$ showed the effluent to be free of Cl^-. The solutions of the salts of the dinucleoside monophosphates from the ion-exchange operation were lyophilized, and the solid materials were dried over P_2O_5 at room temperature under vacuum for at least 24 hours.

Polynucleotides. -- The polyuridylic acid used in the purine binding study was obtained from Sigma Chemical Company as the ammonium salt, type I. The molecular weight was reported by Sigma to be greater than 100, 000. Schwarz BioResearch, Inc., Orangeburg, N. Y., provided the polyuridylic acid used in the adenosine binding study. This material was in the form of the potassium salt, and the molecular weight was reported by Schwarz to be 100, 000 (determined by viscosity). These poly U samples were used without further purification, and were not examined for contaminants. Both samples were converted to the sodium salt, lyophilized and dried over P_2O_5 as described above.

Bases, Nucleosides, and Nucleotides. -- Purine was obtained from Cyclo Chemical Corp., Los Angeles. The purine-8, 9-D was prepared by heating a solution of purine in D_2O at 50°-60°C in a water bath for 48 hours to effect exchange of the H_8 and H_9 protons. [89]

The solution was lyophilized to obtain the solid. Both purine and purine-8,9-D were sublimed in vacuo before use. The following nucleosides and nucleotides were obtained as A grade materials from Calbiochem, and were used without further purification: adenosine, cytidine, uridine, thymidine, 2′-deoxyuridine, cytidine-5′-monophosphate, and uridine-5′-monophosphate (disodium salt). The 5′-CMP was converted to the sodium salt on a Dowex 50W-X8 column and lyophilized, and all the nucleosides and nucleotides were dried over P_2O_5 as described above.

Miscellaneous. -- Deuterium oxide (D_2O), 99.7%, was supplied by Columbia Organic Chemicals Co., Inc., Columbia, S. C. Deuterated phosphoric acid, D_3PO_4, was prepared by adding reagent grade P_2O_5 (J. T. Baker Chemical Co., Phillipsburg, N. J.) to D_2O. Tetramethylsilane (TMS) supplied by Aldrich Chemical Co., and tetramethylammonium chloride from Matheson, Coleman, and Bell were used as references for chemical shift measurements.

2. Methods

All solutions were prepared by weight, and concentrations are expressed in moles of solute per liter of D_2O, which is equivalent to moles/liter, M, at the concentrations used in this work. Solution volumes ranged from ~0.3 to ~1.0 ml. Measurements of pH were made using a Leeds and Northrup 7401 pH meter equipped with a No. 12413 miniature electrode assembly. This apparatus was standardized to read pH in H_2O solutions. To measure pD in D_2O

solutions, the standard correction[90] was applied: pD = pH (meter reading) + 0. 40. The pD of the D_2O solutions was lowered by adding small aliquots of 4 M HCl in the CpC study (which would have a slight effect on the validity of the pD measurements), and by adding D_3PO_4 in the ApU-UpA study.

The proton chemical shifts in the work done at 60 Mcps (TpT, TpdU, dUpT, and 0. 08 M CpC studies) are referred to external TMS in CCl_4 solution, and are expressed in cps. No corrections for bulk magnetic susceptibility effects have been applied to these data. The absolute chemical shifts at 60 Mcps are accurate to ± 0. 5 - 1. 0 cps, and the chemical shift differences between closely spaced resonances in the same spectrum are accurate to ~± 0. 2 cps.

In the work at 100 Mcps, the chemical shifts were measured relative to external TMS in a capillary tube contained in the sample tube, and are generally expressed in ppm. A small amount of tetramethylammonium chloride was added to the solutions used for the variable temperature and purine binding experiments to serve as an internal standard. This allowed corrections to be made for changes in the difference in bulk magnetic susceptibility between the TMS capillary and the D_2O solution with changes in temperature or purine concentration. The corrections are quite large in the temperature studies, ~20 cps (0. 20 ppm) between 6° and 86°C, but amount to only 3. 0 - 3. 5 cps at 1. 0 M concentration of added purine. The uncontrolled sample temperature in the studies at 100 Mcps was nominally ~ 29°C.

3. Instrumentation

Four NMR spectrometers were utilized in the course of the work reported here. A Varian A-60 spectrometer was used for the study of TpT, TpdU and dUpT. The probe temperature was ~30°C for this work, and was not controlled. In the investigation of CpC at 0.08 M concentration, Varian A-60A and A-56/60 instruments were used. In both of these spectrometers the normal probe temperature was 36° to 40°C. The A-56/60 was equipped with a variable temperature probe and Varian V-6040 controller, which was used for the temperature study of 0.08 M CpC + 0.96 M purine. A Varian C-1024 computer of average transients (CAT) was used to enhance the signal-to-noise ratio of the spectra in the CpC study, particularly for the broad purine resonances.

The work at 100 Mcps was carried out on a Varian HA-100 spectrometer operated in the frequency sweep mode. TMS in a sealed capillary provided the field/frequency lock signal, as well as serving as the external reference for the chemical shift measurements. Chemical shifts were measured to ± 0.1 cps by counting the frequency difference between the reference and sweep oscillators with a Hewlett-Packard 5512-A frequency counter. In the variable temperature work, the sample temperature was controlled to ± 1°C by means of a Varian V-4343 variable temperature controller, and was determined using the methanol and ethylene glycol samples and calibration curves supplied by Varian. A C-1024 time-averaging computer was used for spectrum enhancement.

III. PMR STUDIES OF DINUCLEOSIDE MONOPHOSPHATES

The results of investigations of several dinucleoside monophosphates by proton magnetic resonance spectroscopy are presented here. The first compounds studied were the deoxyribose dinucleoside monophosphates TpT, TpdU and dUpT,[51] and this work subsequently led to studies of the ribose dinucleotides. No attempt has been made to investigate all sixteen of these molecules; rather, several examples of each structural type (purine-purine, purine-pyrimidine, pyrimidine-purine, pyrimidine-pyrimidine) were chosen for detailed investigation. In addition to the work reported in this thesis on ApC, CpA, CpC, ApU, UpA and UpU, studies by J. H. Nelson on ApA, ApG, GpA, ApU, and UpA,[60] and by J. H. Prestegard on ApU and UpA[57] have also been completed in these laboratories.

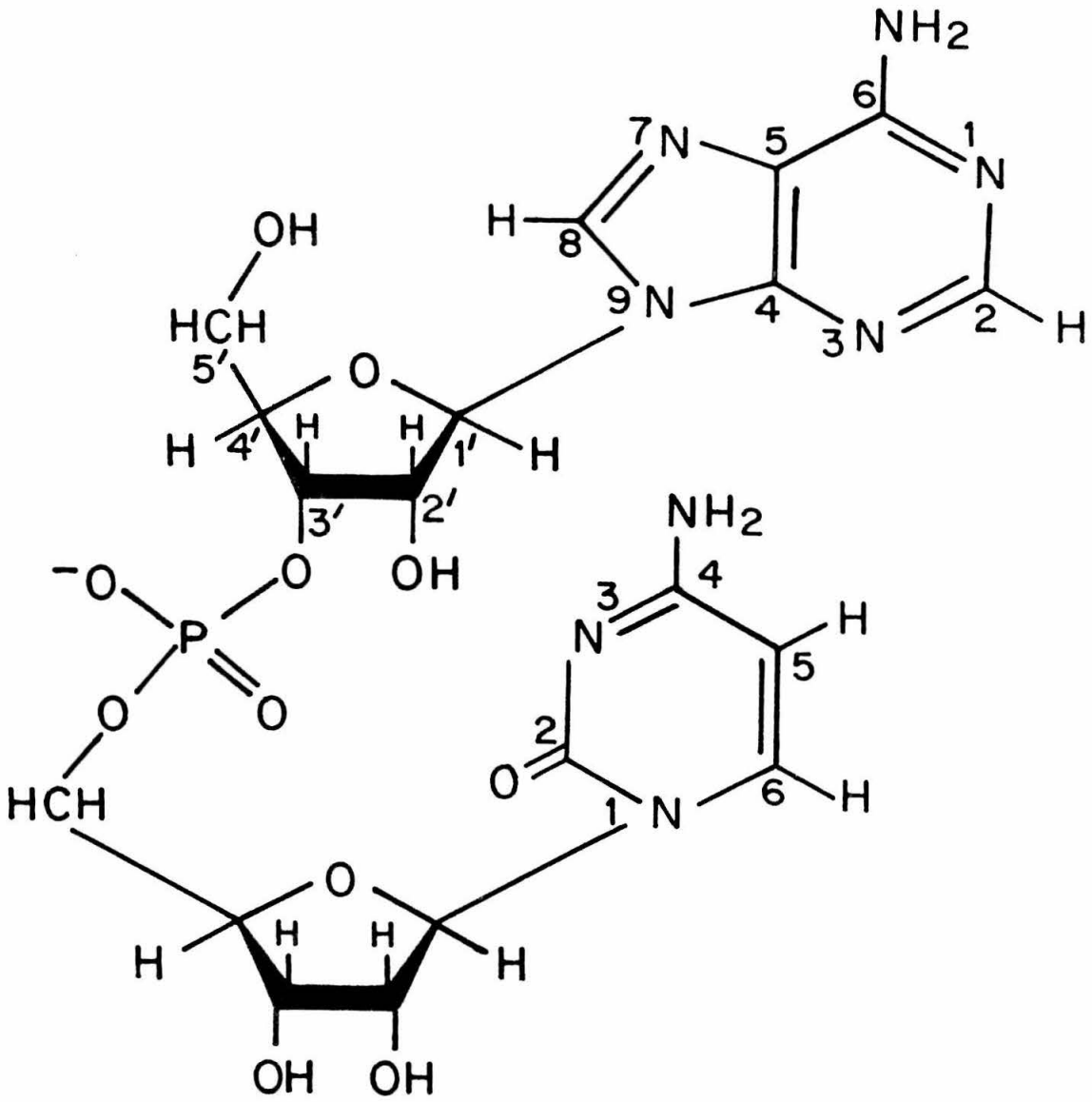
1. Structure and Conformation of Dinucleoside Monophosphates

There are a number of structural and conformational properties which apply in general to all dinucleoside monophosphates. These characteristics, and the nomenclature pertaining to this class of compounds, are discussed in this Section.

The ribose dinucleoside monophosphate with adenosine esterified in the 3′-position and cytidine esterified in the 5′-position, adenylyl (3′→5′) cytidine (ApC), is shown in Figure 3. This structural diagram is only schematic, and bond angles and distances shown here are highly distorted. The numbering scheme using unprimed numbers for the purine and pyrimidine bases applies to the other bases (see Figure 1)

FIGURE 3

Structural diagram of adenylyl (3′→ 5′) cytidine, ApC.


NH2
6
7N
5
1
N
H
8
9N
4
3N
2
H
OH
HCH
5′
O
H
4′
H
H
1′
H
3′
2′
OH
−O
O
P
O
O
O
HCH
NH2
4
3
N
H
5
2
O
1N
6
H
O
H
H
H
H
OH
OH

ApC

as well as to adenine and cytosine. The numbers referring to the furanose ring of ribose (or 2′-deoxyribose) are primed, as shown, and apply not only to the carbon atoms but also to the substituents bonded to them. In referring to a particular atom or group in a dinucleotide, it is necessary to specify the particular nucleoside as well as the skeletal position. In this thesis, the nucleoside is distinguished by a capital letter enclosed in parentheses: (A), (G), (C), (U), or (T) following the skeletal designation. This convention is inadequate for dinucleotides containing two identical bases, and in this instance the position of phosphate attachment is cited as (3′) or (5′). Thus the hydrogen atom attached to carbon atom $C_{1'}$ of the cytidine ribose ring in ApC would be denoted $H_{1'}$ (C), the analogous proton in CpC would be designated $H_{1'}$ (5′), and so on.

The problem of determining the conformation of a dinucleoside monophosphate is a complicated one. As is evident in Figure 3, there are a number of bonds about which rotation may occur. The relative conformation of the two bases can be affected by rotations about the glycosidic bonds (N_1 of pyrimidine base to $C_{1'}$ of ribose, N_9 of purine base to $C_{1'}$ of ribose), and the following bonds of the ribose-phosphate backbone: $C_{3'}$-$O_{3'}$ of the 3′-nucleoside, two O-P bonds, $O_{5'}$-$C_{5'}$ and $C_{5'}$-$C_{4'}$ of the 5′-nucleoside. In addition to rotations about these seven bonds, there are several possibilities for the conformation of the ribose rings, adding to the complexity of the situation. Of course, many of the possible dinucleoside monophosphate conformations would be highly unfavorable because of repulsive non-bonded interactions

between atoms.

The conformations of the ribofuranose ring are generally discussed in terms of displacement of either $C_{2'}$ or $C_{3'}$ above or below the (approximate) plane formed by the four atoms, $O_{1'}$, $C_{1'}$, $C_{4'}$, and $C_{3'}$ or $C_{2'}$. Displacement of $C_{2'}$ or $C_{3'}$ toward the $C_{5'}$ side of the five membered sugar ring is called endo and displacement of either of these atoms toward the side opposite $C_{5'}$ is termed exo. The resulting ribose conformations are then $C_{2'}$-endo, $C_{2'}$-exo, $C_{3'}$-endo and $C_{3'}$-exo. Rotation about the glycosidic bond has been categorized as either syn or anti. In the syn conformation, the six membered ring of a purine base or the C_2-O_2 carbonyl group of a pyrimidine base is located above the ribose ring; in the anti conformation, proposed for the double helix of DNA, C_8 of a purine or C_6 of a pyrimidine is situated above the ribose ring.

Haschemeyer and Rich[91] have summarized the results of several X-ray diffraction crystal structure studies of nucleosides and nucleotides, and have calculated the distances between non-bonded atoms as a function of rotation about the glycosidic bond for purine and pyrimidine nucleosides. They concluded that pyrimidine nucleosides would be more stable in the anti conformation for both $C_{2'}$-endo and $C_{3'}$-endo ribose conformations. Purine nucleosides, according to their analysis, could adopt either syn or anti conformations for $C_{2'}$-endo puckering of ribose (or deoxyribose), while the anti conformation is more favorable with $C_{3'}$-endo sugar conformation from the standpoint of minimizing repulsive non-bonded interactions. Of the crystal

structures examined by Haschemeyer and Rich, only deoxyadenosine showed an exo sugar conformation ($C_{3'}$-exo).

Conformations of the ribose-phosphate backbone resulting from rotations about the $C_{3'}$-$O_{3'}$, $C_{5'}$-$C_{4'}$, and $O_{5'}$-$C_{5'}$ bonds of a hypothetical $C_{3'}$-endo nucleotide monomer unit have been calculated by Sasisekharan et al.,[92] using a hard-sphere approximation for non-bonded contacts. Rotations about the P-$O_{3'}$ and $O_{5'}$-P bonds required to specify the repeat of this monomer unit in space (or the dinucleoside monophosphate conformation) were not considered. They found that only a small number of conformations for the monomer unit were free of non-bonded contacts according to this crude model. They also found that anti conformations with regard to rotation about the glycosidic bond were favored for both purine and pyrimidine nucleosides, with conformations of pyrimidine nucleosides being particularly restricted.

2. Adenylyl (3′→5′) Cytidine and Cytidylyl (3′→5′) Adenosine

The sequence isomers ApC and CpA have been studied in aqueous solution by pmr under varying conditions of concentration and temperature, in an effort to determine the nature and the extent of intra- and intermolecular association of these dinucleotides.

2.1. Pmr Spectra of ApC and CpA

The 100 Mcps pmr spectra at 29°C of 0.010 M solutions of the sodium salts of ApC and CpA are presented in Figures 4 and 5. The region of the adenine H_8 and H_2 and cytosine H_6 protons is shown in Figure 4 a and b, and the region of the cytosine H_5 and ribose $H_{1'}$

FIGURE 4

Time-averaged 100 Mcps pmr spectra in the region of adenosine H_8 and H_2 and cytidine H_6 resonances (-8.90 to -8.00 ppm from TMS capillary). Each spectrum is the sum of 31 scans. (a) 0.010 M ApC; (b) 0.010 M CpA; (c) 0.010 M adenosine; (d) 0.010 M cytidine.

ApC and CpA (sodium salts), ADENOSINE and CYTIDINE; 0.010M.
H_8, H_2 and H_6 Proton Resonances

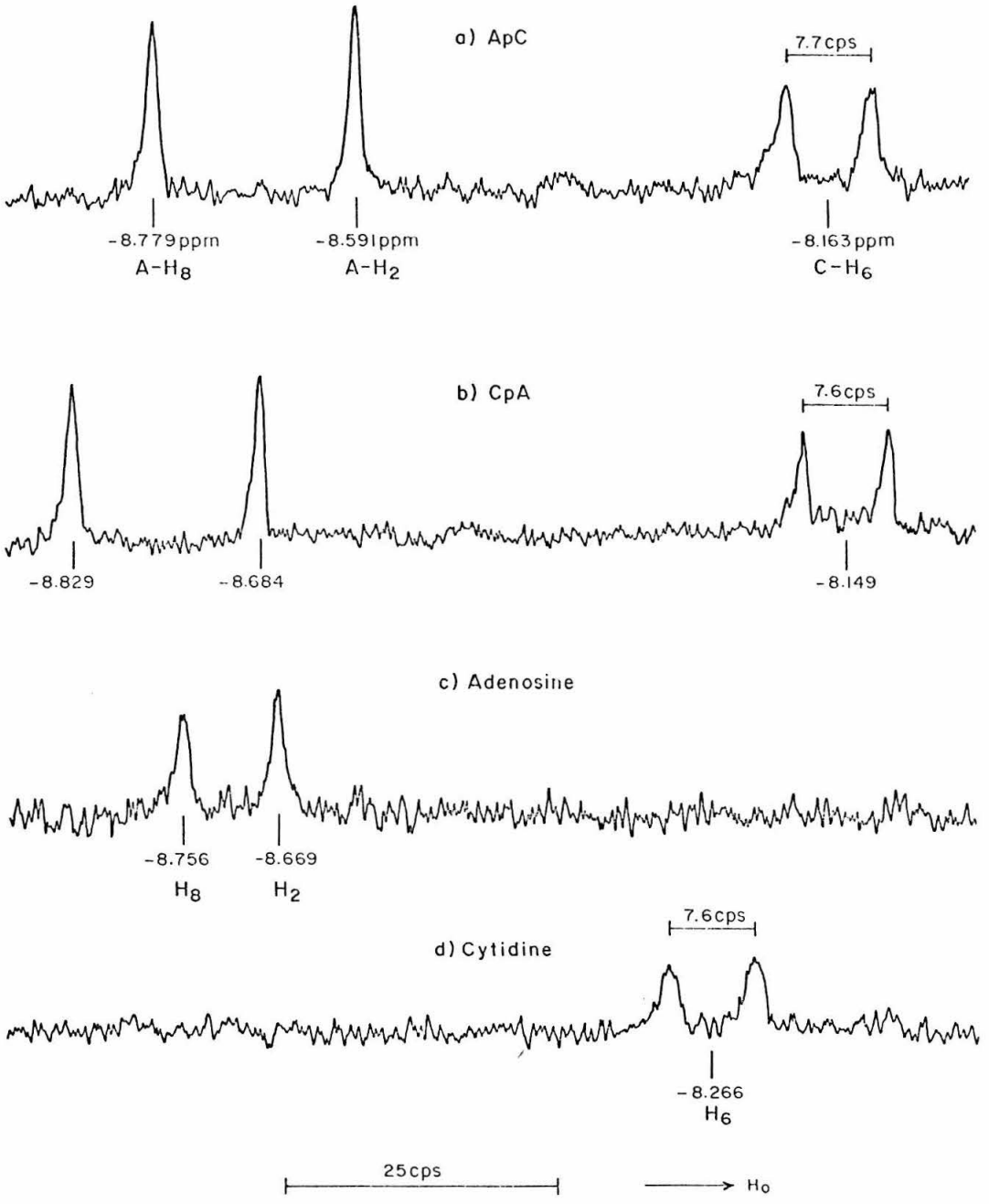
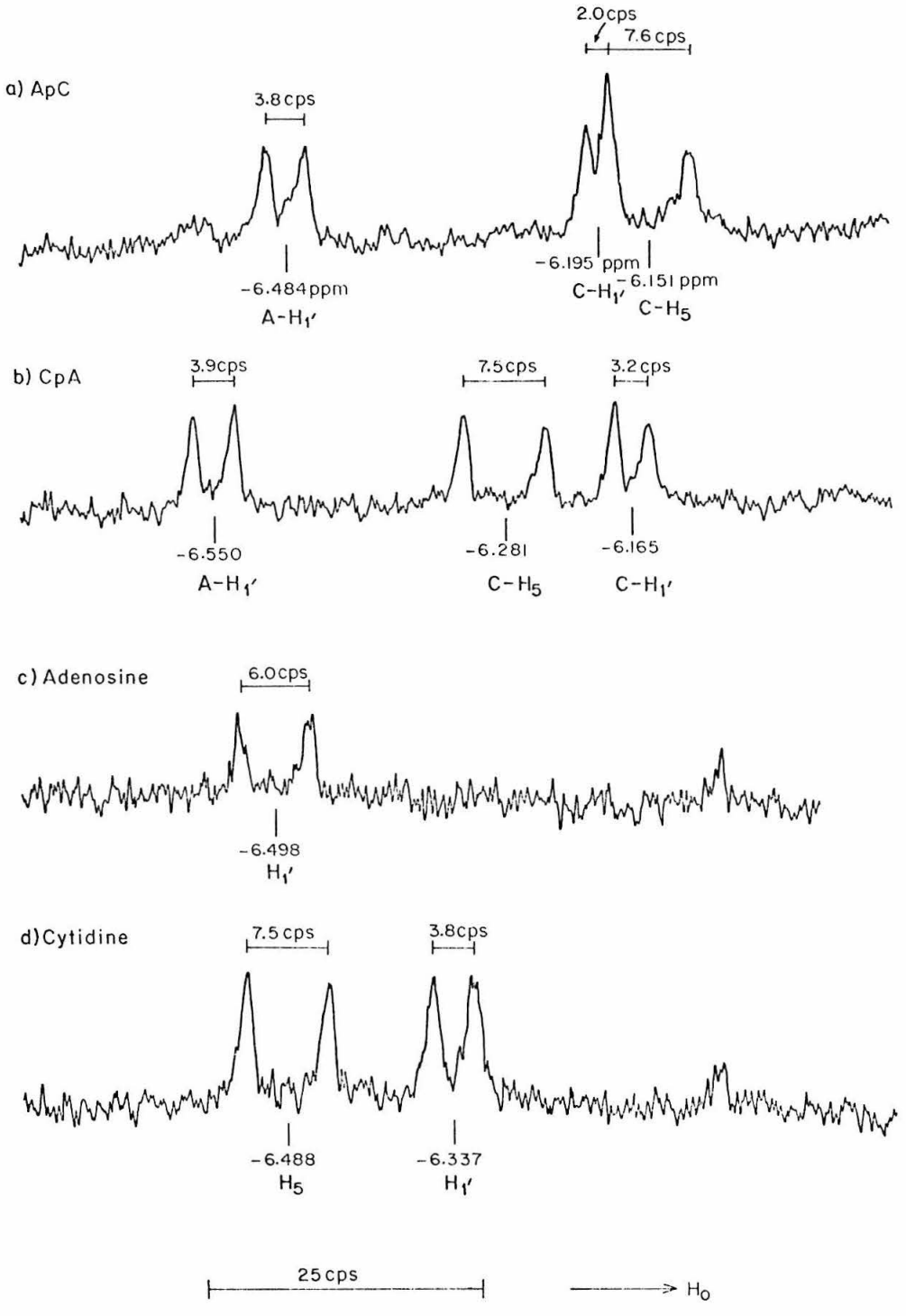


FIGURE 5

Time-averaged 100 Mcps pmr spectra in the region of cytidine H_5 and $H_{1'}$ and adenosine $H_{1'}$ resonances (-6.80 to -5.90 ppm from TMS capillary). Each spectrum is the sum of 31 scans. (a) 0.010 M ApC; (b) 0.010 M CpA; (c) 0.010 M adenosine; (d) 0.010 M cytidine.

ApC and CpA(sodium salts), ADENOSINE and CYTIDINE; 0.010 M.
H_5 and $H_{1'}$ Proton Resonances.



protons is depicted in Figure 5 a and b. For comparison, the spectra of the adenosine and cytidine nucleosides in the same spectral regions under similar experimental conditions are shown in sections c and d of these figures.

2. 1. 1. Assignment of resonances

The assignment of the dinucleotide resonances depicted in Figures 4 and 5 is straightforward from comparison with the nucleoside spectra. As in the case of other purine derivatives,[22] the adenine H_8 protons of ApC and CpA are readily distinguished from the H_2 protons by exchange with deuterium of the H_8 protons upon equilibration in D_2O at elevated temperatures. In this manner, the resonance at lowest field has been assigned to the H_8 proton in both ApC and CpA. The cytosine H_6 and H_5 protons are spin-spin coupled to give doublets, with the coupling constant $|J_{H_5-H_6}| = 7.6$ cps in both ApC and CpA as well as in the monomeric cytidine nucleoside. The ribose $H_{1'}$ resonances are doublets from coupling with the $H_{2'}$ protons, and the coupling constants, $|J_{H_{1'}-H_{2'}}|$, are seen to change markedly in going from the nucleosides to the dinucleotides. In both ApC and CpA, the lower field $H_{1'}$ doublet can be assigned to the adenosine nucleoside. Because of the ring-current magnetic anisotropy of the adenine base, the ribose $H_{1'}$ resonance of the adenosine nucleoside is expected to appear at lower field relative to those of the pyrimidine nucleosides.

In addition to the resonances shown in Figures 4 and 5, the ribose $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$ and $H_{5''}$ protons give rise to resonances occurring in the spectral region from -4. 20 to -5. 20 ppm. There is

extensive spin-spin coupling among these protons by complex schemes, and the resonances due to the 3′- and 5′-esterified nucleosides do not coincide, so that assigning the resonances in this region would be immensely difficult. Therefore this spectral region was not studied to any extent. The hydroxyl and amino group protons do not give distinct resonances because of rapid exchange of these protons with the solvent, D_2O.

2. 1. 2. Discussion of chemical shifts

All of the monitored resonances of ApC and CpA are shifted from their spectral positions in the component nucleosides. This is due in part to the effect of the singly charged phosphate group, and resonances of analogous protons on 3′- and 5′-esterified nucleosides are affected differently because of this difference in phosphate attachment. The influence of the phosphate group on the proton chemical shifts is primarily a consequence of its negative charge and resulting electric field.[93] The properties of the neighboring base can also have an effect on the chemical shifts of protons of a particular base in a dinucleotide. The adenine base in both ApC and CpA possesses a large ring-current magnetic anisotropy which can produce upfield shifts of the proton resonances of the neighboring cytosine base. The cytosine base, however, appears from previous work[28] to have a negligibly small magnetic anisotropy.

Recently, Prestegard and Chan[94] have demonstrated that the chemical shifts of the adenine H_8 proton and the uracil H_6 proton are dependent upon the rotational conformation of the base relative to the

ribose ring in the nucleosides and mononucleotides. This work showed that the angle of rotation of the base about the glycosidic bond is related to the conformation of the ribose ring to which the base is attached. In the dinucleotides, the intramolecular stacking interaction between the two bases can affect both the angle of rotation of each base relative to its ribose ring and the conformation of the ribose moieties. Hence the factors which affect the chemical shifts of the adenine (and presumably guanine) H_8 protons and those of the uracil (and cytosine) H_6 protons can be quite complex in the dinucleotides.

Cytidine Protons. -- The effect of the phosphate group on the chemical shifts of the cytidine H_6, H_5 and $H_{1'}$ protons of ApC and CpA can approximately be cancelled out by referring the chemical shifts of these protons to those of the corresponding 3′- or 5′-esterified cytidine nucleosides of CpC (from Section 5). In Table III are summarized the chemical shifts of these cytidine protons in 0. 010 M ApC, CpA and CpC (all sodium salts), and the differences in the chemical shifts resulting from the substitution of adenine for cytosine as the adjacent base in ApC and CpA are indicated. The cytidine resonances in ApC and CpA can be seen to be shifted appreciably upfield from their corresponding spectral positions in CpC.

The upfield shifts observed for the cytidine H_5 resonances in ApC and CpA compared to CpC must have their origin primarily in the ring-current magnetic anisotropy of the adjacent adenine base, since the resonances of these protons in the pyrimidine nucleosides and nucleotides have been shown to be insensitive to the conformation of the base relative to the ribose ring.[94] On the basis of the work by Prestegard

TABLE III. Shifts of cytidine proton resonances resulting from the substitution of adenine for cytosine as the adjacent base in a dinucleoside monophosphate (concentration 0. 010 M).

	$\underline{H_6}$		$\underline{H_5}$		$\underline{H_{1'}}$	
	(3′)	(5′)	(3′)	(5′)	(3′)	(5′)
	ppm		ppm		ppm	
ApC		-8. 163		-6. 151		-6. 195
CpA	-8. 149		-6. 281		-6. 165	
CpC	-8. 308	-8. 358	-6. 400	-6. 408	-6. 242	-6. 331
Shift	+0. 159	+0. 195	+0. 119	+0. 257	+0. 077	+0. 136

and Chan, however, one would expect the chemical shifts of the cytosine H_6 protons in ApC and CpA to be influenced by the conformation of the cytosine base relative to the ribose ring, as well as by the ring-current magnetic anisotropy of the adenine base.

Prestegard and Chan found that the uridine H_6 resonance shifted upfield as the ribose $H_{1'}$-$H_{2'}$ coupling constant, $|J_{H_{1'}-H_{2'}}|$, became smaller with the addition of salt to a solution of the nucleoside or nucleotide. A decrease in $|J_{H_{1'}-H_{2'}}|$ is indicative of a change in average ribose conformation from 2′-endo toward 3′-endo ring puckering (see the discussion in Section 2. 3. 1). Consideration of CPK molecular models indicates that the conformation of the pyrimidine base relative to the ribose ring is quite restricted when the ribose conformation is 2′-endo, with the H_6 proton quite close to the ether oxygen of the furanose ring. A change in ribose conformation toward 3′-endo allows a greater range of angular rotation about the glycosidic bond for the base, and the H_6 proton would on the average be farther from the ether oxygen atom. The change in the chemical shift of the H_6 proton with changing ribose conformation was attributed to the magnetic anisotropy of the ether oxygen. It is felt by Prestegard and Chan that H_6 is deshielded when in close proximity to the ether oxygen, so that the H_6 resonance shifts downfield as the base-ribose conformation about the glycosidic bond is constrained to bring H_6 and the ether oxygen atom close to one another.

In the dinucleotides, the intramolecular stacking interaction between the two bases is expected to be an important factor in deter-

mining the conformation of the bases relative to the ribose rings. Examination of models of ApC and CpA indicates that for effective base-base overlap, the pyrimidine bases must assume conformations which would bring H_6 close to the ether oxygen. Hence if base-base overlap were to serve as the important criterion for the extent of the base-stacking interaction, one would expect the H_6 proton to be, on the average, more nearly eclipsed with the ether oxygen of the ribose ring the stronger the stacking interaction between the two bases. Since the intramolecular base-stacking interaction is expected to be stronger in ApC and CpA than in CpC, the H_6 resonances would be expected to be at lower fields in ApC and CpA than in CpC on the basis of conformation about the glycosidic bond.

As indicated by the data in Table III, however, the cytosine H_6 resonances in both ApC and CpA are at appreciably higher fields than the corresponding resonances of CpC at 29°C. It is evident, then, that the cytidine H_6 protons of both ApC and CpA are also exposed to the ring-current magnetic anisotropy of the adjacent adenine bases to a large degree. The appreciable ring-current shift observed for the cytosine H_6 and H_5 resonances would seem to indicate that ApC and CpA tend to assume conformations in which the adenine and cytosine bases are intramolecularly stacked to a significant extent at 29°C in aqueous solution.

The factors affecting the chemical shifts of the $H_{1'}$ (C) protons in ApC and CpA are also complex. As indicated above, effects arising directly from the location of the phosphate attachment are expected to

be compensated for when the chemical shifts of these protons are referred to the corresponding protons in CpC. From geometrical considerations, the ring-current magnetic anisotropy of the adjacent adenine base is expected to shift the cytidine $H_{1'}$ resonance further upfield in ApC than in CpA, if the bases are intramolecularly stacked to a comparable extent. This trend is clearly indicated by the chemical shift data for these protons in Table III.

However, Prestegard and Chan pointed out that the chemical shifts of the ribose $H_{1'}$ protons are also sensitive to the conformation of the ribose ring and the angle of rotation of the base about the glycosidic bond. Briefly, they observed that the 2'-hydroxyl group of ribose exerts a shielding effect on $H_{1'}$ and the 2-keto oxygen of the pyrimidine base deshields $H_{1'}$, when the $H_{1'}$ proton is close to these groups. The $H_{1'}$ proton is closer to the 2'-hydroxyl group when the ribose ring is in the 3'-endo conformation than when it is in the 2'-endo conformation. Since the intramolecular base-stacking in a dinucleotide is expected to restrict the conformation of the base about the glycosidic bond so that the 2-keto group is rotated away from the $H_{1'}$ proton, the base-stacking interaction is seen to lead to upfield shifts for the $H_{1'}$ resonances.

The greater degree of intramolecular base-stacking expected in ApC and CpA compared to CpC may contribute to the observed upfield shift of the $H_{1'}$ protons of these dinucleotides relative to those of CpC as a consequence of the magnetic anisotropy of the 2-keto group, adding to the effect of the adjacent adenine base. The ribose conformations of

the 5′-esterified cytidine nucleosides of ApC and CpC are almost identical, judging from the respective $H_{1'}$-$H_{2'}$ coupling constants, hence a difference in the contribution of the 2′-hydroxyl group to the $H_{1'}$ chemical shifts is small. In comparing the $H_{1'}$ (C) proton of CpA with the $H_{1'}$ (3′) proton of CpC, it is noted that the difference in $H_{1'}$-$H_{2'}$ coupling constants ($|J_{H_{1'}-H_{2'}}|$ = 3.2 cps for CpA and 2.3 cps for CpC) indicates that the ribose conformation is more 2′-endo in CpA than in CpC. (The connection between $|J_{H_{1'}-H_{2'}}|$ and ribose conformation is discussed in Section 2.3.1). This would cause $H_{1'}$ to be further away from the 2′-hydroxyl group in CpA than in CpC, and would contribute to shifting $H_{1'}$ downfield in CpA relative to CpC. This difference in ribose conformation could help account for the smaller upfield shift for $H_{1'}$ of CpA compared to ApC, when referring both of these dinucleotides to CpC.

Adenosine Protons. -- The effect of a neighboring cytosine base on the chemical shifts of the adenosine protons of ApC and CpA can be assessed by comparing the chemical shifts of these protons with those of the corresponding protons in adenosine-3′-monophosphate and adenosine-5′-monophosphate. The chemical shifts of the adenosine protons in these molecules are strongly concentration dependent, hence it is necessary to make comparisons at extremely low concentrations. The infinite dilution chemical shifts for the H_8, H_2 and $H_{1'}$ protons of 3′-AMP and 5′-AMP (singly-charged sodium salts) have been reported by Schweizer et al.,[27] and are compared with the infinite-dilution chemical shifts obtained in Section 2.2 for ApC and CpA in Table IV.

TABLE IV. Shifts of adenosine proton resonances resulting from the esterification of cytidine to adenosine 3′ and 5′ monophosphates. Data on 3′-AMP and 5′-AMP from results of Schweizer et al.[27]

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

The data indicate that esterification of cytidine to 3′-AMP and 5′-AMP causes the H_2 resonance of ApC and the H_8 resonance of CpA to be shifted upfield by about 0. 08 ppm, and has only a small effect on the other monitored adenosine resonances. A similar chemical shift difference for the adenine H_8 protons of the dinucleotide UpA and 5′-AMP has also been noted,[56] and might suggest that the phosphate group is on the average constrained further away from the adenine base in the dinucleoside monophosphate than in the nucleotide. The sizeable upfield shift observed for the adenine H_2 proton in ApC compared to 3′-AMP is surprising, in view of the relative lack of sensitivity of the adenine H_2 resonance to the nature of the ribose backbone,[56] and the generally accepted view[28] that the cytosine base has a negligibly small magnetic anisotropy. The present observations would seem to indicate that the cytosine base can have a small influence on the magnetic environment of a proton located in its immediate vicinity.

2. 2. Concentration Dependence

The effect of concentration on the pmr spectra of ApC and CpA was studied in order to determine the extent of intermolecular association of these dinucleotides, and to ascertain the effects of this association on chemical shifts of the various protons. As discussed in Part I, Section 2. 1, monomeric bases and nucleosides associate extensively in aqueous solution by base-stacking, and it would be expected that the dinucleoside monophosphates would exhibit similar behavior. Knowledge of the extent and the nature of the intermolecular association processes is important in assessing the results of

experiments designed to investigate only the intramolecular interaction between the two bases of a dinucleotide.

The concentration dependence of the chemical shifts of the monitored protons of ApC and CpA was studied over the concentration range 0.0025 M to 0.100 M at 29°C, and the results are summarized in Figures 6 and 7. All the resonances are shifted to higher fields with increasing dinucleotide concentration, with the adenine base protons exhibiting the largest shifts. The extrapolated infinite-dilution chemical shifts and the concentration shifts at 0.10 M are summarized in Table V. Changes in the bulk magnetic susceptibility of the solution with concentration are extremely small over the concentration range investigated (~ 0.2 cps), and no corrections have been applied to these data. The ribose coupling constants $|J_{H_1'-H_2'}|$ are independent of concentration from 0.0025 M to 0.100 M.

The upfield shifts observed with increasing dinucleotide concentration indicate that ApC and CpA self-associate intermolecularly by base-stacking. Since the cytosine base has only a negligible magnetic anisotropy, and the proton chemical shifts of the monomeric cytidine nucleoside are not concentration dependent despite the intermolecular base-stacking of cytidine demonstrated in osmotic studies,[28] the observed concentration shifts for ApC and CpA must be a consequence of the ring-current magnetic anisotropy of the adenine base. The large concentration shifts exhibited by the adenine ring protons indicate considerable A-A base-stacking in the intermolecularly associated species. In the case of ApC, the cytosine base protons are only

FIGURE 6

Chemical shifts (at 100 Mcps) of adenosine H_8 and H_2 and cytidine H_6 protons of ApC and CpA as a function of concentration at 29°C. ApC: H_8 ○, H_2 □, H_6 △; CpA: H_8 ●, H_2 ■, H_6 ▲.

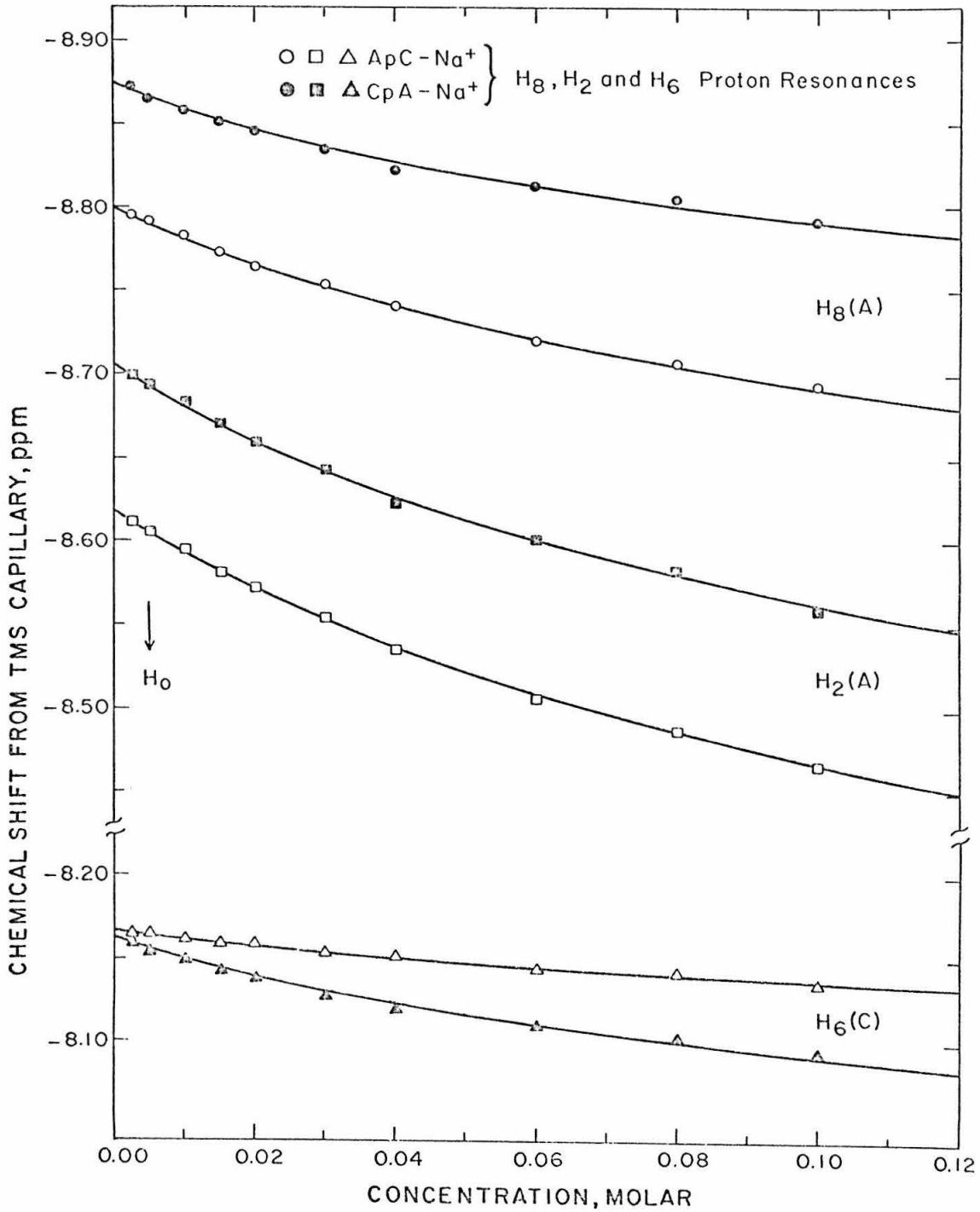

-8.90
-8.80
-8.70
-8.60
-8.50
-8.20
-8.10
CHEMICAL SHIFT FROM TMS CAPILLARY, ppm
ApC - Na+
CpA - Na+
H8, H2 and H6 Proton Resonances
H8(A)
H2(A)
H6(C)
Ho
0.00
0.02
0.04
0.06
0.08
0.10
0.12
CONCENTRATION, MOLAR

FIGURE 7

Chemical shifts (at 100 Mcps) of cytidine H_5 and $H_{1'}$ and adenosine $H_{1'}$ protons of ApC and CpA as a function of concentration at 29°C. ApC: H_5 Δ, $H_{1'}$ (A) ○, $H_{1'}$ (C) □; CpA: H_5 ▲, $H_{1'}$ (A) ●, $H_{1'}$(C) ■.

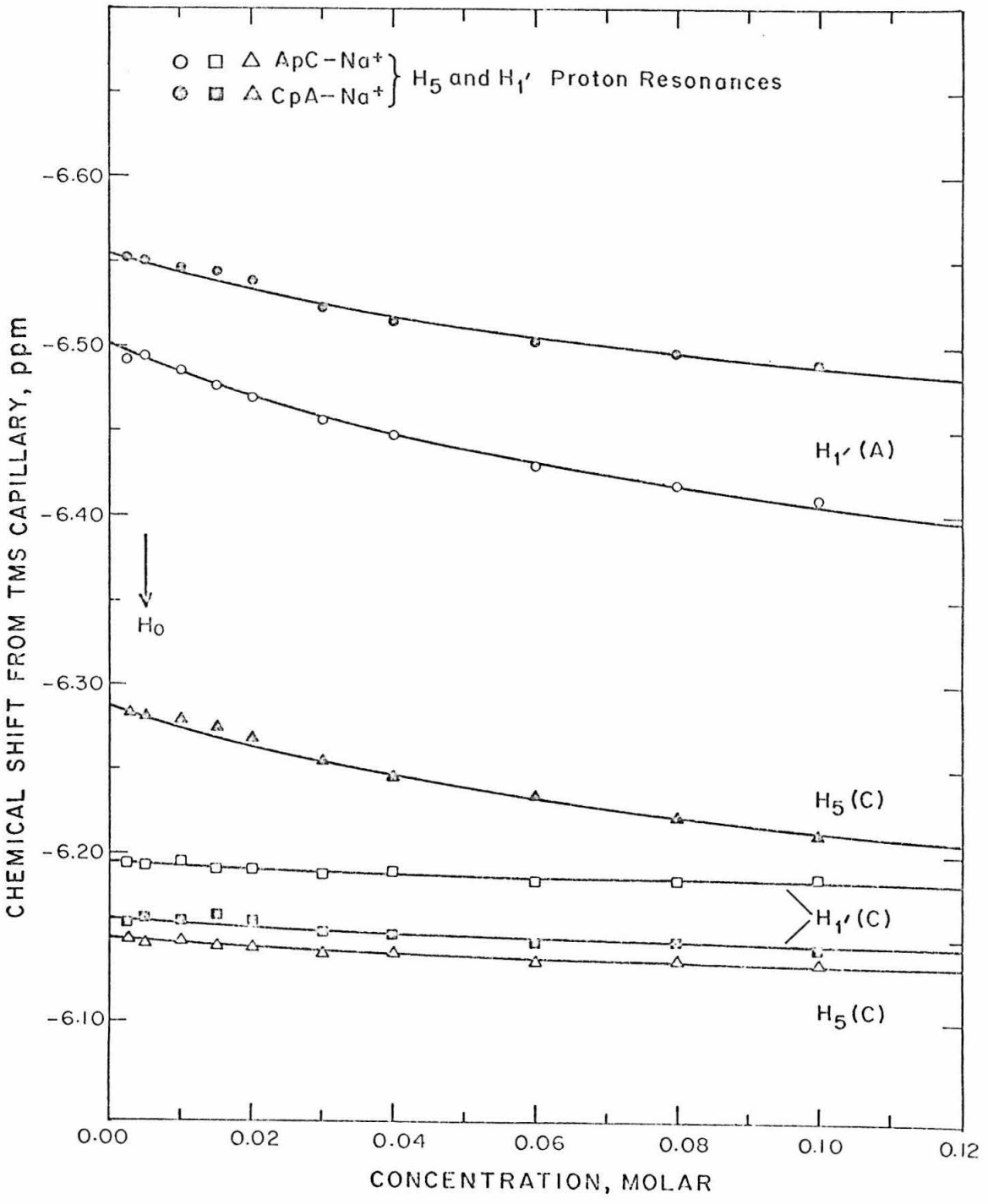

ApC–Na+
CpA–Na+
H5 and H1′ Proton Resonances
-6.60
-6.50
-6.40
-6.30
-6.20
-6.10
H1′ (A)
H0
H5 (C)
H1′ (C)
H5 (C)
CHEMICAL SHIFT FROM TMS CAPILLARY, ppm
0.00
0.02
0.04
0.06
0.08
0.10
0.12
CONCENTRATION, MOLAR

TABLE V. Extrapolated infinite dilution chemical shifts and infinite dilution to 0.10 M shifts for ApC and CpA proton resonances at 29°C.

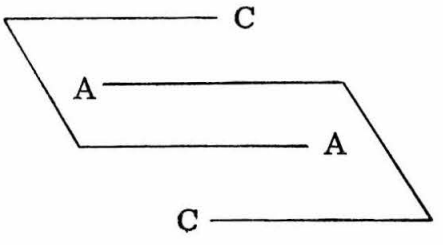
Proton	Chemical Shift at Infinite Dilution		Shift from Infinite Dilution to 0.10 M	
	ApC	CpA	ApC	CpA
	ppm	ppm	ppm	ppm
H_8 (A)	-8.800	-8.874	0.107	0.082
H_2 (A)	-8.619	-8.706	0.152	0.146
H_6 (C)	-8.167	-8.163	0.031	0.069
$H_{1'}$(A)	-6.501	-6.556	0.091	0.066
H_5 (C)	-6.149	-6.287	0.013	0.075
$H_{1'}$(C)	-6.194	-6.161	0.008	0.016

slightly affected by the intermolecular association, implying that little A-C base-stacking is involved in the complex or complexes formed. With CpA on the other hand, a considerably greater degree of intermolecular A-C base-stacking would appear to be indicated by the larger concentration shifts observed for the cytosine H_6 and H_5 protons. This sequence-dependent behavior is rather surprising, and a consideration of the several possible types of intermolecular complexes may be helpful in understanding these results.

If a dinucleotide is strongly stacked intramolecularly, it will self-associate primarily by stacking of the external or exposed faces of the bases of the folded molecule. In both ApC and CpA, a large tendency for A-A base-stacking is to be expected, followed by A-C base-stacking, which in turn is more favorable than C-C base-stacking. The concentration shifts observed for the cytosine H_6 and H_5 resonances may be due in part to A-C stacked intermolecular complexes of this type, with the differences in the concentration shifts for these protons between ApC and CpA reflecting differences in the A-C base-stacking tendencies as a result of geometric effects and steric restrictions on A-C base overlap in the associated species.

If the two bases in ApC and CpA are, however, not strongly stacked intramolecularly, self-intercalated dimers might also be formed. In such complexes, a base of one molecule would be inserted between the two bases of another dinucleotide molecule. Again, because of the greater tendency for A-A base-stacking, the intercalated complexes of ApC or CpA are expected to involve insertion of the

adenine base primarily. Such a self-intercalated ApC dimer may be represented schematically as follows:



The formation of these intermolecular complexes can also lead to concentration shifts for the cytosine proton resonances. These shifts will reflect both the ring-current magnetic anisotropy effects of the incorporated adenine base, and the accompanying reduction in the intramolecular ring-current effect of the adjacent adenine base as a result of the formation of the intercalated complex. Since the cytidine protons in ApC experience a larger ring-current effect from the adjacent adenine base in the intramolecular stack than in CpA (see discussion in Section 2. 3), and since the incorporated adenine base in the intercalated complex is expected to shift the cytidine proton resonances in both dinucleotides by similar amounts, the larger concentration shifts for the cytidine resonances in CpA may not be unexpected.

On the basis of the concentration data, it is not possible to ascertain the relative importance of the various intermolecular complexes which may contribute to the observed cytidine proton concentration shifts. However, it is probable that both types of inter-

molecular complexes considered here are present to some extent in these solutions.

Although the dinucleoside monophosphates, like the simpler bases and nucleosides, may associate intermolecularly to form dimers, trimers, tetramers and higher associated species in aqueous solution, only the dimer would probably be of significance at dinucleotide concentrations below 0.10 M. Accordingly, the concentration shifts have been interpreted in terms of a dimerization equilibrium for the intermolecular self-association process.

First, the following quantities are defined:

m = concentration of monomeric dinucleotide, moles/ℓ.

d = concentration of dinucleotide dimer, mole/ℓ.

M = total stoichiometric concentration of dinucleotide, moles/ℓ.

= m + 2d.

The dimerization constant K is as follows:

$$\frac{d}{m^2} = K\ (\ell/\text{mole})$$

or

$$\frac{d}{(M-2d)^2} = K. \qquad (1)$$

The quadratic expression is solved for d to give:

$$d = \frac{(4\ KM + 1) - (8\ KM + 1)^{\frac{1}{2}}}{8\ K} \qquad (2)$$

Assuming that the reversible dimerization equilibrium is rapid on the NMR timescale, the observed chemical shift of a given proton is a weighted average of the chemical shifts in monomer and dimer environments:

$$\delta_{obs.} = \delta_m f_m + \delta_d f_d \qquad (3)$$

where:

$\delta_{obs.}$ = observed chemical shift, ppm
δ_m = chemical shift in monomer, ppm
δ_d = chemical shift in dimer, ppm
f_m = mole fraction of dinucleotide as monomer
f_d = mole fraction of dinucleotide as dimer

Rearranging (3),

$$\delta_{obs.} = \delta_m (1 - f_d) + \delta_d f_d$$

$$= \delta_m + (\delta_d - \delta_m) f_d \qquad (4)$$

Thus the observed chemical shift of a given proton depends linearly on the fraction of dinucleotide which is dimerized. The factor $(\delta_d - \delta_m)$, the chemical shift difference between monomer and dimer environments for a given proton, is called the dimerization shift. The fraction of dinucleotide molecules involved in dimer formation is simply: $f_d = \frac{2d}{M}$.

Thus the chemical shift observed for a particular proton can be expressed as a function of total dinucleotide concentration M by:

$$\delta_{obs.} = \delta_m + (\delta_d - \delta_m) \frac{(4KM + 1) - (8KM + 1)^{\frac{1}{2}}}{4KM} \qquad (5)$$

This expression contains the chemical shift in the monomer, the dimerization shift, and the dimerization constant as parameters. Expression (5) is equally valid when several different dimer species are present, i. e. :

$$\frac{d_1}{m^2} = K_1$$

$$\frac{d_2}{m^2} = K_2$$

$$\vdots$$

etc.,

and it is not possible to determine from the data whether one or more equilibria are involved. In this case, the overall dimerization constant K is the sum of the formation constants for all the dimer species, and the dimerization shift $(\delta_d - \delta_m)$ represents the weighted mean over all these species:

$$K = \sum_i K_i$$

$$(\delta_d - \delta_m) = \frac{\sum_i (\delta_{d_i} - \delta_m) K_i}{\sum_i K_i},$$

where K_i and δ_{d_i} denote the formation constant and dimerization shift for the i <u>th</u> dimer species.

A computer least-squares fit of the concentration data to (5)

yielded the results given in Table VI. The δ_m values used were obtained by extrapolating the chemical shifts to infinite dilution (Table V). In this analysis, the best fit for a particular value of K was determined by treating $(\delta_d - \delta_m)$ as an adjustable parameter, and K was varied from 0.5 to 8.0 ℓ/mole in increments of 0.1 ℓ/mole. The value of K giving the lowest mean square deviation for a given proton was selected as the "best K" for that proton. Fitting of the cytidine H_6, H_5 and $H_{1'}$ protons of ApC and the cytidine $H_{1'}$ proton of CpA to (5) was not attempted, as these resonances shift little with concentration, and the relative experimental error would be large. The value of K giving the lowest <u>total</u> mean square deviation for a particular set of protons was also determined, and is termed the "best average" K. In the case of ApC, the "best average" K does not differ appreciably from the "best K" for each proton. Although the K giving the best fit varies a great deal depending on the proton resonance considered in the case of CpA, the "best average" K's for the adenosine and cytidine moieties are quite consistent. The dimerization shifts reported in Table VI are those obtained using the "best average" K for all protons.

The dimerization constants of 2.5 ℓ/mole for ApC and 2.7 ℓ/mole for CpA by the above procedure indicate that the intermolecular self-association tendencies of these two dinucleoside monophosphates are very similar. These values are to be compared with the self-association constants of 4.5 $molal^{-1}$ for adenosine[19] and 0.9 $molal^{-1}$ for cytidine,[17] obtained previously by osmotic studies. Since the

TABLE VI. Values of dimerization constant K (liters/mole) giving the best fit for various ApC and CpA proton chemical shift *vs* concentration plots, the best average value for a set of protons, and dimerization shifts calculated using the best average dimerization constant.

Proton	ApC			CpA		
	Best K	Best Avg. K	Dimerization Shift	Best K	Best Avg. K	Dimerization Shift
			ppm			ppm
H_8 (A)	2.7	2.5	0.402	4.0	2.7	0.297
H_2 (A)	2.4		0.565	2.5		0.511
$H_{1'}$(A)	3.3		0.353	2.2		0.240
H_6 (C)	--		0.112	4.5	2.7	0.257
H_5 (C)	--		0.054	1.6		0.262
$H_{1'}$(C)	--		0.037	--		0.053

dimerization of ApC or CpA involves primarily A-A stacking, a factor of ~2 reduction in the dimerization constant from the value for the adenosine nucleoside is to be expected, since only one face of the adenine base would be accessible for intermolecular A-A base stacking if the dinucleotide is intramolecularly stacked. Finally, the dimerization shifts obtained for the adenine proton resonances of ApC and CpA are comparable to those reported by Chan et al. for purine[22]: H_6, 0.59 ppm; H_2, 0.68 ppm; H_8, 0.47 ppm. This also tends to indicate that the treatment of intermolecular self-association presented here is a reasonable one.

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

2.3. Temperature Dependence

In order to study the intramolecular base-stacking interactions in ApC and CpA, the proton magnetic resonance spectra of these dinucleoside monophosphates were studied as a function of temperature. In connection with these experiments there are two very important

TABLE VII. Fraction of dinucleoside monophosphate dimerized at several concentrations.

Dinucleoside Monophosphate Concentration	Fraction in Dimer	
	K = 2. 5 (ApC)	K = 2. 7 (CpA)
0. 010 M	0. 046	0. 049
0. 050	0. 172	0. 181
0. 100	0. 268	0. 280
0. 200	0. 382	0. 395

experimental considerations. The first of these involves the necessity of working at as low a dinucleoside monophosphate concentration as is feasible, to minimize the intermolecular base-stacking effects demonstrated in the previous Section. The intermolecular association process is certainly temperature dependent, and could completely mask the intramolecular base-stacking of interest here if the concentration of dinucleoside monophosphate were appreciable. On the other hand, the NMR method is not very sensitive and a compromise between the elimination of intermolecular effects and a reasonable time for the accumulation of spectra is necessary. A concentration of 0. 010 M for the dinucleoside monophosphate was selected as representing a reasonable compromise of these two requirements. At this concentration, the accumulation of about 30 successive scans is necessary to obtain a spectrum with a reasonably good signal-to-noise ratio. Less than 5% of the dinucleoside monophosphate is dimerized at 0. 010 M concentration, and the chemical shifts of the various ApC and CpA protons differ from the infinite-dilution values by less than 3 cps at 29°C.

The second experimental consideration is the choice of an appropriate reference compound for the chemical shift measurements. It might appear that any internal standard would suffice to compensate for changes in bulk diamagnetic susceptibility differences between the D_2O solution and the TMS capillary (which provides the lock signal) with temperature. However, various possible reference compounds exhibit different temperature dependences in D_2O solution with respect to the TMS capillary. This behavior suggests that changes in

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

The effect of temperature on the chemical shifts of the adenosine H_8 and H_2 protons from the TMS capillary was determined by J. H. Prestegard[95] for a 3.2×10^{-3} M adenosine solution, and these data served as a reference for the adenosine H_8 and H_2 resonances of ApC and CpA. The low adenosine concentration in the reference solution rendered intermolecular A-A base-stacking effects unimportant over the entire temperature range investigated. A 0.030 M solution of cytidine was studied as a function of temperature, and the chemical shifts of the H_6, H_5 and $H_{1'}$ protons were measured relative to internal $N(CH_3)_4^+$. The use of $N(CH_3)_4Cl$ in the ApC and CpA solutions made possible a comparison of cytidine chemical shifts in the dinucleoside monophosphates and the cytidine nucleoside, with the proton resonance of the $N(CH_3)_4^+$ ion serving as an intermediate reference. As the C-C base stacking interaction does not affect the chemical shifts of the

cytidine protons, it was possible to use a rather high cytidine concentration in the reference solution. It was not feasible to add adenosine and cytidine to the ApC and CpA solutions to serve as direct internal standards, because the spectra of the nucleosides and the dinucleoside monophosphates overlap.

The 100 Mcps pmr spectra of 0.010 M solutions of ApC and CpA were determined at a number of temperatures between 6° and 86°C. The temperature dependence of the chemical shifts observed for the adenosine H_8 and H_2 and cytidine H_6, H_5, and $H_{1'}$ protons of ApC and CpA relative to corresponding protons of the nucleosides are summarized in Figures 8 and 9. The cytidine resonances in both ApC and CpA are seen to occur at higher fields than in the cytidine nucleoside at low temperatures, and as expected, all of the monitored cytidine resonances in the two dinucleotides are shifted downfield relative to the cytidine nucleoside as the temperature is increased. Over the temperature range investigated, the cytidine H_5, H_6 and $H_{1'}$ proton resonances shift by 0.33, 0.19, and 0.24 ppm, respectively, in ApC, and by 0.17, 0.03, and 0.18 ppm, respectively, in CpA. With the exception of the H_2 resonance of ApC, the monitored adenosine resonances in these dinucleotides exhibit almost no shifts relative to the adenosine nucleoside with temperature. This H_2 resonance shifts downfield by 0.09 ppm as the temperature is increased from 6° to 86°C.

Although the intermolecular self-association of the dinucleotides increases as the temperature is lowered, this is expected to have a negligible effect on the chemical shifts of the ApC and CpA protons at

FIGURE 8

Chemical shifts (at 100 Mcps) of ApC protons from the corresponding protons of the adenosine and cytidine nucleosides. H_8 ●, H_2 ▲, H_5 Δ, H_6 □, $H_{1'}$ (C) ○.

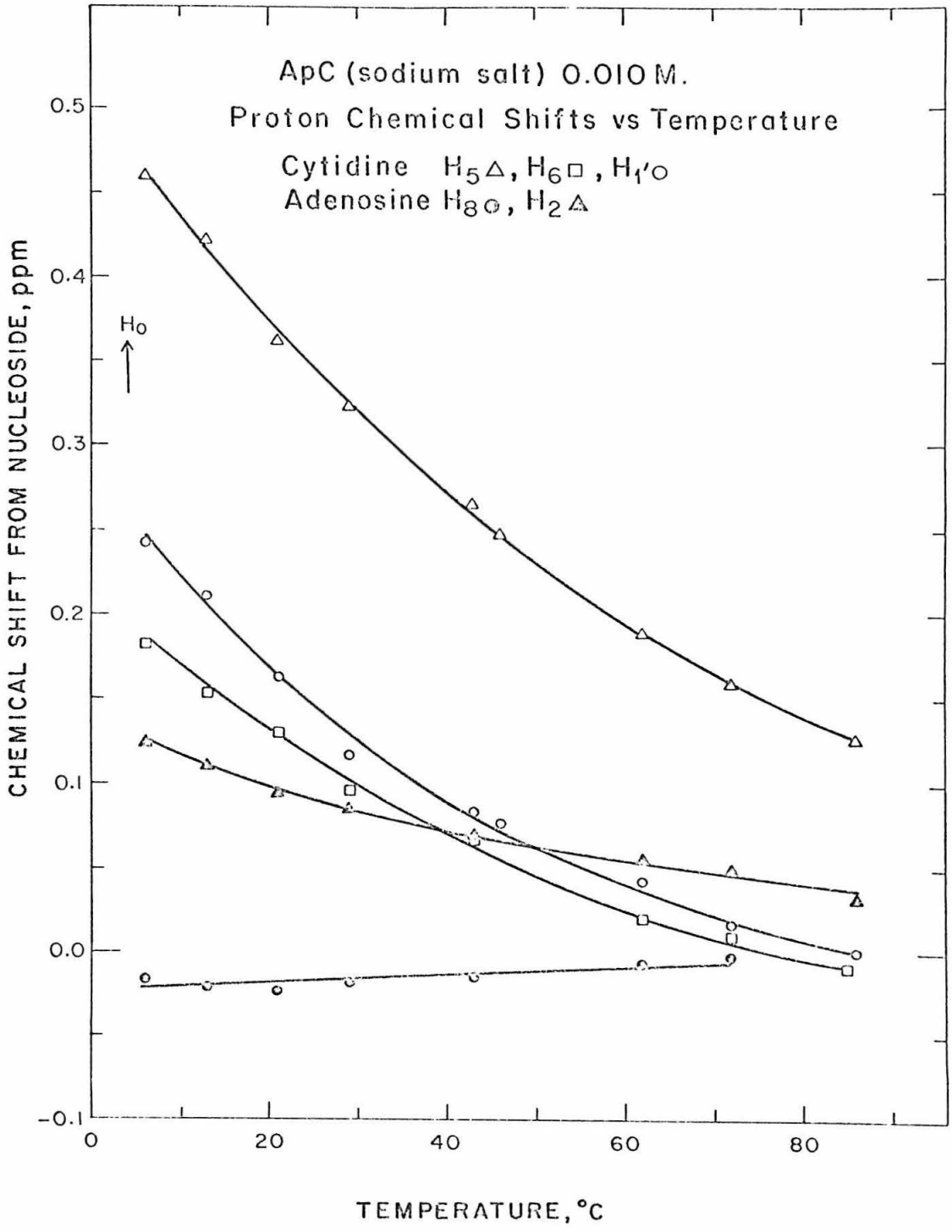
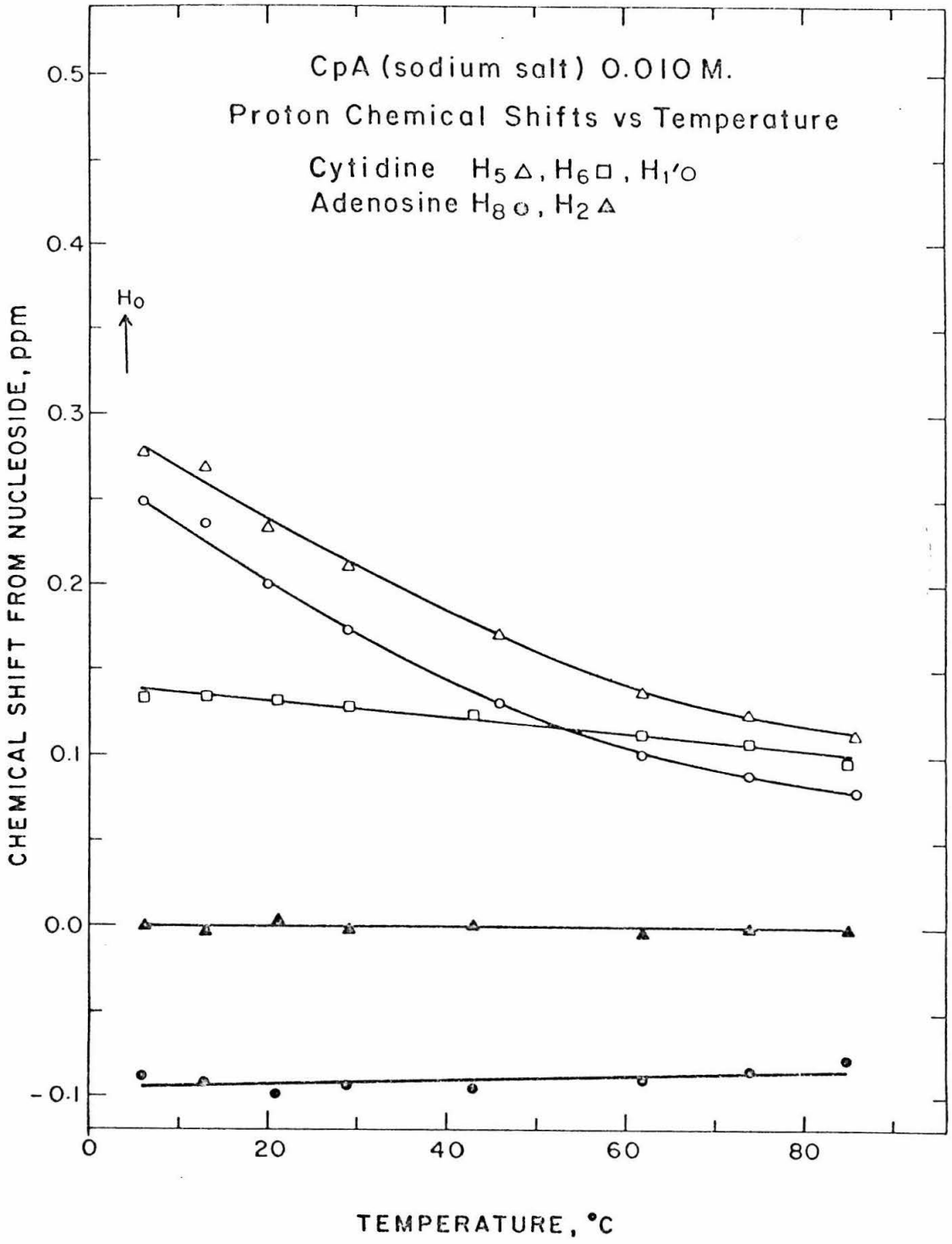

ApC (sodium salt) 0.010 M.
Proton Chemical Shifts vs Temperature
Cytidine H5△, H6□, H1'○
Adenosine H8○, H2△
Ho
CHEMICAL SHIFT FROM NUCLEOSIDE, ppm
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
20
40
60
80
TEMPERATURE, °C

FIGURE 9

Chemical shifts (at 100 Mcps) of CpA protons from the corresponding protons of the adenosine and cytidine nucleosides. H_8 ●, H_2 ▲, H_5 Δ, H_6 □, $H_{1'}$ (C) ○.


CpA (sodium salt) 0.010 M.
Proton Chemical Shifts vs Temperature
Cytidine H5 △, H6 □, H1'○
Adenosine H8 ○, H2 △
Ho
CHEMICAL SHIFT FROM NUCLEOSIDE, ppm
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
20
40
60
80
TEMPERATURE, °C

this concentration. Assuming an enthalpy for dimerization of -8.0 kcal/mole, the dimerization constants would change from ~ 2.5 ℓ/mole at 29°C to ~ 7.5 ℓ/mole at 6°C. Approximately 12% of the dinucleotides would then be dimerized at 6°C compared to 5% at 29°C, and this could account for a shift of the adenosine resonances by ~ 3-4 cps and of the cytidine resonances by ~ 2 cps to higher field with decreasing temperature.

It is reasonable to assume that the observed shifts reflect changes in the conformations of the dinucleoside monophosphates as the intramolecular base-stacking interaction is disrupted with increasing temperature. However, in view of the dependence of the chemical shifts of the base protons and the ribose $H_{1'}$ protons upon the conformation of the ribose ring and upon the conformation of the base about the glycosidic bond, care must be taken not to interpret the observed temperature shifts solely in terms of the magnetic anisotropy of the neighboring base. It is to be expected that changes in the degree of intramolecular base-stacking of a dinucleoside monophosphate would be accompanied by changes in the conformation of the ribose-phosphate-ribose backbone. It is necessary to consider these conformational changes in order to correctly interpret the shifts of the various monitored proton resonances with temperature.

2.3.1. Ribose Conformation

A sensitive indication of the conformation of the ribose ring is provided by the coupling constant between $H_{1'}$ and $H_{2'}$. The values

of $|J_{H_1'-H_2'}|$ for both the adenosine and cytidine moieties in ApC and CpA at a number of temperatures are summarized in Table VIII. These coupling constants are seen to be highly temperature dependent, essentially doubling in magnitude between 6° and 86°C. (Note that the splitting of the H_1'(C) doublet in ApC is difficult to measure accurately, as it overlaps the H_5(C) doublet--see Figure 5). Since the $H_1'-H_2'$ coupling constants are virtually independent of temperature in the nucleosides and mononucleotides, the observed variations in these ribose coupling constants of ApC and CpA with temperature must clearly result from changes in the intramolecular base-stacking interaction. The $|J_{H_1'-H_2'}|$ values in ApC and CpA are much smaller than those in the corresponding nucleosides or mononucleotides at low temperatures, increasing to approach these values at high temperatures.

The magnitude of the coupling constant between vicinal protons (protons bound to adjacent carbon atoms, $H_1-C_1-C_2-H_2$) is dependent upon the dihedral angle between the $H_1-C_1-C_2$ plane and the $C_1-C_2-H_2$ plane, as has been shown by Karplus.[96] As was mentioned in Section 3. 1, no exo ribose conformations have been observed in X-ray crystallographic studies of nucleosides and nucleotides with the sole exception of 2'-deoxyadenosine.[91] Thus C_2'-endo and C_3'-endo conformation for the ribose ring would be expected for these molecules (and the dinucleotides) in aqueous solution as well. Jardetzky[97] has considered molecular models of the ribose moiety in its several possible conformations, and has found dihedral angles between the H_1' and H_2' protons of 150° for C_2'-endo and 115° for C_3'-endo conformations. Using the Karplus formula for vicinal couplings, coupling

TABLE VIII. Spin-spin coupling constants $|J_{H_{1'}-H_{2'}}|$ between the $H_{1'}$ and $H_{2'}$ protons of the ribose rings of ApC and CpA at several temperatures. Accuracy of measurement ± 0.1 cps except where approximate value is given (± ~0.3 cps).

Temp. °C	ApC		CpA	
	A	C	A	C
	cps	cps	cps	cps
6	~2.5	~1.8	2.8	2.1
13	3.1	~2.0	~3.0	2.5
20			3.4	2.7
21	3.3	~2.4		
29	3.9	--	3.8	3.3
43	4.1	~2.6		
46	4.3	2.8	4.4	3.6
62	4.5	~3.5	4.7	~4.2
72	4.7	~3.0		
74			4.8	~3.7
86	4.8	--	4.8	4.4

constants $|J_{H_1'-H_2'}|$ of 6.9 cps for $C_{2'}$-endo and 1.7 cps for $C_{3'}$-endo were calculated. The nucleosides and mononucleotides all exhibit coupling constants $|J_{H_1'-H_2'}|$ having intermediate values, and a somewhat arbitrary distinction would have to be made in order to classify them as having a $C_{2'}$-endo or $C_{3'}$-endo ribose conformation. Actually, the dihedral angle between $H_{1'}$ and $H_{2'}$ may vary from molecule to molecule for a given ribose conformation, depending on the various non-bonded interactions in each particular molecule. The dihedral angles determined by Jardetzky thus represent idealized cases to some extent. It may be possible that the ribose ring of a given nucleoside or nucleotide alternates between well-defined 2'-endo and 3'-endo conformations, and that the observed $|J_{H_1'-H_2'}|$ coupling constants represent a weighted average ribose conformation.

In view of the work of Jardetzky,[97] it can be concluded from the data of Table VIII that the ribose rings of both the adenosine and cytidine moieties of ApC and CpA favor the $C_{3'}$-endo conformation at low temperatures, where the bases of the dinucleotide are intramolecularly stacked to the greatest extent. At high temperatures, where the degree of intramolecular base-stacking is smaller, the $C_{2'}$-endo conformation is preferred. Similar behavior for the temperature dependence of the ribose coupling constants $|J_{H_1'-H_2'}|$ of ApA, ApA-c-p, and GpA has been observed by Hruska and Danyluk,[55] and their interpretation is similar to that offered here.

The stability of the 3'-endo ribose conformation at low temperatures for the 3'-nucleoside can be rationalized by examination

of molecular models of ApC and CpA in stacked conformations. This suggests that when the ribose conformation of the 3′-nucleoside moiety is 2′-endo, strong steric repulsion between the 2′ - >CHOH group of the 3′-nucleoside and the base and ribose ring of the 5′-nucleoside would hinder base-base overlap. The 3′-endo ribose conformation for the 3′-nucleoside would alleviate this problem.

2. 3. 2. Discussion of chemical shifts

Because of the conformational changes induced in the ribose-phosphate backbone by the intramolecular stacking interaction, and in view of possible effects on the conformations of the bases about their glycosidic bonds, it appears that only the temperature shifts observed for the cytosine H_5 and adenine H_2 protons can safely be interpreted in terms of the magnetic anisotropy of the neighboring base alone. These protons are located somewhat further away from the ribose-phosphate backbone than are H_6 of cytosine and H_8 of adenine, and the resonances for these protons do not appear to be particularly sensitive to the conformation of the ribose ring or the angle of rotation about the glycosidic bond. Thus the shifts observed for these protons with temperature may be used to deduce information about the intramolecular interaction between the two bases directly.

The cytosine H_5 resonance in ApC is 0. 46 ppm upfield from that of the cytidine nucleoside at 6°C, primarily because of the ring-current magnetic anisotropy of the neighboring adenine base. The large downfield shift observed with increasing temperature, indicative

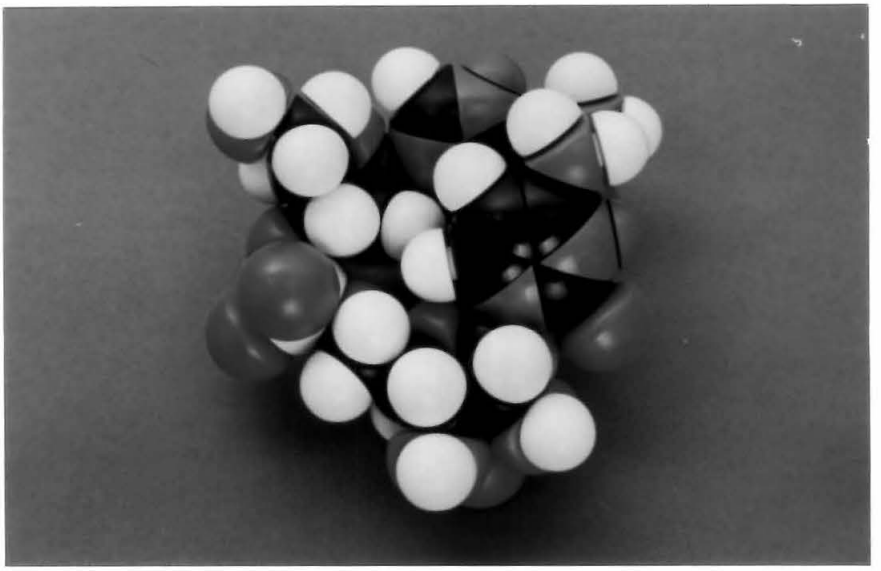
of a decrease in the extent of intramolecular base-stacking in ApC, is thus not unexpected. The ring-current of a neighboring adenine base is not expected to produce an upfield shift greater than 0.5 to 0.7 ppm for the protons of the cytosine base with the most favorable base-base overlap in the intramolecular stack. It may therefore be concluded that the two bases in ApC are rather strongly stacked (~70%) at low temperatures (6°C). The temperature shift observed for the cytosine H_5 proton of CpA is somewhat smaller than that for ApC, apparently reflecting the smaller exposure of this proton to the ring-current of the adjacent adenine base (0.28 ppm at 6°C). The observed shift of the adenine H_2 proton in ApC with temperature would seem to be indicative of a small magnetic anisotropy for the cytosine base which had not been detected in previous work.[28] However, no temperature shift is observed for the adenine H_2 proton in CpA.

It is interesting to note that at 86°C, the cytosine H_5 resonances of ApC and CpA are still at significantly higher fields than that of the cytidine nucleoside (0.15 ppm for both ApC and CpA). This observation indicates that both ApC and CpA are not completely destacked at 86°C, and that there is still a residual base-base interaction at this temperature.

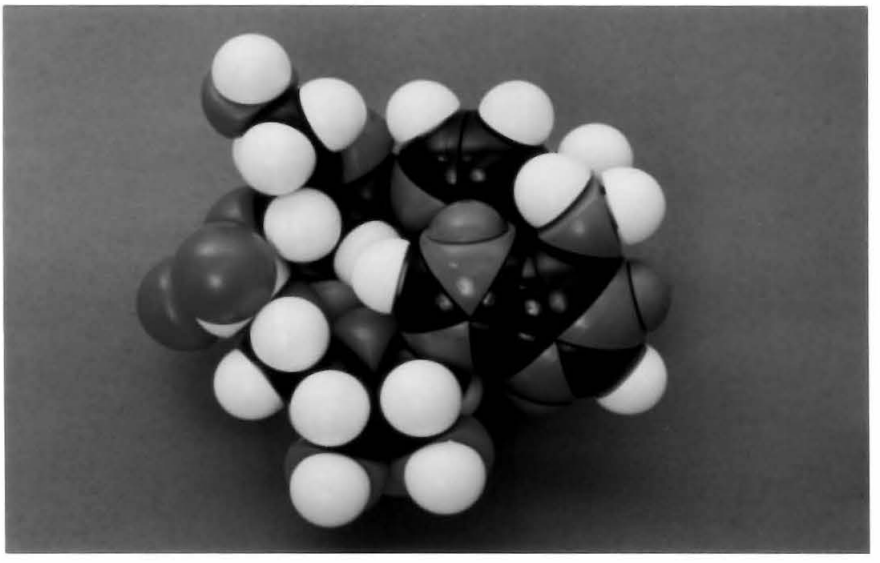
The differences between ApC and CpA in the chemical shifts of the cytosine H_5 and adenine H_2 protons relative to the nucleosides at 6°C, and the corresponding differences in the shifts of these resonances with temperature, most likely arise from differences in the conformations of these molecules. The temperature shifts for

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

A consideration of CPK molecular models of ApC and CpA is helpful in resolving this question. In Figure 10 are depicted the most probable stacked conformations of ApC and CpA, as viewed along the axis of the ribose-phosphate backbone in the direction of 5′- to 3′-phosphate esterification. In these models, the extent of A-C base overlap has been maximized, while avoiding strong repulsive non-bonded interactions. Both of the nucleoside moieties in these models are in the more stable anti conformations with respect to rotation about the glycosidic bond, and the ribose-phosphate-ribose backbones are in a conformation approximating that found in double-helical DNA. For steric reasons, the base of the cytidine nucleoside can only exist in



(a) ApC



(b) CpA

FIGURE 10. Photographs of CPK molecular models of ApC and CpA in hypothetical "stacked" conformations. (a) ApC; (b) CpA.

the anti conformation; however, it appears that the intramolecular base-stacking interaction can also stabilize the less favorable syn conformation in the case of the adenosine nucleoside.

In the stacked ApC model, the cytosine base is able to overlap the six-membered ring of the adenine base quite well, and the cytosine protons (particularly H_5) are situated directly above the adenine ring where they would be exposed to a rather strong magnetic field from the adenine ring-current. In the stacked CpA model, only partial overlap of the cytosine and adenine bases is possible in this conformation; the cytosine H_5 and H_6 protons are well away from the adenine base and would thus experience a smaller magnetic field from the adenine ring-current. Rotation of the adenine base by $\sim 180^\circ$ about the glycosidic bond to give the syn conformation would allow a greater adenine-cytosine base overlap, but the cytosine protons would still not be exposed to the adenine ring-current effect to the extent that they are in ApC. Also, in these hypothetical stacked conformations of ApC and CpA, only the adenine H_2 proton of ApC is sufficiently close to the cytosine base to be affected by its small magnetic anisotropy.

Thus it appears quite likely that the observed differences in the temperature shifts for the cytosine H_5 and adenine H_2 protons between ApC and CpA reflect steric and geometric factors which affect the exposure of these protons to the magnetic anisotropy of the neighboring base, although a difference in the intramolecular A-C base-stacking tendencies between ApC and CpA is also possible. However, a comparison of the $H_{1'}$-$H_{2'}$ coupling constant data between ApC and CpA

(Table VIII) would seem to indicate that the stacking tendencies between the adenine and cytosine bases are quite comparable in the two sequence isomers.

The interpretation of the shifts for the cytosine H_6 and $H_{1'}$ protons of ApC and CpA with temperature is not quite so straightforward as that for the cytosine H_5 and adenine H_2 protons, since, in addition to the ring-current magnetic anisotropy of the neighboring base, other factors can contribute to these observed shifts. The chemical shift of the H_6(C) proton is dependent upon the angle of rotation of the cytosine base relative to the ribose ring about the glycosidic bond. If, when the dinucleotide becomes destacked, the cytosine base can rotate so that the H_6 proton is no longer in close proximity to the ether oxygen (as it appears to be in the stacked conformation), an upfield shift with increasing temperature would be expected for this contribution. Thus the observed temperature shifts for these protons may reflect partial compensation of effects due to changes in the relative conformation of the adenine and cytosine bases (from the adenine ring-current) by changes in the conformation of the cytosine base about its glycosidic bond. As noted above, the shift observed for the H_6(C) proton with temperature is much larger for ApC than for CpA. This is felt to arise primarily from a difference in the exposure of these protons to the ring-current magnetic anisotropy of the neighboring adenine base in the intramolecular stack. Finally, it is noted that although the H_6(C) resonance of CpA is still 0.12 ppm upfield from the H_6 resonance of cytidine at 86°C, the H_6(C)

protons of ApC and the cytidine nucleoside are almost magnetically equivalent at this temperature. This should not be taken to imply that ApC is stacked to a lesser degree than is CpA at this temperature since, in the absence of intramolecular base-stacking, the H_6(C) resonance of ApC should appear $\sim$0. 08 ppm downfield from that of the cytidine nucleoside because of the effect of the phosphate group. This is demonstrated for the H_6 protons of CpC in Section 5. 2.

The shifts observed for the $H_{1'}$(C) protons of ApC and CpA with temperature are quite large, and do not exhibit the sequence dependence shown by the cytosine base protons. The resonances of these $H_{1'}$ protons are appreciably upfield from that of the $H_{1'}$ proton in cytidine at low temperatures. This is somewhat surprising, since the effect of the ring-current magnetic anisotropy of the adjacent adenine base on these protons is not expected to be large. Neither of these protons is located close to the regions of large ring-current fields from the adenine base in intramolecularly stacked ApC or CpA, and $H_{1'}$(C) of CpA in particular is well removed from the adenine base. On the basis of the discussion of the chemical shifts of these protons in Section 2. 1. 2, it seems reasonable to attribute the observed temperature shifts to the conformational changes in the ribose rings and to possible changes in the conformation of the cytosine bases about the glycosidic bonds as the bases of the dinucleotide become destacked with increasing temperature. Both of these contributions are expected to lead to deshielding of the $H_{1'}$ protons as the temperature is increased.

2. 3. 3. Nature of the intramolecular base-stacking interaction

The intramolecular stacking interaction between the two bases of the dinucleoside monophosphates has generally been discussed in terms of a two-state model, [43, 44] in which an equilibrium exists between "stacked" and "unstacked" forms of the molecule. Such a treatment may in fact be a poor approximation to the behavior of the dinucleotides, but regardless of the details of this or any other model which may be proposed to describe the intramolecular base-stacking process, some general features are evident for the chemical shift versus temperature profiles for the cytidine protons of ApC and CpA. Specifically, a sigmoidal variation is expected, with the two asymptotic limits at low and high temperatures denoting the chemical shifts of the proton in the stacked and unstacked environments respectively. Since the intramolecular base-stacking process in the dinucleoside monophosphates is not expected to be a cooperative phenomenon, with a well-defined "melting" over a narrow temperature region as for polynucleotides, [77] the transition between the stacked and unstacked states may extend over a wide temperature range, with the width of the transition region determined primarily by the apparent enthalpy change associated with the stacking process. The sigmoidal curves are also not necessarily symmetrical about the apparent transition temperature, located at the point of maximum slope.

The data presented in Figures 8 and 9 clearly indicate that only a portion of the sigmoidal temperature profile is observable for ApC

and CpA over the available temperature range of ~0°C to ~100°C in D_2O solution. The transition temperature appears to be in the neighborhood of ~10°-~20°C for both ApC and CpA, indicating that primarily the "high temperature" side of the stacked-unstacked transition is observed here. An apparent transition temperature of ~35°C was noted for ApA by Chan and Nelson[56] in their study of this dinucleotide. Furthermore, the width of the transition in ApC and CpA appears to be somewhat broader than that reported for ApA. This indicates that the apparent enthalpy of stacking is somewhat larger for ApA than for ApC and CpA. From these comparisons, it may be concluded that ApA appears to be more strongly stacked than either ApC or CpA at a given temperature.

A more complete knowledge of the chemical shift versus temperature profiles would allow an estimation of the thermodynamic parameters for the intramolecular base-stacking process, within the framework of the two-state model. The use of a concentrated salt solution as the solvent for the dinucleoside monophosphates, such as 4.7 MKF used by Brahms, et al.,[44] allows the temperature range to be extended to ~-20°C. However, it is probable that the intramolecular base-stacking interaction is quite sensitive to the ionic strength of the solution, and the thermodynamic parameters obtained for the system in 4.7 MKF might differ greatly from those appropriate for the system at essentially zero ionic strength. Davis and Tinoco[43] have studied several dinucleoside monophosphates in 25.2% LiCl by ORD, and report that behavior in this solvent is quite similar to that in dilute salt solutions, however.

To summarize the results of this Section, it can be observed that pmr serves as a useful tool for examining the intramolecular base-stacking interaction in ApC and CpA. The effect of the ring-current of the adenine base on the chemical shift of the H_5 proton of the cytosine base provides the best indication of the extent of the intramolecular interaction, with the chemical shifts of the other protons determined by several other factors in addition to the magnetic anisotropy of the adjacent base. The conformations of the ribose rings are also affected by the base-stacking interaction, with 3′-endo ring puckering being preferred in the intramolecular stack. The sequence dependence of the intramolecular A-C base-stacking interaction observed here (greater in ApC than in CpA) is in agreement with the results of optical studies,[41, 44] and it has been possible to make some observations regarding the conformation of the stacked dinucleotide molecules.

3. Pyrimidine-Pyrimidine Dinucleoside Monophosphates

Previous studies of bases and nucleosides by pmr[19, 25, 28] have indicated that, of the naturally occurring bases of DNA and RNA, only adenine (and perhaps guanine) possess a measureable ring-current magnetic anisotropy. The existence of cytosine, uracil, thymine, and their nucleosides in the form of the keto tautomers precludes the circulation of electronic charge responsible for the large magnetic anisotropy shown by adenine. Electron circulation in the six-membered ring of guanine is likewise restricted by the keto group at position 2, and only in the five-membered imidazole ring of guanine would free

circulation of electronic charge be possible. Recent work on the guanine-containing dinucleoside monophosphates ApG and GpA[60] indicates that the ring-current of guanine is indeed smaller than that of adenine.

This distinction between those bases which do and those bases which do not possess a ring-current magnetic anisotropy is most important in regard to the application of pmr spectroscopy to the study of dinucleoside monophosphates. Direct investigation of the intramolecular base-stacking of dinucleotides by monitoring the effect of the ring-current of one base on the chemical shifts of protons of the other base, as reported for ApC and CpA in Section 2. 3, is restricted to those dinucleotides containing at least one purine base. Pmr studies of dinucleoside monophosphates containing two pyrimidine bases would not be expected to yield information regarding intramolecular base-stacking quite so readily by direct study of the effect of temperature on the chemical shifts of the base protons, since the factors controlling these chemical shifts would no longer be dominated by the magnetic anisotropy of the neighboring base. The effects of the intramolecular base-stacking interaction on the conformations of the ribose rings, as indicated by the coupling constant $|J_{H_1'-H_2'}|$ between the ribose H_1' and H_2' protons, and on the conformation of the bases relative to their ribose rings, as indicated by the chemical shifts of the H_6 base protons and H_1' ribose protons, would be of some help in assessing the degree of intramolecular base stacking. However, these properties are less sensitive to the base-stacking interaction than are the chemical shifts

resulting from the magnetic anisotropy of the neighboring base, and also more difficult to interpret in view of the complexity of the factors affecting these properties. Thus it seemed evident from the outset that some additional method for probing the intramolecular base-stacking interaction in pyrimidine-pyrimidine dinucleoside monophosphates by pmr would be quite helpful.

Purine-Binding Studies. -- As has been mentioned previously (Part I, Section 2. 1), unsubstituted purine interacts with the nucleosides cytidine, uridine, and thymidine by base-stacking,[28] with the proton resonances of the nucleosides being shifted upfield as a consequence of the ring-current magnetic anisotropy of purine. The results of this study suggested that purine might serve as a probe molecule in elucidating the degree of intramolecular base-stacking in pyrimidine-pyrimidine dinucleoside monophosphates, and for this reason the interaction of purine with several dinucleotides was investigated.

It was felt that the degree of intramolecular base-stacking might be indicated by a comparison between the magnitude of the purine-induced upfield shift for a given proton in the dinucleotide and that for the analogous proton in the corresponding nucleoside under comparable conditions. If the intramolecular base-stacking forces were weak and the dinucleotide were essentially unstacked, purine would be expected to interact with the dinucleotide by stacking on both faces of each base, producing upfield shifts for the protons of these bases comparable to those observed in the purine-nucleoside interactions by Schweizer, Chan, and Ts'o.[28] If, however, the intramolecular base-stacking

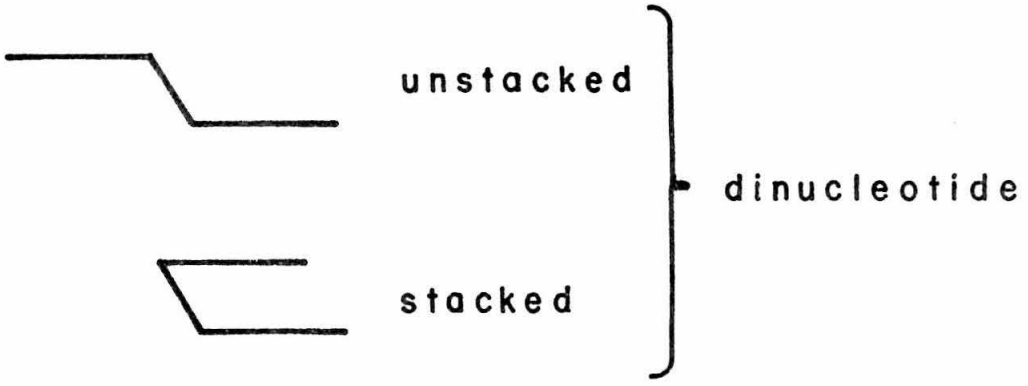
forces were strong and the dinucleotide were stacked, the added purine could interact with the dinucleotide by stacking on only the single exposed face of each base. A purine molecule stacked on the external face of the base of the 3′-nucleoside, for example, would not be expected to produce a very large shift for protons of the 5′-nucleoside, because of the $\frac{1}{r^3}$ dependence of the magnetic anisotropy effect. [24] Therefore this "insulation effect" would be expected to result in purine-induced shifts for the proton resonances of the dinucleotide about half as large as those observed for the monomeric nucleosides.

The two cases are shown schematically in Figure 11, where the two pyrimidine bases of the dinucleotide are represented by horizontal bars and the ribose-phosphate-ribose backbone is represented by the diagonal segment connecting them. This diagram illustrates the expected behavior in the limit of high purine concentration. Intermediate purine-dinucleotide complexes, in which not every exposed face of the dinucleotide would have purine bound to it, would of course also be present in the purine-dinucleotide solution. Since purine self-association is extensive at high purine concentrations, [18, 21] purine dimers, trimers, and higher polymeric species would be present in the solution as well as monomeric purine molecules. It must be noted that each of these aggregates could stack with the dinucleotide. Accordingly, the symbol: —— P in Figure 11 represents any purine species in solution.

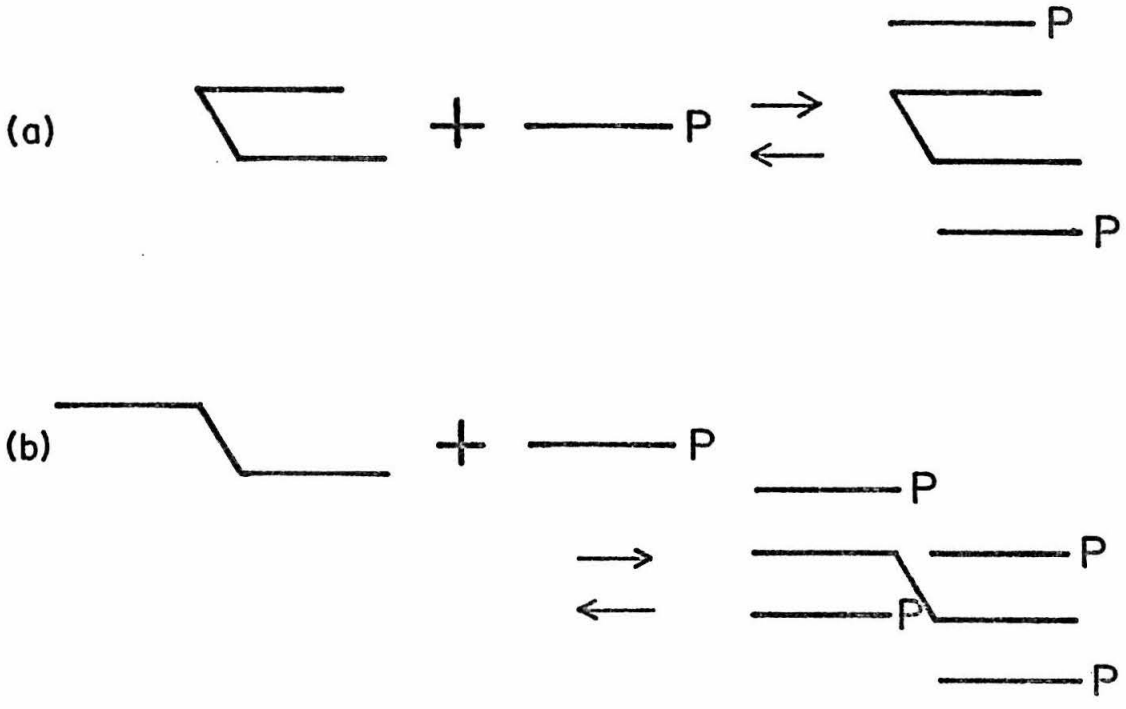
The above discussion indicates the motivation for the initial experiments on the interaction of purine with the deoxyribose dinucleo-

FIGURE 11

Schematic representation of the interaction of purine with a dinucleoside monophosphate: (a) Stacking of purine species on the exposed base faces of a stacked dinucleotide; (b) stacking of purine species with the bases of an unstacked dinucleotide.



P purine (monomer, dimer, etc.)



tides TpT, TpdU and dUpT. The results of these studies, described in the next Section, indicated that the interaction of purine with dinucleoside monophosphates is an interesting phenomenon in its own right, in addition to serving to probe the intramolecular base-stacking interaction in dinucleotides. For this reason, the binding of purine to several other dinucleoside monophosphates was studied in considerable detail, as described in succeeding Sections of Part III of this thesis.

4. Thymidylyl (3′→5′) thymidine, Thymidylyl (3′→5′) 2′-deoxyuridine, and 2′-Deoxyuridylyl (3′→5′) thymidine

The three deoxyribose dinucleoside monophosphates available for study--TpT, TpdU, and dUpT--were pyrimidine-pyrimidine species. It should be noted that the nucleoside 2′-deoxyuridine is not a naturally occurring constituent of DNA, so TpdU and dUpT are not representative of actual adjacent segments in DNA. However this was the only pair of sequence isomers involving deoxyribose available.

This study of TpT, TpdU and dUpT was completed before the studies of the other dinucleoside monophosphates reported in this thesis were begun. Consequently, the data are somewhat less complete, and the experimental conditions less carefully chosen here than in the subsequent work. The three dinucleotides were obtained in the form of solutions of the ammonium salts in D_2O, and although the pD's of these solutions were not measured, they were probably about 5 or so because of hydrolysis of the ammonium ion. Since neither thymine nor uracil has a pK below 9.1,[98] and the pK of

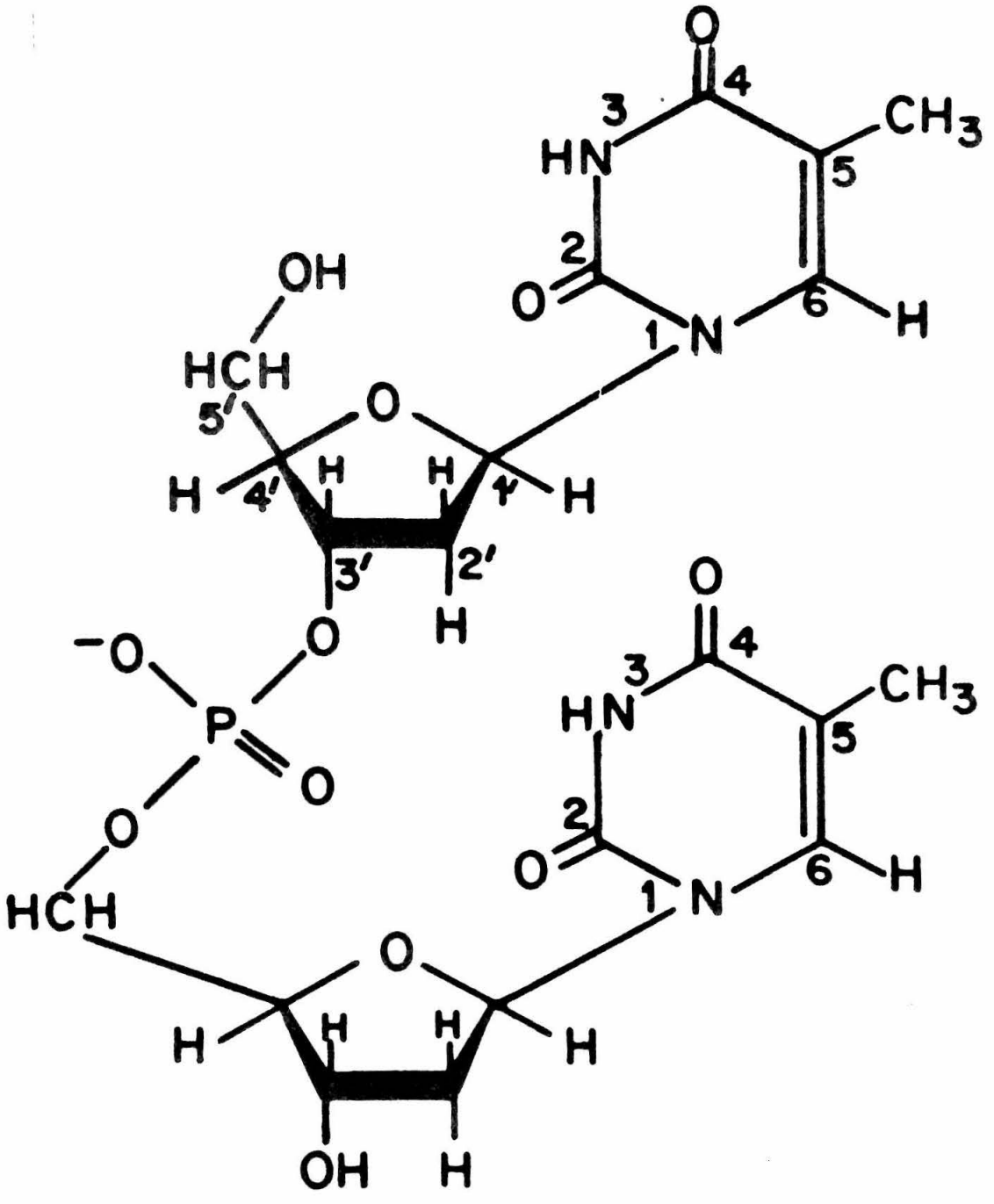
purine is 2.4,[99] complications arising from partial protonation of a base (as for CpC in Section 5.3.3) or of purine (in the purine-binding experiments) do not occur here. The available NMR instrumentation (A-60 spectrometer, no time-averaging computer or variable temperature provisions) required rather high concentrations of dinucleotide (~ 0.1 M) for a satisfactory signal-to-noise ratio. The results of Section 2.2 suggest that the dinucleoside monophosphates are extensively dimerized at these concentrations. However, the chemical shifts of the proton resonances of TpT, TpdU and dUpT are not expected to be concentration dependent in view of the negligibly small magnetic anisotropies exhibited by the thymine and uracil bases.[28] The intermolecular self-association processes undoubtedly compete with the purine binding to some extent, although the purine-dinucleotide interaction must dominate at high purine concentrations.

4.1. TpT

The structure of TpT is shown in Figure 12; the two thymidine nucleosides are non-equivalent because of the asymmetric phosphate attachment. The 60 Mcps pmr spectrum of a 0.13 M solution of TpT (ammonium salt), in the regions of the thymine H_6 and methyl protons and ribose $H_{1'}$ protons, is presented in Figure 13. The same spectral regions for 0.2 M thymidine under similar conditions are shown for comparison. The two H_6 protons and the two sets of CH_3 protons in TpT have essentially the same magnetic environment, since separate resonances for protons of the 3′- and 5′-nucleosides are not observed. The H_6 resonance in TpT is noticeably broader than that in thymidine,

FIGURE 12

Structural diagram of thymidylyl (3′→ 5′) thymidine, TpT.



TpT

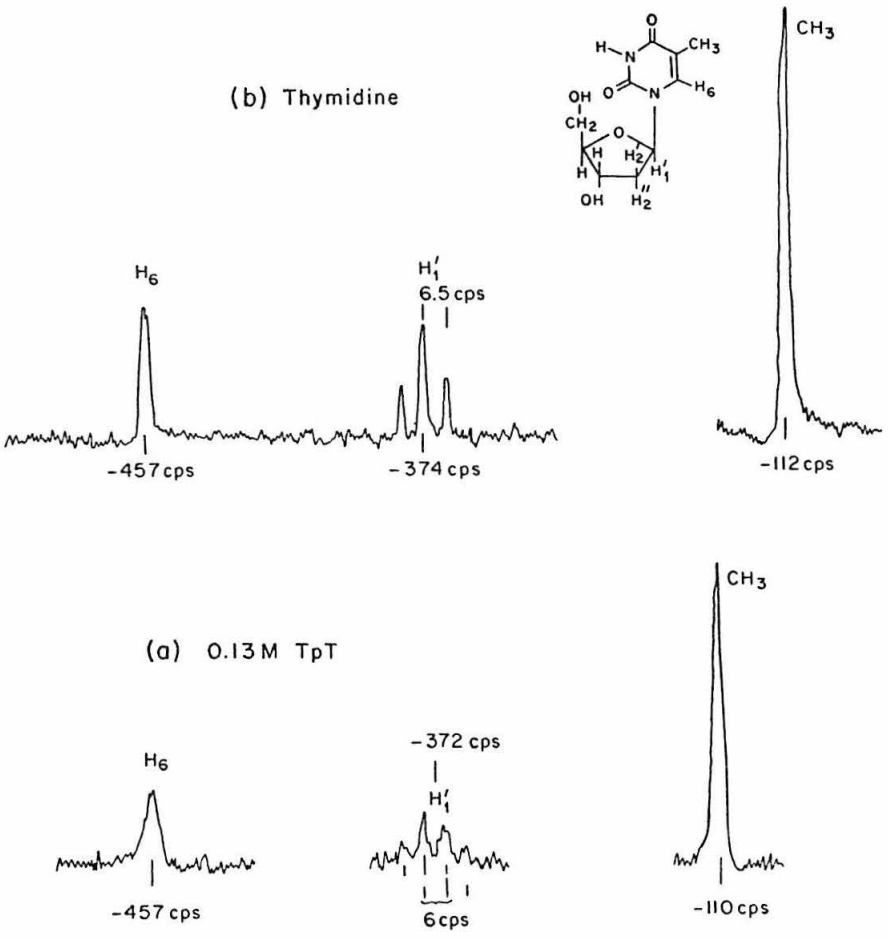


FIGURE 13

Pmr spectra at 60 Mcps of: (a) 0.13 M TpT, and (b) 0.2 M thymidine in the regions of the CH_3, H_6 and $H_{1'}$ protons

thereby suggesting that the two H_6 protons in TpT may not have exactly the same chemical shift.

The two $H_{1'}$ protons of the dinucleoside monophosphate are clearly not magnetically equivalent, and the resonances for these protons consist of a superposition of two 1:2:1 triplets (from coupling with $H_{2'}$ and $H_{2''}$). The chemical shift between the two $H_{1'}$ protons is about 6 cps and is roughly equal to the spin-spin coupling constant between $H_{1'}$ and $H_{2'}/H_{2''}$, yielding the observed 1:3:3:1 quartet. There is little chemical shift difference between protons of TpT and corresponding protons of the thymidine nucleoside. As in the ApC-CpA study of Section 2, the spectral region containing the ribose $H_{2'}$, $H_{2''}$, $H_{3'}$, $H_{4'}$, $H_{5'}$ and $H_{5''}$ proton resonances was not studied because of its complexity. The hydroxyl and amino group protons exchange rapidly with those of the solvent, contributing to the HOD resonance.

The interaction of purine with TpT was investigated with the aim of probing the degree of intramolecular base-stacking in this dinucleotide, as outlined in Section 3. Upon addition of purine to the TpT solution, the resonances of the monitored thymidine protons are shifted to higher fields. From the direction of the shifts, and a comparison of the magnitudes of the induced shifts for the base protons and ribose $H_{1'}$ protons, it is concluded that the mode of interaction between purine and TpT is vertical stacking of the heterocyclic bases, as in the purine-nucleoside interaction.[28] The thymine H_6 and CH_3 peaks are split into two sets of resonances of equal intensity, as shown in Figure 14. It might be noted that the H_6 and CH_3 resonances of both TpT and thymidine show a slight splitting in the absence of purine due

FIGURE 14

Effect of purine on the 60 Mcps pmr spectrum of 0. 13 M TpT. (a) purine/TpT concentration ratio = 2/1 (0. 26 M purine); (b) purine/TpT = 8/1 (1. 04 M purine).

FIGURE 15

Effect of purine on the thymine CH_3 proton resonances of 0. 13 M TpT for purine/TpT concentration ratios of 4/1, 6/1, and 8/1.

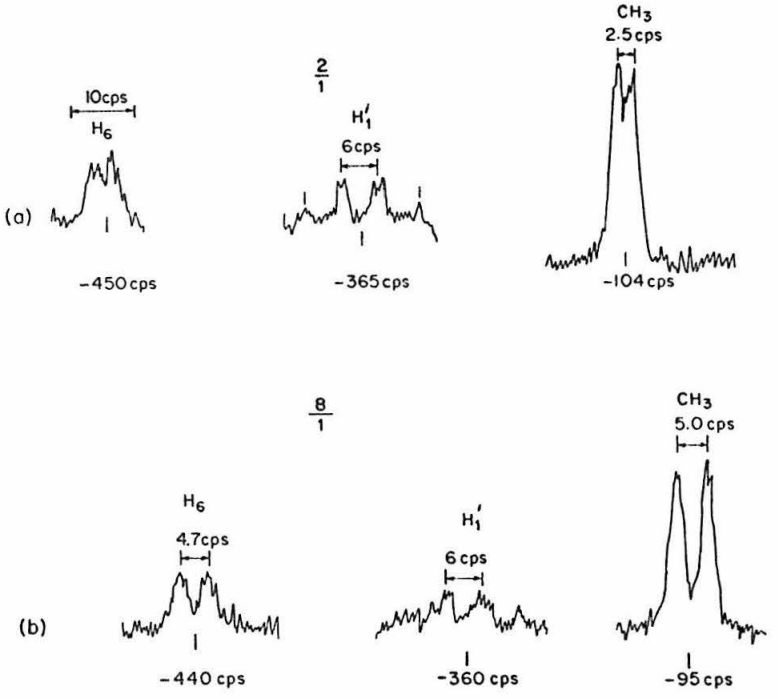


FIGURE 14

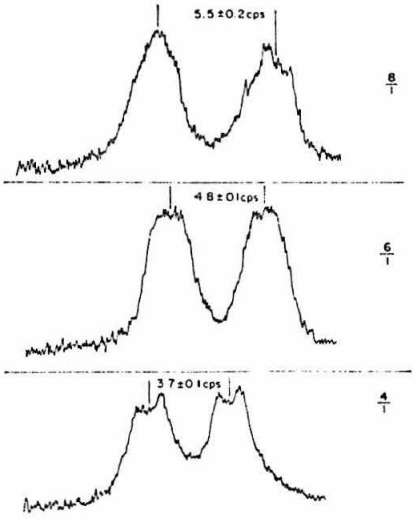


FIGURE 15

to mutual spin-spin coupling. The coupling constant is about 0. 5 to 1. 0 cps, and this spin-spin splitting can still be resolved after purine has been added to the TpT solution, as shown for the CH_3 resonances in Figure 15. The purine-induced splitting is dependent upon the purine concentration, as shown for the CH_3 resonances in Figure 16 and for the H_6 resonances in Figure 17, and reaches a maximum of about 5. 0 cps at the highest purine concentration studied (1. 3 M). The purine-induced chemical shift difference is much smaller for the $H_{1'}$ protons, with a maximum relative shift of about 1 cps. The observed purine-induced shifts for the monitored proton resonances of TpT are summarized in Table IX.

Since the addition of purine to a solution of thymidine does not result in splitting of the thymine resonances, and the two sets of thymine resonances in TpT are of equal intensity over a wide range of purine concentration, it is possible to conclude that the addition of purine to TpT has resulted in the formation of a purine-dinucleoside monophosphate complex in which the two bases of the dinucleoside monophosphate are not equally exposed to the magnetic perturbation produced by the bound purine. This is of course a consequence of the asymmetric phosphate attachment and the resulting non-equivalence of the two thymidine moieties. It is not immediately evident whether the protons experiencing the greatest purine-induced shifts are those of the 3′- or the 5′-nucleoside. This question is resolved by examining the sequence isomers TpdU and dUpT, which provide the basis for the assignments shown in Figures 16 and 17.

FIGURE 16

Plot of purine-induced shifts for thymine CH_3 resonances of 0.13 M TpT, at 60 Mcps.

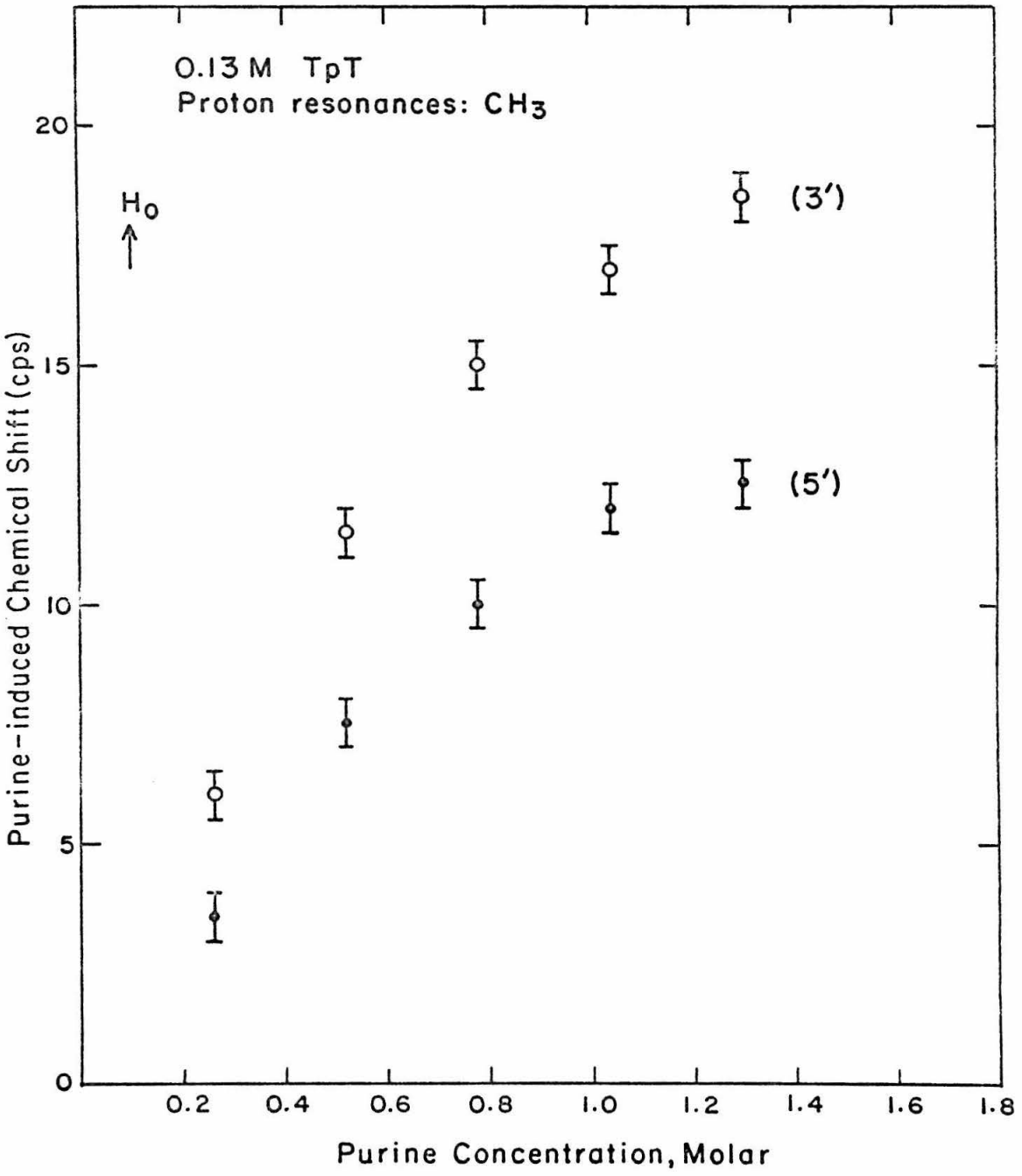

0.13 M TpT
Proton resonances: CH3
Ho
(3')
(5')
Purine-induced Chemical Shift (cps)
Purine Concentration, Molar
0
5
10
15
20
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8

FIGURE 17

Plot of purine-induced shifts for thymine H_6 resonances of 0.13 M TpT, at 60 Mcps.

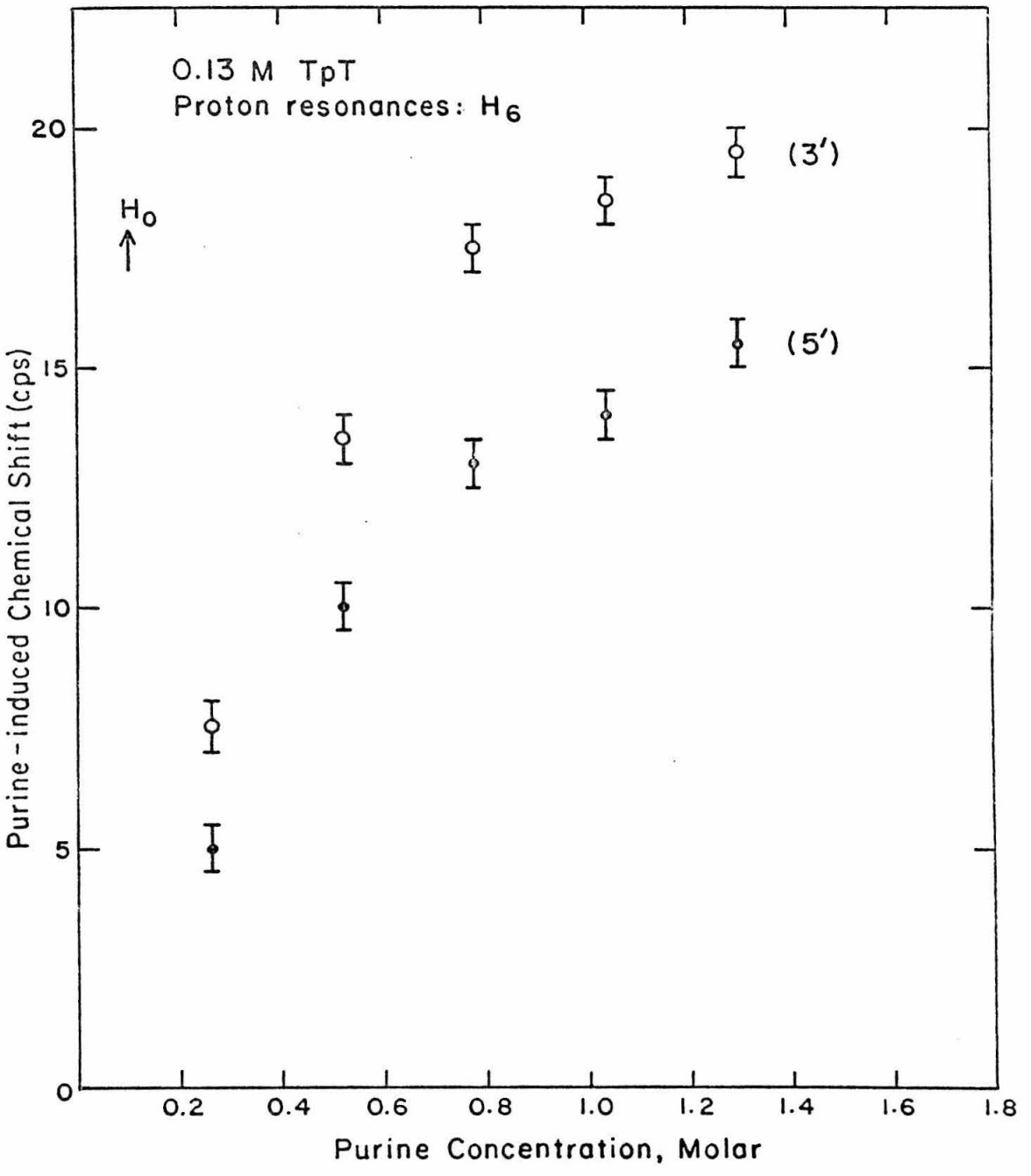

0.13 M TpT
Proton resonances: H6
H0
(3')
(5')
Purine-induced Chemical Shift (cps)
0
5
10
15
20
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Purine Concentration, Molar

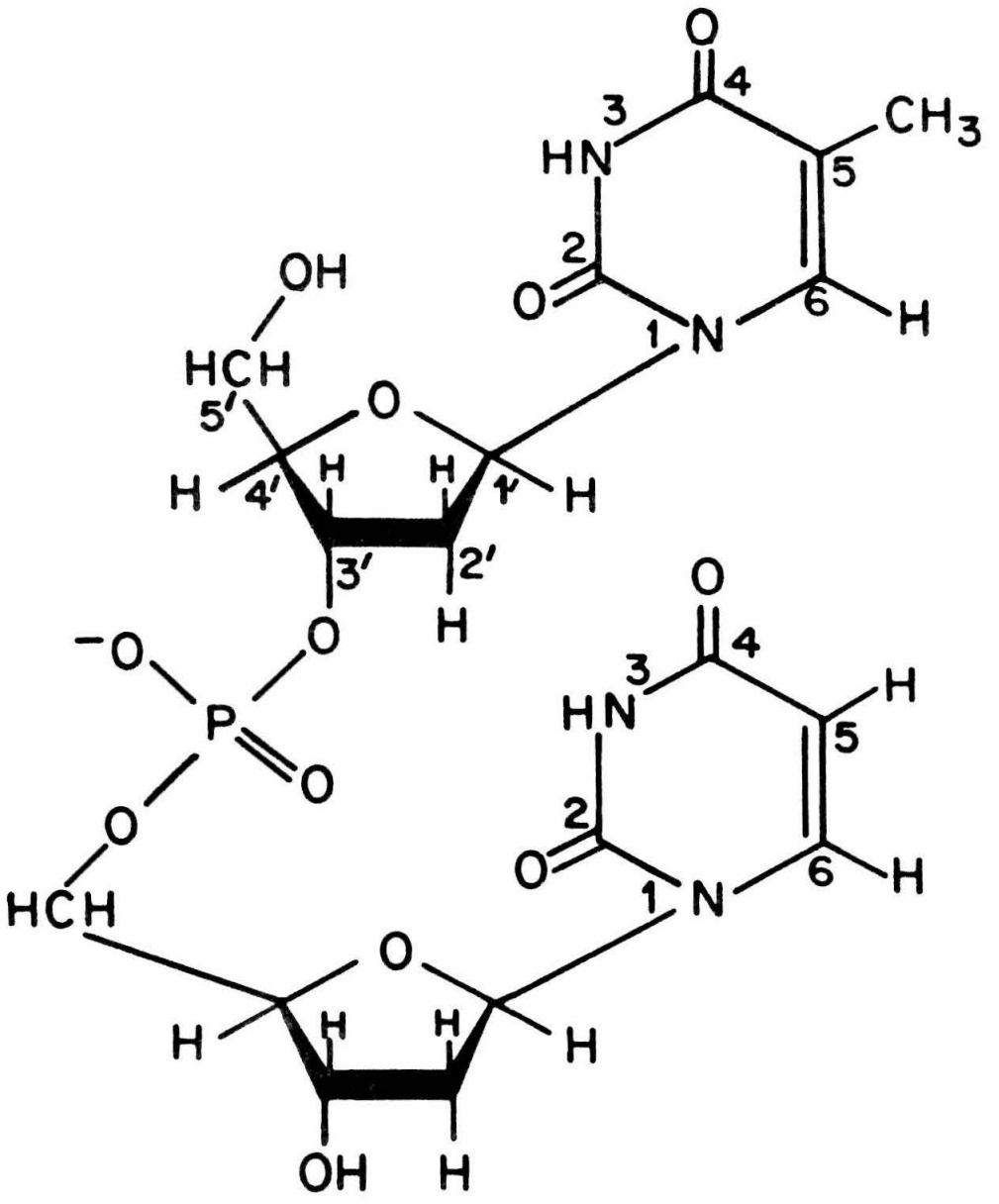
TABLE IX. Purine-induced shifts for proton resonances of 0.13 M TpT, cps at 60 Mcps.

Purine Concentration	CH_3		H_6		$H_{1'}$	
0.26 M	3.5	6.0	5.0	7.5	3.5	4.5
0.52	7.5	11.5	10.0	13.5	8.0	8.5
0.78	10.0	15.0	13.0	17.5	11.0	11.5
1.04	12.0	17.0	14.0	18.5	12.0	12.0
1.30	12.5	18.5	15.5	19.5		

4. 2. TpdU and dUpT

The pmr spectra of a 0. 19 M solution of TpdU, Figure 18, and a 0. 16 M solution of dUpT (both ammonium salts) were also examined. The chemical shifts of the thymine and uracil ring protons and the ribose $H_{1'}$ protons at 60 Mcps are given in Table X, along with those for the thymidine and 2'-deoxyuridine nucleosides for comparison. The chemical shift differences resulting from incorporation of the nucleosides into the dinucleoside monophosphates are seen to be rather small. The two sets of $H_{1'}$ resonances for each dinucleoside monophosphate are triplets, with $|J_{H_{1'}-H_{2'}/H_{2''}}| \cong 6.5$ cps, and these triplets overlap one another both in the absence and presence of added purine.

The addition of purine to these two solutions causes the monitored proton resonances to be shifted to higher fields, as expected. The overlapping $H_{1'}$ resonances shift relative to one another, making accurate chemical shift and coupling constant measurements for these protons impossible. The purine-induced shifts of the base proton resonances are summarized for TpdU in Table XI and for dUpT in Table XII. From these data, it is observed that the purine-induced shifts for thymine are larger for those protons on the base of the 3'-nucleoside. This is illustrated more clearly for the CH_3 protons by Figure 19 and for the H_6 protons by Figure 20. Similarly, the purine-induced shift for H_5 of uracil is greater with dUpT (3'-nucleoside) than with TpdU (5'-nucleoside), as shown in Figure 21, while the uracil H_6 protons of the two dinucleoside monophosphates are shifted about



TpdU

FIGURE 18

Structural diagram of thymidylyl (3′ → 5′) 2′-deoxyuridine, TpdU

TABLE X. Chemical shifts of TpdU, dUpT, thymidine and 2′-deoxyuridine protons, cps at 60 Mcps.

Compound	$H_6(T)$	$CH_3(T)$	$H_6(U)$	$H_5(U)$	$H_{1'}$
TpdU	-454. 5	-110. 5	-468. 5	-350. 0	-374 -371
dUpT	-456. 5	-111. 5	-465. 5	-347. 0	-375 -369
T	-457	-112			-372
dU			-469	-351	-374

TABLE XI. Purine-induced shifts for proton resonances of 0.19 M TpdU, cps at 60 Mcps.

Purine Concentration	Thymine CH_3	Thymine H_6	Uracil H_5	Uracil H_6
0.19 M	6.5	7.0	4.5	4.5
0.38	9.5	10.0	6.5	6.5
0.76	15.0	15.5	10.0	9.5
1.04	18.5	20.0	14.0	13.5
1.52	21.5	22.5	15.5	14.5
1.90	23.0	24.0	17.0	14.5

TABLE XII. Purine-induced shifts for proton resonances of 0.16 M dUpT, cps at 60 Mcps.

Purine Concentration	Thymine		Uracil	
	CH_3	H_6	H_5	H_6
0.16 M	4.5	4.0	4.0	3.5
0.32	6.5	7.0	6.5	5.0
0.64	10.5	11.5	11.5	9.5
0.96	14.0	15.0	15.0	13.0
1.28	15.5	17.0	18.0	14.5
1.60	17.0	18.5	18.5	15.0

FIGURE 19

Plot of purine-induced shifts for thymine CH_3 resonances of 0.19 M TpdU and 0.16 M dUpT, at 60 Mcps.

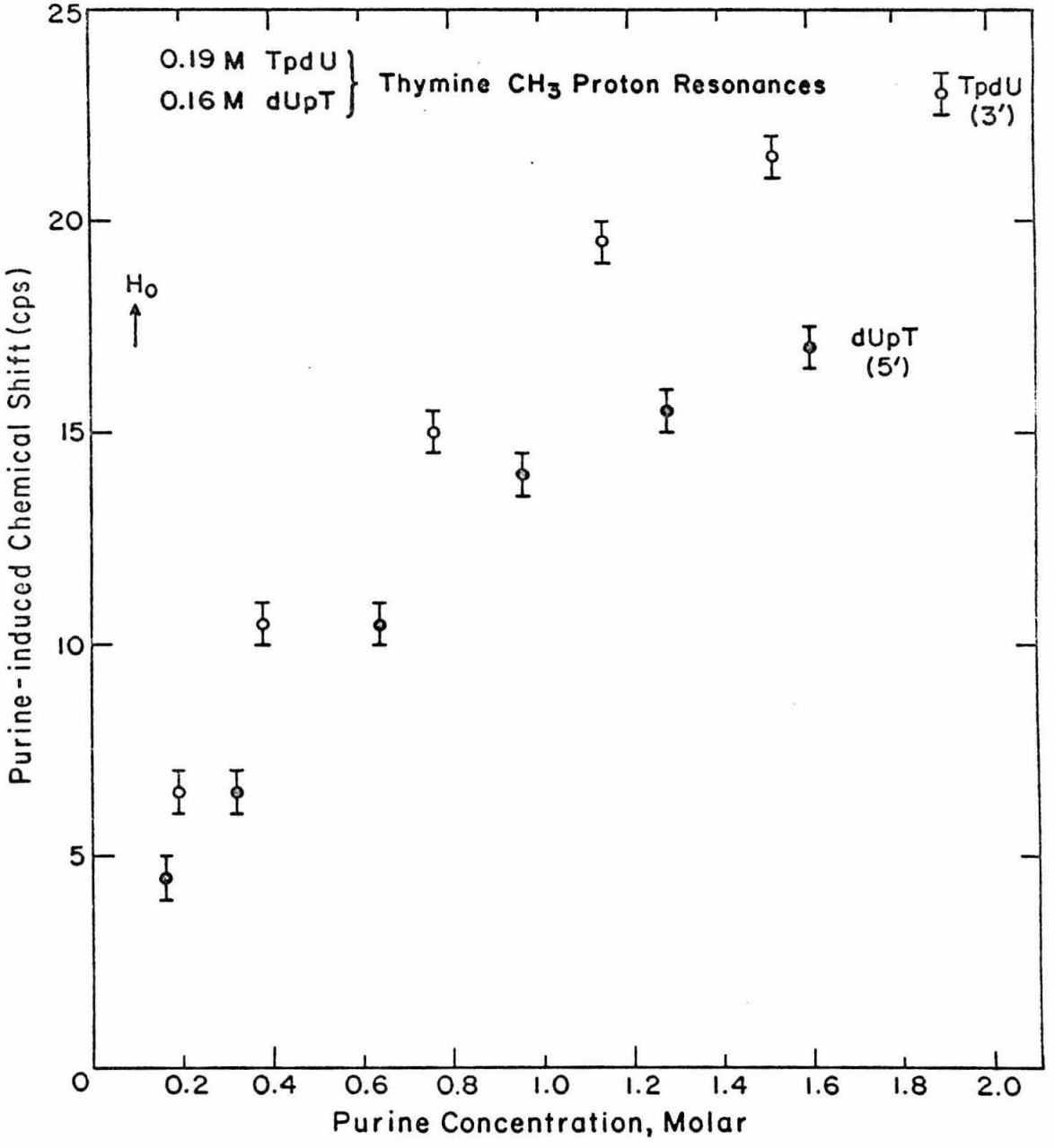

0.19 M TpdU
0.16 M dUpT
Thymine CH3 Proton Resonances
TpdU (3')
dUpT (5')
Ho
Purine-induced Chemical Shift (cps)
Purine Concentration, Molar
0
5
10
15
20
25
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0

FIGURE 20

Plot of purine-induced shifts for thymine H_6 resonances of 0.19 M TpdU and 0.16 M dUpT, at 60 Mcps.

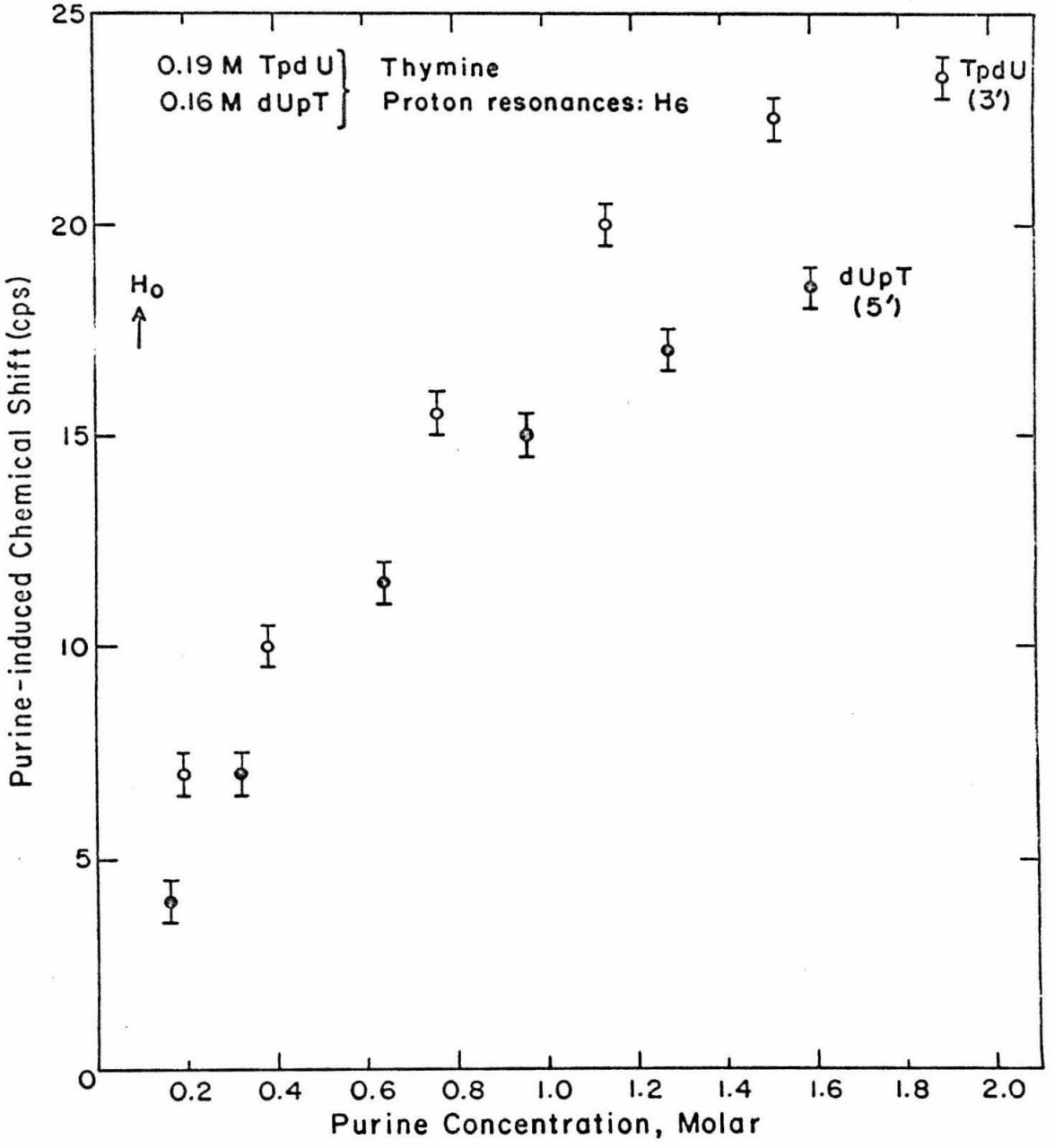

0.19 M TpdU
0.16 M dUpT
Thymine
Proton resonances: H6
TpdU (3')
dUpT (5')
Ho
Purine-induced Chemical Shift (cps)
Purine Concentration, Molar
0
5
10
15
20
25
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0

FIGURE 21

Plot of purine-induced shifts for uracil H_5 resonances of 0.19 M TpdU and 0.16 M dUpT, at 60 Mcps.

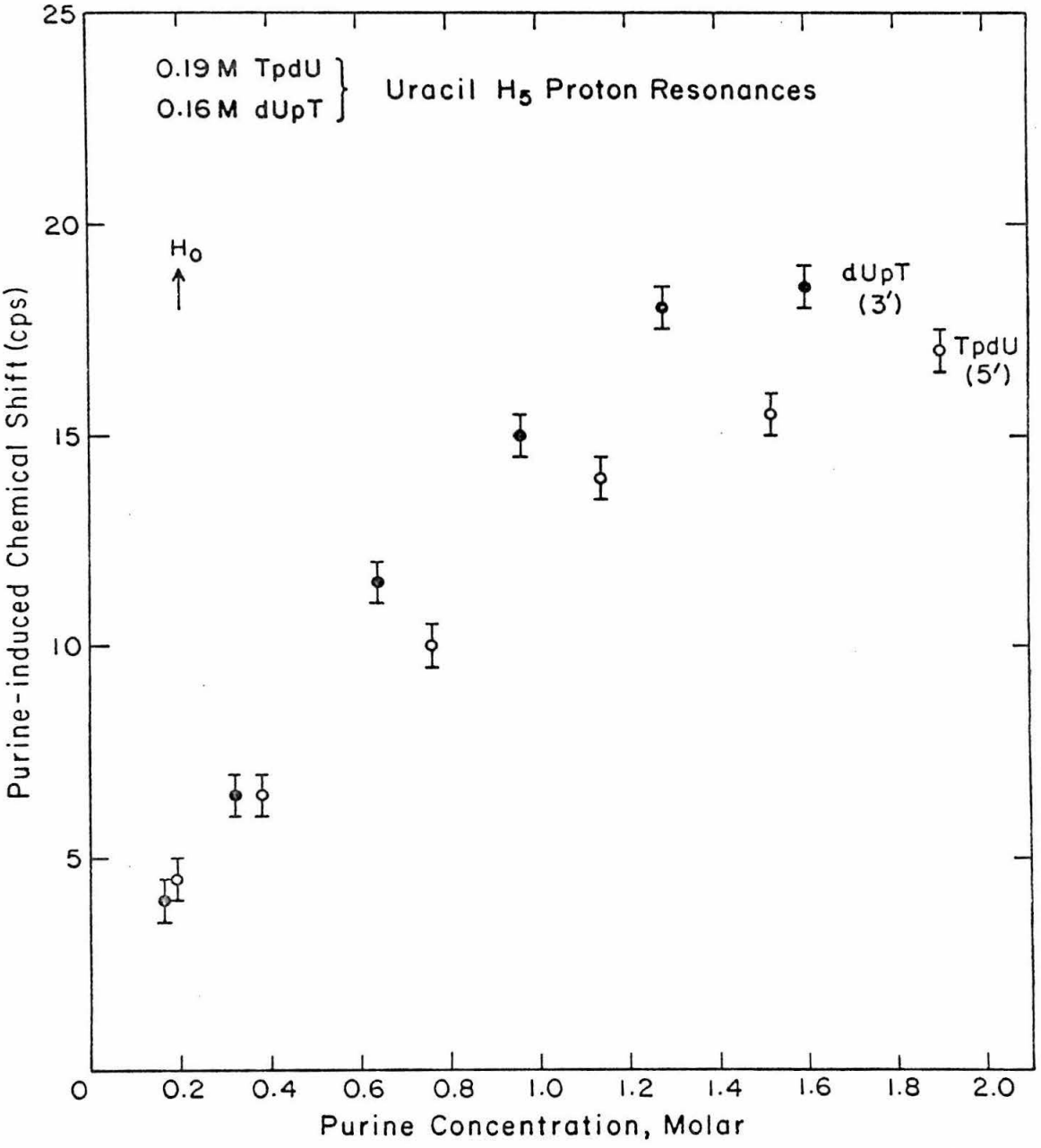
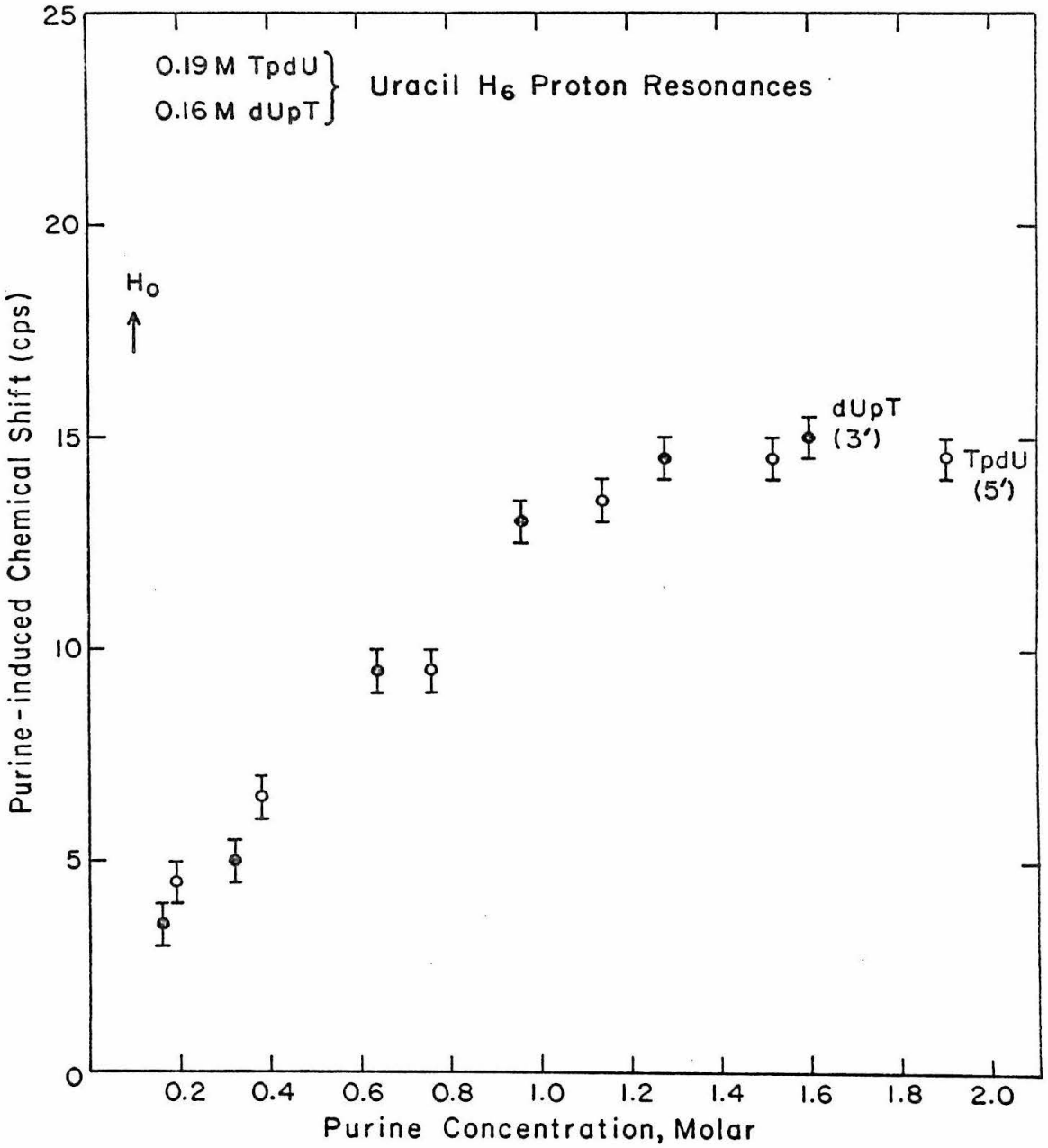

0.19 M TpdU
0.16 M dUpT
Uracil H5 Proton Resonances
Ho
dUpT (3')
TpdU (5')
Purine-induced Chemical Shift (cps)
Purine Concentration, Molar
0
5
10
15
20
25
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0

FIGURE 22

Plot of purine induced shifts for uracil H_6 resonances of 0.19 M TpdU and 0.16 M dUpT, at 60 Mcps.


0.19 M TpdU
0.16 M dUpT
Uracil H6 Proton Resonances
Ho
dUpT (3')
TpdU (5')
Purine-induced Chemical Shift (cps)
Purine Concentration, Molar
0
5
10
15
20
25
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0

equally by added purine, Figure 22. These results lead to the conclusion that the nature of the purine-dinucleotide complex is such that the base protons of the 3′-nucleoside experience a greater ring-current magnetic anisotropy effect from the bound purine than do those of the 5′-nucleoside. This conclusion is supported by the results of the study of purine binding to ApU and UpA described in Section 7.

It is also noted that the purine-induced shifts for the thymine base protons in TpdU and dUpT are larger than those for the uracil base protons, as well as larger than those for the corresponding base protons in TpT. This is probably due to a stronger base-stacking interaction between purine and thymine than between purine and uracil. Such appears to be the case with the purine-nucleoside interactions,[28] where it has been reported that the purine-induced shifts for the H_6 and $H_{1'}$ protons of uridine are 60-70 percent of the corresponding shifts for thymidine.

4.3. Nature of the Complex between Purine and TpT, TpdU, and dUpT

The purine-induced shifts of the thymidine and uridine resonances reported by Schweizer, et al.,[28] from their purine-nucleoside interaction study are listed in Table XIII to permit a comparison with the purine-induced shifts for corresponding protons of TpT, TpdU and dUpT. Because of large differences between the concentrations of the nucleosides and the dinucleoside monophosphates (expressed here in terms of the total base concentration, which is the appropriate

TABLE XIII. Comparison of purine-induced shifts (in cps at 60 Mcps) for proton resonances of thymidine, uridine, TpT, TpdU and dUpT. The data on the nucleosides are from Schweizer, Chan and Ts'o. [28]

Compound	Base Conc.	Purine Conc.	Thymine Protons			
			CH_3	Ratio	H_6	Ratio
Thymidine	0.1 M	1.0 M	20.4	1.00	24.1	1.00
TpT	0.26	1.04	12.0	0.59	14.0	0.58
			17.0	0.83	18.5	0.77
TpdU	0.38	1.14	18.5	0.91	20.0	0.83
dUpT	0.32	0.96	14.0	0.69	15.0	0.62
			Uracil Protons			
			H_5	Ratio	H_6	Ratio
Uridine	0.11	1.0	18.9	1.00	14.1	1.00
TpdU	0.38	1.14	14.0	0.74	13.5	0.96
dUpT	0.32	0.96	15.0	0.79	13.0	0.92

comparison if the dinucleotides are unstacked) quantitative determination of the relative extent of purine-base interaction is not possible. However, it is quite apparent from Table XIII that the purine-induced shifts (at ~1.0 M purine) for the protons of the dinucleotides are considerably more than half as large as those for corresponding protons of the nucleosides. The purine-induced shifts for the thymine CH_3 and H_6 protons of TpT average about 68% of those for the thymidine nucleoside; the shifts for TpdU are about 87% and for dUpT about 66% of the thymidine values. Similarly, the shifts for the uracil base protons in TpdU and dUpT are about 85% of those for the uridine nucleoside. If the concentrations of the dinucleoside monophosphates were more comparable to those of the nucleosides (0.1 M), these ratios would be even greater.

Since the purine-induced shifts for protons of the dinucleoside monophosphates are more than half as large as those for the nucleosides, it is possible to conclude that the situation represented in Figure 11a, in which purine stacks on the exposed faces of stacked dinucleotide molecules, does not accurately depict the actual behavior of these purine-dinucleotide systems. Rather, complexes between purine and TpT, TpdU and dUpT in which purine interacts with both faces of each base of the dinucleotide must be at least as prevalent in these solutions.

A most interesting aspect of the interaction of TpT, TpdU and dUpT with purine is the effect of the dinucleoside monophosphates on the linewidths of the purine resonances. The three purine protons bound to carbon atoms give narrow (< 1.0 cps) resonances at

concentrations as high as 2 M or more. The chemical shifts of these protons are highly concentration dependent because of purine self-stacking, and in 0. 2 M solution they are: -522 cps for H_6, -514 cps for H_2 and -497 cps for H_8. The proton attached to N_9 of purine undergoes rapid exchange with the solvent (D_2O) and does not give a distinct resonance. In the presence of the dinucleotides, the purine resonances are significantly broadened, particularly at low purine concentrations where the fraction of purine bound to the dinucleotide would be highest. The monitored proton resonances of the dinucleoside monophosphates remain narrow throughout the concentration range of added purine, however. The purine proton chemical shifts appear to be little affected by the dinucleoside monophosphates, with these resonances shifting upfield with increasing concentration as expected.

The linewidths* of the three purine proton resonances in the solutions studied are listed in Table XIV, and Figure 23 shows the purine proton resonances in the presence of TpdU, TpT, and dUpT at a purine/dinucleotide concentration ratio of 2/1. It is evident from these data that the three purine resonances are not equally broadened; the order of this effect, namely $H_6 > H_8 > H_2$, is clearly shown for TpdU in Figure 24 and also holds for TpT and dUpT. Furthermore, the purine line-broadening effect is most pronounced for TpdU and least evident for dUpT, with TpT showing intermediate behavior. This trend is depicted for the purine H_6 resonance in Figure 25. The resonances narrow with increasing purine concentration, presumably

*All linewidths reported in this thesis are the measured full linewidths at half height in cps.

TABLE XIV. Purine proton resonance linewidths in the presence of TpdU, TpT and dUpT. (Full linewidth at half-height in cps.)

Purine Conc.	Purine Resonance Linewidths H_6	H_2	H_8	Dinucleotide
0.19 M	~10.	~3.	~6.	0.19 M TpdU
0.38	6.0	2.4	3.4	
0.76	4.0	1.6	2.4	
1.04	3.2	1.5	2.0	
1.52	2.6	1.3	1.6	
0.26 M	4.0	2.2	2.4	0.13 M TpT
0.52	2.7	1.7	1.9	
0.78	2.4	1.4	1.6	
1.04	1.9	1.1	1.5	
0.16 M	~ 1.5	~1.0	~2.0	0.16 M dUpT
0.32	1.6	1.4	1.2	
0.64	1.4	1.0	1.2	
0.96	1.2	1.0	1.0	
1.28	1.1	0.8	0.8	

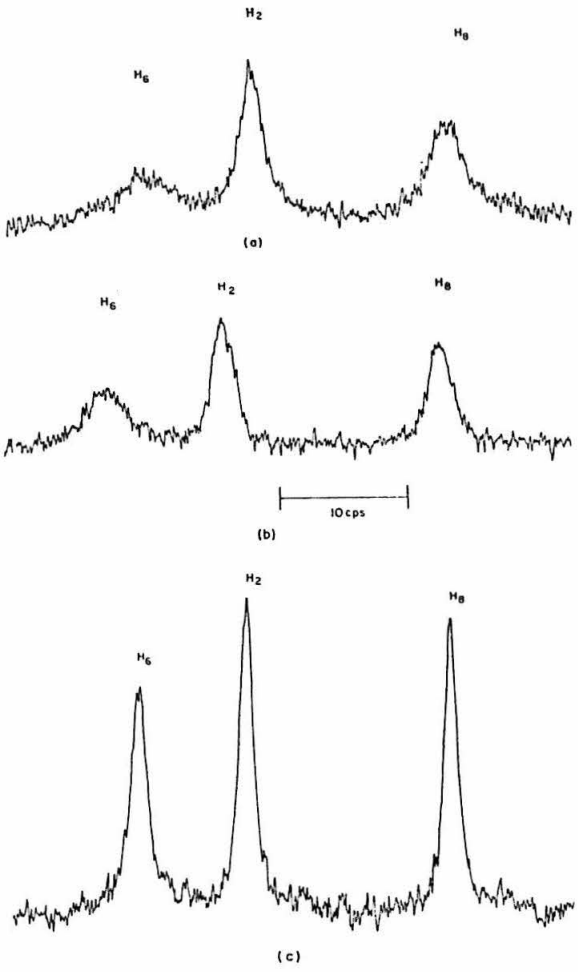


FIGURE 23

Effect of deoxyribose dinucleoside monophosphates on the pmr spectrum of purine. Purine/dinucleoside monophosphate concentration ratio = 2/1. (a) 0.19 M TpdU; (b) 0.13 M TpT; (c) 0.16 M dUpT.

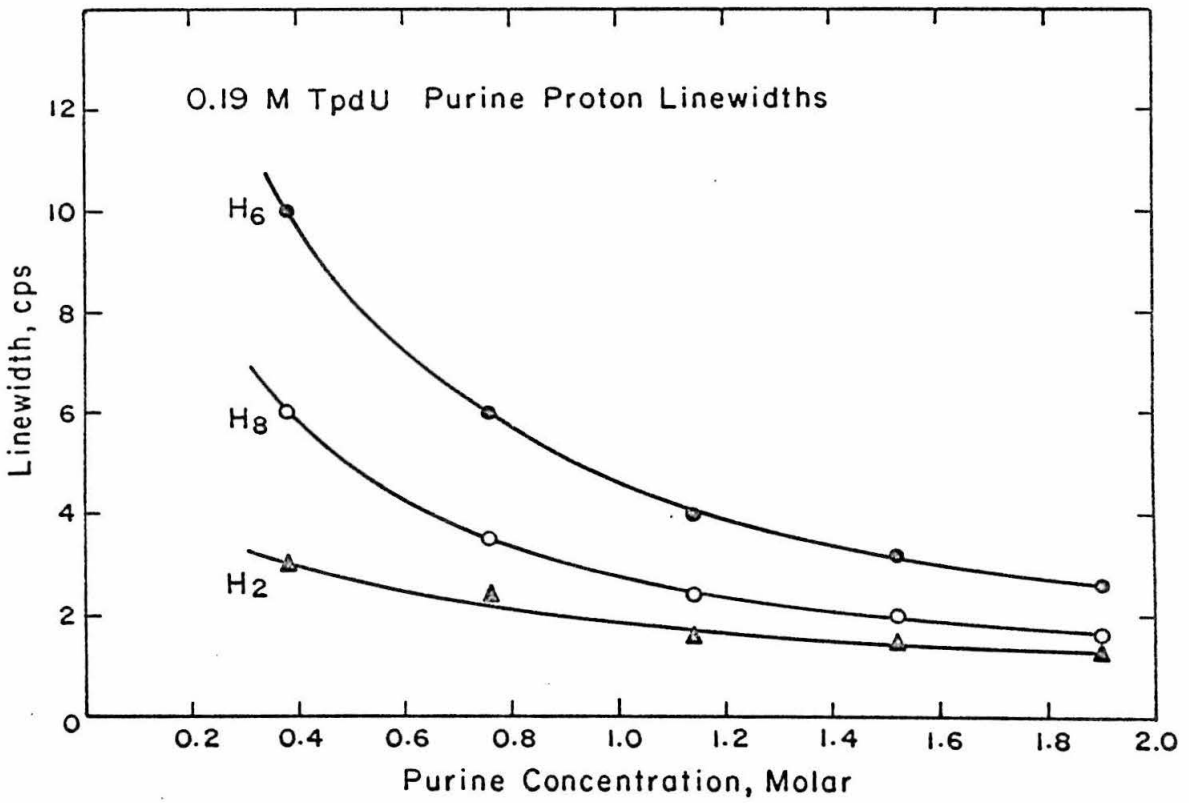


FIGURE 24

Purine proton resonance linewidths in the presence of 0.19 M TpdU, as a function of purine concentration.

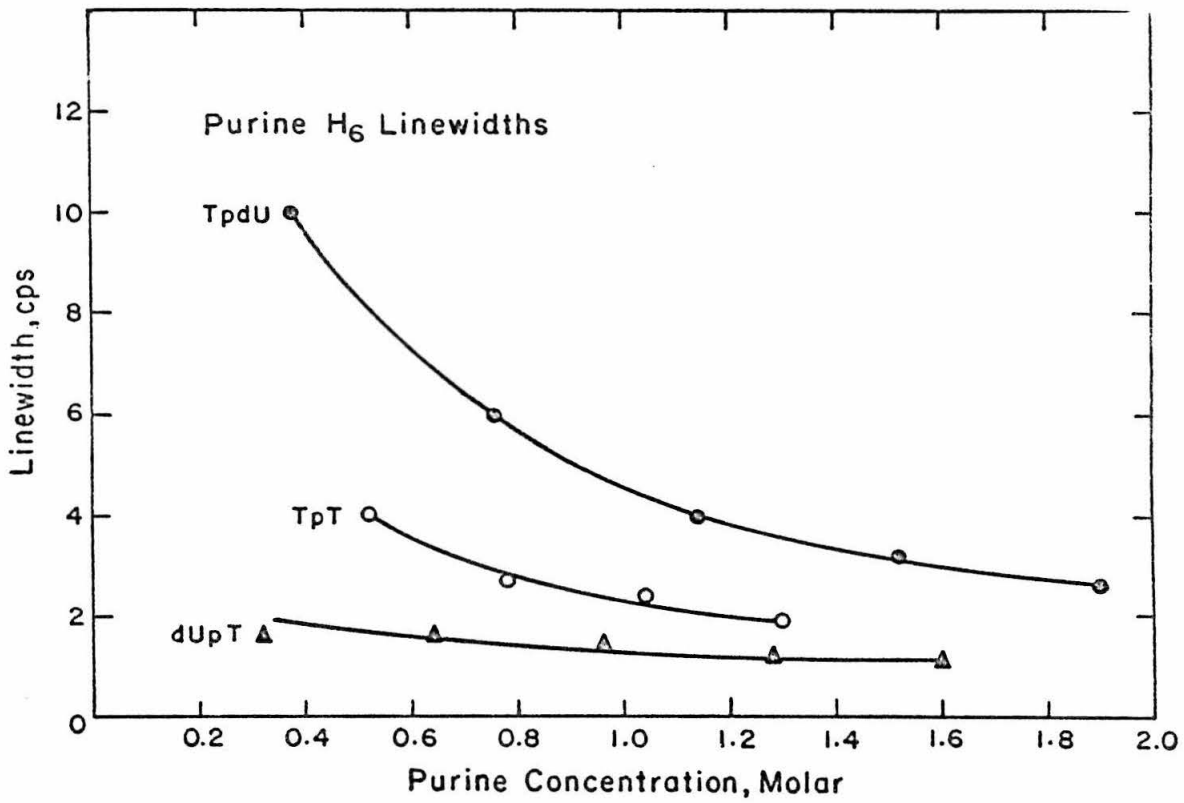


FIGURE 25

Purine H_6 proton resonance linewidths in the presence of 0.19 M TpdU, 0.13 M TpT, and 0.16 M dUpT, as a function of purine concentration.

because of rapid exchange of purine molecules between bound and free environments and a consequent averaging of the linewidths for purine in these two environments.

It is significant that no broadening of the purine proton resonances by the presence of the nucleosides thymidine, uridine, and cytidine was reported by Schweizer, Chan, and Ts'o.[28] Furthermore, Figure 54 of Section 6.3 clearly shows that the linewidths of the purine proton resonances in a 0.12 M purine solution are unaffected by the presence of uridine at a concentration of 0.10 M. Subsequent work has shown that purine proton resonance line broadening also occurs when purine interacts with the ribose dinucleoside monophosphates CpC, UpU, ApU, and UpA, as well as with polyuridylic acid, as reported in the following Sections of this thesis. Thus the purine line broadening phenomenon observed here appears to be a general feature of the interaction of purine with molecules which possess two or more bases linked by a sugar-phosphate backbone.

The structures of di-, oligo-, and polynucleotides allow the formation of a complex quite unlike those formed between purine and monomeric bases, nucleosides and nucleotides. This complex is one in which a single purine molecule inserts or <u>intercalates</u> between two adjacent bases of a dinucleotide or higher oligomer. The formation of such an intercalated complex between purine and a dinucleoside monophosphate is depicted in Figure 26. It is proposed that the formation of purine-dinucleotide intercalated complexes of this type, in which a monomeric purine molecule interacts with the two bases of the dinucleotide in such a way as to bring them together to form a

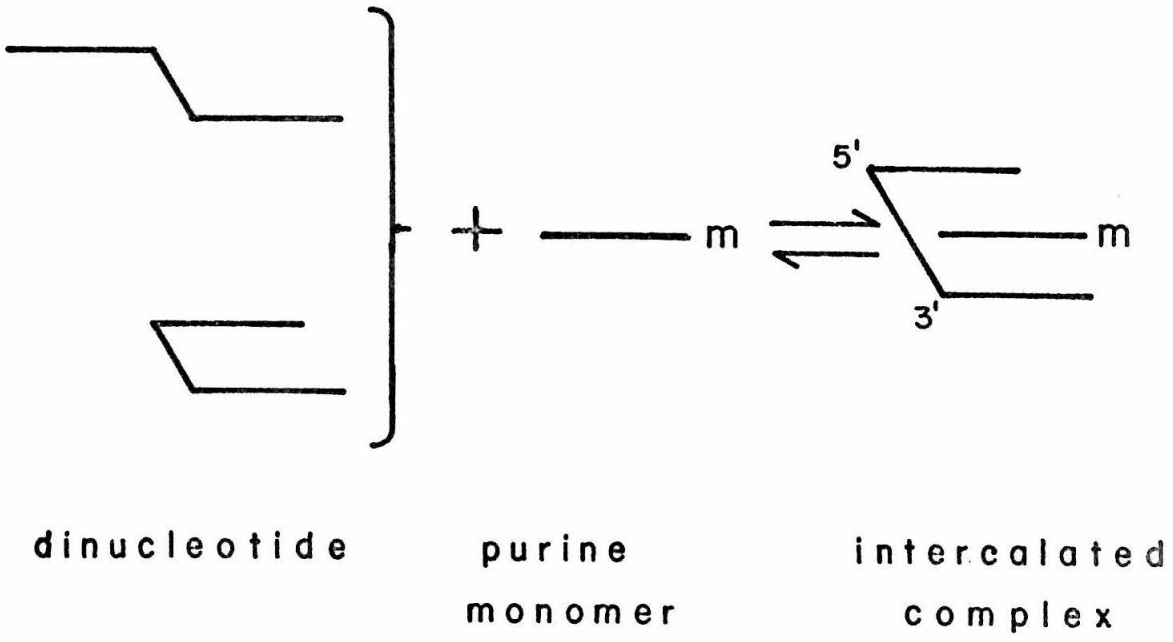


FIGURE 26

Schematic representation of the formation of an intercalated complex involving a dinucleotide molecule and a monomeric purine molecule.

sandwich-like stack, is a unique and important consequence of the binding of purine to dinucleotides--and to higher oligomers and polymers as well.

There are two aspects of the interaction of purine with TpT, TpdU, and dUpT, as well as with the other dinucleotides to be discussed, which tend to support this hypothesis. First of all, there is little reason to expect that purine would shift the base proton resonances of 3′- and 5′-esterified nucleosides by different amounts if the dinucleotides were unstacked and purine species were stacking on both faces of the pyrimidine rings. Moreover, if the purine-induced chemical shift differences can be accounted for in this manner, it is difficult to understand why the ribose $H_{1'}$ resonances are shifted so little relative to one another. In the proposed intercalated complex, the asymmetric phosphate attachment results in the two bases of the dinucleotide being brought into non-equivalent positions, so that the protons of a given base (thymine, uracil, cytosine, etc.) experience slightly different effects from the ring-current magnetic anisotropy of an intercalated purine molecule depending on whether the base is attached to the 3′- or 5′-nucleoside.

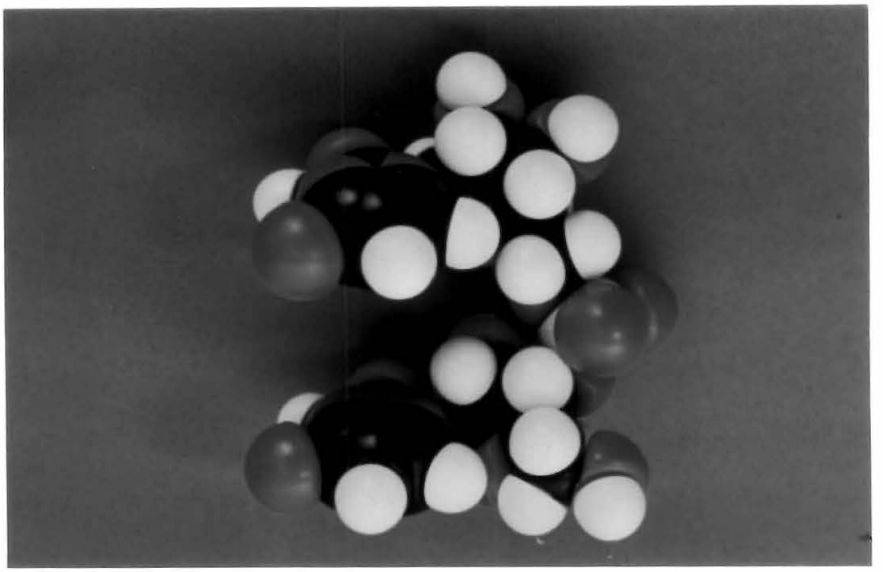
This can be understood if the conformation of the sugar-phosphate-sugar backbone of the dinucleotide in the intercalated complex is similar to that found in double helical DNA. Consideration of CPK molecular models of the dinucleotides indicates that, to accommodate the inserting purine molecule, some extension and untwisting of the sugar-phosphate backbone must occur. In the resulting conformation, the base of the 3′-nucleoside is more exposed to the

intercalated purine molecule than is the base of the 5′-nucleoside, so that the base protons of the 3′-nucleoside would on the average experience a larger effect from the ring-current magnetic anisotropy of the purine molecule. This situation is illustrated schematically in Figure 26, but it is perhaps more helpful to consider the photographs of CPK molecular models of UpU in Figure 27. A model of the UpU molecule with the bases parallel to one another and the ribose-phosphate-ribose backbone extended to accommodate an incorporated purine molecule is shown in Figure 27a; the purine-UpU intercalated complex is depicted in Figure 27b. The greater degree of overlap of the purine molecule with the base of the 3′-nucleoside (lower base) than with the base of the 5′-nucleoside (upper base) is apparent in these photographs.

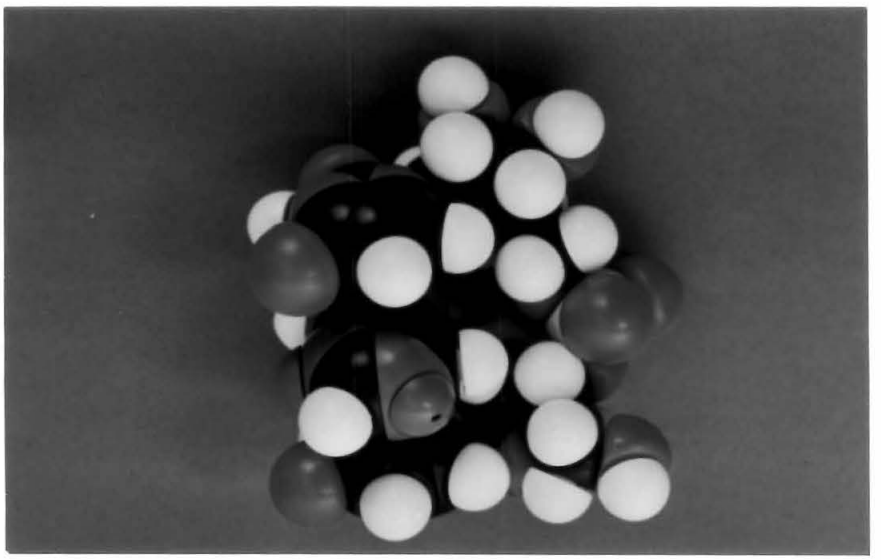
The second aspect of the purine-dinucleotide interaction which lends credence to the idea of purine intercalation is, of course, the purine line broadening phenomenon. It seems unlikely that stacking of purine on the exposed base faces of a stacked or an unstacked dinucleotide molecule could result in a broadening of the purine resonances if a similar interaction between purine and monomeric bases, nucleosides, and nucleotides does not have this effect. Thus the purine line broadening has been attributed to formation of the intercalated complex. Furthermore, the structure of such a complex suggests a possible mechanism for the observed purine line broadening. It can be seen from Figure 27 that the protons of the purine molecule incorporated in the intercalated complex are placed in close

FIGURE 27

Photographs of CPK molecular models representing the formation of a purine-UpU intercalated complex. (a) the UpU molecule with the ribose-phosphate-ribose backbone extended to accommodate a purine molecule; (b) the purine-UpU intercalated complex.



(a)



(b)

proximity to several protons of the sugar-phosphate backbone of the dinucleotide; in particular, the $H_{2'}$, $H_{3'}$, $H_{5'}$, and $H_{5''}$ protons of the 3′-nucleoside. It is proposed that the purine protons experience strong dipolar magnetic fields from these sugar protons, and that fluctuations in these local magnetic fields cause rapid nuclear spin relaxation of the purine protons, thus broadening the resonances of the purine protons. The various aspects of the purine line broadening mechanism and the nature of the purine-dinucleotide intercalated complex are more fully treated in Section 8, and the discussion of the sequence dependence of the effect and the degree of broadening of the individual purine proton resonances is deferred to that Section.

5. Cytidylyl (3′→5′) cytidine

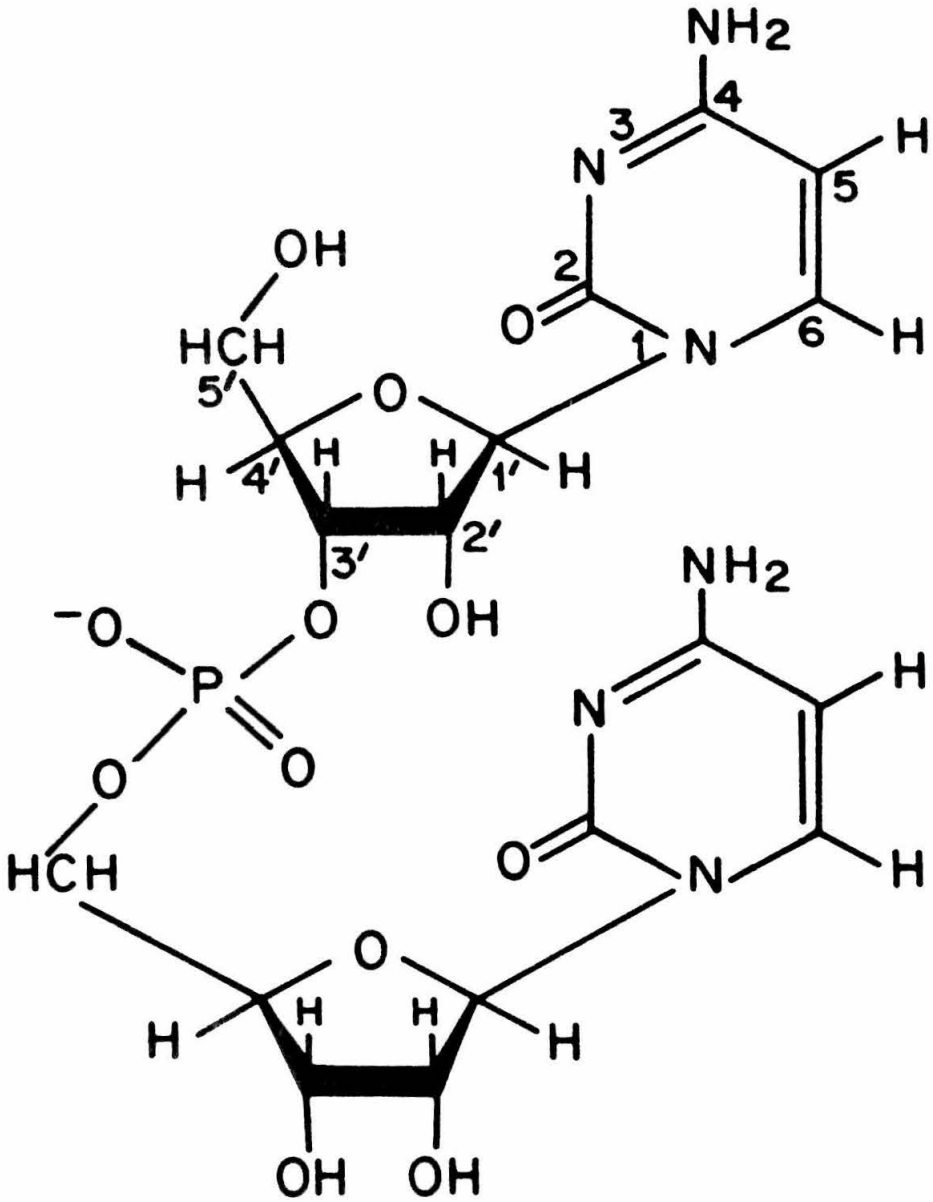
The ribose dinucleoside monophosphate CpC, shown in Figure 28, was investigated at several concentrations and temperatures by pmr spectroscopy. In addition, the interaction of this dinucleotide with purine was examined under several conditions of dinucleotide concentration, pD, and temperature.

5.1. Pmr Spectrum of CpC

The 100 Mcps pmr spectrum at 29°C of a 0.010 M solution of the sodium salt of CpC, in the regions of the cytosine H_6 proton and the cytosine H_5 and ribose $H_{1'}$ protons, is shown in Figure 29. The spectrum of a 0.10 M cytidine solution in the same spectral regions under similar conditions is included for comparison. The cytosine

FIGURE 28

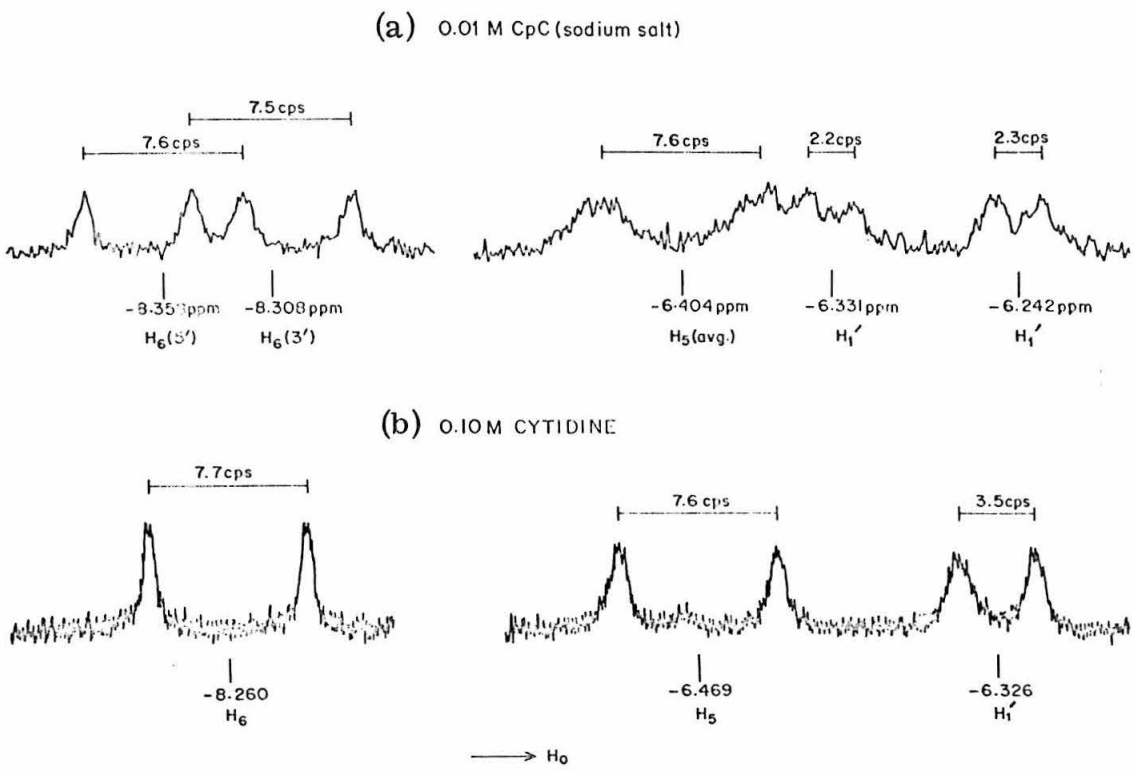
Structural diagram of cytidylyl (3′→ 5′) cytidine, CpC.


NH2
4
3
N
H
5
OH
2
O
N
6
H
HCH
1
5′
O
H
H
H
4′
1′
H
2′
3′
O
OH
−O
O
P
O
O
NH2
H
N
O
N
H
HCH
O
H
H
H
OH
OH

CpC

FIGURE 29

(a) Time-averaged 100 Mcps pmr spectrum of CpC in the regions of the H_6 and H_5 and $H_{1'}$ proton resonances (sum of 25 successive scans). (b) Single-scan spectrum of 0.10 M cytidine in the same spectral regions.


(a) 0.01 M CpC (sodium salt)
7.5 cps
7.6 cps
-8.358 ppm
-8.308 ppm
H6(5')
H6(3')
7.6 cps
2.2 cps
2.3 cps
-6.404 ppm
-6.331 ppm
-6.242 ppm
H5(avg.)
H1'
H1'
(b) 0.10 M CYTIDINE
7.7 cps
-8.260
H6
7.6 cps
3.5 cps
-6.469
-6.326
H5
H1'
Ho

H_6 and H_5 protons are spin-spin coupled to give doublets, with $|J_{H_5-H_6}| = 7.6$ cps for both bases of CpC as well as in the monomeric cytidine nucleoside. The ribose $H_{1'}$ resonances are doublets from coupling with the $H_{2'}$ protons; the coupling constants $|J_{H_{1'}-H_{2'}}|$ are seen to be smaller in CpC (2.2-2.3 cps) than in the cytidine nucleoside (3.5 cps). As with the other dinucleotides studied, the protons of the amino and hydroxyl groups do not give distinct resonances because of rapid exchange with the solvent. Similarly, the resonances of the ribose $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, and $H_{5''}$ protons in the spectral region from -4.20 to -5.20 ppm were not studied in any detail because of the inherent complexity of this spectral region.

Due to asymmetric esterification of the ribose moieties of the two cytidine nucleosides by the phosphate group, the two cytidines in CpC are not geometrically equivalent. This is reflected in the chemical shift differences between corresponding cytidine protons observed in the pmr spectrum of CpC. The two H_6 protons have a chemical shift difference of 0.050 ppm (5.0 cps at 100 Mcps), and the ribose $H_{1'}$ protons show a difference in chemical shift of 0.090 ppm. The cytosine H_5 protons probably have a slight magnetic non-equivalence also (of the order of ~ 0.010 ppm), but this splitting is obscured by the overlap of these rather broad resonances.

5.1.1. Assignment of resonances

No direct experimental assignment of the resonances of similar protons to the particular 3'- or 5'-esterified nucleoside was attempted. However it is possible to make tentative assignments of these

resonances, based on indirect evidence resulting from study of CpC and other molecules. The results of the purine binding experiments to be discussed in Section 5. 3 indicate that the H_6 proton of the 3′-nucleoside resonates at higher field than that of the 5′-nucleoside in CpC. It also appears from the purine binding studies that, assuming a chemical shift difference of 0. 008 ppm between the H_5 proton resonances, the resonance at higher field is that of the 3′-nucleoside.

The ribose $H_{1'}$ resonance occurring at higher field (-6. 242 ppm) has been assigned to the 3′-nucleoside, and the $H_{1'}$ resonance at lower field (-6. 331 ppm) has been assigned to the 5′-nucleoside. The factors pointing to this assignment for the $H_{1'}$ resonances are somewhat more involved than those indicating the assignments of the H_6 and H_5 resonances. First of all, the ribose $H_{1'}$ proton resonance in cytidine-3′-monophosphate (disodium salt, pD = 7. 5) is at higher field than that of cytidine-5′-monophosphate (disodium salt, pD = 7. 8), as shown in Table XV. Secondly, the $H_{1'}$ proton of the cytidine residue in ApC would be expected to feel the effects of the ring-current magnetic anisotropy of the adjacent adenine base to a greater degree than would the $H_{1'}$(C) proton of CpA, based on consideration of molecular models of the dinucleotides. Thus in going from CpC to ApC and CpA the $H_{1'}$(5′) proton of cytidine should be shifted upfield more than the $H_{1'}$(3′) cytidine proton. As shown in Table III, Section 2. 1, the present assignment is consistent with this prediction. Finally, in studies of the binding of purine to the sequence isomers ApU and UpA (Section 7) and ApG and GpA, [60] the purine-induced shift for the $H_{1'}$ proton of a

TABLE XV. Chemical shift differences for cytidine protons between CpC and cytidine 3′- and 5′-monophosphates.

	H_6		H_5		$H_{1'}$	
	(3′)	(5′)	(3′)	(5′)	(3′)	(5′)
	ppm		ppm		ppm	
CpC (0. 01 M)	-8. 308	-8. 358	-6. 400	-6. 408	-6. 242	-6. 331
3′-CMP-$(Na^+)_2$ (0. 1 M)	-8. 330		(Broad)		-6. 385	
5′-CMP-$(Na^+)_2$ (0. 1 M)		-8. 530		-6. 577		-6. 450
Shift	+0. 022	+0. 172		+0. 168	+0. 143	+0. 119

given nucleoside is greater when the nucleoside is 5′-esterified than when it is 3′-esterified. Since the purine-induced shift for the H_1' proton of CpC which resonates at the lower field is greater than the shift for the higher field H_1' proton (see Section 5. 3), the assignment presented here also seems justified by this comparison of purine-induced shifts.

5. 1. 2. Discussion of chemical shifts

As is the case with the other dinucleoside monophosphates studied, the monitored proton resonances of CpC are shifted from their spectral positions in the cytidine nucleoside. The several factors important in determining the chemical shifts of protons in the dinucleotides were discussed in connection with ApC and CpA in Section 2. 1, and a number of these apply to CpC as well. One contribution which was extremely significant for ApC and CpA, the magnetic anisotropy of the neighboring base, would seem to be much less important for CpC since the magnetic anisotropy of the cytosine base appears to be quite small.

The electric field produced by the phosphate group is of course expected to be quite important in shifting the proton resonances in CpC relative to their positions in the cytidine nucleoside, with protons of the 3′- and 5′-esterified nucleosides affected differently because of the asymmetric phosphate attachment. Thus it seems appropriate to compare the chemical shifts of the CpC protons with the corresponding protons of the cytidine 3′- and 5′-monophosphates. These data are shown in Table XV. It can be seen that the cytidine H_6, H_5, and H_1'

resonances in CpC are all at higher fields than in 3′-CMP and 5′-CMP. In making this comparison, however, it is important to note that the phosphate groups in the mononucleotides are doubly charged at the pD's used here (7.5 for 3′-CMP and 7.8 for 5′-CMP). It would be more appropriate, of course, to compare the proton resonances of CpC with those of singly charged mononucleotides. Unfortunately, the titration curves for the bases and the phosphate groups of the CMP's overlap to an appreciable extent. The pK's for the second dissociation of the phosphate groups in 3′-CMP and 5′-CMP are 6.0-6.2,[98] and the pK for protonation of the cytosine base is ~4.3 in these nucleotides, so that at a pD where the phosphate group is singly charged (pD below ~4.5) a significant fraction of the cytosine bases would be protonated also. Because of this situation, it is necessary to make the comparison between the chemical shifts for protons of CpC and those of the mononucleotides with doubly negative phosphate groups to avoid complications arising from partial protonation of the cytosine bases.

The electric field resulting from the negatively charged phosphate group is expected to shift the proton resonances of the nucleotides to lower fields relative to the nucleosides, and this effect should be larger when the phosphate group carries a double negative charge than when it is singly charged. This is borne out by a comparison of the chemical shifts of the 3′-CMP and 5′-CMP protons with those of cytidine. As seen from Table XV, the H_6 resonance in 5′-CMP is at considerably lower field than that in 3′-CMP or in CpC. It appears that the phosphate group in 5′-CMP can more closely

approach the protons of the cytosine base than can the phosphate group in 3′-CMP or in CpC (assuming that the dinucleotide is rather strongly stacked), and this could account for the extremely low field position of the H_6 resonance in 5′-CMP.

From the preceding discussion, the difference in the charge on the phosphate group between CpC and the mononucleotides would be expected to have a lot to do with the chemical shift differences between the CpC protons and those of 3′-CMP and 5′-CMP. On the basis of the phosphate effect, the CpC resonances would be expected to occur at fields intermediate between those for the cytidine nucleoside and for the doubly charged cytidine monophosphates. However, the H_5 and $H_{1'}$ (3′) resonances are at even higher fields in CpC than in the cytidine nucleoside.

A possible rationalization of the rather high field positions of the $H_{1'}$ resonances in CpC involves the effects of the magnetic anisotropies of the 2-keto group of the cytosine base and the 2′-hydroxyl group of the ribose ring. If CpC is intramolecularly stacked to an appreciable extent, the conformations of the bases relative to their ribose rings would be restricted in such a way as to cause the keto groups to be, on the average, further away from the $H_{1'}$ protons in the dinucleotide than in the monomeric nucleoside. Since proximity of $H_{1'}$ to the 2-keto group of a pyrimidine base causes the proton to be deshielded (shifted downfield),[94] this behavior would cause the $H_{1'}$ protons of CpC to resonate at higher fields than in cytidine. The work of Prestegard and Chan,[94] discussed in Section 2. 1, indicates that the 2′-hydroxyl group exerts a shielding effect on $H_{1'}$, with the proton

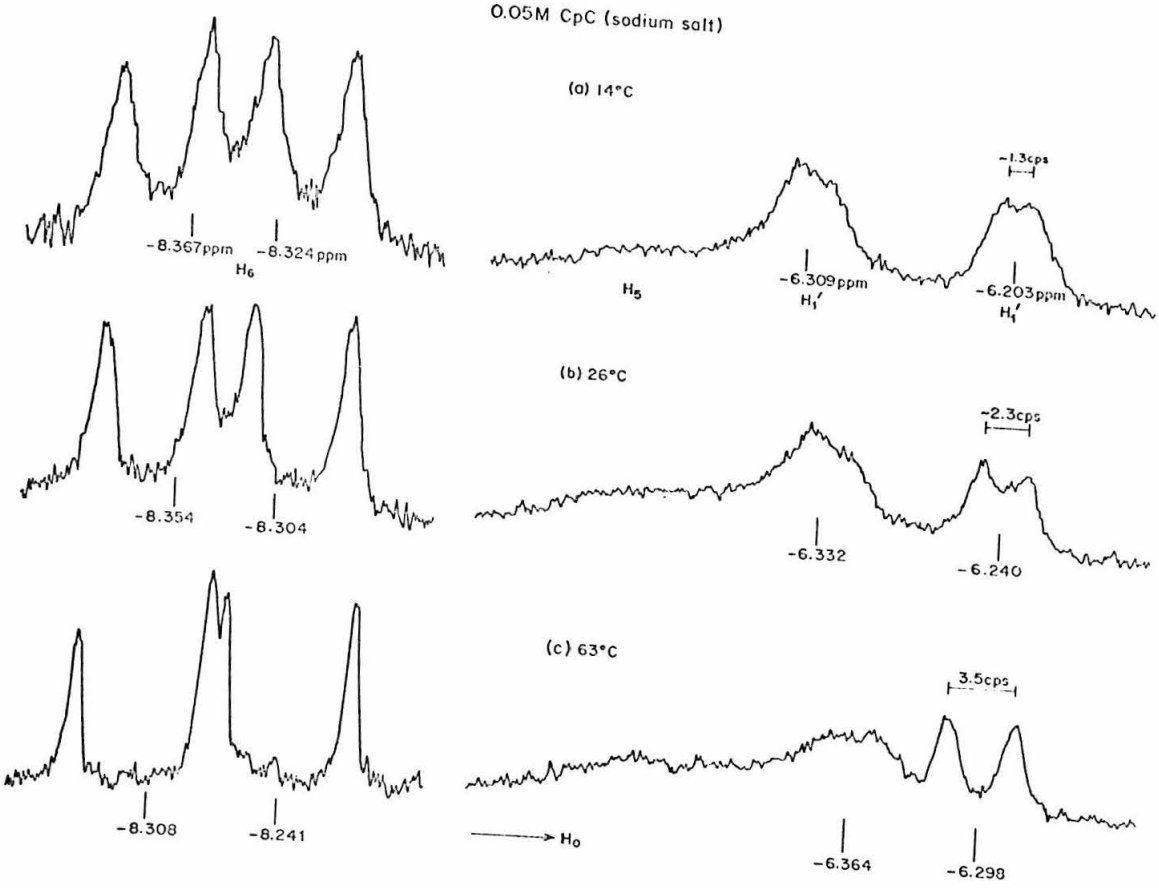
resonance experiencing an upfield shift from proximity to 2′-OH. The observation of smaller values of the coupling constants $|J_{H_1'-H_2'}|$ in CpC than in cytidine indicates that the 2′-hydroxyl groups are closer to the H_1' protons in the dinucleotide than in the monomeric nucleoside, and this could contribute to the shielding of the H_1' protons in addition to the effect of the 2-keto group.

It is difficult to see how the H_5 resonances could be at higher fields in CpC than in the cytidine nucleoside on the basis of the conformation of the cytosine bases relative to the ribose rings, or on the basis of the conformation of the ribose ring itself. As mentioned in Section 2. 3, the cytosine base appears to have a shielding effect on protons located in its vicinity, and it is possible that the H_5 protons in CpC experience an upfield shift due to the magnetic anisotropy of the neighboring cytosine base. In view of the apparent magnitude of this effect noted in the ApC-CpA study, it is somewhat surprising that the H_5 resonances appear at such high fields, however.

Concentration Dependence. -- The chemical shifts of the H_6 and H_1' resonances (as well as the spin-spin coupling constants $|J_{H_5-H_6}|$ and $|J_{H_1'-H_2'}|$) remain constant as the concentration of CpC is raised from 0. 010 M to 0. 05 M, as a comparison of Figure 30b with Figure 29a clearly shows. The H_5 resonances, already quite broad at 0. 010 M, broaden greatly with increasing dinucleotide concentration; the H_1' and H_6 resonances appear to broaden slightly. In view of the self-association of the cytidine nucleoside[17] and of the dinucleotides ApC and CpA (as discussed in Section 2. 2), it can be concluded that CpC undoubtedly self-associates intermolecularly by

FIGURE 30

Cytosine H_6 and H_5 and ribose $H_{1'}$ resonances at 100 Mcps for 0. 05 M CpC (sodium salt). Time-averaged spectra; sum of 15 scans. (a) 14°C, (b) 26°C, (c) 63°C. Chemical shifts compensated for bulk magnetic susceptibility differences.


0.05M CpC (sodium salt)
(a) 14°C
-8.367ppm
-8.324ppm
H6
H5
-6.309ppm
H1'
-6.203ppm
H1'
1.3cps
(b) 26°C
-8.354
-8.304
-6.332
-6.240
2.3cps
(c) 63°C
-8.308
-8.241
Ho
-6.364
-6.298
3.5cps

base-stacking. The lack of a concentration dependence for the proton chemical shifts of CpC is in line with the lack of an appreciable magnetic anisotropy for the cytosine base. The large effect of CpC concentration on the linewidths of the H_5 resonances is felt to be a result of the formation of self-intercalated intermolecular dimers, with the resonances being broadened by the magnetic dipolar relaxation mechanism responsible for the broadening of the resonances of an intercalated purine molecule. It appears from consideration of molecular models of CpC that the H_5 proton of an intercalated cytosine base could be brought into very close proximity to the ribose protons of the ribose-phosphate backbone. Thus the H_5 proton in such a complex would experience the strong dipolar magnetic fields produced by the ribose protons, and if the correlation time for motion of the cytosine base of one dinucleotide relative to the backbone of the other dinucleotide were long enough, this could provide a strong relaxation mechanism for the H_5 proton. This possibility is further discussed in Section 5. 3.

5. 2. Temperature Dependence

The temperature dependence of the pmr spectrum of CpC is expected to be somewhat less informative as regards the intramolecular base-stacking interaction than was the temperature study of ApC and CpA reported in Section 2. 3. The lack of an appreciable magnetic anisotropy on the part of the cytosine base makes the chemical shifts of the base protons in CpC dependent on a variety of other factors, such as the conformations of the bases relative to the ribose rings, and thus

more difficult to interpret in terms of the intramolecular base-stacking interaction.

The effect of temperature on the 100 Mcps pmr spectrum of a 0.05 M solution of CpC (sodium salt) is depicted in Figure 30. The chemical shifts at 14°C and 63°C reported here have been corrected relative to those at 26°C to compensate for changes in the bulk magnetic susceptibility difference between the TMS reference capillary and the D_2O solution, with $N(CH_3)_4Cl$ serving as the internal reference. It is evident from the spectra of Figure 30 that the H_6 proton resonances are rather broad at 14°C, and become narrower as they shift upfield (relative to $N(CH_3)_4^+$) with increasing temperature, with $H_6(3')$ showing a greater upfield shift than $H_6(5')$. Although it is not possible to tell whether the H_5 proton resonances shift with temperature, they do appear to narrow very slightly as the temperature is increased. At this concentration (0.05 M), the H_5 resonances appear as a very broad region of absorption overlapping $H_{1'}(5')$ and tailing downfield from this resonance. The broadening of the H_6 resonances observed at lower temperatures may be an indirect consequence of the rapid spin relaxation for the H_5 protons, reflecting partial collapse of the spin-spin multiplets for the H_6 resonances.

The ribose $H_{1'}$ resonances of CpC are shifted to lower fields as the temperature is raised, with $H_{1'}(3')$ exhibiting the larger shift. The spin-spin coupling constant $|J_{H_{1'}-H_{2'}}|$ for the $H_{1'}(3')$ proton increases with temperature, and this resonance also appears to narrow somewhat. The $H_{1'}(5')$ proton resonance, on the other hand, seems to become broader as the temperature is raised, but this apparent broadening may

just be a consequence of an increase in $|J_{H_1'-H_2'}|$ for this proton or a result of its overlapping the broad H_5 resonances.

The behavior of the coupling constant $|J_{H_1'-H_2'}|$ for the 3′-nucleoside of CpC (the splitting of the $H_1'(3')$ resonance) permits some conclusions regarding the degree of intramolecular C-C base-stacking in this dinucleotide. The fact that this coupling constant is significantly smaller in CpC than in the cytidine nucleoside ($|J_{H_1'-H_2'}|$ = 3.5-3.8 cps) or in 3′-CMP ($|J_{H_1'-H_2'}|$ = 3.6 cps) at low temperature indicates that the conformation of the ribose ring of the 3′-nucleoside is more 3′-endo in CpC than in the monomers. In view of the relationship between ribose conformation and the degree of intramolecular base-stacking in a dinucleotide discussed in Sections 2.1 and 2.3, it would appear that CpC is rather strongly stacked at low temperatures. Furthermore, a comparison of the change in $|J_{H_1'-H_2'}|$ for the 3′-cytidine moieties of CpC and CpA is also illuminating. Between 14°C and 63°C, this coupling constant changes from ~1.3 cps to 3.5 cps in CpC and from ~2.5 cps to ~4.2 cps in CpA (see Table VIII, Section 2.3). The fact that this ribose coupling constant is both smaller in CpC than in CpA and shows a greater change with temperature suggests that the intramolecular base-stacking interaction in CpC is perhaps stronger than that in CpA. However it is also possible that the conformations of the intramolecularly stacked CpC and CpA molecules are quite different, owing to differences in the geometry of the two bases relative to one another as a consequence of the shapes of these bases. Thus differences in the changes in ribose

coupling constants with temperature between these dinucleotides do not necessarily represent differences in the degree of intramolecular base-stacking tendencies.

In order to discuss the effect of temperature on the chemical shifts of the monitored CpC protons, it is appropriate to use the cytidine nucleoside as a reference for the chemical shift measurements. A 0. 010 M solution of CpC was studied at several temperatures, as at this concentration the H_5 resonances, though still rather broad, are narrow enough to permit measurement of the average chemical shift of the two H_5 protons. The chemical shifts of the averaged H_5 resonance and the $H_{1'}$ resonances of CpC relative to 0. 03 M cytidine at the same temperature are listed in Table XVI. The resonances of the H_6 protons of CpC were monitored over a larger range of temperature, and these chemical shifts are displayed in Figure 31. These data show that the H_6 resonances of CpC shift upfield relative to cytidine with increasing temperature, while the H_5 and $H_{1'}$ resonances of CpC shift to lower fields relative to the monomeric nucleoside with increasing temperature. At high temperatures, it is observed that (as depicted in Figure 31) the $H_6(5')$ resonance levels off $\sim$ 0. 08 ppm downfield from the H_6 resonance of cytidine, while the $H_6(3')$ resonance appears to level off at essentially the same chemical shift as the cytidine H_6 proton. This is a consequence of the deshielding effect of the phosphate group on the $H_6(5')$ proton, as noted for 5'-CMP in Table XV.

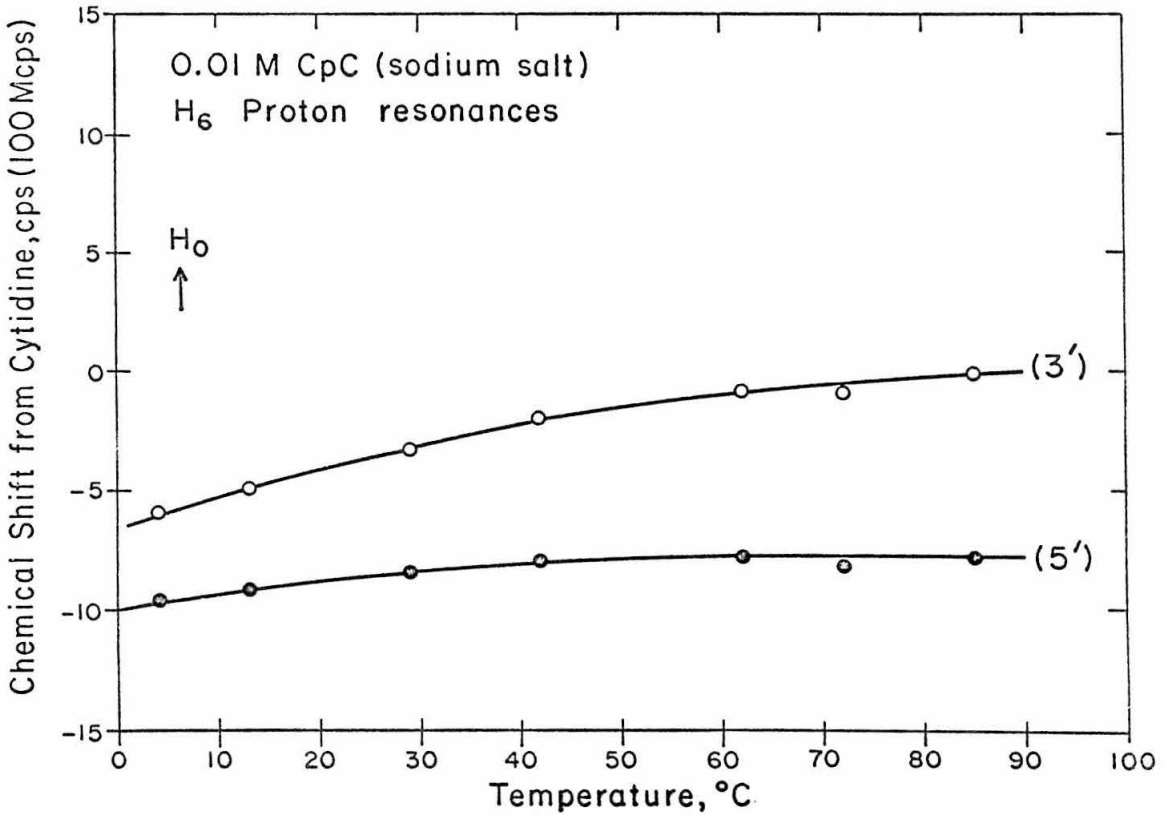
These observations can be rationalized rather neatly on the basis of the decrease in the intramolecular stacking interaction in CpC

TABLE XVI. Chemical shifts of 0. 01 M CpC proton resonances with reference to 0. 03 M cytidine at the same temperature.

Temperature	H_5 (avg.)	$H_{1'}(3')$	$H_{1'}(5')$
27°C	+ 0. 086 ppm	+ 0. 095 ppm	+ 0. 006 ppm
46°	+ 0. 081	+ 0. 064	- 0. 011
72°	+ 0. 041	+0. 025	- 0. 039

FIGURE 31

Chemical shifts (at 100 Mcps) of the H_6 protons of CpC relative to H_6 of 0. 03 M cytidine as a function of temperature.


0.01 M CpC (sodium salt)
H6 Proton resonances
Ho
(3′)
(5′)
Chemical Shift from Cytidine, cps (100 Mcps)
Temperature, °C
15
10
5
0
-5
-10
-15
0
10
20
30
40
50
60
70
80
90
100

with increasing temperature, in terms of the various magnetic anisotropies (cytosine base, 2-keto group, furanose ether oxygen) discussed in Section 2. 1. The ~4. 5 cps shift of the averaged H_5 resonances to lower field with increasing temperature could be a result of a decrease in the exposure of these protons to the magnetic anisotropy of the adjacent cytosine base as the intramolecular C-C stack is disrupted. A greater freedom of rotation of the cytosine bases relative to their ribose rings about the glycosidic bonds could contribute to shifts of the H_6 resonances to higher fields and of the $H_{1'}$ resonances to lower fields with increasing temperature. As the intramolecular base-stacking interaction is disrupted, the cytosine bases would no longer be restricted to conformations in which the H_6 protons and the furanose ether oxygen atoms ($O_{1'}$) are nearly eclipsed; thus, on the average, the H_6 proton would be further from the $O_{1'}$ atom and the $H_{1'}$ proton would be closer to the 2-keto group. Since these groups deshield protons in their vicinities, [94] the H_6 resonances would be expected to shift upfield, and the $H_{1'}$ resonances downfield, with a decrease in intramolecular base-stacking in CpC. The increase in the ribose coupling constant $|J_{H_{1'}-H_{2'}}|$ with temperature indicates that the $H_{1'}$ proton would also be situated further from the 2'-OH group as the dinucleotide is destacked. Since proximity of $H_{1'}$ to 2'-OH results in a shielding of the $H_{1'}$ proton, the observed change in ribose conformation with increasing temperature could also contribute to the downfield shift of the $H_{1'}$ resonances.

5. 3. Purine Binding Studies

The results of the study of the binding of purine to the deoxyribose dinucleoside monophosphates TpT, TpdU and dUpT reported in Section 4 suggested that further investigations of the interaction of this base with other dinucleotides would be of interest both from the standpoint of probing the intramolecular base-stacking interaction in the dinucleotide and with regard to further defining the nature of the proposed purine-dinucleotide intercalated complex. For this reason, the interaction of purine with CpC was studied in considerable detail.

5. 3. 1. Low CpC concentration-0. 010 M

In view of the extensive intermolecular self-association demonstrated for ApC and CpA (Section 2. 2) and the evidence that CpC self-associates as well (Section 5. 1), it was deemed advisable to investigate the purine-CpC interaction at a low dinucleotide concentration to minimize the complexities of CpC dimerization which would tend to compete with the purine binding processes. Accordingly, the effect of added purine on the 100 Mcps pmr spectrum of a 0. 010 M solution of the sodium salt of CpC was investigated. The resonances of the six monitored cytidine protons of CpC were all shifted to higher fields as purine was added, and these purine-induced shifts are summarized in Table XVII. The H_6 resonance at higher field (-8. 308 ppm in the absence of purine--see Figure 29) is shifted more than the lower field H_6 resonance as purine is added. By analogy with

TABLE XVII. Purine-induced shifts of the proton resonances of 0.010 M CpC (sodium salt), ppm. (Measurements made at 100 Mcps.)

Purine Conc.	Purine-Induced Shift					
	H_6		H_5		$H_{1'}$	
	(3′)	(5′)	(3′)	(5′)	(3′)	(5′)
	ppm	ppm	ppm	ppm	ppm	ppm
0.05 M	0.040	0.030	0.042	0.031	0.022	0.021
0.10	0.078	0.062	0.082	0.059	0.036	0.036
0.15	0.100	0.078	0.123	0.091	0.060	0.062
0.20	0.120	0.095	0.146	0.110	0.072	0.074
0.25	0.144	0.114	0.172	0.132	0.081	0.081
0.30	0.162	0.128	0.191	0.144	0.088	0.091
0.40	0.188	0.152	0.223	0.177	0.104	0.111
0.50	0.216	0.179	0.263	0.208	0.127	0.137
0.60	0.238	0.196	0.281	0.225	0.129	0.143
0.80	0.276	0.233	0.327	0.262	0.148	0.165
1.00	0.305	0.260	0.361	0.294	0.165	0.186

the results of the TpdU-dUpT study of Section 4. 2 and the ApU-UpA study of Section 7.2, in which the base proton resonances of the 3′-nucleoside show the larger shifts, the CpC H_6 resonance at higher field is assigned to the 3′-nucleoside, as indicated in Figure 29. A plot of the purine-induced shifts for the H_6 resonances is shown in Figure 32. The linewidths of these resonances remain constant (at ~1. 0 cps) throughout the concentration range of added purine.

The H_5 resonances narrowed considerably as purine was added to the CpC solution, although some of this apparent narrowing may just be a consequence of the separation of the two overlapping H_5 doublets. The chemical shift difference between the two H_5 resonances was clearly resolved as 0. 023 ppm at 0. 05 M purine concentration, and the components of these resonances were as narrow as those for the H_6 resonances under the same conditions. From this observation, it was estimated that the chemical shift difference between the H_5 resonances in the absence of purine (unresolved in Figure 29) is about 0. 008 ppm. As was found for the H_6 protons, the H_5 resonance at higher field shows a greater upfield shift as purine is added. Again, by analogy with the TpdU-dUpT and ApU-UpA behavior, the H_5 resonance exhibiting the greater purine-induced shift is assigned to the 3′-nucleoside. The purine-induced shifts for the H_5 protons are shown in Figure 33. The linewidths of the H_5 resonances remain constant as the purine concentration is increased above 0. 05 M. The abrupt narrowing of the H_5 resonances with the first addition of purine to the CpC solution suggests that the binding of purine to the dinucleotide reduces the extent of the intermolecular self-association of CpC believed responsible for the H_5

FIGURE 32

Plot of the purine-induced shifts for the H_6 proton resonances of 0.010 M CpC (sodium salt), cps at 100 Mcps.

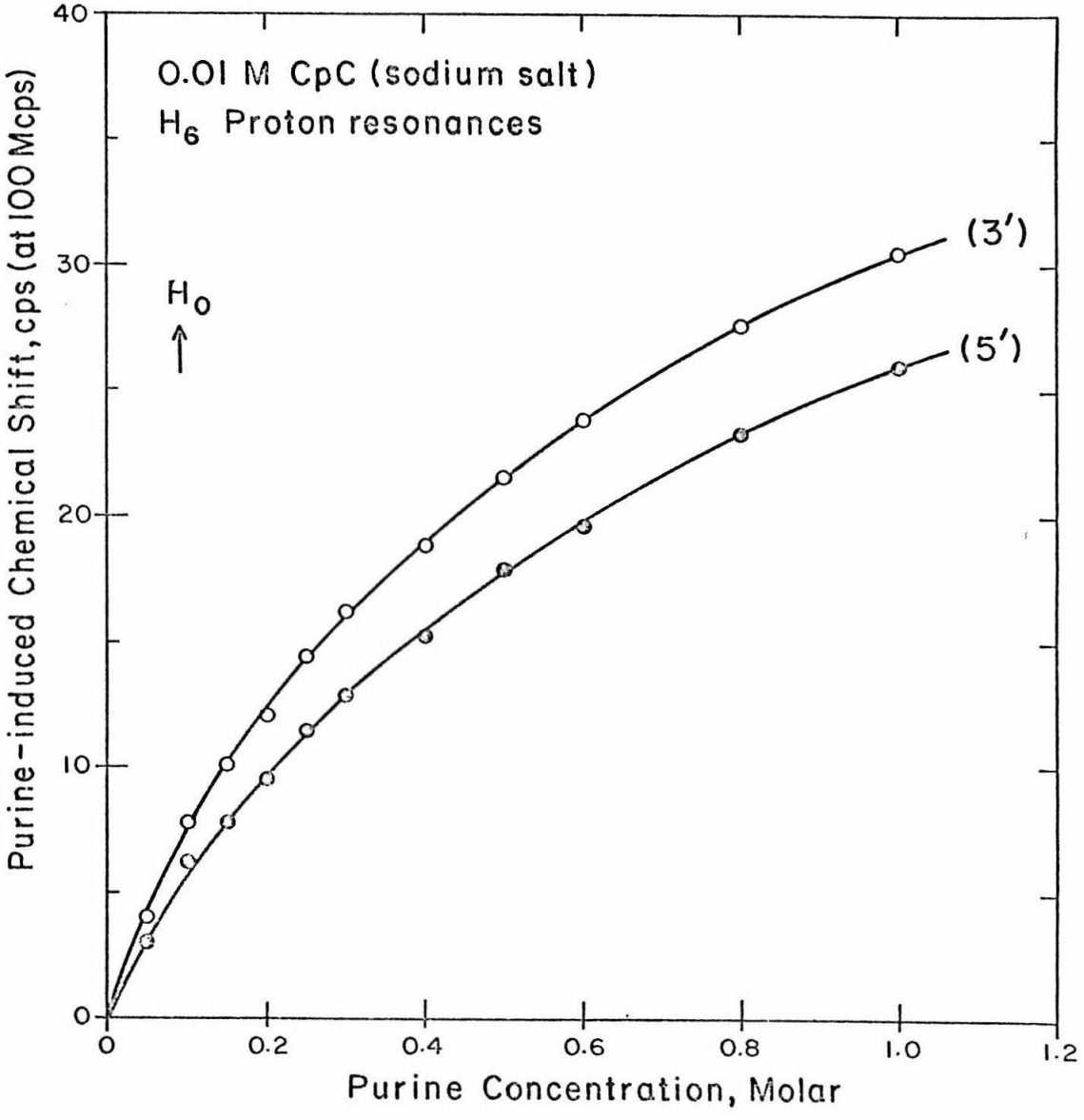
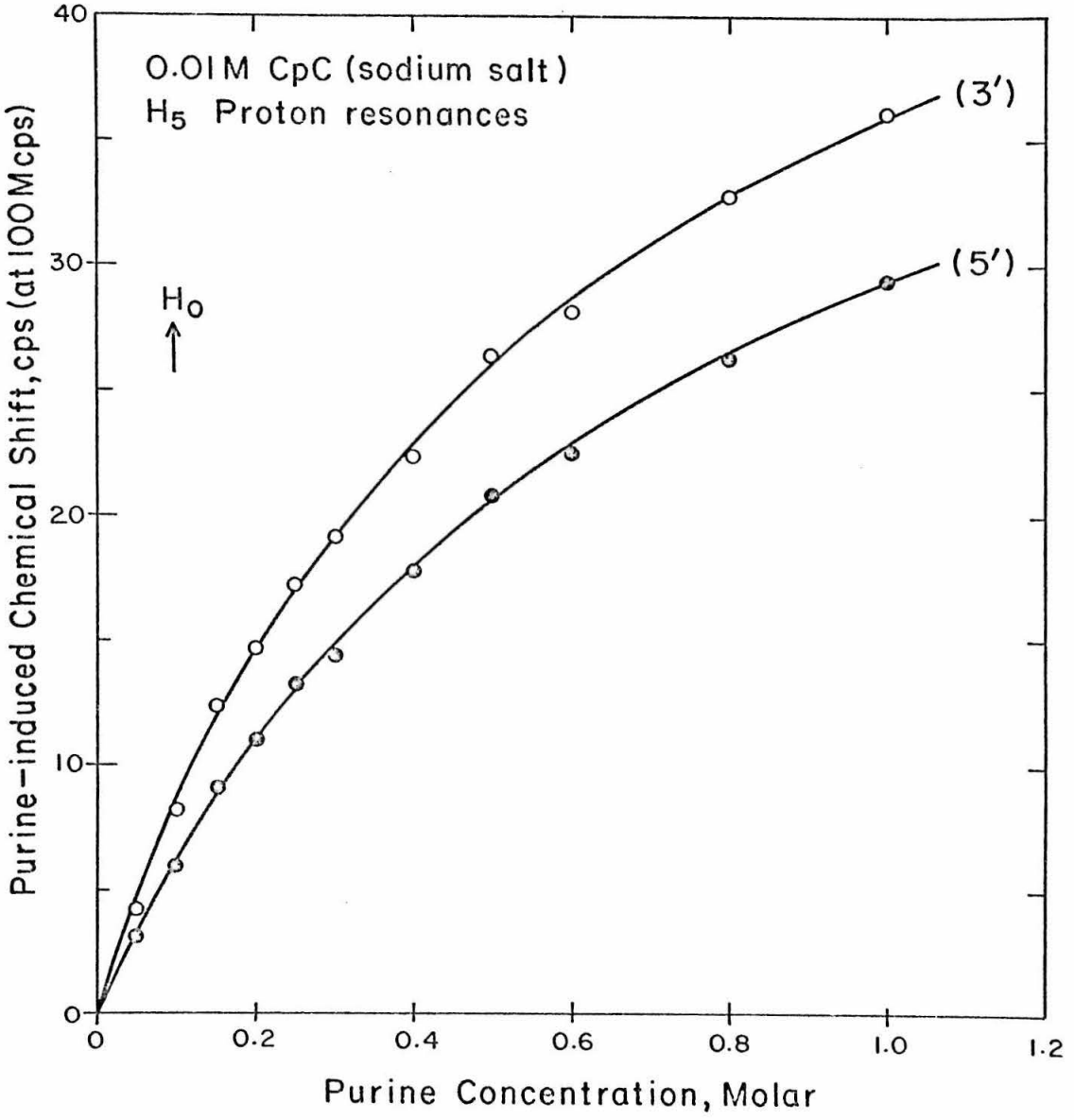

0.01 M CpC (sodium salt)
H_6 Proton resonances
H_0
(3′)
(5′)
Purine-induced Chemical Shift, cps (at 100 Mcps)
Purine Concentration, Molar
0
10
20
30
40
0
0.2
0.4
0.6
0.8
1.0
1.2

FIGURE 33

Plot of the purine-induced shifts for the H_5 proton resonances of 0. 010 M CpC (sodium salt), cps at 100 Mcps.


0.01 M CpC (sodium salt)
H5 Proton resonances
(3′)
(5′)
H0
Purine-induced Chemical Shift, cps (at 100 Mcps)
Purine Concentration, Molar
0
10
20
30
40
0
0.2
0.4
0.6
0.8
1.0
1.2

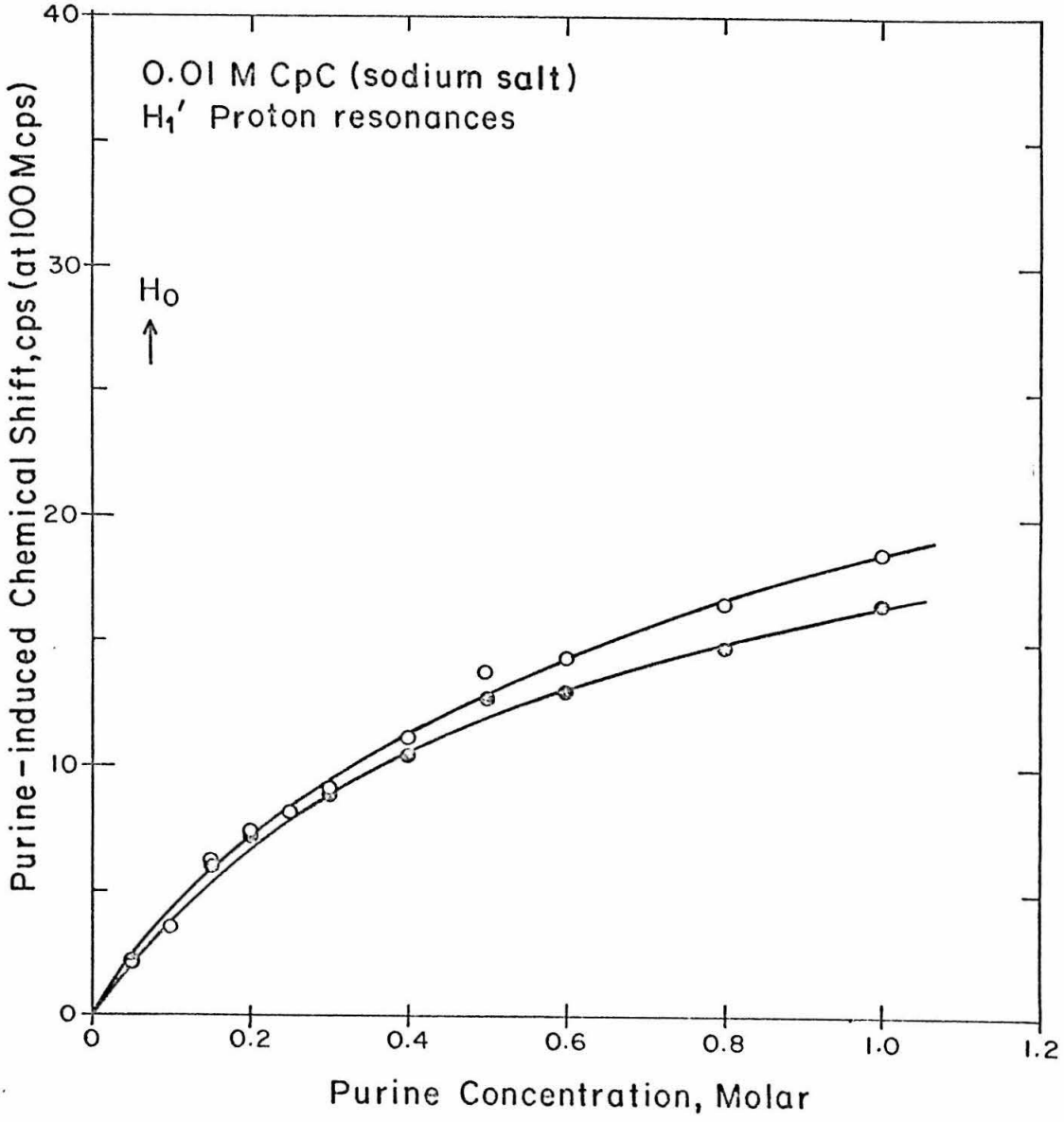
line broadening.

The resonances of the ribose $H_{1'}$ protons of CpC also experience purine-induced shifts to higher fields. The $H_{1'}$ resonance at lower field was shifted slightly more than the higher field resonance, and this observation, coupled with the results of the other purine-dinucleotide binding studies, contributed to the assignment of the lower field $H_{1'}$ resonance to the 5'-nucleoside as discussed in Section 5. 1. The line-widths of the $H_{1'}$ resonances do not change significantly as purine is added, and the purine-induced shifts for these protons are shown in Figure 34. Although accurate measurements of the coupling constants $|J_{H_{1'}-H_{2'}}|$ are not possible because the $H_{1'}$ doublets overlap the H_5 resonances, these coupling constants do appear to increase slightly as purine is added. This observation would indicate that a slight change in the ribose conformation (toward 2'-endo) accompanies the binding of purine to the dinucleotide (see discussion in Section 2. 3. 1). This behavior of the $H_{1'}$-$H_{2'}$ coupling constants was also noted by Chan and Nelson[56] in their study of the ribose dinucleoside monophosphate ApA.

A comparison between the purine-induced shifts for the CpC proton resonances reported here and those for the cytidine nucleoside found by Schweizer, Chan, and Ts'o[28] indicates that purine interacts with both faces of each cytosine base in CpC to an appreciable extent. At 1. 0 M purine concentration, the H_6 resonances of 0. 01 M CpC are shifted ~83% as much as is H_6 of 0. 11 M cytidine. The H_5 and $H_{1'}$ protons are shifted ~71% and ~76% of the corresponding shifts for cytidine respectively. As discussed by Schweizer, et al., in

FIGURE 34

Plot of the purine-induced shifts for the $H_{1'}$ proton resonances of 0.010 M CpC (sodium salt), cps at 100 Mcps; $H_{1'}$ (3′) ●, $H_{1'}$ (5′) ○.


0.01 M CpC (sodium salt)
H1' Proton resonances
Ho
Purine-induced Chemical Shift, cps (at 100 Mcps)
Purine Concentration, Molar
0
10
20
30
40
0
0.2
0.4
0.6
0.8
1.0
1.2

connection with the purine-nucleoside binding study,[28] the order of the purine induced shifts ($H_5 > H_6 > H_{1'}$) probably reflects steric factors controlling the exposure of a particular base proton to the ring-current magnetic anisotropy of an adjacent purine molecule.

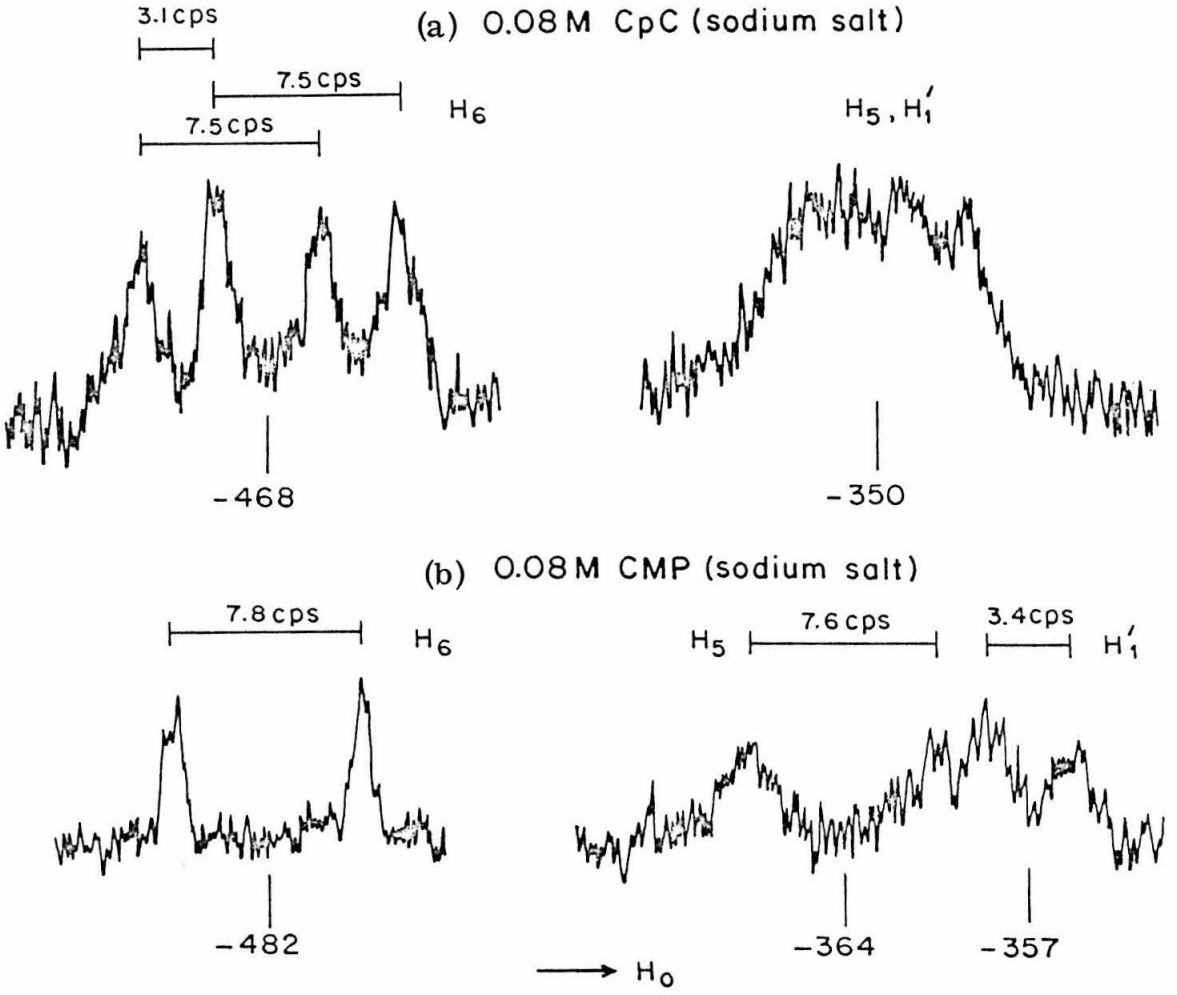
In this study of the interaction of purine with 0. 010 M CpC, it was observed that the purine proton resonances are slightly broadened at low purine concentrations, and that the resonances narrow as the purine concentration is increased. This is believed to result from formation of a purine-CpC intercalated complex as proposed in Section 4. At so low a dinucleotide concentration as 0. 010 M, only a very small fraction of the purine present in the solution would be incorporated in complexes with the dinucleotide. In order to observe the effects of this complex formation on the purine proton resonances, it is necessary to study purine-dinucleotide solutions with a relatively high dinucleotide concentration, so that an appreciable fraction of the purine is bound. Thus the purine-binding study at low CpC concentration discussed here and the studies at higher CpC concentration reported in Sections 5. 3. 2 and 5. 3. 3 complement one another, in that the effects of this interaction on the CpC resonances are best observed at a high purine/CpC concentration ratio while the effects on the purine resonances are most evident at a low purine/CpC concentration ratio.

5. 3. 2. 0. 08 M CpC - sodium salt

The 60 Mcps pmr spectrum of a 0. 08 M solution of the sodium salt of CpC (pD 7. 50) in the regions of the cytosine H_6 and H_5 and ribose $H_{1'}$ proton resonances is shown in Figure 35. Tracings of the same

FIGURE 35

Pmr spectra at 60 Mcps of: (a) 0.08 M CpC (sodium salt), and (b) 0.08 M cytidine-5′-monophosphate (sodium salt) in the regions of the H_6, H_5, and $H_{1'}$ proton resonances. Chemical shifts given in cps from external TMS.


3.1 cps
(a) 0.08 M CpC (sodium salt)
7.5 cps
7.5 cps
H6
H5 , H1′
-468
-350
(b) 0.08 M CMP (sodium salt)
7.8 cps
H6
H5
7.6 cps
3.4 cps
H1′
-482
-364
-357
Ho

spectral regions under similar conditions for a 0. 08 M solution of the sodium salt of 5′-CMP (pD = 7. 80) are included for comparison. The chemical shift reported for the H_6 protons of CpC is the mean of the chemical shifts for the H_6 doublet of the 3′-nucleoside (-466. 5 cps) and that of the 5′-nucleoside (-469. 5 cps). The chemical shift difference between the two H_6 protons of CpC (3. 1 cps at 60 Mcps = 0. 052 ppm) is essentially identical with that found for a 0. 010 M CpC solution at 100 Mcps (see Figure 29). The H_5 resonances of CpC are so broad as to be unobservable at this dinucleotide concentration, as discussed in Section 5. 1. The $H_{1'}$ resonances are also rather broad at this CpC concentration, and since the chemical shift difference between these protons is expected to be only ~ 5. 4 cps at 60 Mcps, they overlap one another so that the separate components of the two doublets are not resolved. The chemical shift reported here (-350 cps) represents the mean value for the $H_{1'}(3')$ and $H_{1'}(5')$ protons.

It should be noted that the rather broad resonances observed for the H_5 and $H_{1'}$ protons of 5′-CMP are believed to be a consequence of the overlap between the titration regions for the cytosine base and the phosphate group, [100] and unrelated to the broadening of the H_5 resonances of CpC observed at higher dinucleotide concentrations. The H_5 and $H_{1'}$ resonances of cytidine-5′-monophosphate are broad at pD values at which either the cytosine base or the phosphate group (or both) are partially protonated, and quite narrow at higher or lower solution pD. Since this behavior is absent with the dinucleotides (because the phosphate group is doubly esterified, having a pK below ~ 1. 0), it will not be discussed further.

The addition of purine to the CpC solution causes the monitored cytidine proton resonances to be shifted to higher fields as a consequence of the purine-CpC base-stacking interaction. The mean purine-induced shifts for both H_6 protons and for both $H_{1'}$ protons are reported in Table XVIII. The $H_6(3')$ doublet exhibits a greater purine-induced shift than the $H_6(5')$ doublet, in accord with the results of Section 5. 3. 1, and Figure 36 shows the H_6 resonances at several concentrations of added purine. A plot of the purine-induced shifts for the individual H_6 doublets is given in Figure 37, and the purine-induced chemical shift difference for the H_6 protons is plotted in Figure 38. Since the $H_{1'}$ resonance at lower field, $H_{1'}(5')$, is expected to have a larger purine-induced shift than $H_{1'}(3')$ with increasing purine concentration (see Section 5. 3. 1), the two $H_{1'}$ doublets move closer together as purine is added and it is not possible to resolve them. The cytosine H_5 proton resonances of CpC remain broad beyond detection by high-resolution techniques throughout the concentration range of added purine, presumably because the extensive intermolecular self-association of CpC is not entirely disrupted by competition from the formation of the purine-CpC complexes. A comparison of the purine-induced shifts for the H_6 and $H_{1'}$ protons between Tables XVII and XVIII indicates that, at a given purine concentration, the shifts for the 0. 08 M CpC solution are slightly smaller than those for the 0. 010 M CpC solution. This is to be expected, since the fraction of CpC involved in complexes with purine is smaller at the higher dinucleotide concentration.

TABLE XVIII. Mean purine-induced shifts of the H_6 and $H_{1'}$ proton resonances of 0. 08 M CpC (sodium salt); cps at 60 Mcps, and ppm. The purine induced chemical shift difference (splitting) for the H_6 protons is also given.

Purine Conc.	H_6 Resonances Shift		Splitting	$H_{1'}$ Resonances Shift	
	cps	ppm	cps	cps	ppm
0. 08 M	2. 5	0. 04	0. 7	2. 0	0. 03
0. 16	5. 0	0. 08	1. 1	3. 5	0. 06
0. 24	7. 0	0. 12	1. 3	5. 0	0. 08
0. 32	8. 5	0. 14	1. 5	5. 5	0. 09
0. 40	9. 5	0. 16	1. 7	6. 5	0. 11
0. 48	11. 0	0. 18	1. 9	7. 0	0. 12
0. 64	13. 0	0. 22	2. 1	9. 5	0. 16
0. 80	14. 5	0. 24	2. 2	10. 0	0. 17
0. 96	15. 5	0. 26	2. 3	10. 5	0. 18
1. 28	18. 0	0. 30	2. 4	11. 5	0. 19
1. 60	19. 5	0. 32	2. 5	12. 5	0. 21

FIGURE 36

Tracings of the H_6 proton resonances of 0. 08 M CpC (sodium salt) at several concentrations of added purine: (a) 0. 16 M purine, (b) 0. 48 M purine, (c) 0. 80 M purine, (d) 1. 28 M purine. Chemical shifts given in cps from external TMS.

Cytosine H_6 Resonances — 0.08 M CpC (sodium salt)

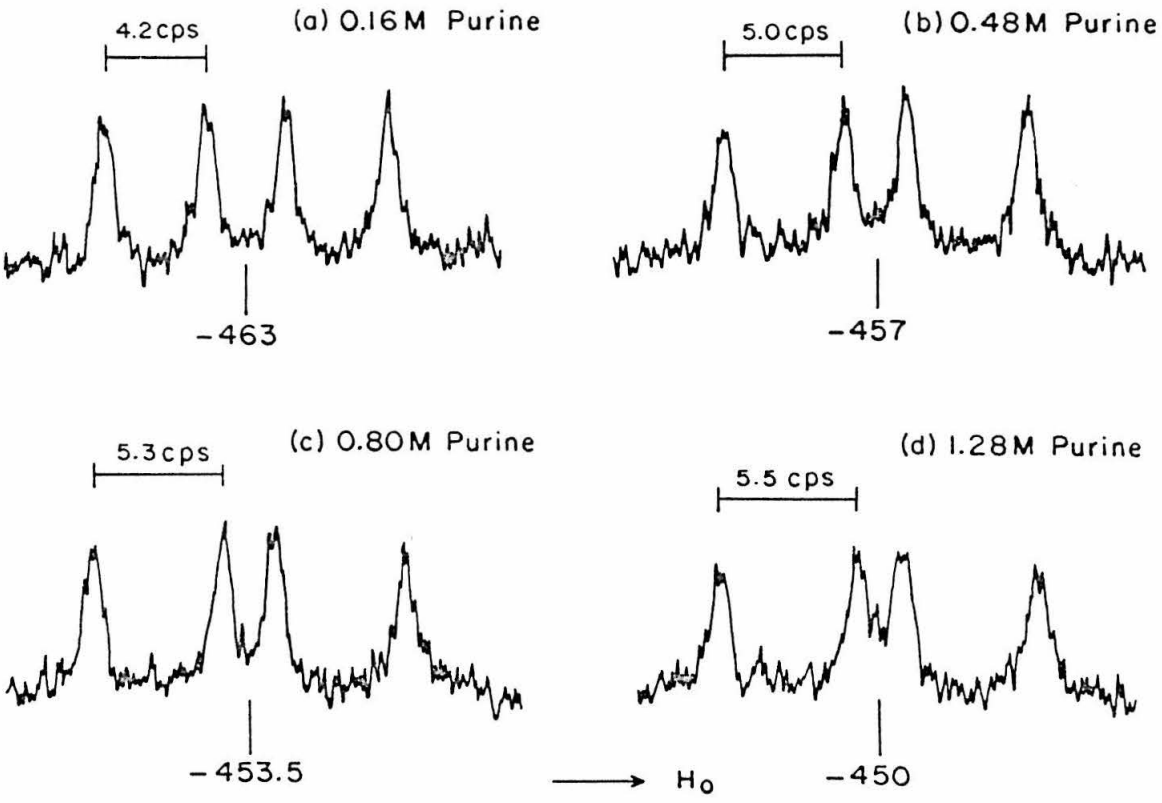


FIGURE 37

Plot of the purine-induced shifts for the H_6 proton resonances of 0. 08 M CpC (sodium salt), cps at 60 Mcps.

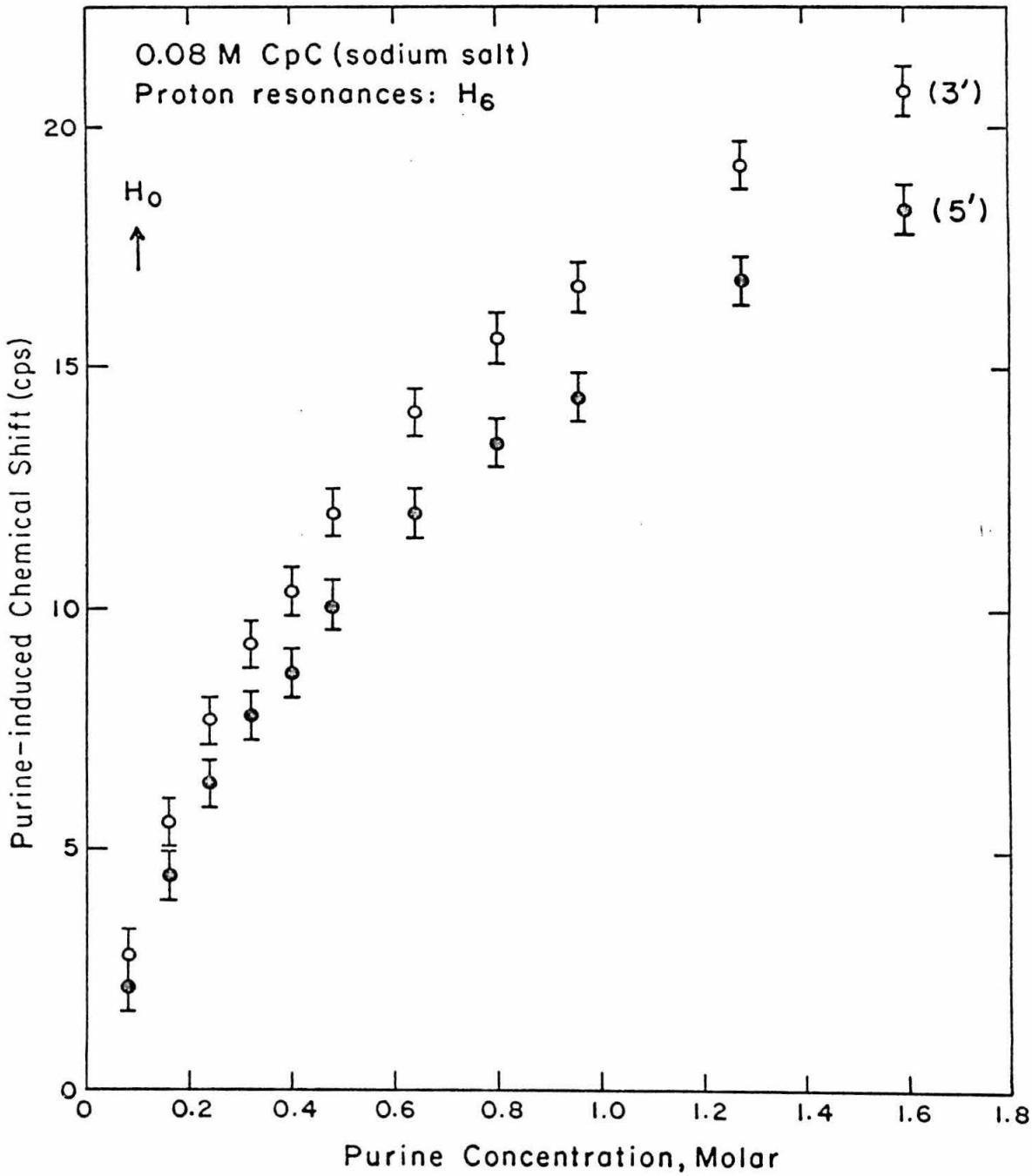
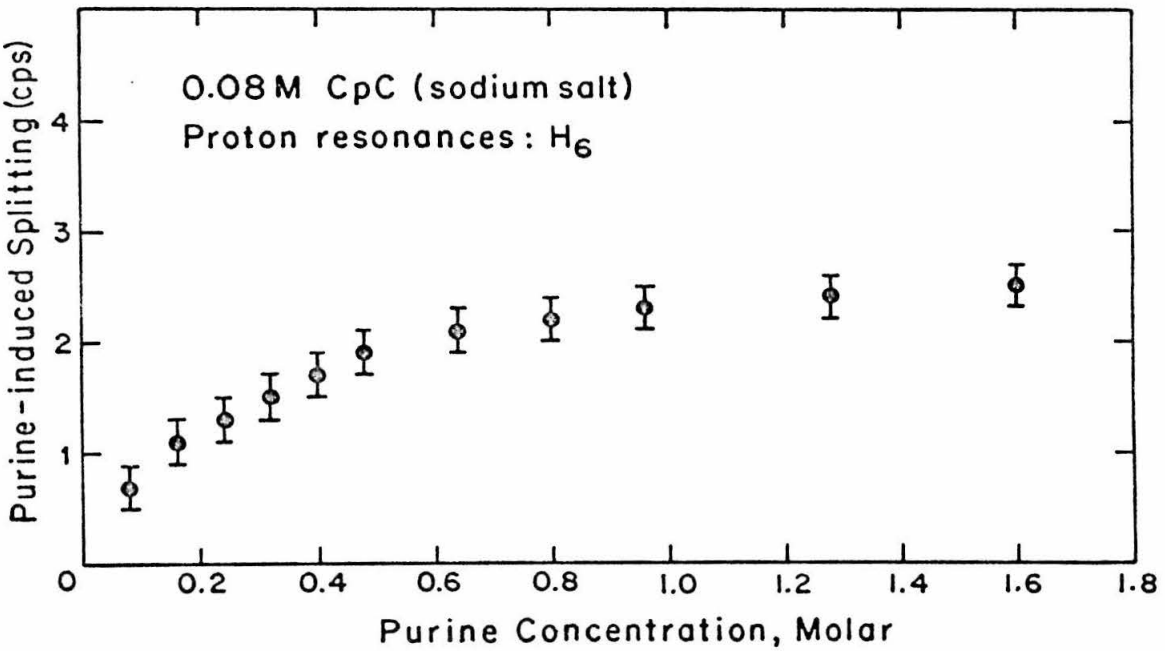

0.08 M CpC (sodium salt)
Proton resonances: H6
(3')
(5')
H0
Purine-induced Chemical Shift (cps)
Purine Concentration, Molar
0
5
10
15
20
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8

FIGURE 38

Plot of the purine-induced chemical shift difference (splitting) between the two H_6 protons of 0. 08 M CpC (sodium salt), cps at 60 Mcps.


0.08 M CpC (sodium salt)
Proton resonances: H_6
Purine-induced Splitting (cps)
0
1
2
3
4
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Purine Concentration, Molar

The purine proton resonances are markedly broadened in the presence of 0. 08 M CpC at pD = 7. 50. As discussed in Section 5. 3. 1, the purine line broadening is more pronounced at higher CpC concentration since a larger fraction of the purine is involved in complex formation with the dinucleotide. The linewidths of the purine proton resonances are given in Table XIX. At the lowest purine concentration studied, 0. 08 M, the H_6 and H_8 resonances are too broad to be measured accurately; these resonances also overlap that of the H_2 proton. Tracings of the purine spectrum in the presence of 0. 08 M CpC (sodium salt) at several purine concentrations are shown in Figure 39. It is evident from these data that the specificity of the purine line broadening phenomenon here is the same as in the studies of the interaction of purine with the deoxyribose dinucleoside monophosphates TpdU, TpT, and dUpT reported in Section 4; namely, H_6 is affected more than H_8, with the H_2 resonance broadened the least. At a given purine concentration, the purine resonances are broader in the presence of CpC than in the presence of the deoxyribose dinucleotides. Since the concentrations of TpT, TpdU, and dUpT were about double that of CpC, the intrinsic purine linewidths in the purine-dinucleotide intercalated complex must be considerably greater with CpC than with the deoxyribose dinucleotides studied. This aspect is discussed more fully in Section 8.

5. 3. 3. 0. 08 M CpC - ammonium salt

The interaction of purine with the ammonium salt of CpC was also investigated at 60 Mcps. As mentioned in Section 4, a solution

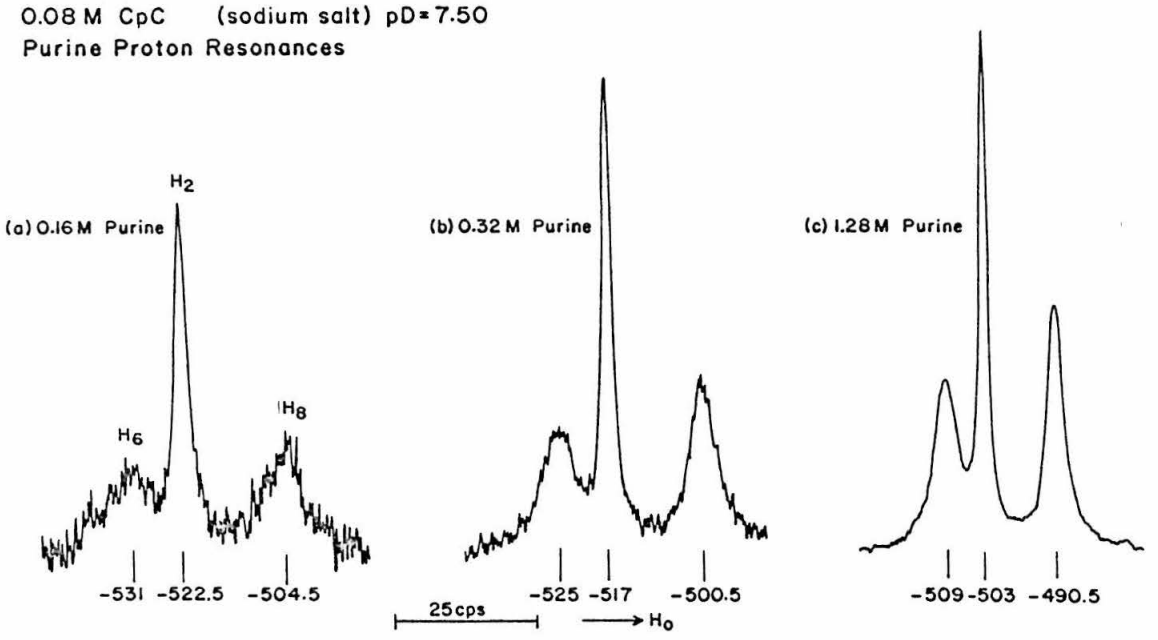
TABLE XIX. Purine proton resonance linewidths in the presence of 0.08 M CpC (sodium salt, pD = 7.50). (Full linewidth at half-height in cps, measured at 60 Mcps).

Purine Conc.	Purine Resonance Linewidths		
	H_6	H_2	H_8
0.08 M	--[a]	~5 cps	--[a]
0.16	~12 cps	3.0	~9 cps
0.24	~10	2.2	6.5
0.32	8	2.0	6.0
0.40	7	2.0	5.2
0.48	7.0	1.7	4.2
0.64	6.0	1.6	3.9
0.80	5.5	1.5	3.6
0.96	5.1	1.5	3.5
1.28	5.2	1.6	3.5
1.60	5.9	1.9	3.8

[a]Too broad to measure accurately.

FIGURE 39

Pmr spectrum of purine at 60 Mcps in the presence of 0.08 M CpC (sodium salt): (a) 0.16 M purine, (b) 0.32 M purine, (c) 1.28 M purine. Chemical shifts given in cps from external TMS.


0.08 M CpC (sodium salt) pD = 7.50
Purine Proton Resonances
(a) 0.16 M Purine
H2
H6
H8
-531 -522.5 -504.5
(b) 0.32 M Purine
-525 -517 -500.5
25 cps
Ho
(c) 1.28 M Purine
-509 -503 -490.5

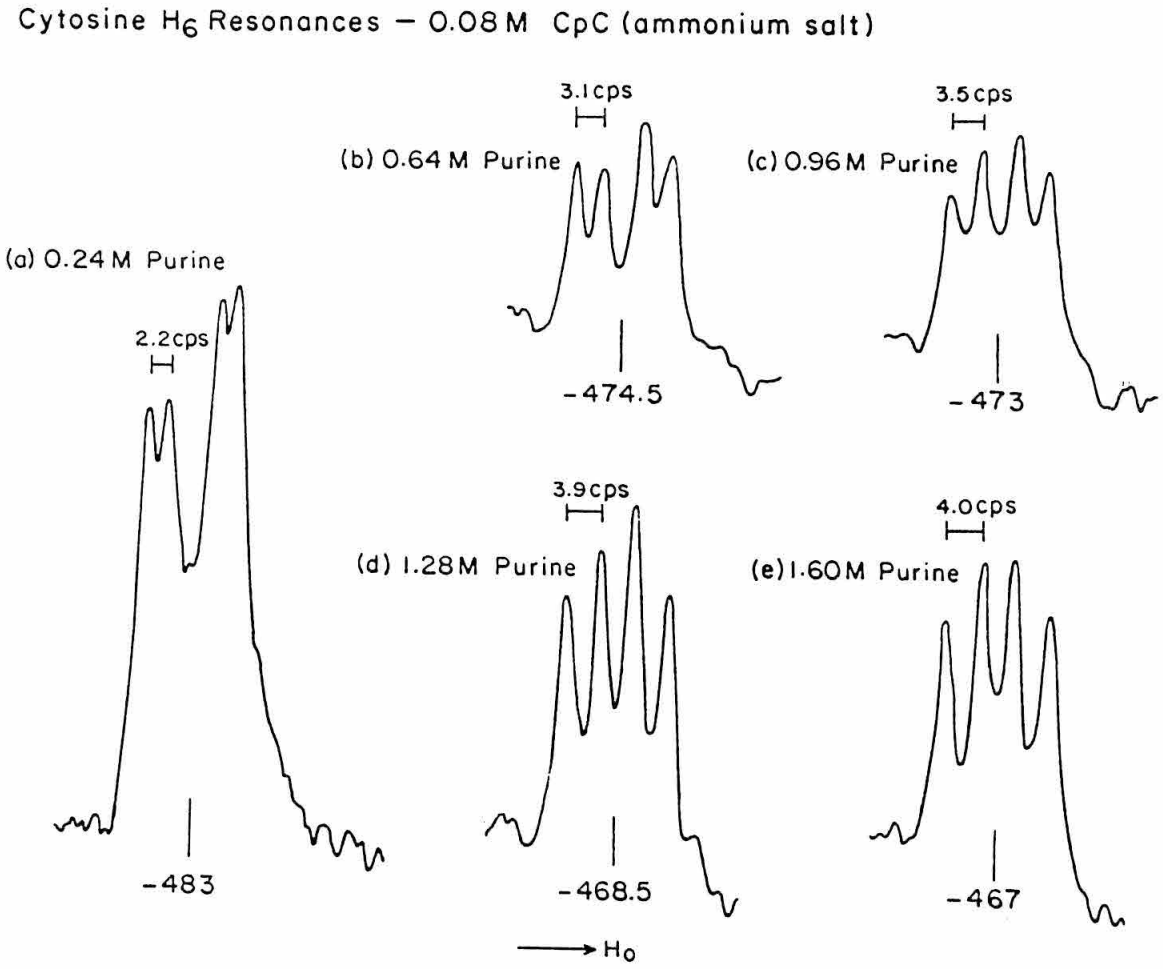
of the ammonium salt of a dinucleoside monophosphate has a pD of about 5 as a result of the acidic hydrolysis of the NH_4^+ ion. Since the cytosine base has a pK of about 4. 3, [98] a fraction of the bases of CpC will be protonated in a solution of the ammonium salt of this dinucleotide in the absence of a buffer system operative above pD 6 or so. This was the case in the CpC (ammonium salt)--purine system studied here, and the occurrence of protonated CpC molecules has been shown to have a profound effect on the purine-CpC intercalated complex.

The 60 Mcps pmr spectrum of the ammonium salt of CpC is quite similar to that of the sodium salt at 0. 08 M dinucleotide concentration. The resonances here are somewhat broader, however, and the chemical shift difference between the two H_6 protons is smaller and could not be resolved. In addition, the H_6 proton resonances are at lower fields in this solution (-486 cps) than with the sodium salt (-468 cps) as a consequence of the protonation of the cytosine base. The H_5 resonances are broad beyond detection and, as with the sodium salt, it was not possible to resolve the $H_{1'}$ doublets of the 3′- and 5′-esterified nucleosides.

The addition of purine to this solution causes the monitored H_6 and $H_{1'}$ proton resonances to be shifted to higher fields, and the purine-induced shifts are comparable to those reported in Table XVIII for the sodium salt study. The H_6 resonances became narrower as purine was added, and the chemical shift difference between the $H_6(3')$ and $H_6(5')$ doublets was resolved, as shown in Figure 40. It can be seen, by comparison of this Figure with Figures 35 and 36, that the

FIGURE 40

Time-averaged H_6 proton resonances of 0. 08 M CpC (ammonium salt) at several concentrations of added purine: (a) 0. 24 M purine, 14 scans; (b) 0. 64 M purine, 10 scans; (c) 0. 96 M purine, 19 scans; (d) 1. 28 M purine, 21 scans; (e) 1. 60 M purine, 21 scans. Chemical shifts at 60 Mcps given in cps from external TMS.


Cytosine H_6 Resonances – 0.08 M CpC (ammonium salt)
(a) 0.24 M Purine
2.2cps
-483
(b) 0.64 M Purine
3.1cps
-474.5
(c) 0.96 M Purine
3.5cps
-473
(d) 1.28 M Purine
3.9cps
-468.5
(e) 1.60 M Purine
4.0cps
-467
H_o

chemical shift difference between the two H_6 protons is quite a bit smaller for the ammonium salt than for the sodium salt of CpC at the same purine concentration. The results of the study of the effect of pD on the purine-CpC interaction to be described below suggest that this is a consequence of the partial protonation of the cytosine bases in the solution of the ammonium salt.

The most pronounced difference between the interactions of the sodium and ammonium salts of CpC with purine is in the effect of the dinucleotide upon the linewidths of the purine resonances. The purine line broadening phenomenon is much more severe for the ammonium salt than for the sodium salt, with the purine spectrum appearing as a single broad peak at purine concentrations below 0. 24 M. The measured (or estimated) purine proton resonance linewidths in the presence of 0. 08 M CpC (NH_4^+) are listed in Table XX and several tracings of the purine spectrum are shown in Figure 41. At 0. 24 M purine concentration, the H_2 resonance is prominent, with the H_8 resonance apparent as a shoulder upfield from H_2. The H_6 resonance in this solution contributes to the tailing of the H_2 resonance to lower field. As the purine concentration is increased, the three purine resonances narrow as a consequence of averaging of the resonances for bound and free purine. However, even at the highest purine concentration studied (1. 60 M), where the purine/dinucleotide concentration ratio is 20/1, the H_6 resonance of purine only appears as a shoulder on the low field side of the H_2 resonance. It is clear that the linewidths reported for the purine resonances in Table XX are quite approximate, and that those for the H_6 resonance are gross estimations. An effort

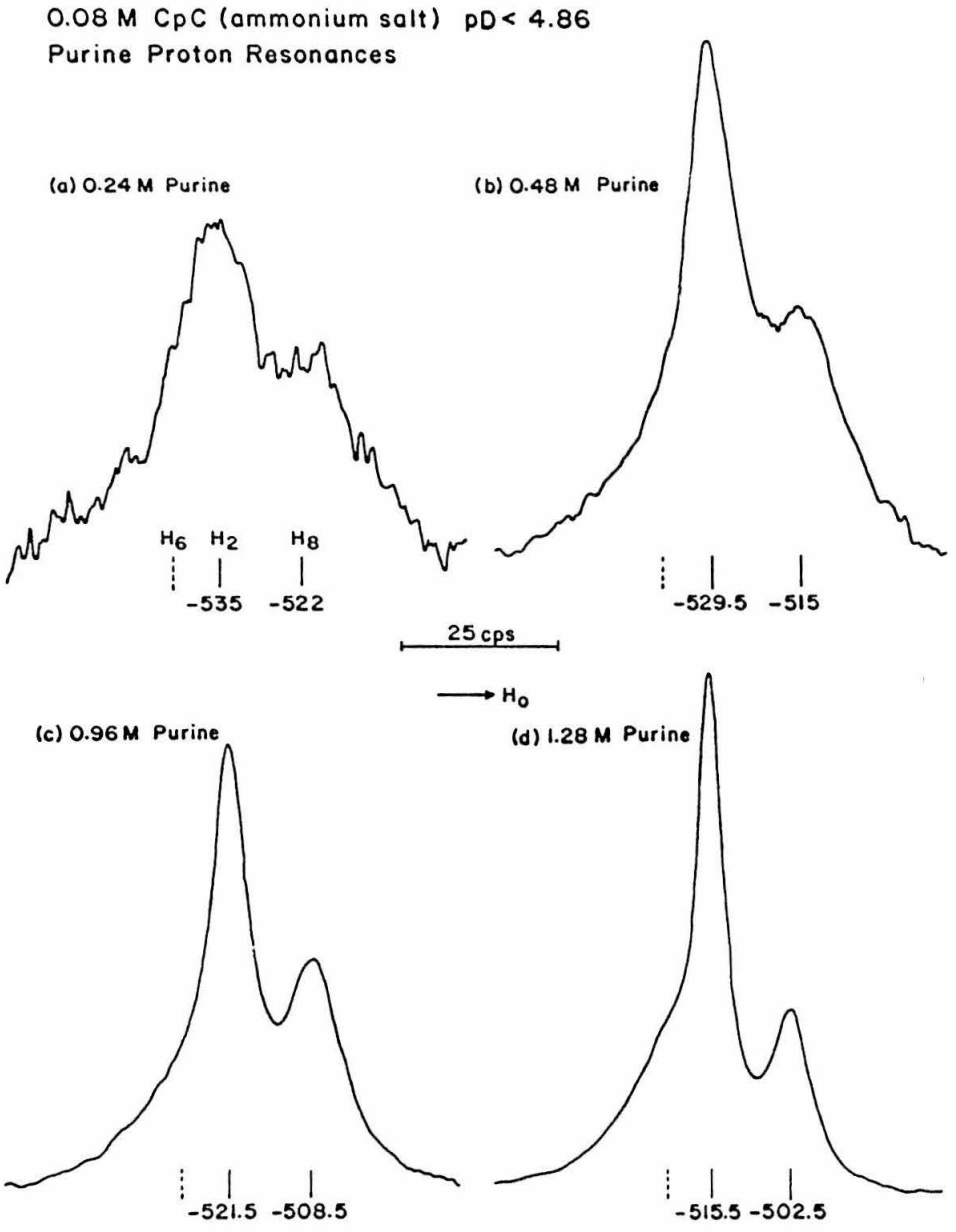
TABLE XX. Purine proton resonance linewidths in the presence of 0.08 M CpC (ammonium salt, pD < 4.86). (Full linewidth at half-height in cps, measured at 60 Mcps).

Purine Conc.	Purine Resonance Linewidths H_6	H_2	H_8
0.24 M	--[a]	~18 cps	~23 cps
0.32	--	~14	~27
0.40	--	~14	~22
0.48	--	10	18
0.64	--	11	15.5
0.80	~16 cps	7.6	13.1
0.96	~15	8.6	12.1
1.28	~14	6.1	8.1
1.60	~12	5.5	7.2

[a]Too broad to even be estimated.

FIGURE 41

Time-averaged pmr spectra of purine at 60 Mcps in the presence of 0. 08 M CpC (ammonium salt): (a) 0. 24 M purine, 14 scans; (b) 0. 48 M purine, 24 scans; (c) 0. 96 M purine, 15 scans; (d) 1. 28 M purine, 21 scans. Chemical shifts given in cps from external TMS.


0.08 M CpC (ammonium salt) pD< 4.86
Purine Proton Resonances
(a) 0.24 M Purine
(b) 0.48 M Purine
H6 H2 H8
-535 -522
-529.5 -515
25 cps
Ho
(c) 0.96 M Purine
(d) 1.28 M Purine
-521.5 -508.5
-515.5 -502.5

was made to underestimate the purine line widths, so the actual linewidths may be somewhat larger than the values given here.

It can be concluded from these results that either the intrinsic linewidths of the purine proton resonances in the purine-CpC intercalated complex are greater in this system than for the sodium salt at pD 7.50 as reported in Section 5.3.2, or a much larger fraction of the purine is incorporated in the complex at the lower pD of the ammonium salt solution. Since the purine-induced chemical shift difference between the H_6 resonances (which is believed to be an indication of the extent of involvement of the dinucleotide in the intercalated complex) is comparable for the two systems, the former interpretation is preferred.

Temperature Dependence. -- The effect of temperature on this CpC-purine interaction was investigated by examining the 0.08 M CpC (ammonium salt) solution containing 0.96 M purine at three temperatures. As shown in Table XXI, the three purine proton resonances in this system narrow considerably as the temperature is increased. This is probably a result of two factors. Since the enthalpy of formation of the purine-dinucleotide intercalated complex must be negative, an increase in temperature reduces the fraction of purine bound to CpC. In addition, the correlation time for the motion of an intercalated purine molecule relative to the dinucleotide is shorter at higher temperatures; consequently the intrinsic linewidths for the proton resonances of the incorporated purine molecule become smaller as the temperature is increased.

TABLE XXI. Effect of temperature on the purine proton resonance linewidths in a 0. 08 M CpC (ammonium salt) solution containing 0. 96 M purine (measured at 60 Mcps).

Temperature	Purine Resonance Linewidths		
	H_6	H_2	H_8
33°C	~20 cps	7. 7 cps	~14 cps
58°	14. 5	5. 0	7. 5
78°	10. 0	3. 4	5. 0

Effect of pD on the Purine-CpC Interaction. -- The pD of the 0. 08 M CpC (ammonium salt)--1. 60 M purine solution was measured at the conclusion of the purine binding study and found to be 4. 86. It is estimated that about 25% of the cytosine bases (pK 4. 3) are protonated in this solution, and that an insignificant fraction of the purine molecules (pK 2. 4) are protonated. The effect of pD on the purine-CpC interaction was further investigated by lowering the pD of this solution by addition of concentrated HCl. The results of these experiments for the cytosine H_6 and H_5 and purine proton resonances are depicted in Figures 42-44. The H_6 resonances shift to lower fields with decreasing pD, with the $H_6(3')$ resonance showing a greater downfield shift than the $H_6(5')$ resonance. The $H_6(3')$ resonance is at higher field than $H_6(5')$ at higher pD values, the two doublets are nearly superimposed at pD 3. 38 (Figure 42b), and $H_6(3')$ appears at lower fields than $H_6(5')$ below pD $\sim$ 2. 9 (Figures 42c, d, and e). The H_5 proton resonances of CpC appear as a broad absorption at pD 4. 00, and they narrow considerably as they shift downfield with decreasing pD. There also appears to be a chemical shift difference between the H_5 doublets of $\sim$ 2. 6 cps at pD 3. 38 (Figure 43b) and $\sim$ 2 cps at pD 2. 87 (Figure 43c), with the two resonances essentially superimposed at pD 0. 87 as shown in Figure 43e. This narrowing of the H_5 resonances is felt to result from a decrease in the intermolecular CpC self-association as the cytosine bases are protonated. The average chemical shifts and the apparent "splittings" of the cytosine H_6 and H_5 protons, as well as the average $H_{1'}$ chemical shift, are listed at several values of pD in

FIGURE 42

Effect of pD on the cytosine H_6 resonances in a solution of 0. 08 M CpC (ammonium salt) and 1. 60 M purine: (a) pD = 4. 00, (b) pD = 3. 38, (c) pD = 2. 87, (d) pD = 2. 30, (e) pD = 0. 87.

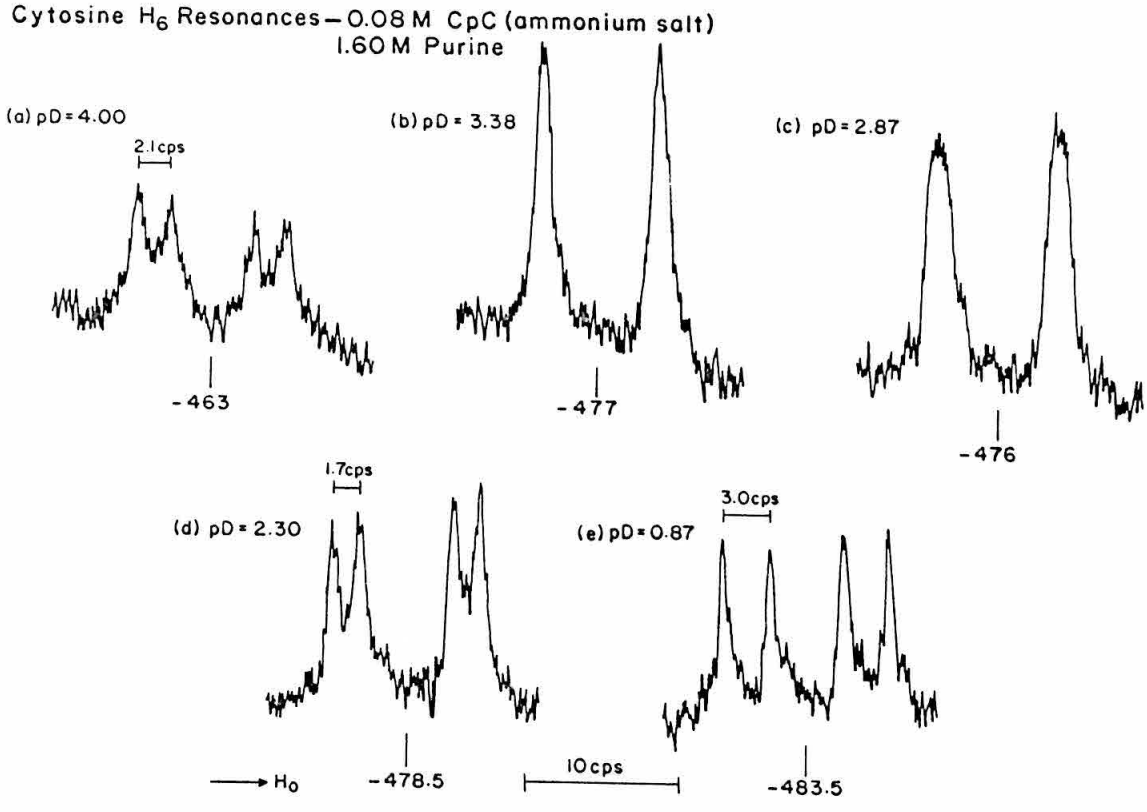

Cytosine H_6 Resonances — 0.08 M CpC (ammonium salt)
1.60 M Purine
(a) pD = 4.00
2.1cps
-463
(b) pD = 3.38
-477
(c) pD = 2.87
-476
(d) pD = 2.30
1.7cps
-478.5
(e) pD = 0.87
3.0cps
-483.5
H_o
10cps

FIGURE 43

Effect of pD on the cytosine H_5 resonances in a solution of 0. 08 M CpC (ammonium salt) and 1. 60 M purine: (a) pD = 4. 00, (b) pD = 3. 38, (c) pD = 2. 87, (d) pD = 2. 30, (e) pD = 0. 87.

Cytosine H_5 Resonances — 0.08 M CpC (ammonium salt)
1.60 M Purine

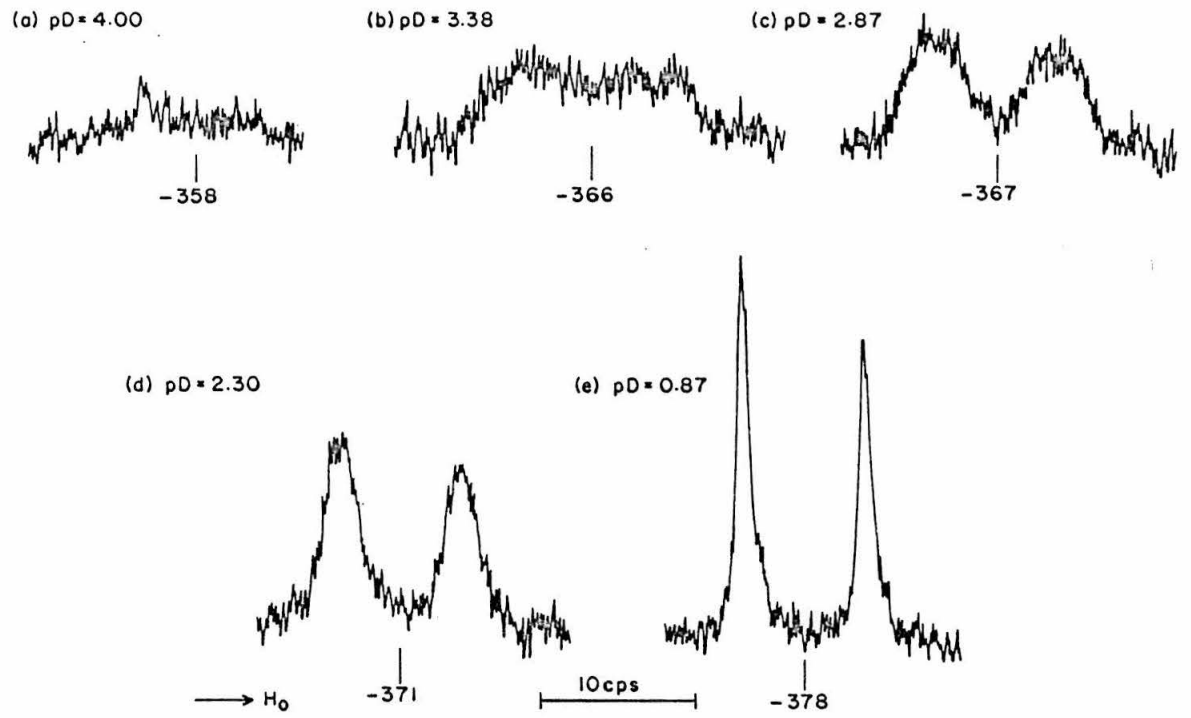


FIGURE 44

Effect of pD on the purine proton resonances in a solution of 0. 08 M CpC (ammonium salt) and 1. 60 M purine: (a) pD = 4. 00, (b) pD = 3. 38, (c) pD = 2. 87, (d) pD = 2. 30, (e) pD = 0. 87.

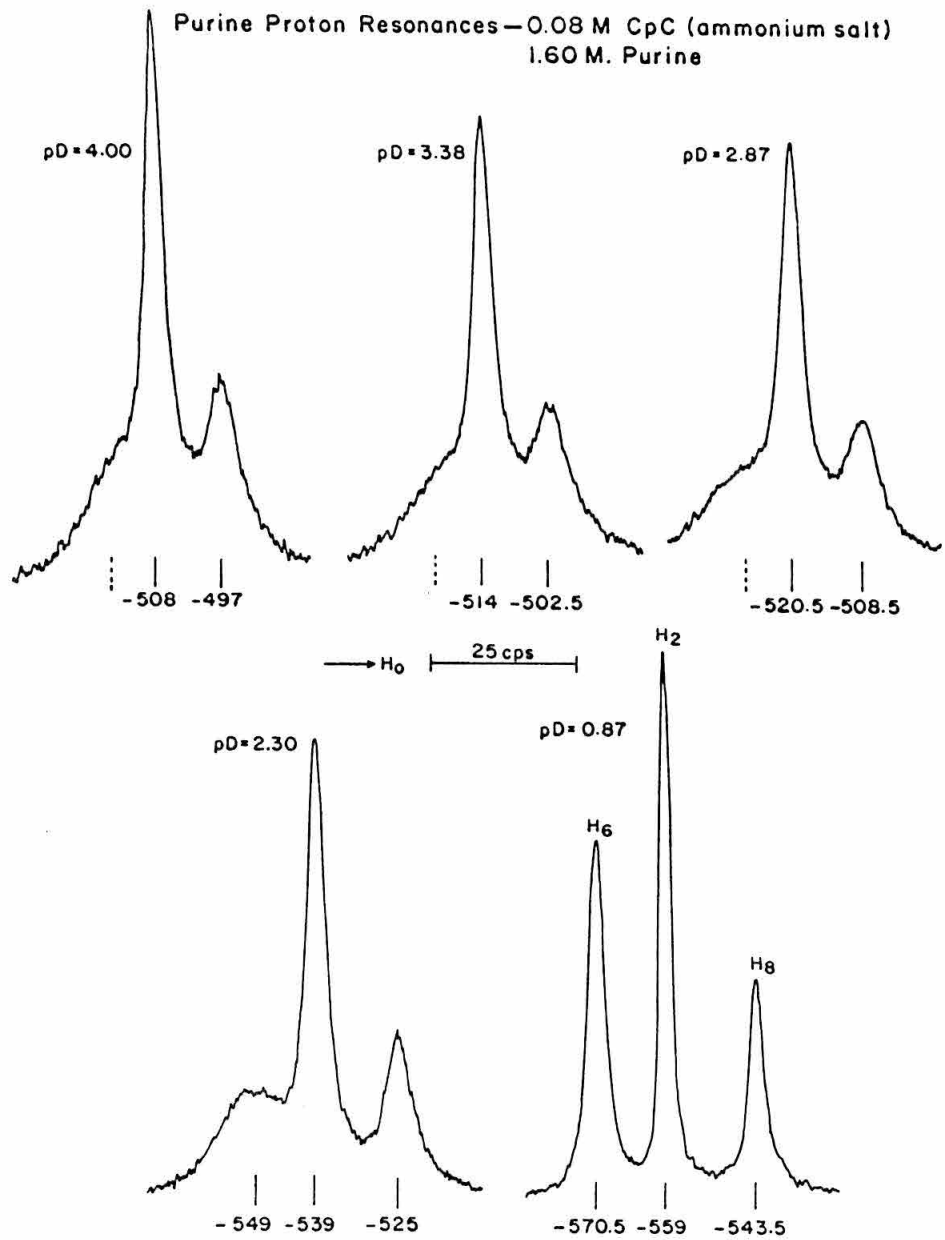

Purine Proton Resonances—0.08 M CpC (ammonium salt)
1.60 M. Purine
pD = 4.00
-508 -497
pD = 3.38
-514 -502.5
pD = 2.87
-520.5 -508.5
→ H₀
25 cps
pD = 2.30
-549 -539 -525
pD = 0.87
H₂
H₆
H₈
-570.5 -559 -543.5

Table XXII. The linewidths of the purine proton resonances in this solution at several pD are given in Table XXIII, and spectral tracings of these resonances are shown in Figure 44.

Although the chemical shift measurements reported in Table XXII are not highly accurate (because no compensation for bulk susceptibility effects has been made), it is apparent that the H_6 resonances (and presumably the H_5 resonances also) move downfield a significant amount between pD 4. 86 and pD 3. 38. By contrast, the $H_{1'}$ resonances only shift by ~2 cps to lower field over this pD range. These shifts are believed to reflect the change in the degree of protonation of the cytosine base, and $H_{1'}$ would be expected to be relatively insensitive to this titration, being attached to the ribose ring. Since the linewidths of the purine resonances do not decrease over this pD range, and in fact appear to increase slightly as the pD is lowered, it is unlikely that the downfield shifts of the cytidine proton resonances between pD 4. 86 and pD 3. 38 are a consequence of a smaller extent of purine complexation. The cytidine resonances shift little between pD 3. 38 and pD 2. 87, and exhibit large shifts to lower fields below pD 2. 87. Furthermore, the $H_{1'}$ resonances, which move only ~2. 5 cps to lower field between pD 4. 86 and pD 2. 87, shift by 6. 0 cps below pD 2. 87. These shifts appear to be a consequence of the dissociation of the purine-dinucleotide complexes as the purine molecules are protonated. The purine proton resonances show large downfield shifts below pD 2. 87, and the magnitudes of these shifts (as well as the chemical shifts of the H_6, H_2 and H_8 protons at pD 0. 87)

TABLE XXII. Chemical shifts, and chemical shift differences between protons of 3′- and 5′-cytidine nucleosides ("splitting"), for a 0.08 M solution of CpC containing 1.60 M purine (measured at 60 Mcps).

pD	H_6 Chem. Shift	H_6 Splitting	H_5 Chem. Shift	H_5 Splitting	$H_{1'}$ Chem. Shift
	cps	cps	cps	cps	cps
4.86	-463	4.2	--	--	-344
4.00	-470	2.1	-358	--	-345.5
3.38	-477	~0	-366	~2.6	-346
2.87	-476	<1	-367	~2	-346.5
2.30	-478.5	1.7	-371	<1	-348
0.87	-483.5	3.0	-378	~0	-352.5

TABLE XXIII. Purine proton resonance linewidths in a 0.08 M CpC solution containing 1.60 M purine (measured at 60 Mcps).

pD	Purine Resonance Linewidths		
	H_6	H_2	H_8
4.86	> 12 cps	5.5 cps	7.2 cps
4.00	> 12	4.0	8.0
3.38	> 12	4.5	9.0
2.87	~16	4.5	7.5
2.30	15	4.0	6.5
0.87	3.9	2.3	3.0

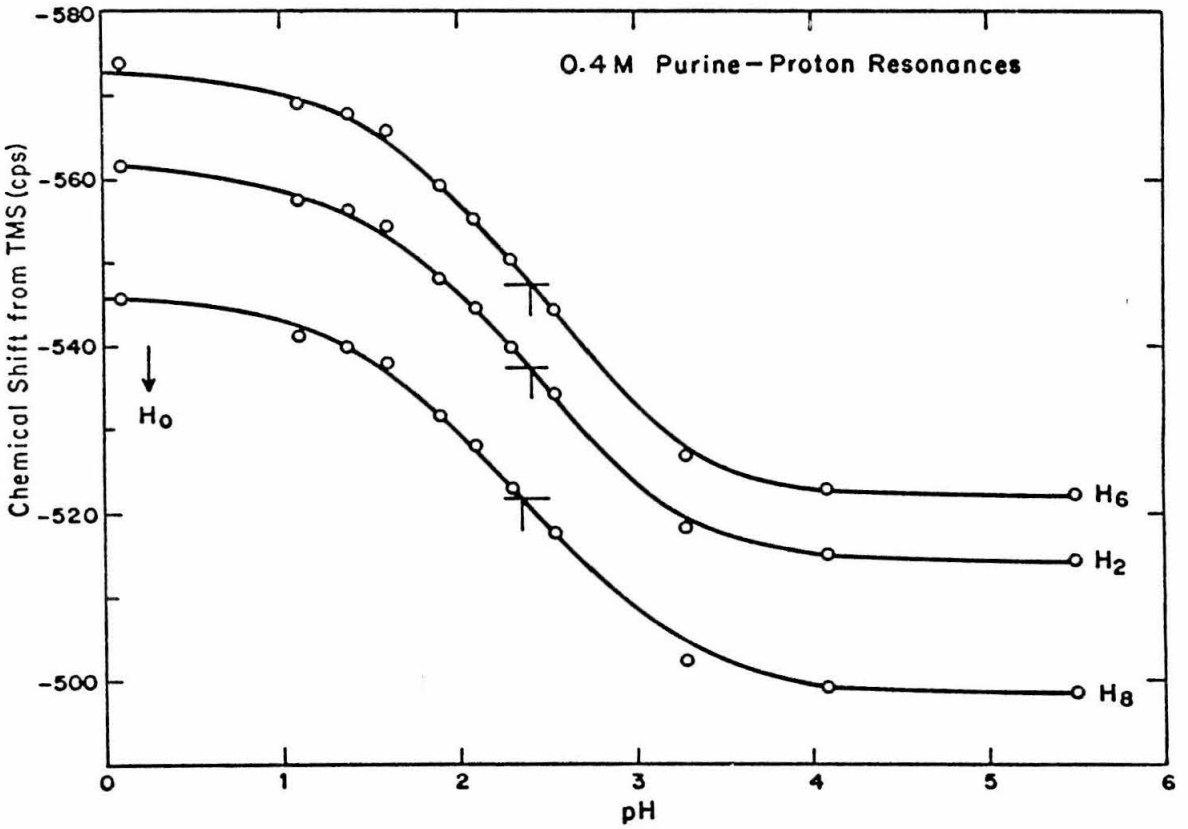
agree well with the pH behavior of a 0.4 M purine solution in the absence of the dinucleotide, shown in Figure 45. The purine resonances begin to narrow appreciably only below pD 2.30, where all of the cytosine bases and more than half of the purine molecules are protonated.

These results indicate that the purine-CpC interaction is at least as strong when all the C bases are protonated as when they are uncharged. In fact, the purine resonance linewidths appear to increase as the pD is lowered to ~3.4, where the maximum degree of cytosine protonation consistent with only a slight degree of purine protonation is attained. The purine-CpC interaction is disrupted only when both the purine molecules and the cytosine bases are protonated, presumably because of electrostatic repulsion between the positively charged species. This behavior of the purine-CpC system with changes in pD is clearly responsible for the difference between the effects of the sodium salt (pD 7.50) and the ammonium salt (pD < 4.86) of CpC on the purine resonance linewidths.

Although comparison of the purine resonance linewidths in the CpC (sodium salt) solution at pD 7.50 with those in the CpC (ammonium salt) solution at pD < 4.86 under comparable conditions shows the purine lines to be broader at the lower pD, this does not necessarily mean that a larger fraction of the purine is involved in the intercalated complex when a significant fraction of the cytosine bases are protonated. The observed purine linewidths depend not only on the fraction of purine bound, but also on the intrinsic linewidths for the purine resonances in the intercalated complex. The linewidths in the

FIGURE 45

Chemical shifts of the purine proton resonances as a function of pH for a 0. 4 M solution of purine in H_2O.


0.4 M Purine – Proton Resonances
Chemical Shift from TMS (cps)
-580
-560
-540
-520
-500
H_o
H_6
H_2
H_8
0
1
2
3
4
5
6
pH

complex are determined by several factors involving the geometry of the complex and the correlation time effective in controlling the relaxation process, and it may be differences in these factors resulting from protonation of the cytosine bases that are responsible for the pD dependence of the observed purine line broadening phenomenon.

6. Uridylyl (3′→5′) Uridine

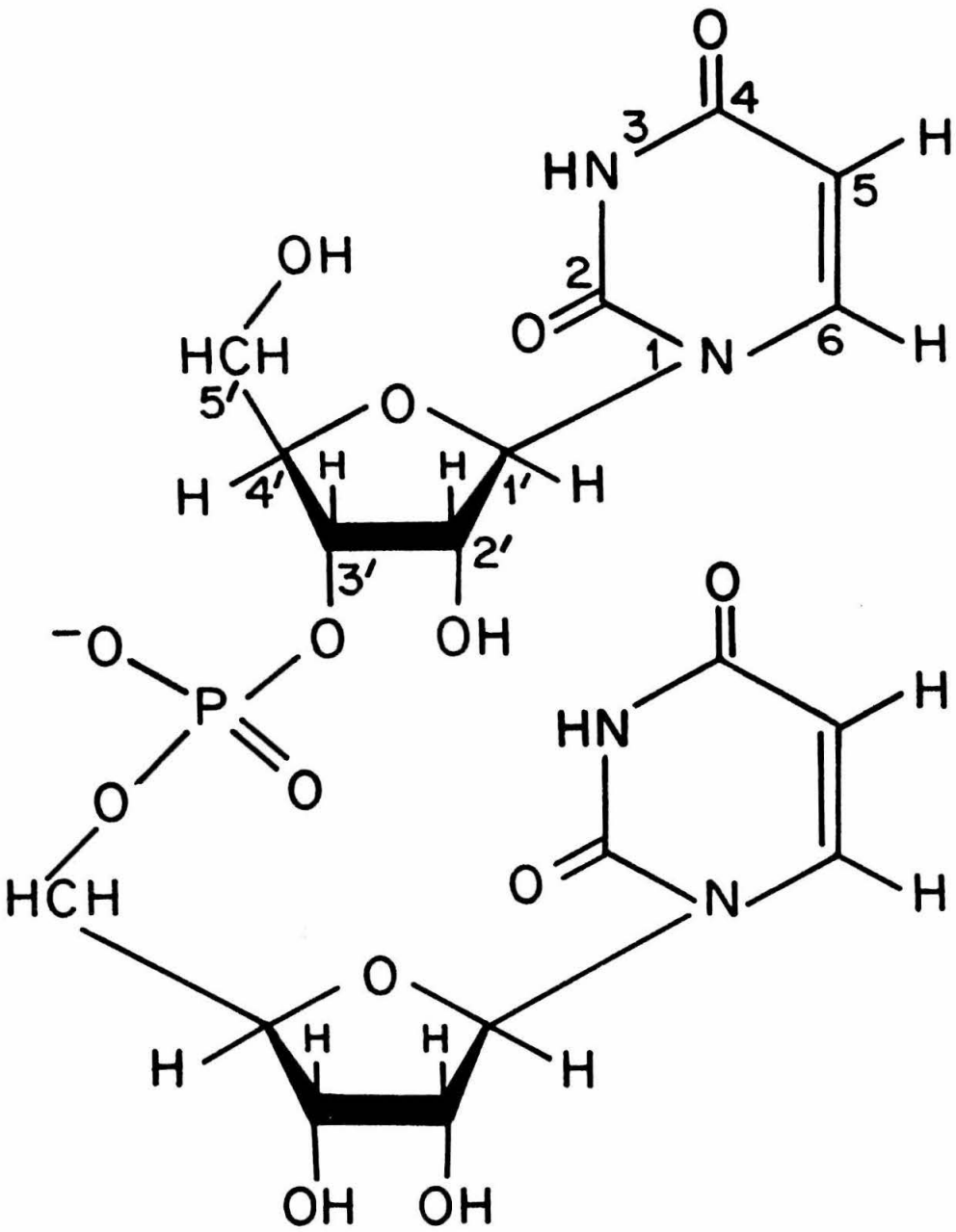
The ribose dinucleoside monophosphate UpU, shown in Figure 46, was investigated at several concentrations and temperatures, and the interaction of this dinucleotide with purine was examined at both high and low UpU concentrations.

6.1. Pmr Spectrum of UpU

The 100 Mcps pmr spectrum at 29°C of a 0.08 M solution of the sodium salt of UpU, in the regions of the uracil H_6 protons and the uracil H_5 and ribose $H_{1'}$ protons, is shown in Figure 47. The spectrum of a 0.10 M uridine solution in the same spectral regions under similar conditions is included for comparison. The uracil H_6 and H_5 protons are spin-spin coupled to give doublets, with the coupling constant $|J_{H_5-H_6}| = 8.0$ cps for both bases of UpU as well as in the monomeric uridine nucleoside. The ribose $H_{1'}$ resonances are doublets from coupling with the $H_{2'}$ protons. Since the $H_{1'}$ resonances of UpU are rather broad here and also overlap the broad H_5 resonances, it is not possible to measure $|J_{H_{1'}-H_{2'}}|$ accurately in this spectrum. However, the higher field $H_{1'}$ doublet can be picked out of the overlapping

FIGURE 46

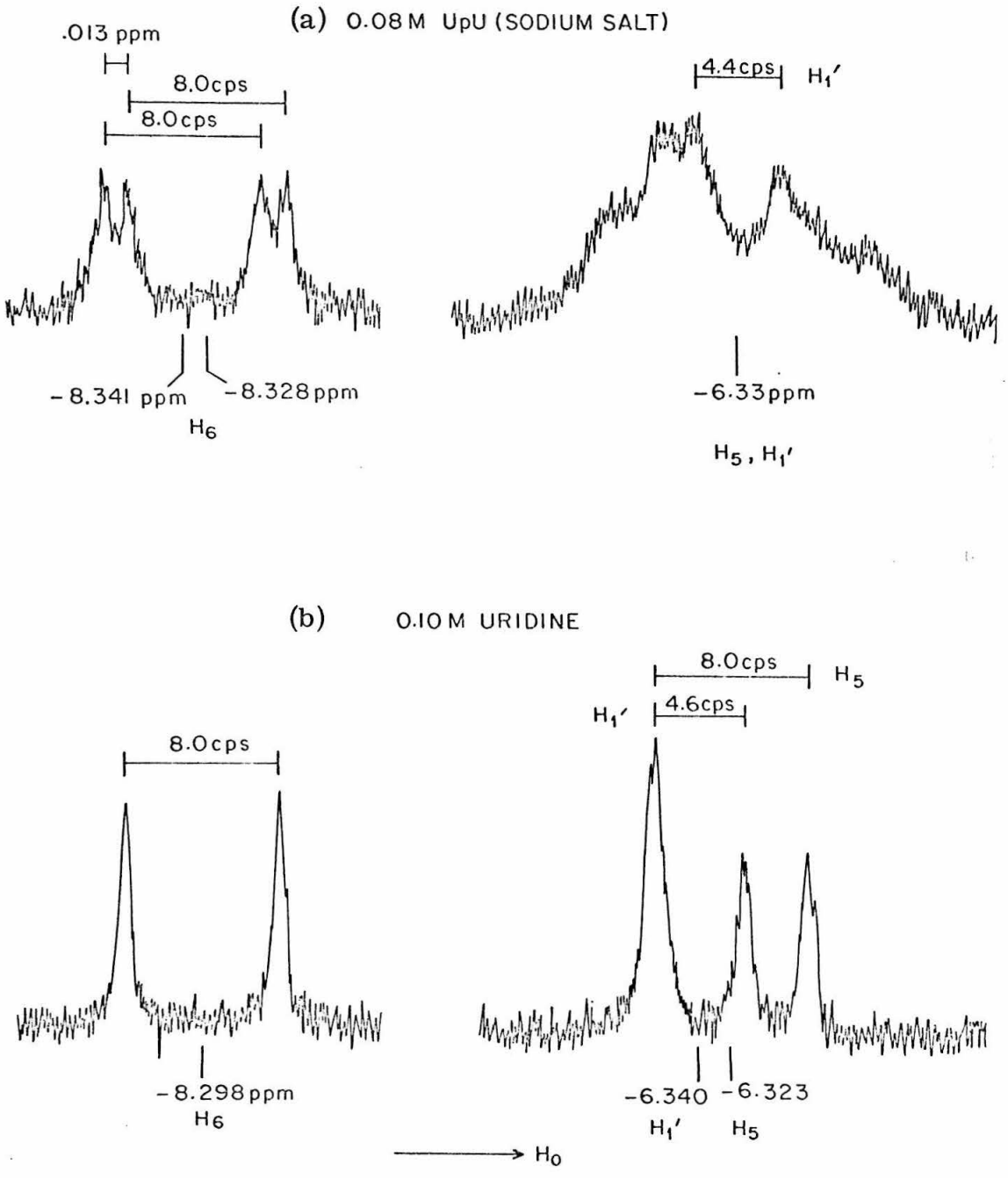
Structural diagram of uridylyl (3′→ 5′) uridine, UpU.



UpU

FIGURE 47

(a) Pmr spectrum of 0. 08 M UpU (sodium salt) at 100 Mcps in the regions of the H_6, and H_5 and $H_{1'}$ proton resonances. (b) Spectrum of 0. 10 M uridine in the same spectral regions under similar conditions.


(a) 0.08 M UpU (SODIUM SALT)
.013 ppm
8.0cps
8.0cps
4.4cps
H1′
-8.341 ppm
-8.328ppm
H6
-6.33ppm
H5, H1′
(b) 0.10 M URIDINE
8.0cps
H5
H1′
4.6cps
8.0cps
-8.298ppm
H6
-6.340
-6.323
H1′
H5
H0

resonances centered at -6. 33 ppm in Figure 47 and its coupling constant $|J_{H_{1'}-H_{2'}}|$ ($\sim$4. 4 cps) is about the same as in the uridine nucleoside (4. 6 cps). As with the other dinucleoside monophosphates studied, the NH and hydroxyl protons of UpU do not give distinct resonances because of rapid exchange with the solvent. Similarly, the spectral region from -4. 20 to -5. 20 ppm, which contains the ribose $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, and $H_{5''}$ proton resonances, was not studied in detail because of its complexity.

As is the case for CpC (Section 5. 1), the non-equivalence of the two nucleoside moieties of UpU resulting from the asymmetric phosphate esterification results in chemical shift differences between corresponding uridine protons in the dinucleotide. The UpU spectrum is somewhat better resolved at 0. 010 M dinucleotide concentration than at 0. 08 M, and the chemical shifts of the monitored uridine protons are listed in Table XXIV. The two H_6 protons are seen to have a chemical shift difference of 0. 013 ppm, and the ribose $H_{1'}$ proton resonances are separated by 0. 048 ppm. The uracil H_5 protons have a magnetic non-equivalence of $\sim$0. 025 ppm which is somewhat obscured by the width of these resonances and by their overlap with the $H_{1'}$ resonances as well as with one another.

6. 1. 1. Assignment of resonances

The assignment of the proton resonances of UpU to the particular 3'- or 5'-esterified nucleoside is indirect, and follows the procedure used to assign the proton resonances of CpC as described

TABLE XXIV. Chemical shift differences for uridine protons between UpU and uridine 3′- and 5′-monophosphates.

	H_6 (3′)	H_6 (5′)	H_5 (3′)	H_5 (5′)	$H_{1'}$ (3′)	$H_{1'}$ (5′)
	ppm		ppm		ppm	
UpU (0.01 M)	-8.332	-8.342	-6.308	-6.333	-6.332	-6.380
3′-UMP-$(Na^+)_2$ (0.05 M)	-8.332		-6.351		-6.383	
5′-UMP-$(Na^+)_2$ (0.10 M)		-8.562		-6.433		-6.446
Shift	0.000	+0.220	+0.043	+0.100	+0.051	+0.066

in Section 5. 1. 1. The results of the purine binding experiments to be discussed in Section 6. 3 indicate that the H_6 resonance at higher field is that of the 3′-nucleoside; similarly, $H_5(3')$ also occurs at higher field than $H_5(5')$. The $H_{1'}$ resonances of UpU, which are resolved better in the temperature and purine binding studies to be described than is shown in Figure 47, have been assigned on the basis of several comparisons. As shown in Table XXIV, the ribose $H_{1'}$ proton resonance in uridine-3′-monophosphate (disodium salt, pD = 8. 1) is at higher field than that in cytidine-5′-monophosphate (disodium salt, pD = 8. 0). Furthermore, the $H_{1'}$ proton of the uridine residue in ApU would be expected to feel the effects of the ring-current magnetic anisotropy of the adjacent adenine base to a greater degree than would the $H_{1'}$ (U) proton of UpA, based on consideration of CPK molecular models of these dinucleotides. Therefore, in going from UpU to ApU and UpA, the $H_{1'}(5')$ proton of uridine should be shifted upfield more than the $H_{1'}(3')$ uridine proton. As shown in Table XXVIII, Section 7. 1, the assignment of the higher field $H_{1'}$ resonance in UpU to the 3′-nucleoside and the lower field $H_{1'}$ resonance to the 5′-nucleoside is consistent with this prediction. Finally, in studies of the binding of purine to the sequence isomers ApU and UpA (Section 7.2) and ApG and GpA, [60] the purine-induced shift for the $H_{1'}$ proton of a given nucleoside is greater when the nucleoside is 5′-esterified than when it is 3′-esterified. Since the purine-induced shift for the $H_{1'}$ proton of UpU which resonates at the lower field is greater than the shift for the higher field $H_{1'}$ proton (see Section 6. 3), the assignment presented in Table XXIV for the $H_{1'}$

resonances of UpU also seems justified on the basis of this comparison of purine-induced shifts.

6. 1. 2. Discussion of chemical shifts

The monitored proton resonances of UpU are shifted from their spectral positions in the uridine nucleoside. This is the case with the other dinucleoside monophosphates as well, and the factors contributing to the chemical shifts of protons in the dinucleotides have been discussed in some detail for ApC and CpA in Section 2. 1, and for CpC in Section 5. 1. As is the case with CpC, the effect of the magnetic anisotropy of the neighboring base on the proton resonances of a given uridine residue in UpU should be of little consequence, as the magnetic anisotropy of the uracil base, like that of cytosine, appears to be negligible. (28)

The influence of the phosphate group on the chemical shifts of the UpU protons is expected to be significant, with protons of the 3′- and 5′-esterified nucleosides affected differently because of this asymmetric phosphate esterification. Consequently, the chemical shifts of the uridine protons in a 0. 01 M UpU solution are compared with those of the corresponding protons of the uridine 3′- and 5′-monophosphates in Table XXIV. The fact that the concentrations of the mononucleotides are appreciably higher than that of UpU in this comparison is of little consequence, since the chemical shifts of the 3′-UMP and 5′-UMP protons (as well as those of UpU) are not concentration dependent. The uridine H_6, H_5 and $H_{1'}$ resonances in UpU are seen to be at higher fields than in the mononucleotides,

although it must be noted that the phosphates groups carry a double negative charge at these pD's, which would cause the UMP protons to be deshielded more than if the phosphate groups were only singly charged. It would have been possible to compare the proton chemical shifts in UpU with those in the singly charged mononucleotides, since the uracil base is not protonated at low pD. However, to be consistent with the comparison of chemical shifts in CpC with those in 3′-CMP and 5′-CMP of Section 5. 1, the results for the uridine mononucleotides with doubly negative phosphate groups are cited here. It is evident, from Tables XVand XXIV, that the chemical shift differences between comparable protons of the dinucleoside monophosphates and the mononucleotides are about half as large for UpU as for CpC. The sole exception to this is the $H_6(5')$ proton, and a possible explanation for the extremely low field position for this proton was discussed in Section 5. 1. 2.

The temperature study of UpU reported in Section 6. 2, as well as the results of several studies by optical and other methods,[41, 43-45] indicate that the intramolecular U-U base-stacking interaction is negligible. Thus it would not appear that the conformations of the uracil bases relative to their ribose rings, or the ring puckering of the ribose moieties, differ appreciably in UpU from their conditions in the monomeric uridine nucleoside. Consequently, the magnetic anisotropies of the furanose oxygen ($O_{1'}$), the 2-keto group, and the 2′-OH group would not be expected to contribute to chemical shift differences for the H_6 and $H_{1'}$ protons between the uridine nucleoside and the dinucleoside monophosphate UpU. Since CpC appears to

be appreciably stacked at the temperature of these studies (~29°C), the effects of these various anisotropies on the chemical shifts of the CpC protons, as discussed in Section 5. 1. 2, would account for the larger upfield shifts for the $H_{1'}$ resonances in going from the cytidine mononucleotides to CpC than from the uridine mononucleotides to UpU. The chemical shifts of the H_5 protons of UpU are essentially the same as in uridine, while they are at somewhat lower fields in the mononucleotides (particularly 5′-UMP). This is probably due to a difference in the conformation of the phosphate group relative to the nucleoside moieties as a consequence of its double esterification, in going from 3′-UMP to 5′-UMP to UpU, rather than to any effects from magnetic anisotropy of the adjacent base.

Concentration Dependence. -- The chemical shifts of the H_6, H_5 and $H_{1'}$ protons remain constant for UpU concentrations between 0. 08 M and 0. 010 M. The H_5 resonances, quite broad at 0. 08 M, are somewhat narrower at 0. 010 M but still cannot be completely resolved. Like the other dinucleotides studied, UpU self-associates intermolecularly in aqueous solution by base-stacking. These processes are probably somewhat less extensive for UpU than for the other dinucleotides, however, since the self-association tendency of the uridine nucleoside is smaller than those for the other nucleosides. [17] The lack of a concentration dependence for the proton chemical shifts of UpU is as expected, in view of the negligible magnetic anisotropy of the uracil base mentioned previously. The large linewidths of the H_5 resonances in UpU are attributed to the formation of self-intercalated

intermolecular dimers, as for CpC, since the H_5 resonances in uridine, 3′-UMP, and 5′-UMP are narrow over a large range of concentration and solution pD. This intermolecular self-association tendency appears to be smaller for UpU than for CpC, since the H_5 resonances are narrower for UpU than for CpC at 0. 08 M dinucleotide concentration.

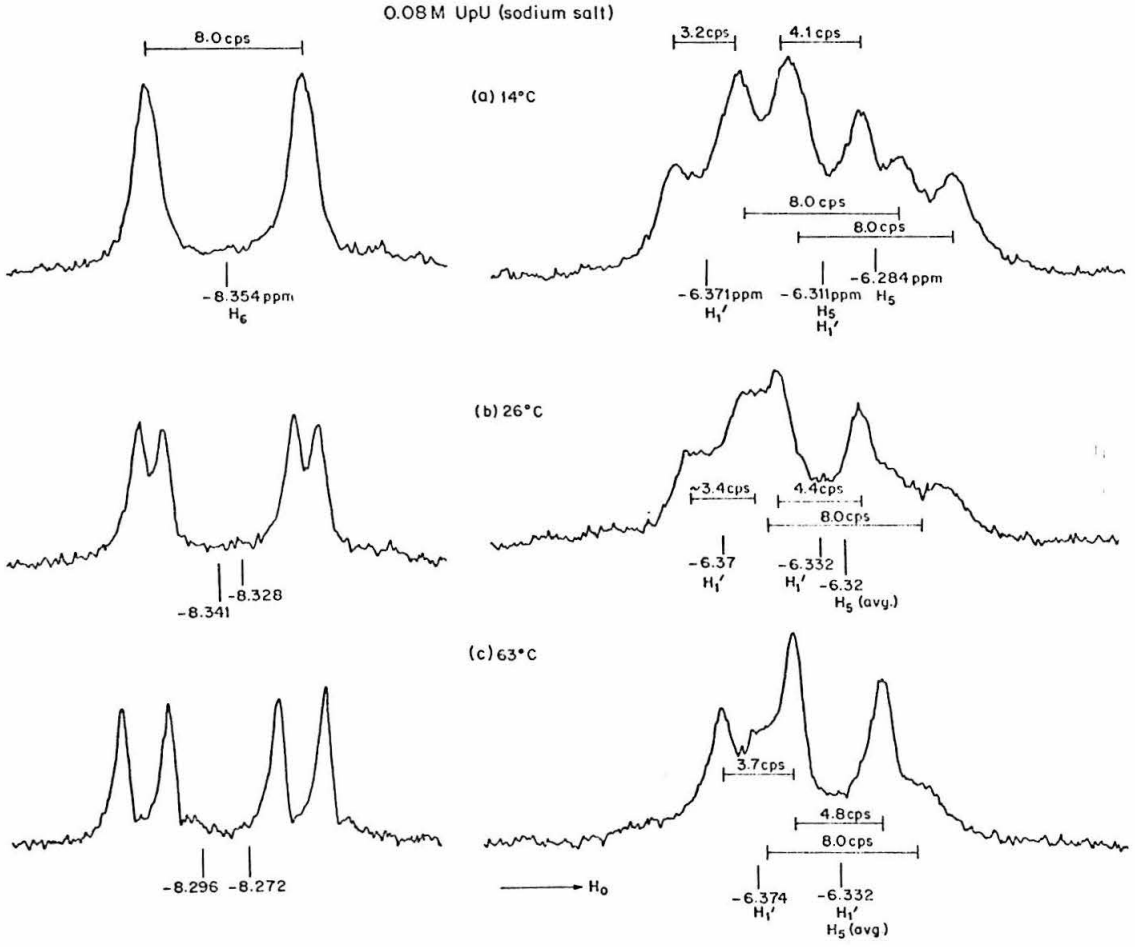
6. 2. Temperature Dependence

The temperature dependence of the pmr spectrum of UpU is, like that of CpC, expected to yield somewhat less information regarding the intramolecular base-stacking interaction than the study of ApC and CpA in Section 2. 3. Yet, in the CpC study (Section 5. 2), the effects of temperature on the conformations of the ribose rings and the chemical shifts of the base protons and ribose $H_{1'}$ protons did demonstrate that there is a significant degree of intramolecular C-C base stacking in this dinucleotide.

Accordingly, the 100 Mcps pmr spectrum of the 0. 08 M UpU (sodium salt) solution was examined at three temperatures, and these spectra are depicted in Figure 48. The chemical shifts at 14°C and 63°C reported here have been corrected relative to those at 26°C through the use of $N(CH_3)_4Cl$ as an internal standard. The two H_6 doublets are seen to have nearly the same chemical shift at 14°C, and both of these resonances move upfield with increasing temperature, with $H_6(3')$ exhibiting the greater shift. The linewidths of the H_6 resonances do not appear to be sensitive to temperature. Although it is difficult to tell just what is happening in the region of the H_5 and

FIGURE 48

Uracil H_6 and H_5 and ribose $H_{1'}$ resonances at 100 Mcps for 0.08 M UpU (sodium salt). Time-averaged spectra; sum of 10 scans. (a) 14°C, (b) 26°C, (c) 63°C. Chemical shifts compensated for bulk magnetic susceptibility differences.


0.08 M UpU (sodium salt)
8.0 cps
-8.354 ppm
H6
(a) 14°C
3.2 cps
4.1 cps
8.0 cps
8.0 cps
-6.371 ppm
H1'
-6.311 ppm
H5
H1'
-6.284 ppm
H5
(b) 26°C
-8.341
-8.328
~3.4 cps
4.4 cps
8.0 cps
-6.37
H1'
-6.332
H1'
-6.32
H5 (avg.)
(c) 63°C
-8.296
-8.272
3.7 cps
4.8 cps
8.0 cps
Ho
-6.374
H1'
-6.332
H1'
H5 (avg)

$H_{1'}$ proton resonances because of the extensive overlapping, it is apparent that the H_5 resonances shift to lower fields somewhat as the temperature is increased. The linewidths of the H_5 resonances also do not appear to change significantly with temperature. The chemical shifts of the ribose $H_{1'}$ protons of UpU change only slightly with temperature. The resonance of the lower field $H_{1'}$ proton, $H_{1'}(5')$, shifts at most ~ 0.003 ppm to lower field in going from 14°C to 63°C, while the $H_{1'}(3')$ proton resonance shifts by about 0.02 ppm, also to lower field. These resonances also appear to become somewhat narrower with increasing temperature, and the coupling constants $|J_{H_{1'}-H_{2'}}|$ are seen to increase by 0.5 to 0.7 cps between 14°C and 63°C.

Although it is not possible to compare the behavior of the H_5 proton resonances with temperature between UpU and CpC because of the broad lines for H_5 in the latter dinucleotide, the behavior of the $H_{1'}$ resonances of UpU, both with regard to the chemical shifts and the coupling constants $|J_{H_{1'}-H_{2'}}|$, is in marked contrast to that observed in CpC. While the $H_{1'}(5')$ resonance in CpC is shifted downfield by 0.055 ppm between 14°C and 63°C, the $H_{1'}(5')$ resonance in UpU exhibits a negligible shift. Similarly, whereas $H_{1'}(3')$ of CpC shifts downfield by 0.095 ppm between these two temperatures, $H_{1'}(3')$ of UpU shifts by only ~ 0.02 ppm to lower field. Also, $|J_{H_{1'}-H_{2'}}|$ for the 3'-nucleoside changes from ~ 1.3 cps to 3.5 cps between 14°C and 63°C in CpC, while in UpU this coupling constant changes from 4.1 cps to 4.8 cps.

From these observations, it can be concluded that the conformations of the ribose rings in UpU change very little with temperature. Furthermore, since the chemical shifts of the H_1' protons are sensitive to the angle of rotation of the bases about the glycosidic bonds as well as to the conformations of the ribose rings, [94] it is apparent that the conformations of the uracil bases relative to their ribose rings in UpU show no significant change with temperature between 14°C and 63°C. Since both the ribose conformation and the orientation of a base relative to its ribose ring in a dinucleoside monophosphate are sensitive to the degree of intramolecular base-stacking of the dinucleotide, as clearly shown in Sections 2. 3 and 5. 2, it can be concluded that the intramolecular base-base interaction in UpU does not change with temperature. The observation that both the chemical shifts and the coupling constants of the H_1' protons in UpU are very similar to their values in the uridine nucleoside suggests further that UpU is essentially completely unstacked throughout the temperature range investigated in the present study.

A comparison of Figure 48 with Figure 30 shows that the H_6 resonances of UpU and those of CpC shift by virtually identical amounts with temperature between 14°C and 63°C, with reference to $N(CH_3)_4^+$. As mentioned in Section 5. 2, however, it is preferable to use the nucleosides as references for the chemical shift measurements, to compensate for shifts arising from changing solvation with temperature and other factors. When this comparison is made, it is found that the $H_6(3')$ resonance of CpC shifts upfield by 0. 043 ppm relative to cytidine,

while the $H_6(5')$ resonance shifts upfield by 0. 019 ppm, between 14°C and 63°C. The $H_6(3')$ resonance of UpU shifts upfield by 0. 022 ppm, and the $H_6(5')$ resonance shows no shift relative to uridine between these two temperatures. Since these shifts are believed to reflect changes in the orientation of the bases relative to the ribose rings as a consequence of a change in the intramolecular base-stacking interaction (Section 5. 2), these observations, too, would seem to indicate that the intramolecular base-stacking interaction is quite a bit smaller in UpU than in CpC.

6. 3. Purine Binding Studies

The interaction between purine and UpU was examined at two dinucleotide concentrations, as reported for CpC in Section 5. 3. The purpose of the study at the lower UpU concentration was to examine the influence of the added purine on the pmr spectrum of the dinucleotide in the absence of intermolecular UpU self-association, while the observation of the effects of complex formation on the purine resonances required a somewhat higher concentration of UpU.

6. 3. 1. Low UpU concentration - 0. 010 M

The addition of purine to a 0. 010 M solution of the sodium salt of UpU caused the six monitored proton resonances of the dinucleotide (measured at 100 Mcps) to be shifted to higher fields as a consequence of the purine-uracil base-stacking interaction. These purine-induced

shifts are shown in Table XXV. The behavior of the proton resonances of UpU is similar to that found for the cytidine protons of CpC, in that the H_6 resonance at higher field in the absence of purine exhibits a larger purine-induced shift than the lower field H_6 resonance. Again, as in the CpC study, the broad H_5 resonances narrowed as purine was added to the solution, and the H_5 doublet at higher field showed the greater purine-induced shift. The chemical shifts of the uracil H_5 protons of UpU in the absence of purine (shown in Table XXIV) were determined by extrapolating the data of these purine binding experiments to zero purine concentration. The narrowing of the H_5 resonances of UpU as a consequence of purine addition is believed due to competition of the purine binding with the intermolecular self-association of the dinucleotide, and the H_5 linewidths are comparable to those of the H_6 and $H_{1'}$ proton resonances at 0.05 M purine concentration. The assignments of the H_6 and H_5 resonances to the 3′- and 5′-esterified nucleosides is, as for CpC, by analogy with the TpdU-dUpT study of Section 4.2 and the ApU-UpA study of Section 7. Plots of the purine-induced shifts for the uracil base proton resonances of UpU are shown in Figures 49 and 50.

The ribose $H_{1'}$ resonance at lower field in UpU, $H_{1'}(5')$, experiences a larger purine-induced shift than for the $H_{1'}(3')$ proton, as seen in Figure 51. It was not possible to measure the coupling constants $|J_{H_{1'}-H_{2'}}|$ very accurately, since the $H_{1'}$ resonances overlap the H_5 resonances (as well as one another) throughout the 0.0 to 1.0 M purine concentration range. These coupling constants did not appear

TABLE XXV. Purine-induced shifts of the proton resonances of 0. 010 M UpU (sodium salt), ppm. (Measurements made at 100 Mcps.)

Purine Conc.	H_6 (3′)	H_6 (5′)	H_5 (3′)	H_5 (5′)	$H_{1'}$ (3′)	$H_{1'}$ (5′)
	ppm	ppm	ppm	ppm	ppm	ppm
0. 05 M	0. 018	0. 016	0. 026	0. 021	0. 016	0. 018
0. 10	0. 039	0. 035	0. 051	0. 042	0. 033	0. 033
0. 15	0. 055	0. 051	0. 077	0. 062	0. 045	0. 049
0. 20	0. 074	0. 068	0. 097	0. 079	0. 059	0. 063
0. 25	0. 087	0. 081	0. 112	0. 091	0. 067	0. 073
0. 30	0. 102	0. 095	0. 128	0. 106	0. 077	0. 087
0. 40	0. 124	0. 115	0. 158	0. 131	0. 093	0. 113
0. 50	0. 139	0. 129	0. 185	0. 152	0. 109	0. 123
0. 60	0. 153	0. 142	0. 202	0. 169	0. 117	0. 132
0. 80	0. 185	0. 171	0. 239	0. 198	0. 134	0. 154
1. 00	0. 208	0. 192	0. 273	0. 224	0. 152	0. 176

FIGURE 49

Plot of the purine-induced shifts for the H_6 proton resonances of 0.010 M UpU (sodium salt), cps at 100 Mcps.

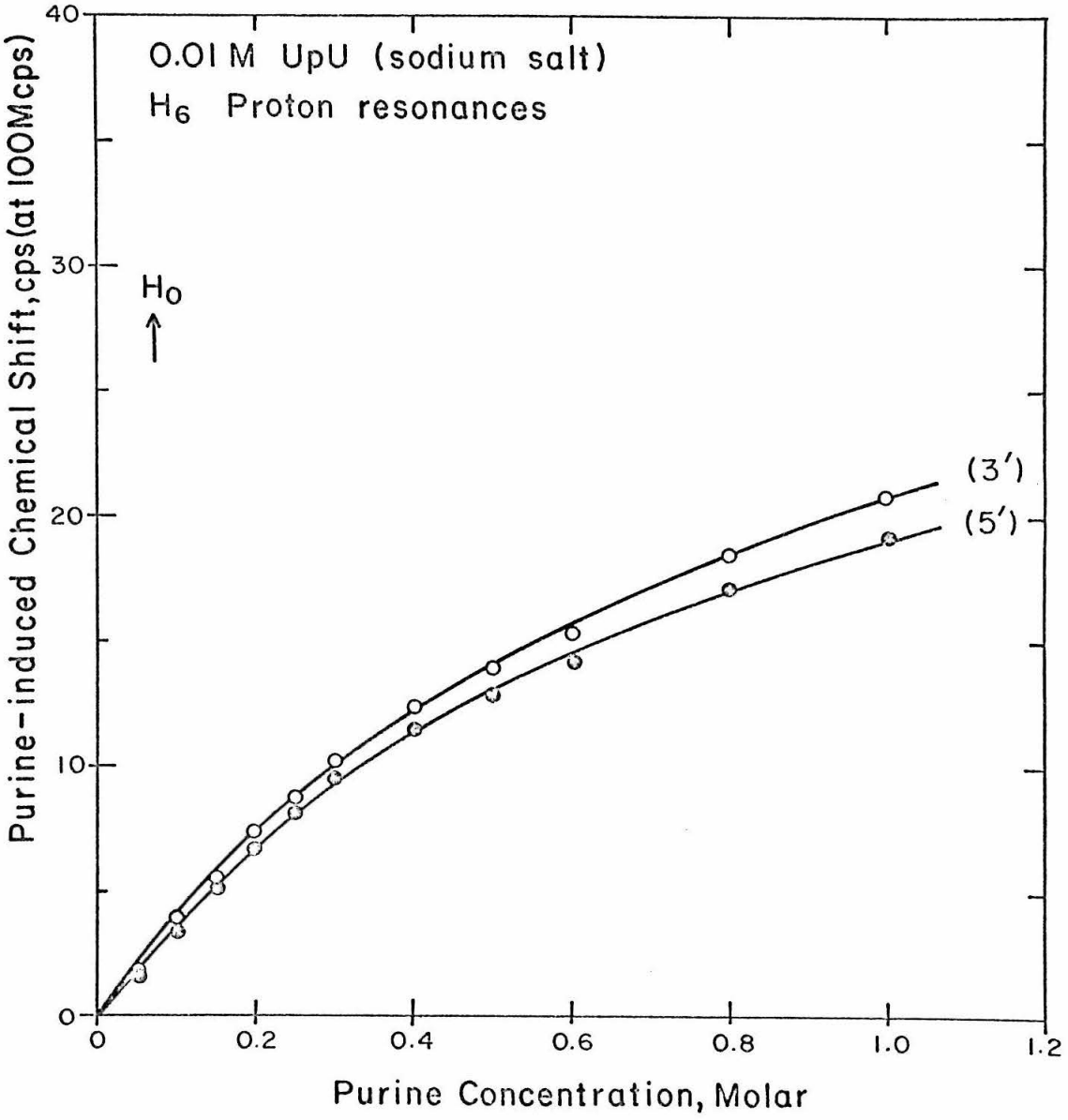

0.01 M UpU (sodium salt)
H6 Proton resonances
Ho
(3′)
(5′)
Purine-induced Chemical Shift, cps (at 100Mcps)
Purine Concentration, Molar
0
10
20
30
40
0
0.2
0.4
0.6
0.8
1.0
1.2

FIGURE 50

Plot of the purine-induced shifts for the H_5 proton resonances of 0. 010 M UpU (sodium salt), cps at 100 Mcps.

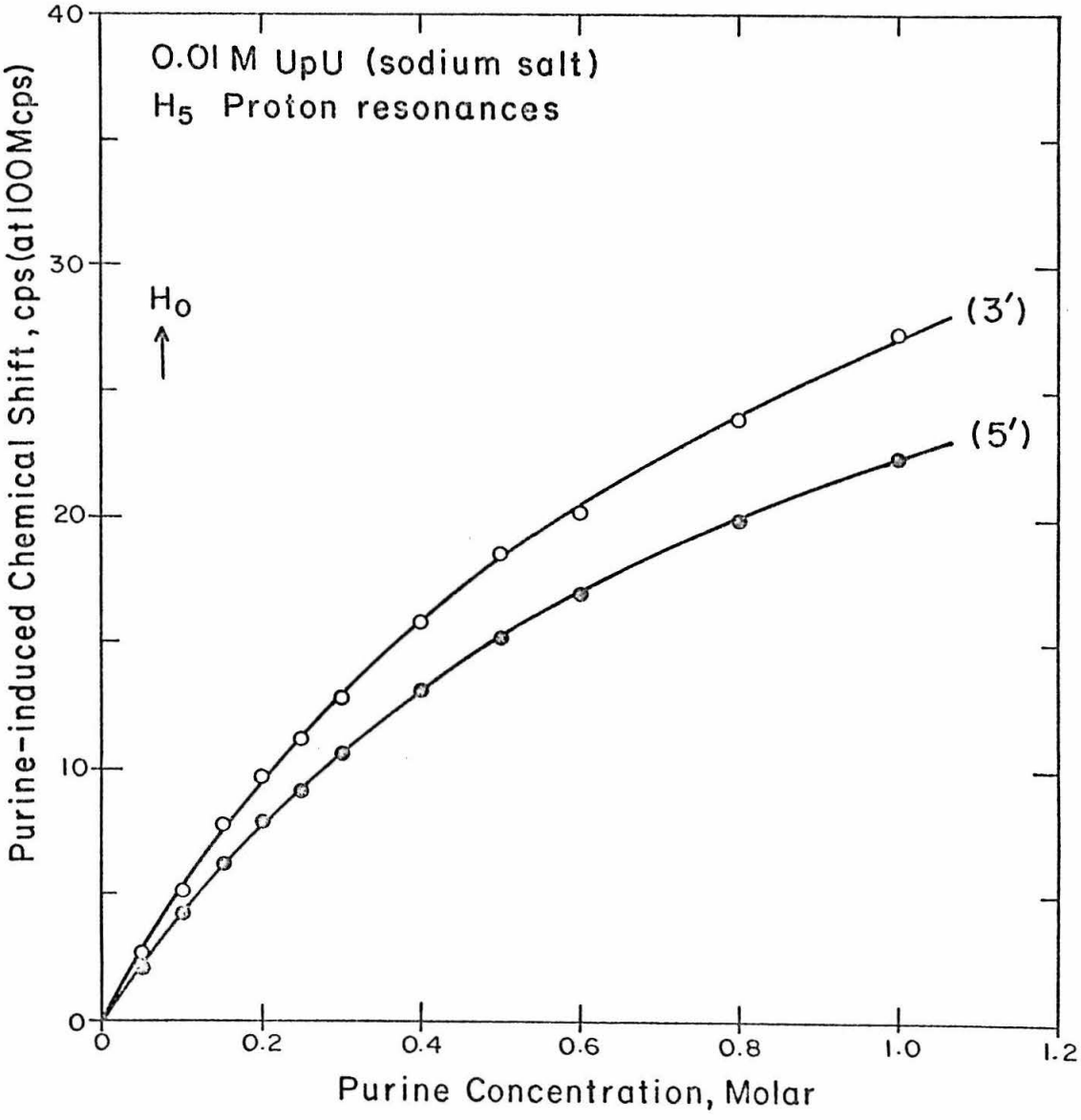
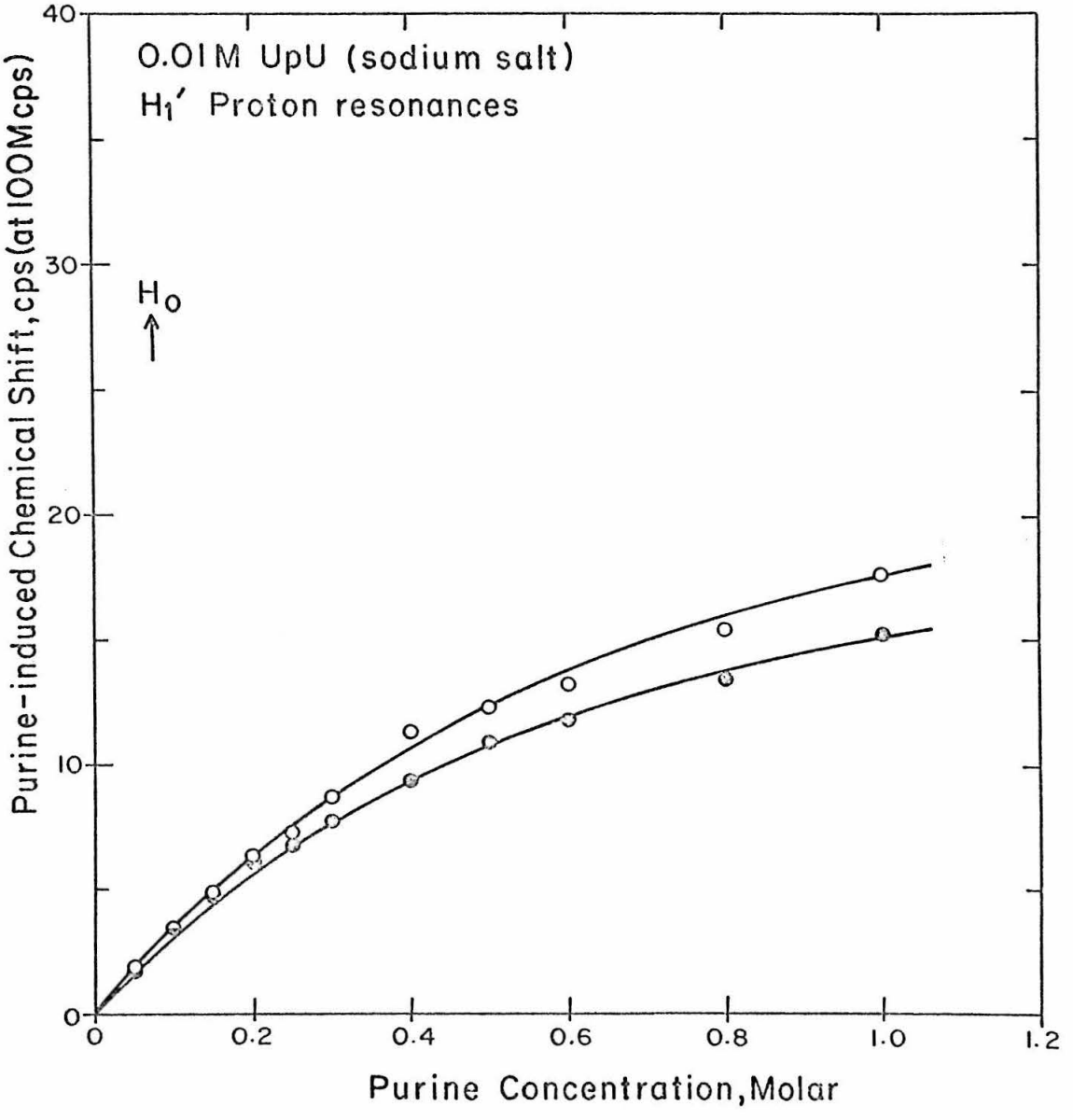

0.01 M UpU (sodium salt)
H5 Proton resonances
Ho
(3′)
(5′)
Purine-induced Chemical Shift, cps (at 100 Mcps)
Purine Concentration, Molar
0
10
20
30
40
0
0.2
0.4
0.6
0.8
1.0
1.2

FIGURE 51

Plot of the purine-induced shifts for the $H_{1'}$ proton resonances of 0.010 M UpU (sodium salt), cps at 100 Mcps; $H_{1'}(3')$ ●, $H_{1'}(5')$ ○.


0.01M UpU (sodium salt)
H1′ Proton resonances
Ho
Purine-induced Chemical Shift, cps (at 100M cps)
Purine Concentration, Molar
0
10
20
30
40
0
0.2
0.4
0.6
0.8
1.0
1.2

to change much in this study, however, and it was concluded that no appreciable changes in the conformations of the ribose rings occur with purine binding. This is in contrast to the results of the study of the binding of purine to CpC reported in Section 5. 3. 1, where a significant change in ribose conformation is seen to occur.

The purine-induced shifts for the uracil H_6 and H_5, and ribose $H_{1'}$ resonances in 0. 010 M UpU are ~85%, ~79%, and ~76%, respectively, of the purine-induced shifts for the analogous protons of the uridine nucleoside (at 0. 11 M concentration) reported by Schweizer, et al., [28] at 1. 0 M purine concentration. This is about the same behavior as found in the CpC-cytidine comparison, and indicates that both faces of each base in the dinucleotide are stacking with purine. A comparison of the corresponding plots of purine-induced shifts for the base and $H_{1'}$ protons between UpU and CpC (Figures 49 and 32, 50 and 33, 51 and 34) shows that both these shifts and the purine-induced chemical shift differences, or "splittings", are quite a bit larger for the base protons in CpC than in UpU; the ribose $H_{1'}$ resonances are shifted a little more in CpC than in UpU by added purine. These findings lead to the conclusion that purine stacks more strongly with the cytosine bases of CpC than with the uracil bases of UpU. This is not an unexpected result, as the purine-induced shifts reported for the cytidine nucleoside are larger than those for the uridine nucleoside, [28] and the self-association tendency is greater for cytidine than for uridine (see Table I).

The purine resonances are somewhat broadened in the presence of 0. 010 M UpU, particularly at low purine concentrations,

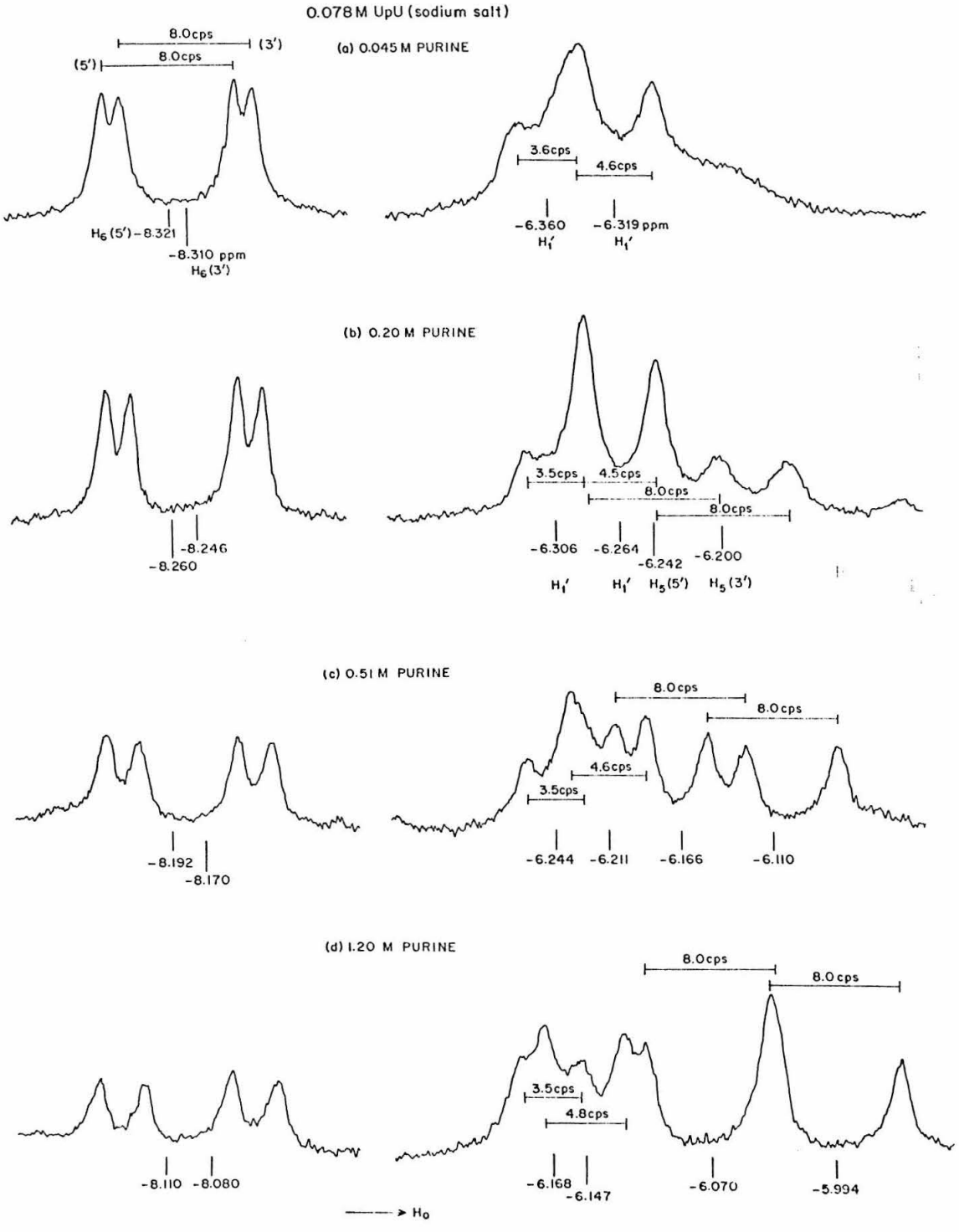
and become narrower as the purine concentration is increased. At 0. 05 M purine concentration, the linewidths are as follows: H_6, 4. 6 cps; H_2, 1. 8 cps; H_8, 3. 6 cps. This suggests that an intercalated complex is formed between purine and UpU, as with the other dinucleoside monophosphates whose interactions with purine have been examined. Therefore, the purine-UpU system was further investigated, at higher dinucleotide concentration, so as to examine the effects of the interaction upon the purine proton resonances.

6. 3. 2. High UpU concentration - 0. 078 M

The interaction of purine with 0. 078 M UpU affected the monitored proton resonances of the dinucleotide to about the same extent as in the study at lower UpU concentration. The UpU proton resonances in this solution at several concentrations of added purine are depicted, in the regions of the uracil H_6 protons and uracil H_5 and ribose $H_{1'}$ protons, in Figure 52. The H_6 resonances are seen to remain narrow as they shift to higher fields with increasing purine concentration, with $H_6(3')$ showing a greater shift than $H_6(5')$. The chemical shifts and coupling constants $|J_{H_{1'}-H_{2'}}|$ for the $H_{1'}$ protons are difficult to measure accurately throughout the entire range of purine concentration, because of the overlap of these resonances with one another and with the H_5 resonances as is evident in Figure 52. At low purine concentrations, the H_5 resonances are broad, and underlie the $H_{1'}$ doublets. At 0. 094 M purine, the high field components of the two H_5 doublets are clearly resolved. As the purine concentration is

FIGURE 52

Time-averaged 100 Mcps pmr spectrum of 0. 078 M UpU (sodium salt) in the regions of the H_6, H_5 and $H_{1'}$ proton resonances in the presence of purine. (a) 0. 045 M purine, 11 scans; (b) 0. 20 M purine, 12 scans; (c) 0. 51 M purine, 15 scans; (d) 1. 20 M purine, 13 scans.


0.078 M UpU (sodium salt)
8.0cps
(3')
8.0cps
(5')
H6 (5') – 8.321
-8.310 ppm
H6 (3')
(a) 0.045 M PURINE
3.6cps
4.6cps
-6.360
H1'
-6.319 ppm
H1'
(b) 0.20 M PURINE
3.5cps
4.5cps
8.0cps
8.0cps
-8.246
-8.260
-6.306
-6.264
-6.242
-6.200
H1'
H1'
H5 (5')
H5 (3')
(c) 0.51 M PURINE
8.0cps
8.0cps
4.6cps
3.5cps
-8.192
-8.170
-6.244
-6.211
-6.166
-6.110
(d) 1.20 M PURINE
8.0cps
8.0cps
3.5cps
4.8cps
-8.110
-8.080
-6.168
-6.147
-6.070
-5.994
→ Ho

increased further, the H_5 resonances become as narrow as those for the H_6 protons, and both components of each doublet are clearly resolved as the H_5 resonances shift upfield relative to the $H_{1'}$ resonances. The purine-induced shifts of the UpU proton resonances in this system are not quite so great as for the solution of lower dinucleotide concentration, but this difference is only ~5% at 1.0 M purine concentration.

The purine proton resonances are, as expected, markedly broadened in this 0.078 M UpU solution. Spectral tracings of the purine resonances in this system at three purine concentrations are shown in Figure 53. At 0.045 M purine, only the H_2 resonance is observed; the H_6 resonance at lower field and the H_8 resonance at higher field are broadened beyond detection. As the purine concentration is increased, the H_6 and H_8 resonances become evident, and all three peaks become narrower. (Note--The apparent shoulder on the high field side of the H_8 resonance in Figure 53b is an artifact, a spinning sideband from the H_2 resonance.) The measured purine proton resonance linewidths for this purine-UpU system are listed in Table XXVI. As a comparison of these data with those for the 0.08 M CpC-purine system reported in Table XIX (Section 5.3.2) clearly shows, the purine resonances are broadened to about the same extent by these two dinucleoside monophosphates. Furthermore, the broadening of the purine resonances is in the order $H_6 > H_8 > H_2$, as for all of the other purine-dinucleotide systems which have been investigated.

As mentioned previously, the nucleosides have no apparent

FIGURE 53

Time-averaged 100 Mcps pmr spectrum of purine in the presence of 0. 078 M UpU (sodium salt). (a) 0. 045 M purine, 28 scans; (b) 0. 094 M purine, 55 scans; (c) 0. 41 M purine, 10 scans.

0.08M UpU (sodium salt) + PURINE.
PURINE PROTON RESONANCES

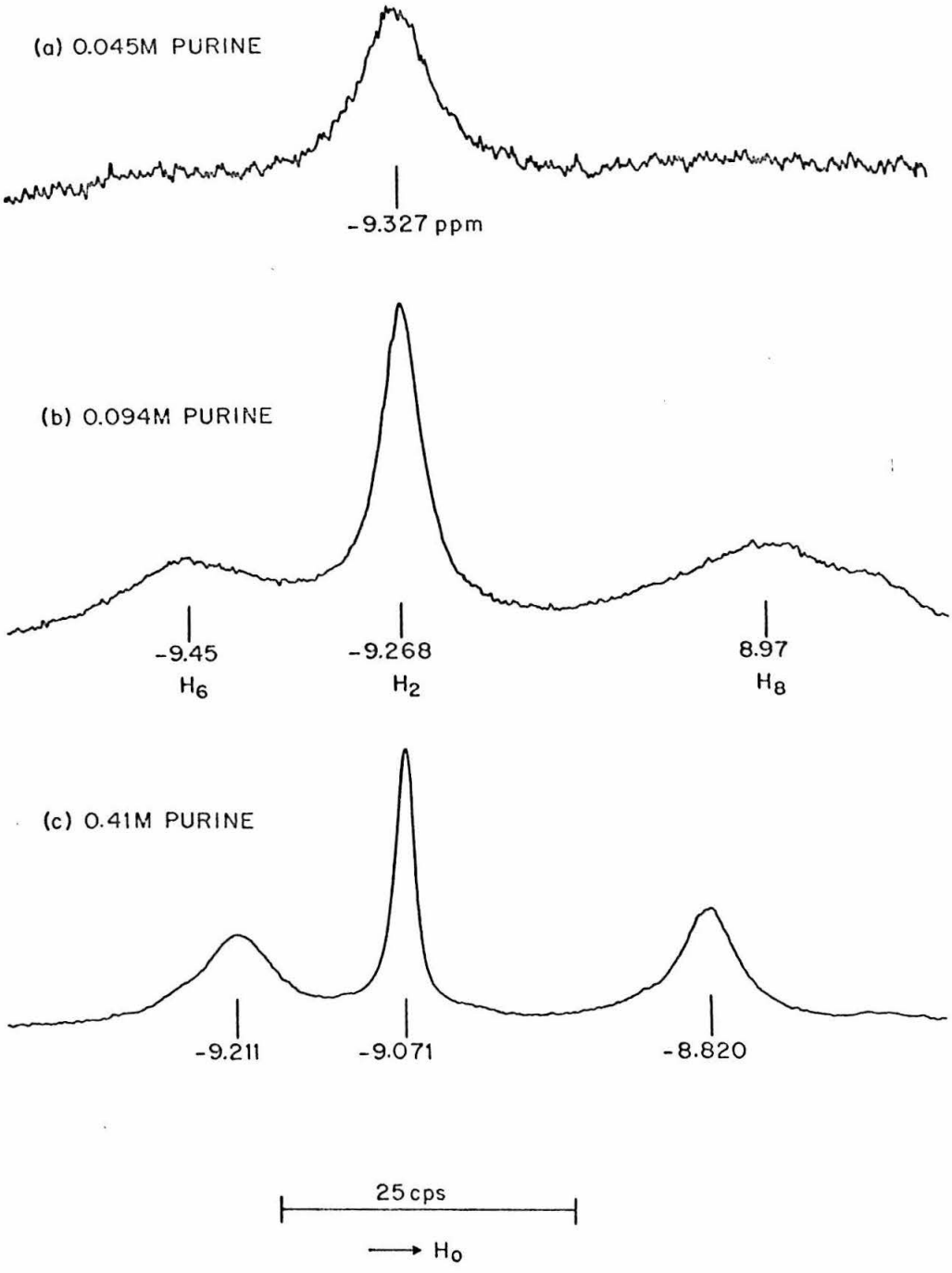


TABLE XXVI. Purine proton resonance linewidths in the presence of 0.08 M UpU (sodium salt). (Full linewidth at half-height in cps, measured at 100 Mcps.)

Purine Conc.	Purine Resonance Linewidths		
	H_6	H_2	H_8
0.045 M	--[a]	6.8 cps	--[a]
0.094	~14 cps	3.8	~14 cps
0.20	~10	2.7	8.6
0.30	~ 8.5	2.4	6.4
0.41	7.6	2.2	5.6
0.51	6.1	2.1	4.9
0.60	5.8	1.7	4.0
0.82	4.7	1.5	3.1
1.00	4.3	1.6	3.1
1.20	3.8	1.4	2.7

[a]Broad beyond detection.

effect on the purine proton resonance linewidths. Figure 54a shows a 100 Mcps pmr spectrum of 0. 15 M purine alone, and the resonances are seen to be narrow, with linewidths of 0. 8 - 1. 0 cps under these conditions. In a solution of 0. 12 M purine containing 0. 10 M uridine, Figure 54b, the purine resonances show no increase in linewidth whatsoever. The occurrence of a base-stacking interaction between purine and the uridine nucleoside is indicated by a shift of the uridine resonances to higher fields as 0. 12 M purine is added to the 0. 10 M uridine solution. These shifts (0. 053 ppm for H_6, 0. 069 ppm for H_5 and 0. 048 ppm for $H_{1'}$) are somewhat larger than those for UpU at comparable purine concentration (Table XXV). These observations clearly demonstrate the importance of the ribose-phosphate backbone in connection with the purine line broadening phenomenon.

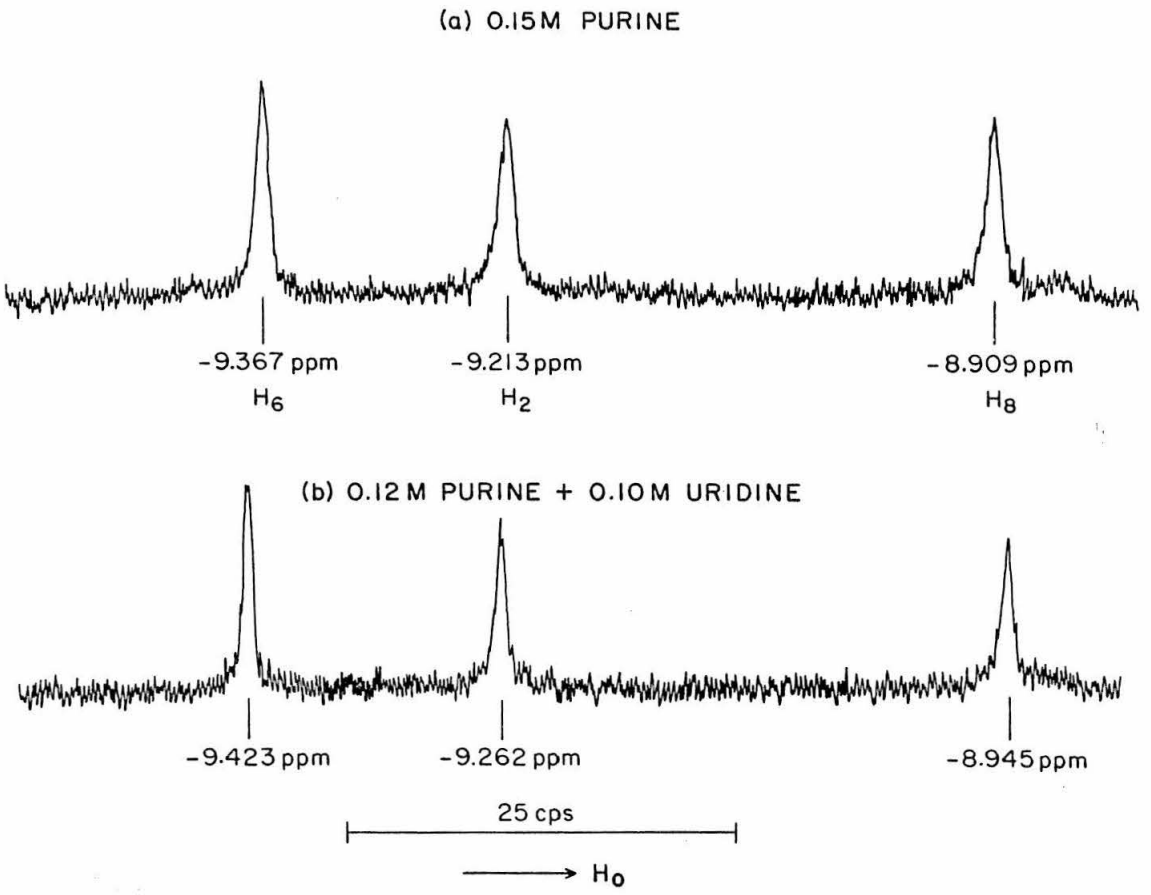
Although the effect of solution pD on the purine-UpU interaction (as reported for the purine-CpC system in Section 5. 3. 3) was not investigated, it might be expected that the purine binding would persist to very low pD, since the uracil bases are not protonated.

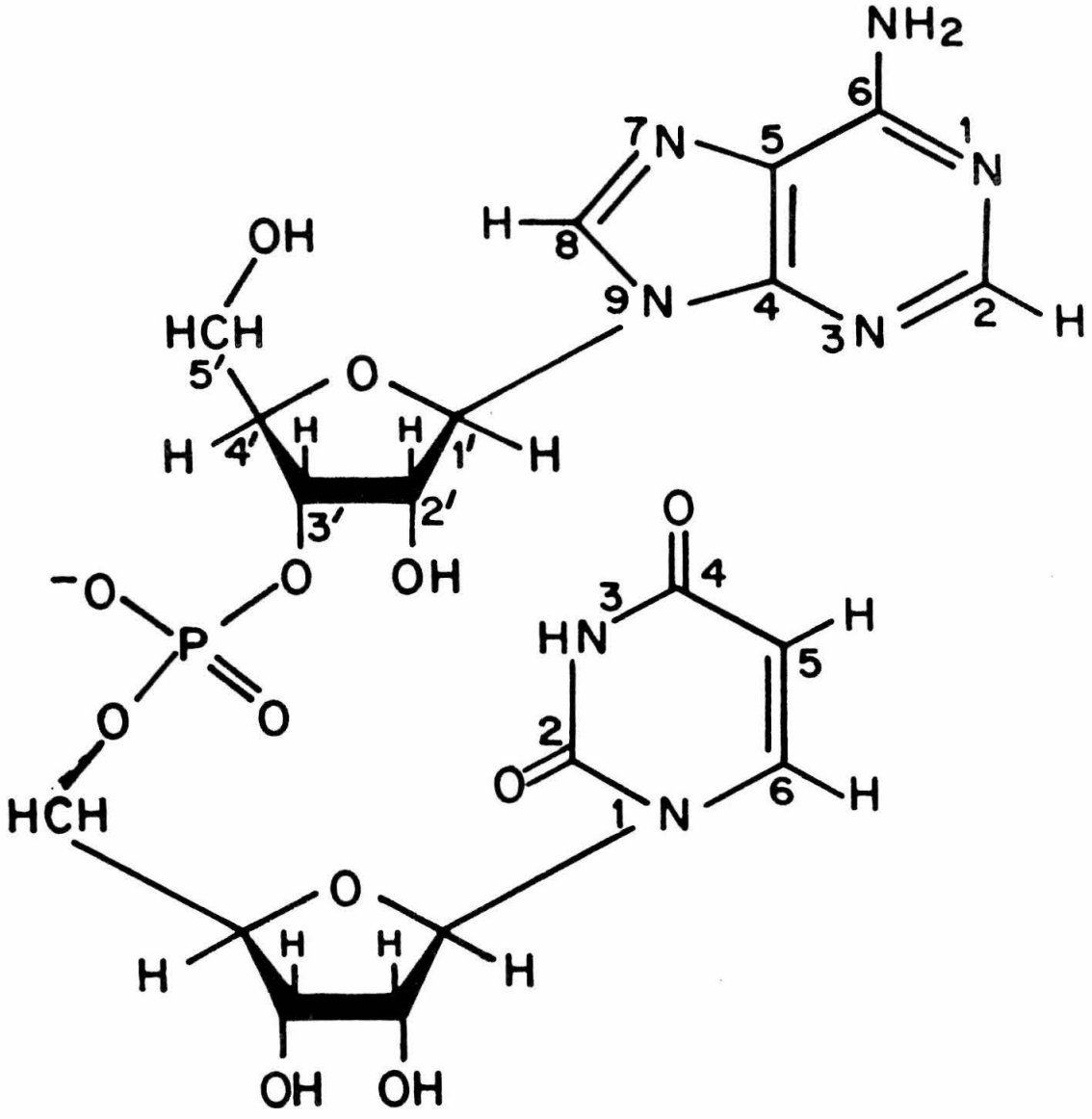
7. Adenylyl (3′→5′) uridine and Uridylyl (3′→5′) adenosine

The sequence isomers ApU (Figure 55) and UpA have been studied in aqueous solution by pmr spectroscopy with regard to the interaction of these dinucleoside monophosphates with purine. The concentration dependent and temperature dependent properties of ApU and UpA have been studied by other workers in this laboratory, [57] so these aspects are not treated in the present discussion.

FIGURE 54

(a) Pmr spectrum of 0.15 M purine at 100 Mcps. (b) Pmr spectrum of 0.12 M purine in the presence of 0.10 M uridine at 100 Mcps.


(a) 0.15M PURINE
-9.367 ppm
H6
-9.213 ppm
H2
-8.909 ppm
H8
(b) 0.12M PURINE + 0.10M URIDINE
-9.423 ppm
-9.262 ppm
-8.945 ppm
25 cps
Ho



ApU

FIGURE 55. Structural diagram of adenylyl (3′ → 5′) uridine, ApU.

7. 1. Pmr Spectra and Assignment of Resonances

The 100 Mcps pmr spectra at 29°C of 0. 010 M solutions of the sodium salts of ApU and UpA were obtained in the regions of the adenine H_8 and H_2 and uracil H_6 protons, and the uracil H_5 and ribose $H_{1'}$ protons. The chemical shifts for these resonances are listed in Table XXVII, along with those for the component nucleosides under comparable conditions. As was the case for ApC and CpA, as reported in Section 2. 1, the assignment of the proton resonances in ApU and UpA is quite straightforward. The adenine H_8 protons were identified by exchange with deuterium at elevated temperatures, and these are the resonances at lowest field in both ApU and UpA. The uracil H_6 and H_5 protons are spin-spin coupled to give doublets, with $|J_{H_5-H_6}|$ = 8. 1 cps in both ApU and UpA as well as in the uridine nucleoside. The ribose $H_{1'}$ resonance at lower field in each of these dinucleotides has been assigned to the adenosine nucleoside, since this proton is expected to feel a deshielding effect from the ring-current magnetic anisotropy of the adenine base. As with the other dinucleoside monophosphates studied, the spectral region containing the other ribose proton resonances was not examined in detail.

The chemical shifts of the monitored adenosine proton resonances of ApU and UpA are quite similar to those for their counterparts in ApC and CpA, shown in Figures 4 and 5 of Section 2. 1. Thus the discussion of the various factors affecting the chemical shifts of these protons in ApC and CpA (Section 2. 1. 2) would appear to apply to ApU and UpA as well. The chemical shifts of the uridine

TABLE XXVII. Proton chemical shifts for 0. 010 M ApU and UpA (sodium salts), 0. 010 M adenosine, and 0. 10 M uridine, in ppm from TMS capillary. (Note--the chemical shifts of the uridine protons are virtually independent of nucleoside concentration.)

Proton	ApU	UpA	Adenosine	Uridine
	ppm	ppm	ppm	ppm
H_8 (A)	-8. 768	-8. 854	-8. 756	
H_2 (A)	-8. 627	-8. 671	-8. 669	
H_6 (U)	-8. 188	-8. 170		-8. 266
$H_{1'}$ (A)	-6. 490	-6. 544	-6. 498	
H_5 (U)	-6. 08	-6. 215		-6. 488
$H_{1'}$ (U)	-6. 203	-6. 172		-6. 337

H_6, H_5 and $H_{1'}$ protons of ApU and UpA are compared with those for the corresponding protons of UpU (from Section 6. 1) in Table XXVIII. It can be seen from these data that the substitution of adenine for uracil as the adjacent base in ApU and UpA causes the uridine resonances in these dinucleotides to be shifted appreciably upfield from their corresponding field positions in UpU. These shifts are a consequence primarily of the ring-current magnetic anisotropy of the adjacent adenine base, although the magnetic anisotropies of various other groups in the molecules also may contribute. A comparison of Table XXVIII with Table III of Section 2. 1 shows that the shifts resulting from replacement of a pyrimidine base by adenine are of about the same magnitude for ApU and UpA as for ApC and CpA. Since the several contributions to these shifts for ApC and CpA have also been discussed in Section 2. 1. 2 this subject will not be dealt with further, as the same treatment applies to ApU and UpA as well.

A study of the temperature dependence of the pmr spectra of ApU and UpA[57] has indicated that there is a significant intramolecular base-stacking interaction in these dinucleotides, although the A-U base-stacking tendency appears to be somewhat smaller than the A-C base-stacking tendency in ApC and CpA (a result which is in agreement with the optical studies of these dinucleotides--see Part I, Section 3. 1).

7. 2. Purine Binding Studies

The sequence isomers ApU and UpA were selected to serve as

TABLE XXVIII. Shifts of uridine proton resonances resulting from the substitution of adenine for uracil as the adjacent base in a dinucleoside monophosphate (concentration 0.010 M).

	(3′) H_6 (5′)		(3′) H_5 (5′)		(3′) $H_{1'}$ (5′)	
	ppm		ppm		ppm	
ApU	-8.188		-6.08		-6.203	
UpA	-8.170		-6.215		-6.172	
UpU	-8.332	-8.342	-6.308	-6.333	-6.332	-6.380
Shift	+0.162	+0.154	+0.093	+0.25	+0.160	+0.177

subjects for an investigation of the interaction of purine with purine-pyrimidine and pyrimidine-purine dinucleotides. The differences in the relative base-stacking tendencies of adenosine and uridine previously reported[17, 20] suggested that the two bases of these dinucleotides would bind purine with quite different affinities, and it was felt that sequence-dependent effects concerning the purine-dinucleotide complexes would be quite apparent with ApU and UpA. Thus the interaction of purine with these sequence isomers was investigated at both high and low dinucleotide concentration.

7. 2. 1. Low dinucleotide concentration - 0. 010 M

Both ApU and UpA have been found to undergo extensive intermolecular self-association in aqueous solution.[57] The dimerization constants derived for these processes are about the same as for the self-association of ApC and CpA reported in Section 2. 2 (~2. 5 l/mole). Furthermore, the chemical shifts of the protons of ApU and UpA are concentration dependent as a result of the intermolecular processes, as with ApC and CpA. Thus it is particularly important that the interaction of purine with these dinucleotides be studied at low dinucleotide concentration, so that the chemical shifts observed as purine is added will represent only the purine-binding process, and will not be complicated by changes in the extent of dinucleotide self-association as a result of competition between the two equilibria.

Accordingly, the effects of added purine on the 100 Mcps pmr spectra of 0. 010 M solutions of the sodium salts of ApU and UpA were

investigated. The purine-induced shifts of the monitored proton resonances to higher fields are summarized in Figures 56-58. The adenine base proton resonances exhibit the largest shifts, followed by the ribose $H_{1'}$ protons of the adenosine moieties of the two dinucleotides. The H_2 resonances of ApU and UpA are shifted by nearly identical amounts, with the purine-induced shifts at 1.0 M purine concentration being 0.559 ppm and 0.550 ppm, respectively. There is a small difference between the purine-induced shifts for the H_8 protons, with H_8 of ApU (3′-nucleoside) being shifted by 0.349 ppm and H_8 of UpA (5′-nucleoside) being shifted by 0.313 ppm at 1.0 M purine concentration. The ribose $H_{1'}$ resonance of adenosine is shifted more when it is attached to the 5′-nucleoside (in UpA) than when it is attached to the 3′-nucleoside (in ApU). These shifts, at 1.0 M purine concentration, are 0.326 ppm and 0.279 ppm, respectively. The proton resonances of the uridine moieties of these two dinucleotides experience much smaller purine-induced shifts to higher fields than do the adenosine resonances. Furthermore, there appears to be a very large sequence dependence for the shifts of the uracil base proton resonances. In each case, a given proton is shifted more when it is attached to the 3′-esterified nucleoside. This effect is most pronounced for the H_5 resonances, as H_5 of UpA is shifted by 0.235 ppm while H_5 of ApU is shifted by only 0.047 ppm at 1.0 M purine concentration. There is also a sizable sequence-dependence to the purine-induced shifts for the H_6 resonances (0.136 ppm in UpA compared to 0.087 ppm in ApU at 1.0 M purine), whereas the uridine $H_{1'}$

FIGURE 56

Plot of the purine-induced shifts for the adenine base proton resonances of 0. 010 M ApU and UpA (sodium salts), cps at 100 Mcps.

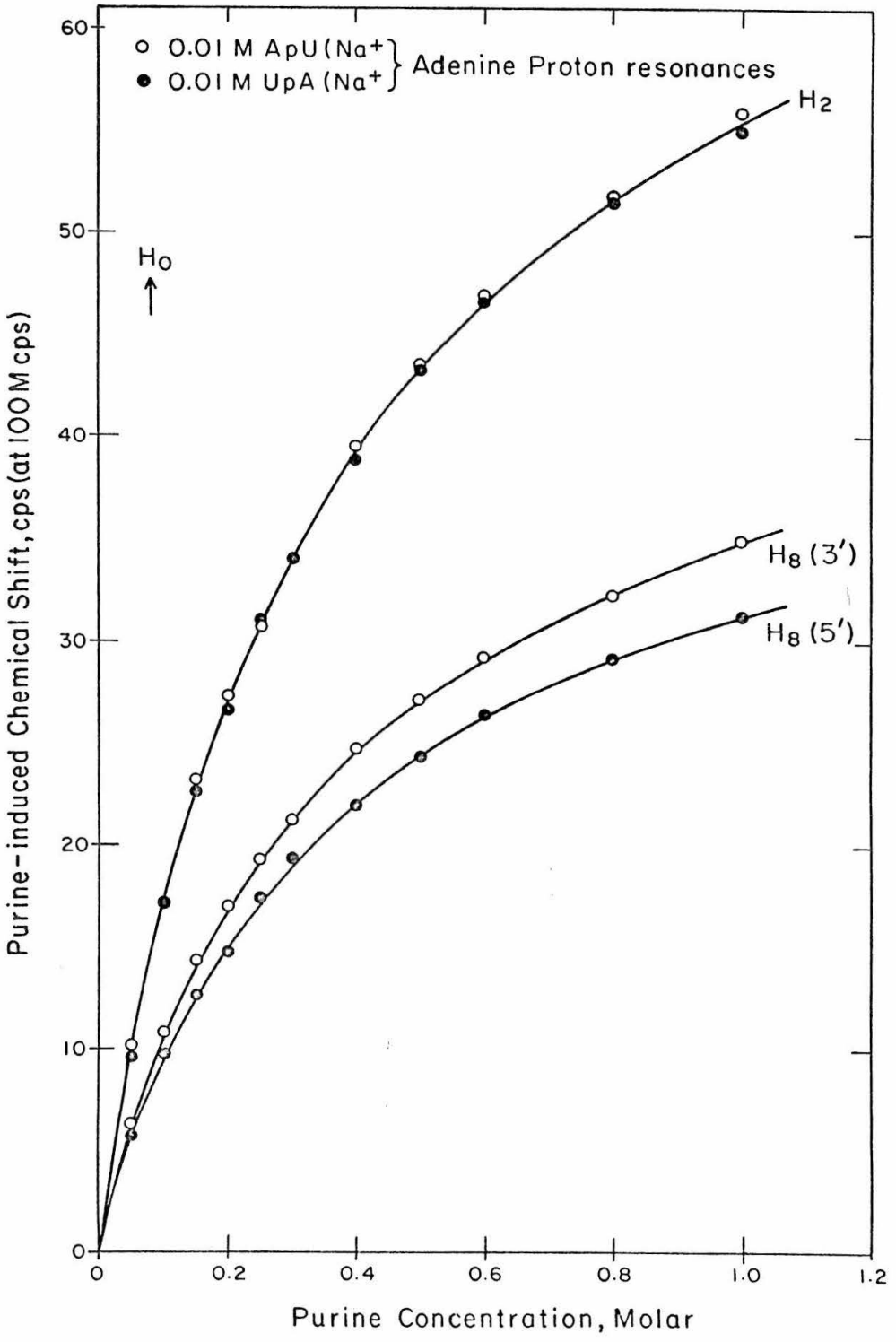

○ 0.01 M ApU (Na+)
● 0.01 M UpA (Na+)
Adenine Proton resonances
H2
H8 (3′)
H8 (5′)
H0
Purine-induced Chemical Shift, cps (at 100 M cps)
Purine Concentration, Molar
0
10
20
30
40
50
60
0
0.2
0.4
0.6
0.8
1.0
1.2

FIGURE 57

Plot of the purine-induced shifts for the uracil base proton resonances of 0. 010 M ApU and UpA (sodium salts), cps at 100 Mcps.

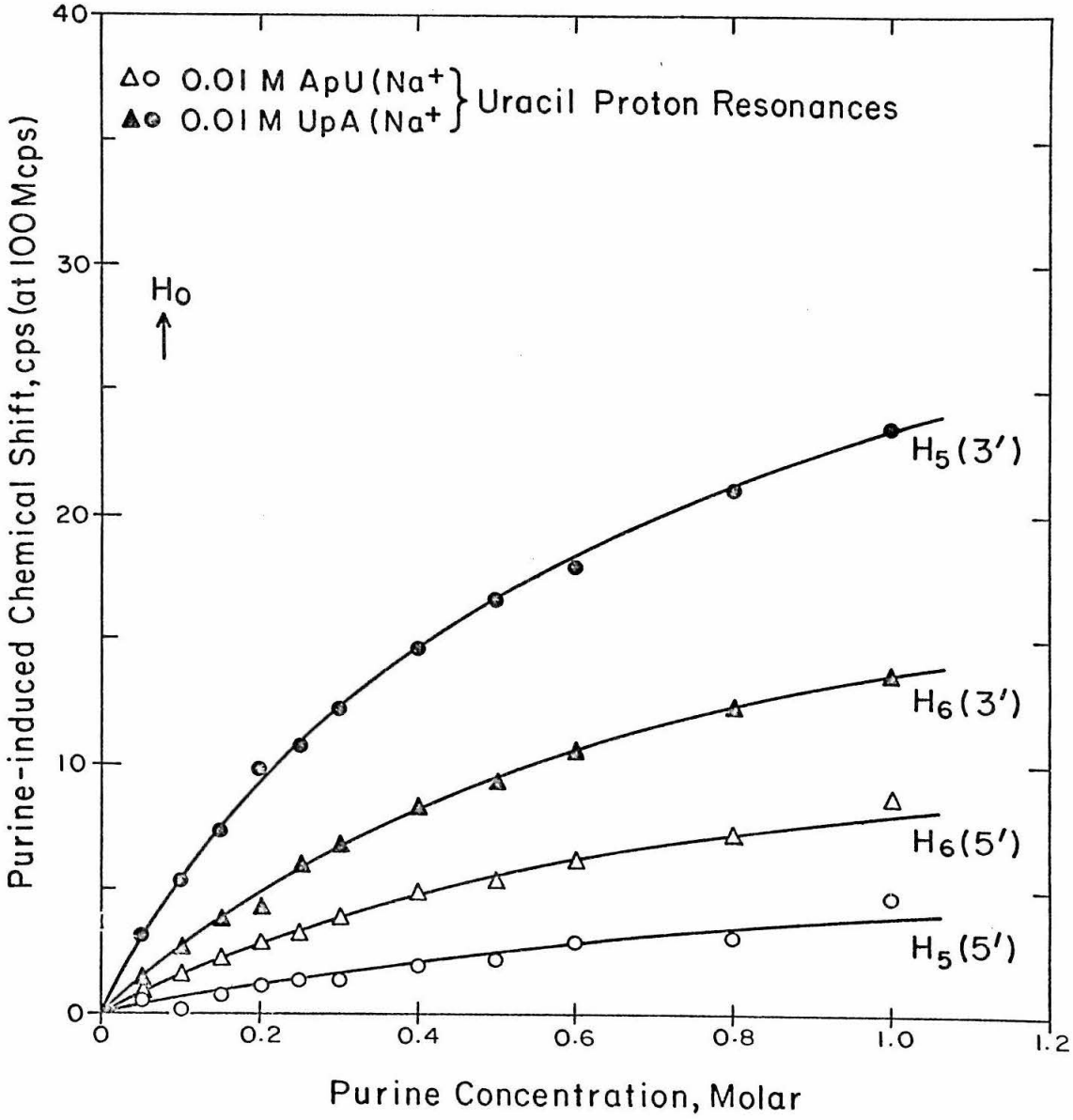
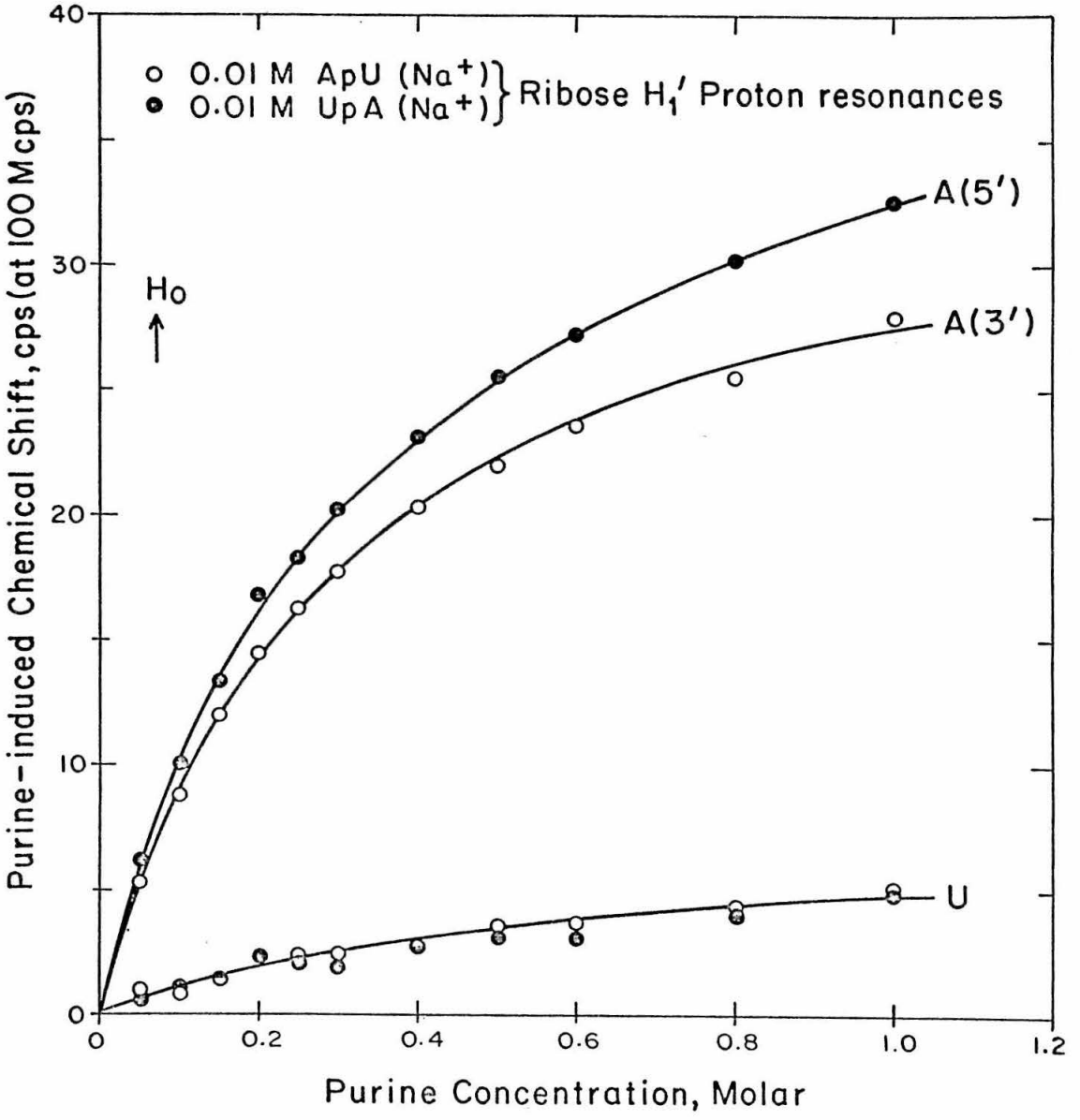

40
30
20
10
0
0.2
0.4
0.6
0.8
1.0
1.2
Δ○ 0.01 M ApU (Na+)
▲● 0.01 M UpA (Na+)
Uracil Proton Resonances
H0
H5 (3′)
H6 (3′)
H6 (5′)
H5 (5′)
Purine-induced Chemical Shift, cps (at 100 Mcps)
Purine Concentration, Molar

FIGURE 58

Plot of the purine-induced shifts for the ribose $H_{1'}$ proton resonances of 0.010 M ApU and UpA (sodium salts), cps at 100 Mcps.


40
30
20
10
0
0
0.2
0.4
0.6
0.8
1.0
1.2
o 0.01 M ApU (Na+)
• 0.01 M UpA (Na+)
Ribose H1' Proton resonances
A(5')
A(3')
U
Ho
Purine-induced Chemical Shift, cps (at 100 Mcps)
Purine Concentration, Molar

resonances experience small and essentially identical shifts in the two dinucleotides.

It is quite obvious that the added purine stacks more strongly with the adenine bases of ApU and UpA than with the uracil bases. This is as expected, in view of the relative self-association tendencies of the purine and pyrimidine nucleosides mentioned previously (see Table I). It is not difficult to understand why there is no sequence dependence for the purine-induced shifts of the adenine H_2 protons. These protons are rather far removed from the point of attachment of the adenine base to its ribose moiety, and purine molecules could freely overlap the six-membered ring of adenine to which this proton is bound without interference by the ribose-phosphate backbone. This would be true for an intercalated purine molecule as well as externally stacked purine species, and as a consequence the purine-induced shifts for H_2 would be quite similar regardless of the position of phosphate esterification. The adenine H_8 proton is rather close to the ribose ring to which this base is attached, and steric restrictions on the geometry with which an interacting purine molecule could approach the five-membered ring of adenine would be expected. As a consequence, it is not surprising that the purine-induced shifts for the H_8 protons are somewhat sequence-dependent.

The large sequence-dependence with regard to the purine-induced shifts shown by the uracil base protons, particularly H_5, is quite surprising in view of the results of the study of UpU reported in Section 6. 3. It is highly unlikely that these differences in purine-

induced shifts between uracil protons of ApU and UpA could be a consequence of the stacking of purine on both faces of each base with the dinucleotides unstacked, or on the exposed face of each base of the stacked dinucleotides. Purine-induced chemical shift differences due to "external" stacking of this type could hardly be greater than those observed for UpU. This sequence dependence is thus attributed to the formation of intercalated complexes, and there are two possible explanations for the observed behavior. First of all, it may be recalled that the purine-induced shift for a given proton of TpdU or dUpT (Section 4. 2) is larger when it is attached to the 3′-esterified nucleoside. This appears to be a consequence of a greater exposure of the base protons of the 3′-nucleoside to the ring-current magnetic anisotropy of an intercalated molecule resulting from steric considerations. Such could also be the case for ApU and UpA.

However, there is an added complication here, in that the uracil protons in these two dinucleotides are exposed to the ring-current magnetic anisotropy of the adjacent adenine base in the absence of purine intercalation. Thus the shielding effect resulting from the intercalation of a purine molecule would be compensated for somewhat by a reduction of the shielding produced by the adenine base, as the dinucleotide "opens up" to accommodate the incorporated purine molecule. Consequently, the net shifts of the uracil proton resonances to higher fields as a result of purine binding would not be expected to be as large for ApU and UpA as for UpU. It is quite easy to account for the very small purine-induced shift for the H_5 resonance of ApU on

this basis. As can be seen from Table XXVIII, this proton experiences a large shielding effect (+0. 25 ppm) from the adjacent adenine base, compared to that felt by the H_5 proton of UpA (+0. 09 ppm). This is quite reasonable in light of the discussion of ApC and CpA in Section 2. 3, where it was shown that the cytosine protons in ApC would be more exposed to the magnetic anisotropy of the adenine base than in CpA, on the basis of the geometries of these dinucleotides in the stacked conformations (see Figure 10). Thus an intercalated purine molecule in ApU would to a large extent merely replace the adenine base as the source of a shielding field for the uracil base protons, and the net upfield shift resulting from purine intercalation would be expected to be rather small. With UpA on the other hand, the uracil base protons feel a much smaller shielding field from the adenine base, so that the net upfield shift produced by an intercalating purine molecule would be correspondingly greater. Of course, shifts of the uracil resonances to higher fields result not only from purine intercalation, but from external stacking of the purine species as well. In the present discussion, it has been assumed that the shifts resulting from external stacking of purine on the uracil bases of ApU and UpA are essentially the same as for UpU.

7. 2. 2. High dinucleotide concentration - 0. 08 M

The interaction of ApU and UpA with purine at high dinucleotide concentration was investigated with the aim determining the effects of complex formation on the purine resonances. As mentioned above, the

presence of competition between dinucleotide self-association and purine binding in these systems would render interpretation of the chemical shifts of the dinucleotide protons rather difficult. For this reason, attention is focused on the purine proton resonances in this Section.

The addition of purine to 0. 08 M solutions of the sodium salts of ApU and UpA results in shifts of the dinucleotide proton resonances to higher fields, and a broadening of the purine proton resonances, as consequences of complex formation. The uridine H_5 resonances in ApU and UpA (and, to a lesser extent, the $H_{1'}$(U) resonance) are quite broad ($\sim$4 cps) in the absence of purine, and become narrow (1-1. 5 cps) as purine is added. Similar behavior was observed for the H_5 protons of CpC (Section 5. 3) and UpU (Section 6. 3), although the H_5 resonances were much broader for these pyrimidine-pyrimidine dinucleotides. This line broadening of the ApU and UpA resonances is also believed to result from formation of intermolecular self-intercalated dimers, with this process being disrupted by the binding of purine to the dinucleotides.

The purine proton resonance linewidths in the presence of 0. 08 M ApU and UpA are listed in Table XXIX. It is seen, by comparison of these values with the purine linewidths in the presence of the other dinucleotides studied (Tables XIV, XIX, XX, XXV), that the purine line broadening effect is significantly less pronounced here than with these other dinucleotides. This is unlikely to be a consequence of a smaller amount of purine being bound in intercalated

TABLE XXIX. Purine proton resonance linewidths in the presence of 0. 08 M ApU and UpA (sodium salts, pD 7. 8-8. 0). (Full linewidth at half-height in cps, measured at 100 Mcps.)

Purine Conc.	Purine Resonance Linewidths H_6	H_2	H_8	Dinucleotide
	cps	cps	cps	
0. 04 M	5. 6	2. 8	3. 2	ApU
0. 08	4. 3	1. 4	~4	
0. 12	1. 8	0. 9	1. 7	
0. 04 M	~6	1. 9	~5	UpA
0. 08	5. 4	1. 6	4. 6	
0. 12	4. 2	1. 3	3. 5	

complexes with ApU and UpA, in view of the relative stacking tendencies of the various bases. However it is not possible to rule out this explanation completely, as the intramolecular A-U base-stacking interactions in ApU and UpA may inhibit the formation of intercalated complexes somewhat. It is believed more likely that the less pronounced purine line broadening observed with ApU and UpA reflects smaller intrinsic linewidths for purine molecules bound in intercalated complexes with these dinucleotides.

Effect of pD on the ApU/UpA-Purine Interaction. -- The pD dependences for the pmr spectra of 0.08 M solutions of ApU and UpA containing 0.12 M purine were examined, to see if the purine line broadening in these systems is sensitive to a charge on a base of the dinucleotide. Since the adenine base has a pK of ~3.7,[98] it will be protonated before purine (pK 2.4) as the pD of the solution is lowered. The linewidths of the purine proton resonances in these dinucleotide solutions are given in Tables XXX and XXXI at several values of solution pD. The behavior in these systems is the same as for the CpC-purine solution as discussed in Section 5.3.3. Namely, the purine resonances broaden with decreasing pD as a base of the dinucleotide is protonated, and begin to narrow only when the purine molecules are protonated as well. The purine-dinucleotide complexes appear to be completely dissociated at low pD, presumably because of electrostatic repulsion between the protonated purine and adenine bases.

Thus it appears that the presence of a charge on the adenine

TABLE XXX. Purine proton resonance linewidths in a 0. 08 M ApU solution containing 0. 12 M purine (measured at 100 Mcps).

pD	Purine Resonance Linewidths		
	H_6	H_2	H_8
8. 00	1. 8 cps	0. 9 cps	1. 7 cps
5. 70	2. 6	1. 2	1. 7
4. 50	3. 6	1. 6	2. 2
3. 35	3. 7	1. 7	2. 4
2. 45	3. 2	2. 0	1. 8
1. 42	1. 3	1. 2	1. 2
0. 95	1. 2	1. 2	1. 3

TABLE XXXI. Purine proton resonance linewidths in a 0. 08 M UpA solution containing 0. 12 M purine (measured at 100 Mcps).

pD	Purine Resonance Linewidths		
	H_6	H_2	H_8
7. 80	4. 2 cps	1. 3 cps	3. 5 cps
4. 65	~7	2. 5	3. 9
4. 20	6. 6	2. 5	4. 0
3. 18	~6	2. 4	3. 8
2. 20	~3. 6	2. 3	~2. 3
1. 20	1. 2	1. 2	1. 4

base either results in a larger tendency for the formation of intercalated complexes between purine and ApU or UpA, or perhaps causes the average geometry of the intercalated purine molecule in the complex or the correlation time controlling the purine proton relaxation to change in such a manner as to lead to increased intrinsic linewidths for the resonances of the intercalated purine molecule. An increase in the extent of the binding of purine to ApU or UpA could conceivably result if protonation of the adenine base causes a decrease in the intramolecular A-U base stacking tendency, thus permitting a greater degree of purine intercalation, or if the stacking interaction between purine and adenine were stronger when adenine is protonated. At the present time, the geometries of the purine-dinucleotide complexes and the factors responsible for the correlation time characterizing the purine proton relaxation mechanism are insufficiently understood to permit an a priori estimate of the linewidths of the purine resonances in these various intercalated complexes.

8. Nature of the Purine-Dinucleotide Intercalated Complex

It is evident from the discussions in the preceding Sections of this thesis that the formation of intercalated complexes between purine and the dinucleoside monophosphates is an important aspect of the interaction between these molecules. It is the purpose of this discussion to deal with several of the properties of this type of complex, and with the purine-dinucleotide interaction in general, in a unified manner.

8. 1. Mode of Purine-Dinucleotide Interaction

An understanding of the binding of purine to dinucleoside monophosphates by base-stacking requires an appreciation of the behavior of purine alone in aqueous solution. As was mentioned previously, purine self-associates extensively in aqueous solution by base-stacking to give dimers, trimers, and higher aggregates as well. According to the work of Ts'o and Chan[18] the association constants for the binding of each additional purine molecule to an aggregate are equal, and they reported a value of 2. 1 $molal^{-1}$ based on osmotic studies at 25°C. More recently, Van Holde and Rossetti[21] have reported a value of 2. 8 l/mole for this process, based on sedimentation studies. As a consequence of this extensive purine self-association, the activity coefficient decreases markedly with increasing purine concentration. The concentration of monomeric purine therefore increases quite slowly with increasing total stoichiometric purine concentration.

As mentioned in Section 3, any of the associated purine species would be expected to stack on the exposed base faces of a stacked dinucleotide molecule, or on both sides of each base of an unstacked dinucleotide. However the formation of a purine-dinucleotide intercalated complex would involve only a monomeric purine molecule, as the two bases of a dinucleotide are restricted by the ribose-phosphate-ribose backbone such that no more highly polymerized purine species could be incorporated in the sandwich-like stack. The purine-induced shifts of the dinucleotide proton resonances to higher fields are a consequence of both externally stacked and

intercalated purine species, while it is suggested that the purine-induced chemical shift difference between analogous protons is due primarily to purine intercalation. Since the purine-induced "splitting" results from an equilibrium with only monomeric purine molecules, this effect would be expected to level off sooner than the total induced shift with increasing purine concentration, since the activity of the monomer increases more slowly than the total activity of all purine species. (18)

This behavior seems apparent in a comparison of the purine-induced chemical shift differences between the H_5 and the H_6 protons of 0. 010 M CpC (sodium salt), shown in Figure 59, with the total purine-induced shifts for these protons, Figures 32 and 33. The plots of purine-induced splitting quite obviously level off more rapidly. Similar behavior is noted also in the 0. 08 M CpC (sodium salt)-purine interaction (Figures 37 and 38), but is less apparent in the 0. 010 M UpU-purine interaction (compare Figure 60 with Figures 49 and 50). These observations are taken to reinforce the idea that the purine-induced chemical shift difference results primarily from purine intercalation. However, it is clear that externally stacked purine species could also affect the protons of 3′- and 5′-esterified nucleosides somewhat differently in such molecules as CpC and UpU, although such differences should be smaller than those resulting from purine intercalation.

FIGURE 59

Plots of the purine-induced chemical shift differences (splittings) between the two H_5 protons and the two H_6 protons of 0.010 M CpC (sodium salt), cps at 100 Mcps.

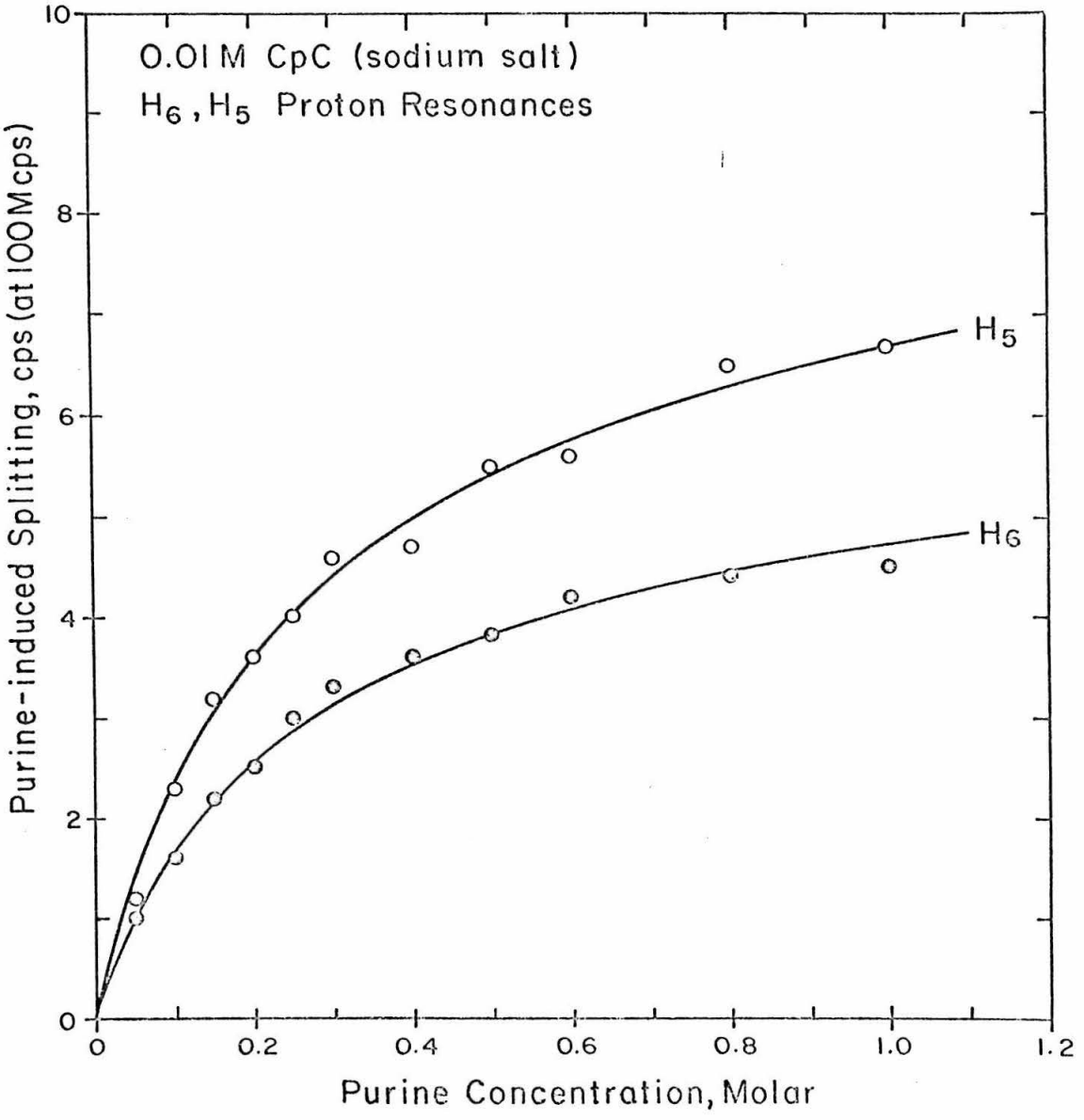
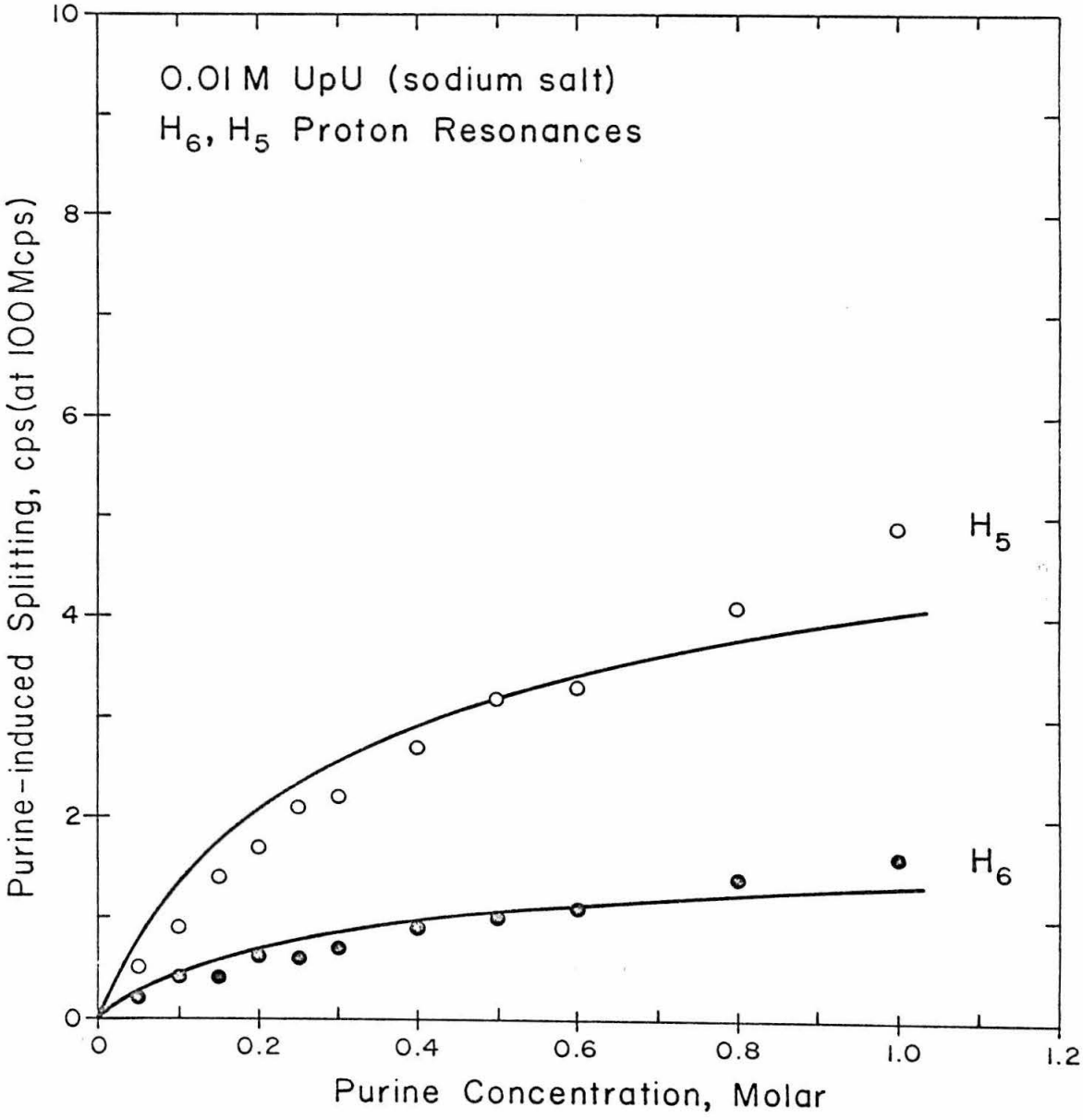

0.01 M CpC (sodium salt)
H6, H5 Proton Resonances
H5
H6
Purine-induced Splitting, cps (at 100 Mcps)
Purine Concentration, Molar
0
2
4
6
8
10
0
0.2
0.4
0.6
0.8
1.0
1.2

FIGURE 60

Plots of the purine-induced chemical shift differences (splittings) between the two H_5 protons and the two H_6 protons of 0.010 M UpU (sodium salt), cps at 100 Mcps.


0.01 M UpU (sodium salt)
H_6, H_5 Proton Resonances
Purine-induced Splitting, cps(at 100Mcps)
Purine Concentration, Molar
H_5
H_6
0
2
4
6
8
10
0
0.2
0.4
0.6
0.8
1.0
1.2

8. 2. Mechanism of the Purine Line-Broadening Phenomenon

There are several factors which could conceivably account for the broadening of the purine proton resonances observed as purine binds to a dinucleoside monophosphate, but most of these can be eliminated from consideration rather easily. For example, the observed purine proton line broadening does not appear to be attributable to a slow rate of exchange of the purine molecules between free and bound environments. The protons of purine molecules incorporated between the bases of a dinucleotide would have slightly different chemical shifts from the protons of unbound purine molecules in solution. If the exchange rate between bound and unbound purine were comparable to the chemical shift difference between the two purine species, the purine resonances could be broadened. [111] However, if this were the case, it would appear that a similar line broadening effect should also be observed for the base protons of the dinucleotide. This is not observed; in every purine-dinucleotide interaction which has been examined, the proton resonances of the dinucleotide show no broadening whatsoever, and in fact often become narrower as purine is added. Furthermore, the long lifetime for a purine molecule in a given environment implied by this interpretation ($\sim 10^{-2}$ sec) is also unreasonable.

It has also been shown that a longer tumbling time for a purine molecule in solution, perhaps as a consequence of its being bound to a dinucleotide molecule, could not in itself account for the observed purine line broadening. Solutions of purine in glycerine, for example,

show narrow purine resonances.[101] Also, ^{14}N NMR studies of purine[102] have indicated that the rotational correlation time for a purine molecule in aqueous solution increases markedly with increasing concentration as a result of the formation of larger purine aggregates, although the purine proton resonances remain quite narrow.

In view of the above discussion, the magnetic dipolar relaxation mechanism for the purine protons in the purine-dinucleotide intercalated complex, as proposed in Section 4. 3, appears even more attractive as the probable cause of the observed purine line broadening. It is believed that the purine protons are exposed to strong magnetic dipolar fields produced by the protons of the ribose-phosphate backbone in the intercalated complex. For these fluctuating local fields to be effective for specific line broadening of the purine proton resonances, the purine protons must be oriented favorably with respect to the ribose protons producing the local fields, and the random process which is responsible for these fluctuations must be charcterized by a correlation time which is of the order of 10^{-10} sec. It is not unreasonable to assume that this correlation time corresponds to the mean lifetime of a purine molecule in the intercalated complex.

If it is indeed a magnetic dipolar coupling between the purine protons and the protons of the ribose-phosphate backbone of the dinucleotide which is responsible for the purine line broadening, the ribose proton resonances of the dinucleotide should also be broadened by this coupling. In this connection, it is noted that the ribose proton resonances of UpU (0. 08 M sodium salt) in the spectral region from

-4. 80 ppm to -4. 20 ppm appear to be broadened somewhat as purine is added to this solution, although this broadening is much less severe than for the purine protons. However, it is expected that the ribose protons would be exposed to the field produced by a single purine proton of an appropriately oriented purine molecule, while the purine proton would experience the fields produced by several ribose protons.

The mechanism for the purine proton resonance line broadening is the least well understood aspect of the purine-dinucleotide interaction, but regardless of the precise cause of this phenomenon, the conclusion that it derives from the formation of a purine-dinucleotide intercalated complex seems inescapable. A number of questions relating to the sequence dependence of this behavior, between such dinucleotides as TpdU and dUpT, and ApU and UpA, remain to be resolved.

Finally, it is observed that the magnitude of the purine resonance line broadening is in the order $H_6 > H_8 > H_2$ for all the purine-dinucleotide systems studied. This suggests that there may be some preference for the purine molecules to be oriented with the H_6 and H_8 protons closer to the ribose protons in the intercalated complex. It does seem somewhat surprising, however, that this geometry would be essentially the same for all of the purine-dinucleotide intercalated complexes.

8. 3. Consideration of the Purine Intercalation Equilibrium

The equilibrium for the interaction of a monomeric purine

molecule with a dinucleotide molecule has been considered, and after making several simplifying assumptions, it has been possible to extract intercalation constants for this purine-dinucleotide binding process from the chemical shift data for CpC and UpU. This treatment neglects the intermolecular self-association of the dinucleotide, and the interaction of purine with the dinucleotide to give complexes not involving purine intercalation have not been considered.

First of all, the following quantities are defined:

C = concentration of purine-dinucleotide intercalated complex, moles/l.

m = concentration of purine monomer, moles/l.

d = concentration of "free" dinucleotide, moles/l.

D = total stoichiometric dinucleotide concentration, moles/l.

and

$$D = d + C.$$

The intercalation equilibrium, involving intercalation constant K_I, is simply

$$\frac{C}{m \cdot d} = K_I \quad (1/\text{mole}) \tag{6}$$

This expression can be rearranged to give the concentration of the intercalated complex as

$$C = \frac{K_I m}{1 + K_I m} D. \tag{7}$$

Thus the concentration of the intercalated complex in a purine-dinucleo-

tide solution depends on the concentration of monomeric purine free in solution, as well as on the intercalation constant and the total stoichiometric dinucleotide concentration.

As shown by Ts'o and Chan, [18] the total purine concentration M in a solution of purine alone is related to the concentration of the purine monomer by:

$$M = \sum_{n=1}^{\infty} n\, m^n\, K_P^{(n-1)} \tag{8}$$

where K_P is the purine self-association constant. In a solution of purine containing a dinucleotide capable of forming an intercalated complex with the purine monomer, (8) must be modified by adding the concentration of the intercalated complex, so that here

$$M = \frac{K_I m}{1 + K_I m} D + \sum_{n=1}^{\infty} n\, m^n\, K_P^{(n-1)} \tag{9}$$

expresses the total stoichiometric purine concentration in a purine-dinucleotide solution in which only 1:1 complexes are considered. Equation (9) can be solved numerically to give m for a given stoichiometric purine concentration M and dinucleotide concentration D, if the intercalation constant K_I and purine self-association constant K_P are provided and the infinite sum is truncated at some finite value N.

It has been assumed, in this analysis, that the purine-induced chemical shift difference δ between two analogous protons of a

dinucleotide with two identical bases is a direct measure of the extent of formation of the purine-dinucleotide intercalated complex. The fraction of the dinucleotide involved in the complex is, from (7), simply:

$$f_C = \frac{K_I m}{1 + K_I m} \qquad (10)$$

Thus the observed purine-induced chemical shift difference δ between two analogous protons is linearly proportional to f_C, the proportionality constant being the intrinsic purine-induced chemical shift difference Δ between these protons in the intercalated complex, and: $\delta = \Delta f_C$.

A least-squares computer fit of (9) to the purine-induced chemical difference data for the base protons of CpC and UpU was made. Briefly, the procedure involves solving (9) for m (given values for D, K_I, and K_P) as a function of stoichiometric purine concentration M, using Δ as an adjustable parameter to give the best fit of f_C (calculated from (10)) to the observed purine-induced chemical shift difference data. This procedure was carried out for several values of K_I, and the value giving the best fit was chosen as the intercalation constant for the particular complex. In this analysis, the value used for the purine self-association constant was 2.4 l/mole, obtained from the work of Van Holde and Rossetti[21] after compensating for the temperature difference (29°C here compared to 25°C in their work) using the enthalpy value they report. Equation (9) was solved by the Newton-Raphson method[103] with the sum in (9) taken to n = 10.

The value of K_I giving the best fit to the purine-induced

chemical shift difference ("splitting") data for the H_5 protons of 0. 010 M CpC was 0. 8; the best fit for the H_6 protons was obtained with K_I = 1. 45. The compromise value of K_I for these two sets of protons giving the lowest total mean square deviation between the calculated behavior and the experimental data was 1. 0 l/mole. This is certainly a reasonable equilibrium constant to expect for the formation of a purine-dinucleotide complex, being quite comparable with the self-association constants for the components of this system. The intrinsic purine-induced chemical shift differences Δ for the CpC-purine complex were calculated to be 0. 37 ppm for the H_5 protons and 0. 26 ppm for the H_6 protons. These values are not out of line with what might be expected on the basis of the magnitudes of the ring-current shifts which could be produced by a purine molecule.[22] These results are rather encouraging, and suggest that this oversimplified model for the formation of a purine-dinucleotide intercalated complex is not wholly unreasonable.

The results obtained from the application of this method to the 0. 010 M UpU data are, however, rather disappointing. The best fits for the purine-induced "splitting" for the H_5 and H_6 resonances are obtained for K_I values less than 0. 1 l/mole, and in this instance the intrinsic purine-induced chemical shift differences giving the best fits are unreasonably large: Δ = 1. 89 ppm for the H_5 protons and Δ = 0. 62 ppm for the H_6 protons. If one assumes that the purine-induced chemical shift differences for the H_5 and H_6 protons of the uracil bases in UpU should be comparable with those for the analogous

protons in CpC, it is found that a value of 0.4 l/mole for the formation constant for the UpU-purine intercalated complex yields $\Delta = 0.50$ ppm for H_5 and $\Delta = 0.16$ for the H_6 protons.

The values of K_I and Δ obtained by this procedure can be put back into (9) and the equation can be solved to give the observed purine-induced chemical shift difference as a function of total stoichiometric purine concentration. These computed curves are shown as the lines in Figures 59 and 60. The agreement between the calculated behavior giving the best fit and the data points is seen to be quite good for CpC, although rather poor for UpU. It must be emphasized that no great deal of significance is claimed for the intercalation constants obtained by this method. Their utility lies principally in the estimation of the intrinsic linewidths for the proton resonances of purine bound in the intercalated complexes, as described in the next Section.

8.4. Purine Resonance Linewidths in the Intercalated Complexes

Since the rate of exchange of purine molecules between bound and free environments is rapid on the NMR time scale, the observed linewidths for the purine resonances in the presence of a dinucleotide represent the weighted average of the linewidths for purine molecules bound in the purine-dinucleotide intercalated complex, and for free purine molecules in the solution. These "free" purine molecules are taken to include also those purine molecules bound to the dinucleotide by external stacking, since the purine resonance linewidths are apparently unaffected by this process.

The relationship giving the observed resonance linewidth is as follows:

$$W_{obs} = f_B\left(\frac{1}{\pi T_{2_B}}\right) + f_o\left(\frac{1}{\pi T_{2_o}}\right) \qquad (11)$$

where:

W_{obs} = observed resonance linewidth, cps.

T_{2_B} = transverse relaxation time for the proton of a bound purine molecule, sec.

T_{2_o} = transverse relaxation time for the proton of a free purine molecule, sec.

f_B = mole fraction of purine bound.

f_o = mole fraction of purine free in solution, $(1 - f_B)$.

Expression (11) is rearranged to give:

$$W_{obs} = f_B\left(\frac{1}{\pi T_{2_B}} - \frac{1}{\pi T_{2_o}}\right) + \frac{1}{\pi T_{2_o}} \quad . \qquad (12)$$

Thus the observed linewidth for a purine proton resonance is proportional to the fraction of purine bound in the intercalated complex, and a plot of the observed linewidth <u>vs</u> the fraction of purine bound should be linear, with a slope of

$$\left(\frac{1}{\pi T_{2_B}} - \frac{1}{\pi T_{2_o}}\right)$$

and an intercept of $\frac{1}{\pi T_{2_o}}$.

From the results of Section 8. 3, it is possible to calculate the fraction of purine bound in the purine-dinucleotide intercalated complex as a function of the total stoichiometric purine concentration. This was done using the intercalation constants K_I derived for the interaction of purine with CpC and UpU, and the results are listed in Table XXXII for 0. 08 M dinucleotide solutions. The observed purine proton resonance linewidths in the 0. 08 M CpC (sodium salt), 0. 08 M CpC (ammonium salt), and 0. 08 M UpU (sodium salt) solutions are plotted vs the calculated fraction of purine bound in Figures 61-63. The lines on these plots were drawn to give the best linear fits to the data points yet give an intercept at $f_B = 0$ of 1 cps, the known linewidth of the resonances of free purine in solution $\left(\frac{1}{\pi T_{2_O}}\right)$. There is a fair amount of scatter to the data, particularly at low concentrations where the accuracy of the linewidth measurements is poor. The slopes of these plots yield the intrinsic linewidths $\left(\frac{1}{\pi T_{2_B}}\right)$ for the three purine protons in the purine-dinucleotide intercalated complexes with CpC (Na^+), CpC (NH_4^+) and UpU (Na^+), and these results are listed in Table XXXIII.

It is noted that the linewidths for intercalated purine determined by this procedure are quite sensitive to the intercalation constant used. If the intercalation constant used were larger, the linewidths obtained would be smaller, and conversely. For example, the purine resonances are broadened comparably by 0. 08 M CpC and UpU (sodium salts), but since the apparent intercalation constant for UpU is smaller than that for CpC, the calculated linewidths for the UpU-purine complex turn out to be more than twice as great as for CpC. Thus the values reported

TABLE XXXII. Mole fractions of purine and dinucleotide bound in the intercalated complexes between purine and CpC and UpU (0. 08 M stoichiometric dinucleotide concentration)

Purine Conc.	Fract. Bound, $K_I = 1.0$		Fract. Bound, $K_I = 0.4$	
	Purine	CpC	Purine	UpU
0. 10	. 0502	. 063	. 0208	. 027
0. 20	. 0385	. 096	. 0161	. 041
0. 30	. 0315	. 118	. 0133	. 051
0. 40	. 0267	. 134	. 0114	. 059
0. 50	. 0233	. 146	. 0100	. 064
0. 60	. 0207	. 155	. 0089	. 069
0. 70	. 0187	. 163	. 0081	. 073
0. 80	. 0170	. 170	. 0074	. 076
0. 90	. 0156	. 176	. 0068	. 079
1. 00	. 0145	. 181	. 0063	. 081

FIGURE 61

Plots of purine resonance linewidths vs calculated mole fraction of purine in the intercalated complex with 0. 08 M CpC (sodium salt). H_6 ●, H_2 Δ, H_8 ○.

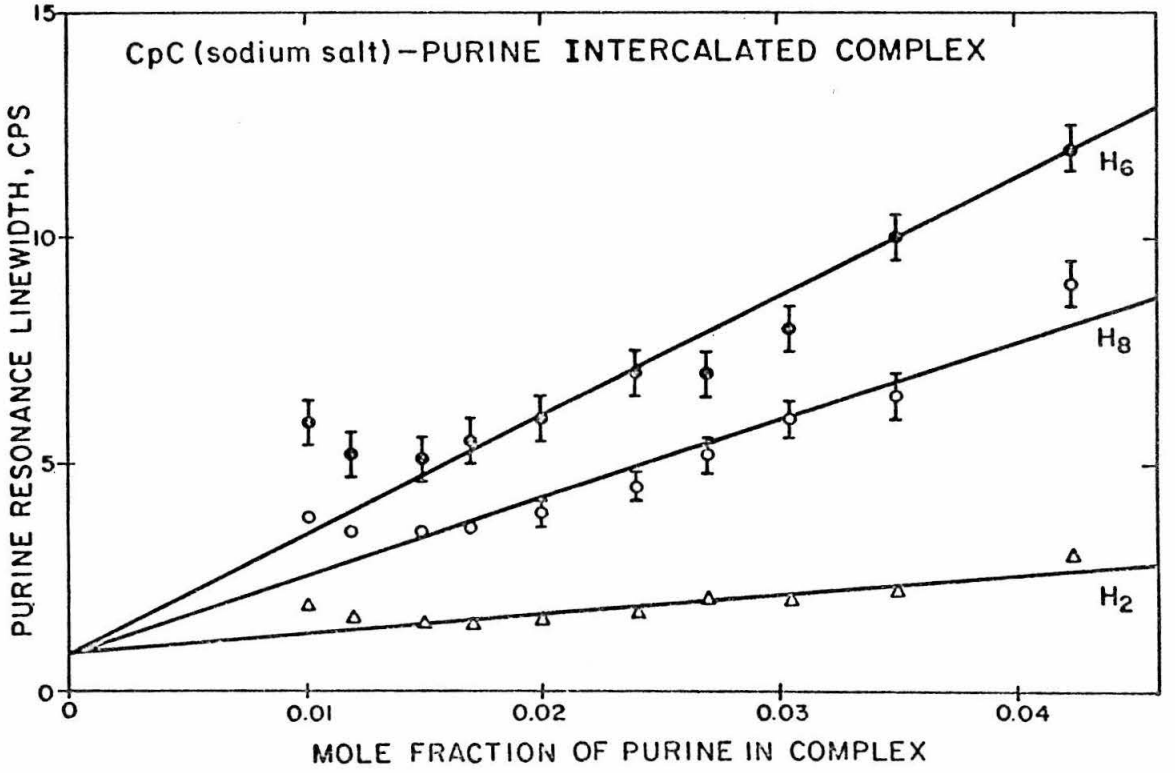

CpC (sodium salt)–PURINE INTERCALATED COMPLEX
PURINE RESONANCE LINEWIDTH, CPS
MOLE FRACTION OF PURINE IN COMPLEX
H6
H8
H2
0
5
10
15
0
0.01
0.02
0.03
0.04

FIGURE 62

Plots of purine resonance linewidths vs calculated mole fraction of purine in the intercalated complex with 0. 08 M CpC (ammonium salt). H_6 ●, H_2 ▲, H_8 ○.

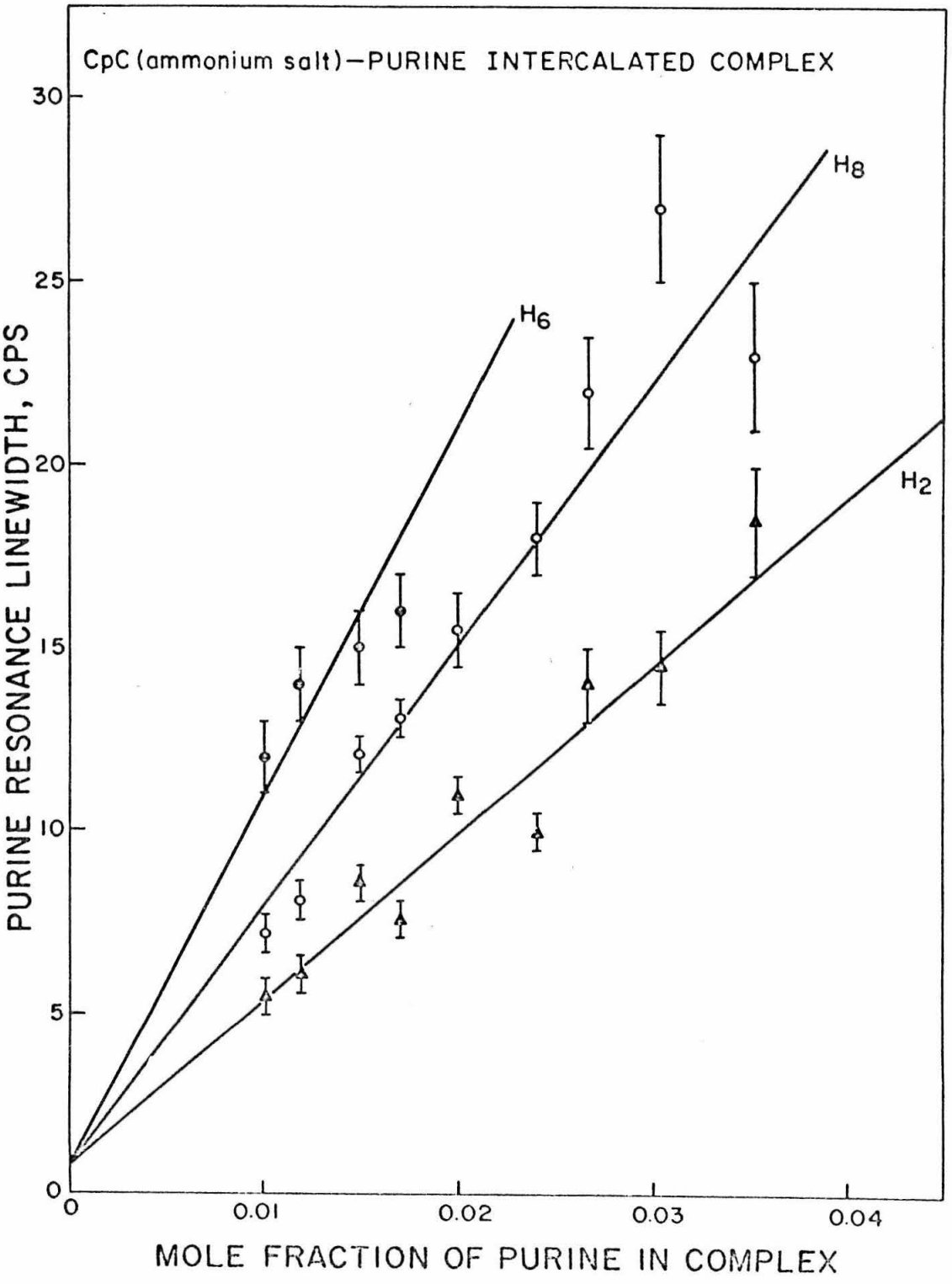

CpC (ammonium salt)—PURINE INTERCALATED COMPLEX
PURINE RESONANCE LINEWIDTH, CPS
MOLE FRACTION OF PURINE IN COMPLEX
H6
H8
H2
0
5
10
15
20
25
30
0
0.01
0.02
0.03
0.04

FIGURE 63

Plots of purine resonance linewidths vs calculated mole fraction of purine in the intercalated complex with 0. 08 M UpU (sodium salt). H_6 ●, H_2 ▲, H_8 ○.

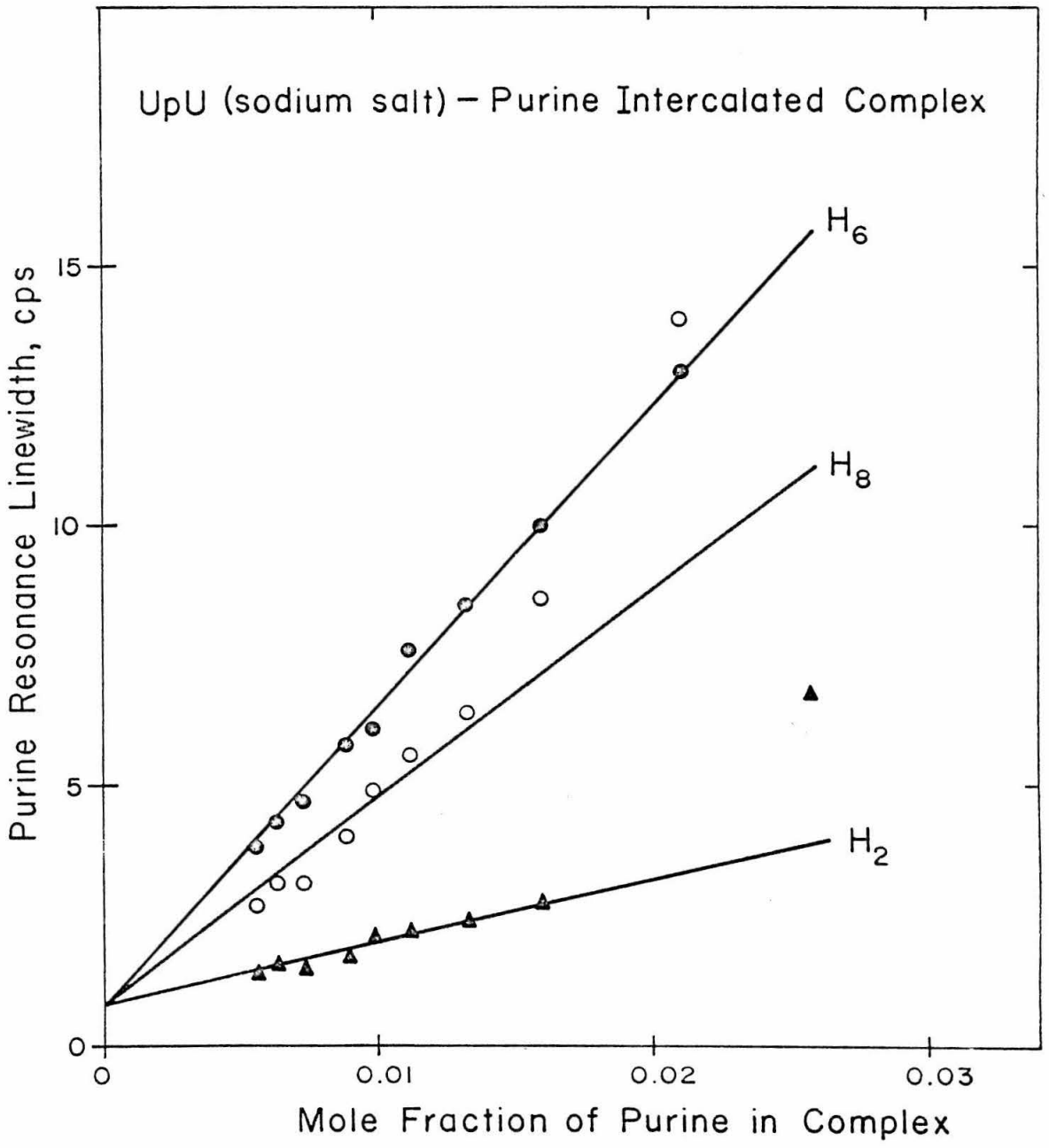

UpU (sodium salt) – Purine Intercalated Complex
H6
H8
H2
Purine Resonance Linewidth, cps
0
5
10
15
0
0.01
0.02
0.03
Mole Fraction of Purine in Complex

TABLE XXXIII. Intrinsic linewidths for the proton resonances of a purine molecule bound in intercalated complexes with CpC and UpU.

Dinucleotide	Linewidth in Complex H_6	H_2	H_8
CpC-Na^+	260 cps	45 cps	175 cps
CpC-NH_4^+	965	445	675
UpU-Na^+	575	120	400

in Table XXXIII are significant only in indicating the order of magnitude of the line broadening effect, which appears to be reasonable in view of the proposed mechanism for this effect.

9. Summary

High-resolution proton magnetic resonance spectroscopy has been demonstrated to be a useful tool for the investigation of the conformational properties of dinucleoside monophosphates in aqueous solution. It has been possible to obtain information by pmr regarding the conformation of the ribose-phosphate backbone of the dinucleotides, as well as the nature of the intramolecular stack, that is not revealed in the optical studies which have been extensively applied to study of these molecules. The pmr properties which are used to monitor the extent of intramolecular base-stacking in the dinucleotides (effect of the magnetic anisotropy of a given base on the chemical shifts of protons of the other base, and conformation of the ribose rings as indicated by the spin-spin coupling constant $|J_{H_1'-H_2'}|$) are complementary to the optical properties of hypochromicity and optical rotation, in that these two methods have different sensitivities to the range of conformations of the two bases relative to one another. However, the results presented here are in general accord with those of the optical studies as to the strengths of the intramolecular base-stacking tendencies of the various dinucleoside monophosphates.

The investigation of the interaction of purine with several dinucleotides has aided in the assignments of the pmr spectra of these

molecules, and has revealed the formation of a complex in which a purine molecule is intercalated between the bases of the dinucleotide. The nature of this purine-dinucleotide intercalated complex has been examined in some detail, and estimates of the binding constants for purine intercalation and the linewidths of the purine proton resonances in the complex have been obtained.

IV. PMR STUDIES OF MONOMER-POLYNUCLEOTIDE INTERACTIONS

Interactions among bases, nucleosides, and dinucleoside monophosphates in aqueous solution have been shown to involve nonspecific base-stacking, as discussed in preceding Sections of this thesis. It is of interest to determine if incorporation of a particular nucleotide in a polymer affects its mode of interaction with monomeric bases and nucleosides in aqueous solution, perhaps favoring hydrogen-bonding over base-stacking interactions. Several investigations of the interaction of guanine oligonucleotides with poly C and adenine oligonucleotides with poly U by optical methods[104-108] have shown that the smaller molecules bind to the polymers cooperatively to form helical structures, presumably analogous to the double and triple helices formed by the complementary polynucleotides. Studies of the adenosine-polyuridylic acid system by infrared spectroscopy,[109] and by equilibrium dialysis, solubility, optical rotation, and hydrodynamic methods[110] have shown that adenosine binds to polyuridylic acid in a cooperative manner with a "melting temperature" near 20°C. The complex involves 1 adenosine

per 2 uracil bases, and is presumably a rigid triple helix.

Proton magnetic resonance spectroscopy has been found to be a particularly useful method for studying the interactions among bases, nucleosides, and dinucleoside monophosphates, as discussed previously, and has allowed hydrogen-bonding to be distinguished from base-stacking as the mode of interaction between bases and base analogs at the monomer level (see Part I, Section 2). Thus the extension of the studies described in Part III of this thesis to the investigation of the interaction of polynucleotides with smaller molecules is expected to yield detailed information regarding the nature of these interactions.

As discussed in Part I, Section 4, several polynucleotides form rigid secondary structures (particularly at lower temperatures) which lead to magnetic dipolar broadening of the proton resonances of these molecules. To obtain the maximum amount of information from monomer-polynucleotide systems by pmr, it is important that the polymer have a minimum of self-structure so that its proton resonances may be well resolved. Polyuridylic acid would appear to be a particularly suitable subject for pmr investigation, as it possesses very little secondary structure at temperatures down to 0°C or so (at low salt concentrations), existing predominantly in the random-coil form.[69-72] Therefore, the interaction of poly U with purine and with adenosine has been investigated by pmr.

1. Binding of Purine to Polyuridylic Acid

Purine was chosen to serve as the monomer component in this

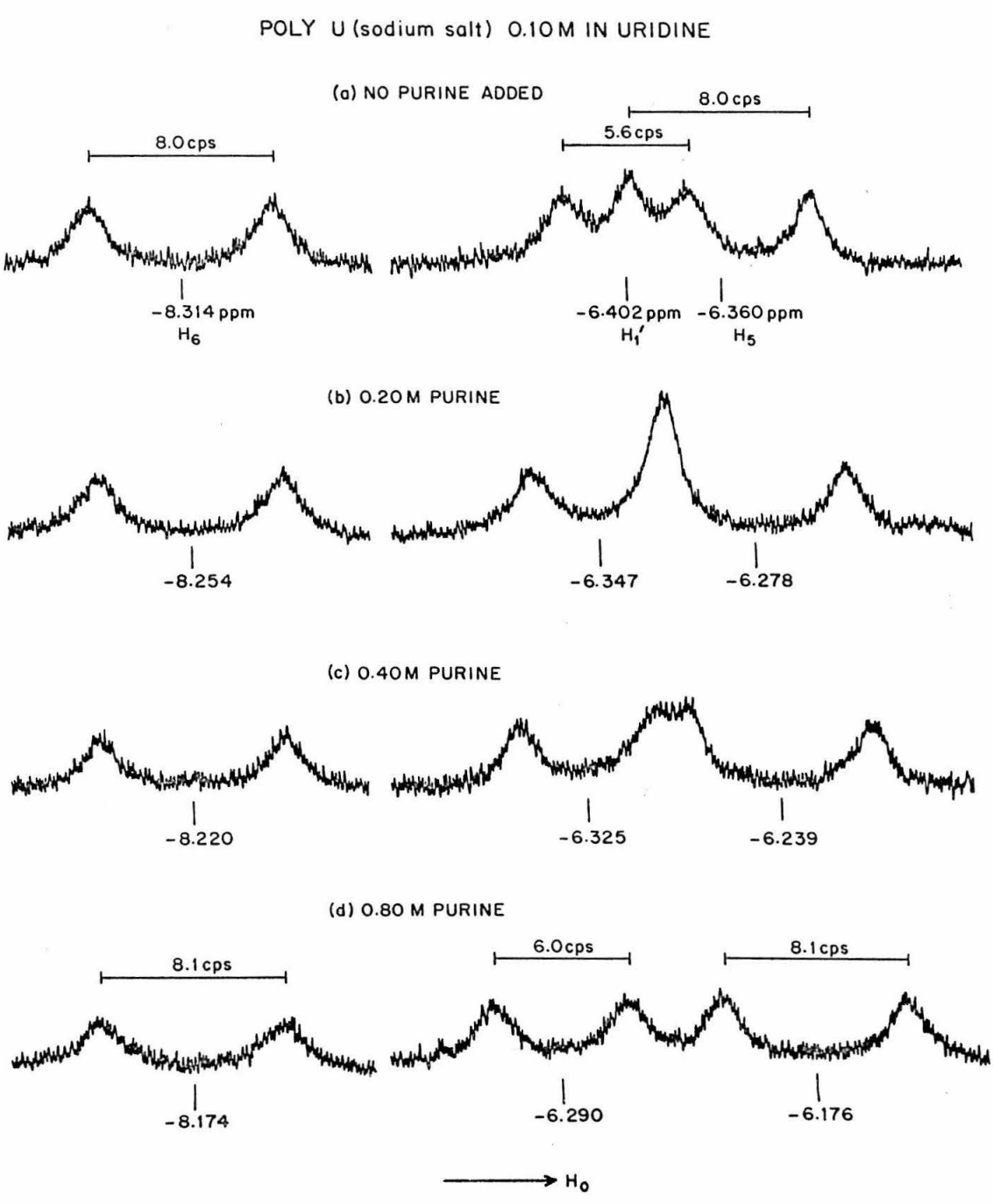
preliminary study, since the interaction of purine with bases, nucleosides, and dinucleoside monophosphates by base-stacking is well understood, and the mode of the purine-poly U interaction should be readily indicated by pmr spectroscopy. This investigation is of particular interest because of the possibility of comparisons of these results with those for the purine-UpU interaction reported in Section 6. 3.

Pmr Spectrum of Polyuridylic Acid. -- The pmr spectrum of poly U (0. 10 M in uridine) at 100 Mcps in the regions of the uracil H_6 and H_5 and ribose $H_{1'}$ resonances is shown in Figure 64a. The H_6 and H_5 resonances are spin-spin doublets with $|J_{H_5-H_6}|$ = 8. 0 cps, and $H_{1'}$ is a doublet from spin-spin coupling with $H_{2'}$, with $|J_{H_{1'}-H_{2'}}|$ = 5. 6 cps. The overlapping H_5 and $H_{1'}$ resonances were not resolved in the earlier studies at 60 Mcps. [80, 81] As seen by comparison of Figure 64a with Figure 47, the three monitored uridine resonances are shifted to lower fields in poly U relative to uridine. $|J_{H_{1'}-H_{2'}}|$ is greater for uridine in poly U than in the uridine monomer, indicating a change in ribose conformation (toward 2'-endo) as the nucleoside is incorporated in the polymer. [97]

The linewidths of the three uridine resonances in poly U are 1. 5 to 2. 0 cps, about twice as great as those for the uridine monomer. This is a result of magnetic dipolar coupling among the magnetic nuclei of the molecule, which is not averaged out as effectively in the polymer as in uridine because of a longer motional correlation time in the polymer. Incomplete averaging of the magnetic dipolar interactions

FIGURE 64

Polyuridylic acid (sodium salt) proton resonances at 100 Mcps; (a) no purine added; (b) 0.20 M purine; (c) 0.40 M purine; (d) 0.80 M purine.


POLY U (sodium salt) 0.10 M IN URIDINE
(a) NO PURINE ADDED
8.0 cps
8.0 cps
5.6 cps
-8.314 ppm
H6
-6.402 ppm
H1'
-6.360 ppm
H5
(b) 0.20 M PURINE
-8.254
-6.347
-6.278
(c) 0.40 M PURINE
-8.220
-6.325
-6.239
(d) 0.80 M PURINE
8.1 cps
6.0 cps
8.1 cps
-8.174
-6.290
-6.176
H0

leads to very large linewidths in highly ordered polymers, broadening proton resonances beyond detection by high-resolution techniques in many cases. The absence of such broad lines for poly U is taken to support a relatively disordered, random coil structure for the polymer under these conditions of temperature and ionic strength. (80)

Resonances of the ribose $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$ and $H_{5''}$ protons occur from about -4.40 to -5.20 ppm, and are broad, poorly resolved bands due to extensive spin-spin coupling and magnetic dipolar broadening of the individual components. Little information could be obtained from this spectral region. Hydroxyl- and NH-proton resonances were not observed because of exchange with the solvent.

Purine-Poly U Interaction. -- The addition of purine to the poly U solution causes the three monitored uridine resonances to be shifted to higher fields, as shown in Figure 64 and Table XXXIV, with the magnitudes of these shifts in the order $H_5 > H_6 > H_{1'}$. These upfield shifts of the uridine resonances indicate that purine interacts with the polymer by stacking with the uracil bases, as in the purine-uridine (nucleoside) and purine-UpU systems, and are a consequence of the ring-current magnetic anisotropy of purine as discussed in Part I, Section 2.1. The purine-induced shifts for the uridine proton resonances of poly U are nearly as large as those observed in the purine-UpU interaction (see Table XXV), thus purine apparently binds to poly U to about the same degree as to UpU. While $|J_{H_5-H_6}|$ was unchanged, $|J_{H_{1'}-H_{2'}}|$ appeared to increase slightly as purine was added to the

TABLE XXXIV. Purine-induced shifts and $H_{1'}$-$H_{2'}$ coupling constant for poly U protons (measured at 100 Mcps).

Purine Conc.	Purine-Induced Shift H_6	H_5	$H_{1'}$	$\lvert J_{H_{1'}-H_{2'}} \rvert$
	ppm	ppm	ppm	cps
0. 027 M	0. 006	0. 009	0. 008	5. 4
0. 054	0. 017	0. 022	0. 018	5. 5
0. 102	0. 036	0. 050	0. 035	5. 6
0. 197	0. 060	0. 082	0. 054	~5. 6
0. 300	0. 084	0. 110	0. 073	~5. 6
0. 400	0. 094	0. 121	0. 077	~6. 0
0. 603	0. 118	0. 154	0. 096	6. 0
0. 802	0. 140	0. 184	0. 112	6. 0
0. 997	0. 150	0. 201	0. 122	5. 9

poly U solution, indicating that a slight change in ribose conformation accompanies purine binding (see discussion in Section 5. 3. 1).

The three observable purine proton resonances are quite narrow in a solution of purine alone, or in a solution of purine containing uridine of the same base concentration as the poly U solution, as shown in Figure 54. In the presence of poly U, however, the purine resonances are extremely broad, as seen in Figure 65 and Table XXXV. At the lowest purine concentration studied, 0. 027 M, the H_2 resonance has a linewidth in excess of 16 cps, while the H_6 and H_8 resonances are broadened beyond detection. All three resonances narrow with increasing purine concentration.

As discussed in preceding Sections of this thesis, the binding of purine to UpU and several other dinucleoside monophosphates also results in purine line broadening of about the same magnitude, and in the order $H_6 > H_8 > H_2$. Thus it seems obvious that the mechanisms involved in producing the purine line broadening in the purine-poly U interaction are the same as in the purine-dinucleotide interactions, and the purine line broadening observed here is attributed to intercalation of monomeric purine molecules between the adjacent uracil bases of poly U. The purine proton linewidths in the complex with poly U appear to be of the same order of magnitude as for the purine-UpU intercalated complex, indicating that the motional correlation times controlling the purine resonance linewidths in these two complexes are comparable. Since the rotational correlation time for

FIGURE 65

Time-averaged purine proton resonances in the presence of poly U; (a) 0. 027 M purine, 70 scans; (b) 0. 10 M purine, 94 scans; (c) 0. 30 M purine, 55 scans.

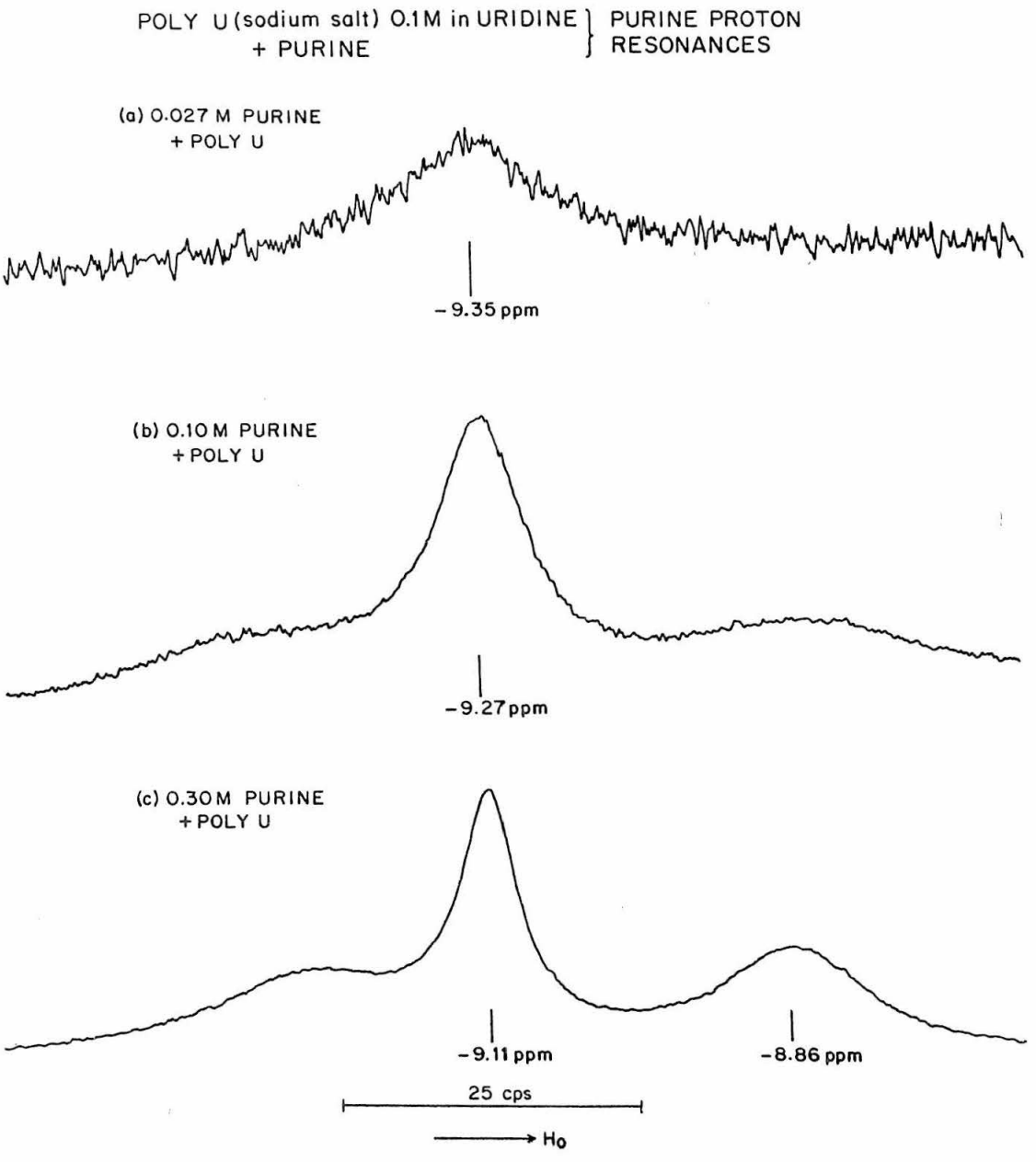

POLY U (sodium salt) 0.1M in URIDINE + PURINE
PURINE PROTON RESONANCES
(a) 0.027 M PURINE + POLY U
-9.35 ppm
(b) 0.10 M PURINE + POLY U
-9.27 ppm
(c) 0.30 M PURINE + POLY U
-9.11 ppm
-8.86 ppm
25 cps
Ho

TABLE XXXV. Purine proton resonance linewidths in the presence of poly U (0.10 M in uridine). (Full linewidth at half-height in cps, measured at 100 Mcps.)

Purine Conc.	Purine Resonance Linewidths		
	H_6	H_2	H_8
	cps	cps	cps
0.027 M	--[a]	~16	--[a]
0.054	--	~13	--
0.102	--	9.2	--
0.197	--	7.2	~17
0.300	--	6.1	~15
0.400	--	5.4	12
0.603	--	4.4	10
0.802	~12	3.7	7.0
0.997	~11	3.4	6.2

[a]Broadened beyond detection.

poly U is considerably longer than for UpU, motion of purine relative to the ribose-phosphate backbone, rather than overall rotation of the purine-poly U complex, determines the correlation time effective in controlling the purine proton linewidths.

Purine strongly self-associates to form stacked dimers, trimers, and higher aggregates, and this process shifts the purine proton resonances to higher field with increasing concentration.[22] At low purine concentrations, the H_2 resonance occurs at lower field in the presence of poly U than in a solution of purine alone, as seen in Table XXXVI. This indicates that poly U has the effect of disrupting the purine association, further evidence that base-stacking is the mode of interaction between purine and poly U.

The intercalation of purine between adjacent uracil bases of poly U does not result in an appreciably more rigid or ordered structure for the polymer. This is indicated by the observation that the poly U proton resonances do not broaden as purine is added, as seen from Figure 64. Formation of a helically ordered hydrogen-bonded purine-poly U complex would result in a rather rigid structure, with consequent broadening of the poly U proton resonances. Thus the purine-poly U complex is a rather weak and labile one, with the polymer remaining in a random coil conformation. The rate of exchange of purine molecules between poly U binding sites and the bulk solution is rapid on the NMR time scale ($> \sim 10^3$ sec^{-1}), since the purine resonances represent an average of bound and free purine, rather than a superposition of broad resonances from bound purine and narrow

TABLE XXXVI. Purine H_2 proton chemical shift in the absence and presence of poly U (0.10 M in uridine). (Measured at 100 Mcps.)

Purine Conc.	H_2 Chemical Shift Poly U	No Poly U	Difference
	ppm	ppm	ppm
0.027 M	-9.355	-9.320	0.035
0.054	-9.321	-9.293	0.028
0.102	-9.267	-9.252	0.015
0.197	-9.180	-9.174	0.006

resonances from free purine. Similarly, the exchange of uracil bases between free and purine-bound environments is rapid as indicated by the lack of separate uridine resonances appearing as a consequence of purine binding.

Although purine does not possess hydrogen-bond complementarity with uracil in the usual Watson-Crick sense, a base-paired structure with two hydrogen-bonds between purine and uracil is possible (Figure 66), and would appear to be fairly stable. Interaction of purine with the uracil bases of poly U by such a scheme does not seem indicated, however, as such a structure could not account for the purine line broadening observed. The large purine-induced upfield shifts for the uridine resonances of poly U are also inconsistent with a purine-uracil interaction by hydrogen-bonding, since this shift requires an overlapping of the planar purine and uracil bases.

2. Interaction of Adenosine with Polyuridylic Acid

The possibility of complementary base-pairing by hydrogen-bonding between adenine and uracil bases makes the adenosine-poly U interaction a particularly interesting one.

Pmr Spectrum of Poly U. -- As in the purine-poly U study, the pmr spectrum of poly U at 30°C, shown in Figure 67a, exhibits resonances for the uracil H_6 and H_5 and ribose $H_{1'}$ protons with linewidths of ~2-3 cps, about three times the linewidths observed for the uridine monomer. The H_6 and H_5 resonances are spin-spin doublets with $|J_{H_5-H_6}| = 8.0$ cps, and $H_{1'}$ is a doublet from spin-spin coupling

FIGURE 66

A possible hydrogen-bonded purine-uracil complex.

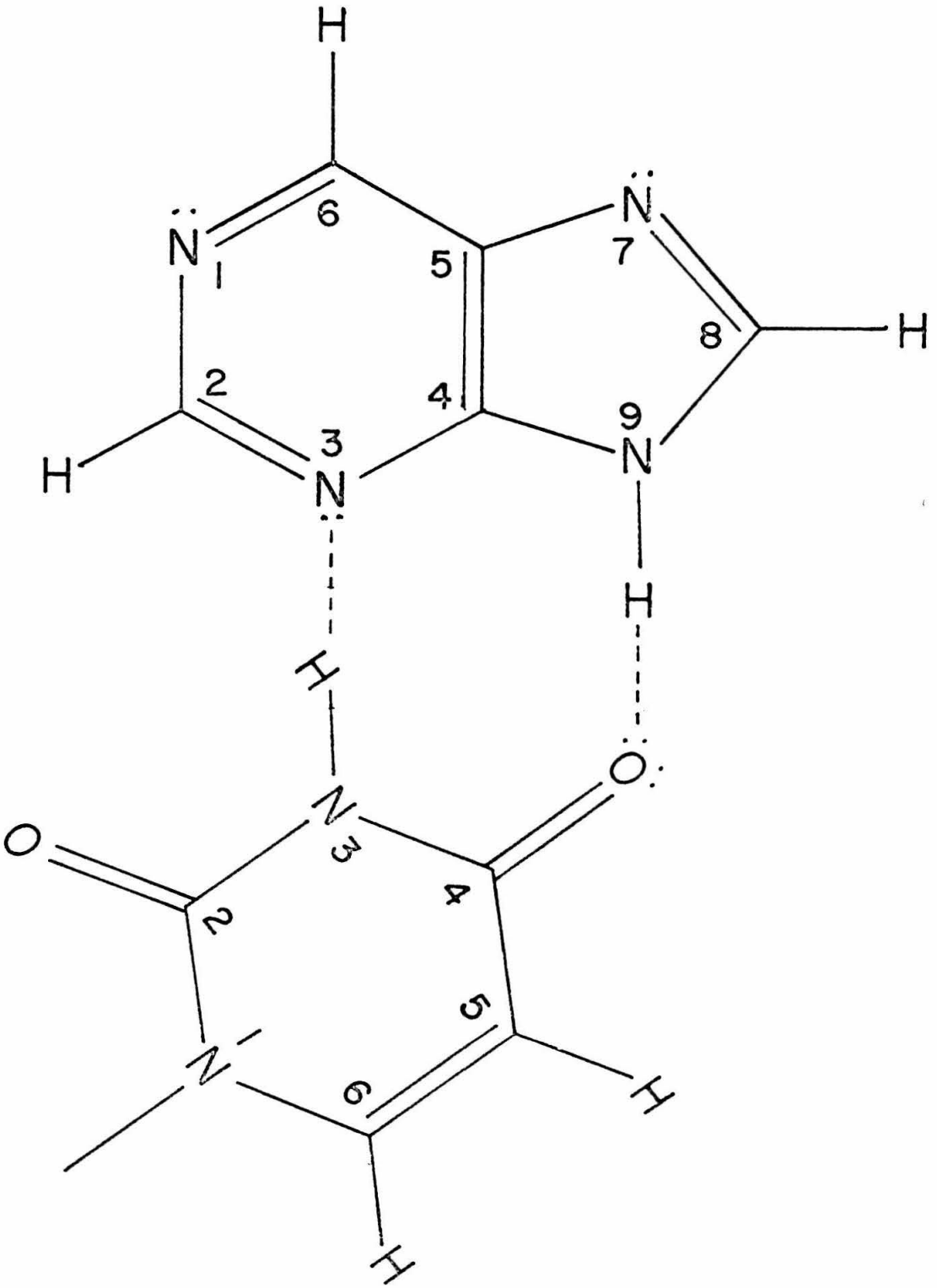
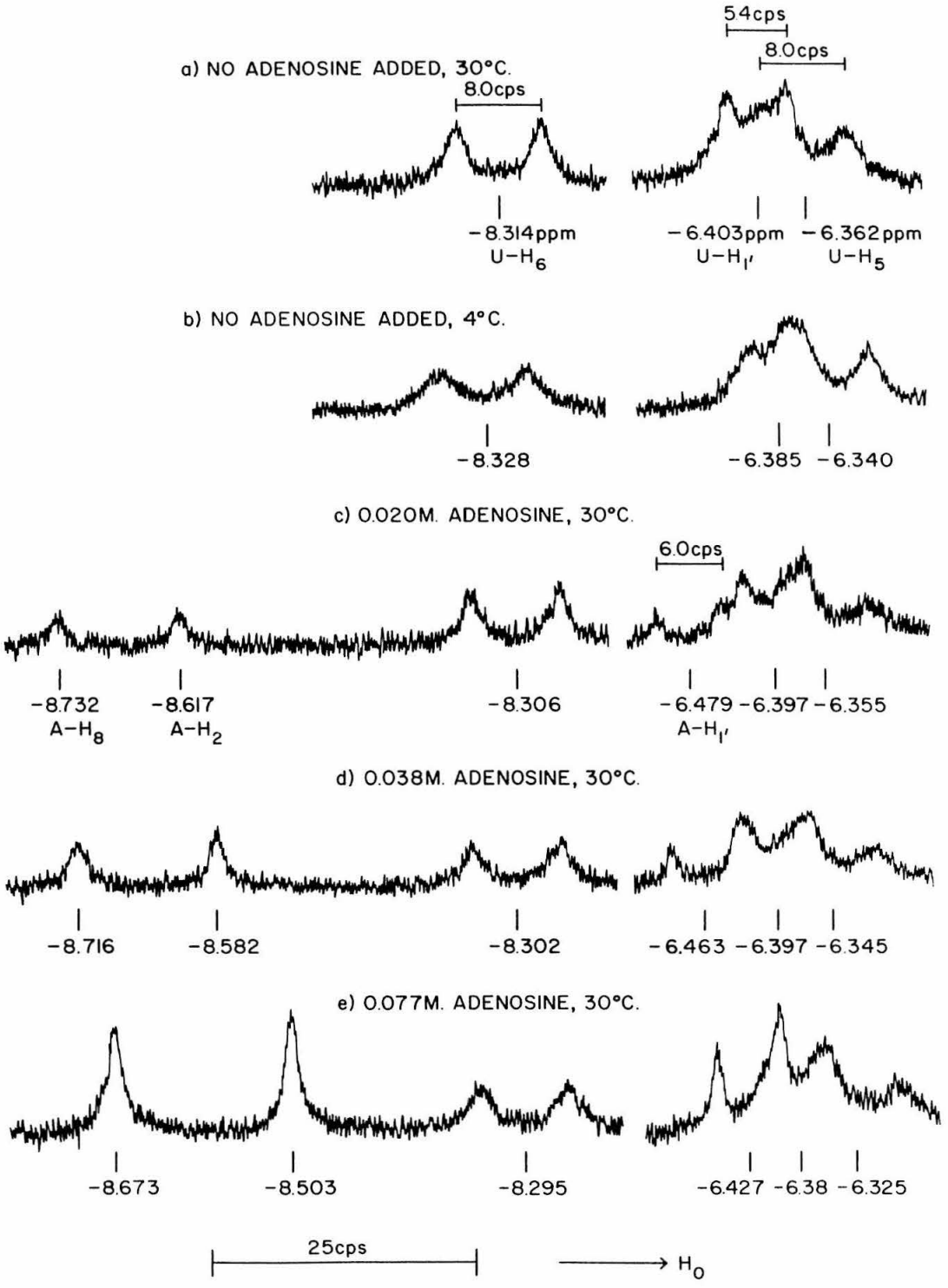

H
N
1
6
5
N
7
8
H
2
4
9
H
3
N
N
H
H
O
O
N
3
2
4
5
N
6
H
H

FIGURE 67

Polyuridylic acid (sodium salt) and adenosine proton resonances at 100 Mcps; (a) no adenosine added, 30°C; (b) no adenosine added, 4°C; (c) 0. 020 M adenosine, 30°C; (d) 0. 038 M adenosine, 30°C; (e) 0. 077 M adenosine, 30°C.

POLY U(sodium salt) 0.078M IN URIDINE



with $H_{2'}$ with $|J_{H_{1'}-H_{2'}}| = 5.4$ cps. As in the other studies reported in this thesis, the spectral region from -4.40 to -5 20 ppm containing the other ribose proton resonances was not examined in detail. At 4°C the linewidths of the H_6, H_5, and $H_{1'}$ resonances are only about 50% greater than at 30°C, as shown in Figure 67b. The chemical shifts change only slightly over this temperature range after correcting for the effect of temperature on bulk susceptibility differences between the TMS reference capillary and the D_2O solution. The coupling constants $|J_{H_5-H_6}|$ and $|J_{H_{1'}-H_{2'}}|$ are virtually independent of temperature over this temperature range. The absence of an increase in the poly U proton resonance linewidths with decreasing temperature indicates that the polymer assumes a relatively disordered, random coil structure over the temperature range 30°C to 4°C under the present conditions of ionic strength and pD.

Adenosine-Poly U Interaction. -- The effect of adding adenosine to the poly U solution at 30°C is depicted in Figures 67c, d, and e. The low solubility of adenosine in D_2O prevented increasing the adenosine concentration beyond ~0.08 M. Even at this concentration the solution was metastable at room temperature and below, with adenosine crystallizing out of solution on standing for an hour or two after initially heating the solution to dissolve the nucleoside. At 30°C, the addition of adenosine can be seen to shift the three monitored uridine resonances to higher fields. The adenosine-induced shifts are summarized in Table XXXVII. These upfield shifts indicate that the adenosine interacts with the polymer by intercalation and stacking of the planar adenine and uracil bases, as in the purine-poly U interaction.

TABLE XXXVII. Adenosine-induced shifts for protons of poly U (0. 078 M in uridine). (Measured at 100 Mcps.)

Adenosine Conc.	Adenosine-Induced Shift		
	H_6	H_5	$H_{1'}$
	ppm	ppm	ppm
0. 020 M	0. 008	0. 007	0. 006
0. 038	0. 012	0. 017	0. 006
0. 077	0. 019	0. 037	~0. 02

The relative order of the adenosine-induced upfield shifts of the poly U resonances ($H_5 > H_6 > H_{1'}$), and the magnitudes of these shifts, agree closely with the results of the purine-poly U binding study (see Table XXXIV). Thus purine and adenosine both stack with the uracil bases of poly U to about the same degree. As in the purine-poly U interaction, the rate of exchange of adenosine and uridine residues between bound and free environments is rapid on the NMR time scale ($> \sim 10^3$ sec^{-1}). This base-stacking interaction, which is estimated to involve ~ 5 to 10% of the uracil and adenine bases in complex formation at these concentrations, was not evident in the previous studies of this system by other physical methods. (109, 110)

The additional resonances appearing in the spectrum after the addition of adenosine to the poly U solution are those of the H_8, H_2, and $H_{1'}$ protons of adenosine, with the assignments indicated. These adenosine resonances are readily distinguished from the poly U resonances on the basis of their spectral positions and their relative intensities. As in uridine, the $H_{1'}$ resonances in adenosine also appears as a doublet due to spin-spin coupling with the $H_{2'}$ proton of the ribose ring. The $H_{1'}$-$H_{2'}$ coupling constant is 6. 0 cps.

The proton resonances of unsubstituted purine, under similar conditions, experience extreme line broadening in the presence of poly U from intercalation of purine between adjacent uracil bases of the polymer. In the present system, at low adenosine concentrations, the H_8 and H_2 resonances are only slightly broadened in the presence of poly U, and become narrower with increasing adenosine concentration.

The bulky ribose group in adenosine apparently hinders the adenine base from assuming the close proximity to the ribose-phosphate backbone of poly U required for the line broadening to occur. The adenosine resonances are also shifted to higher fields with increasing concentration, a result of the extensive self-association of adenosine which has been demonstrated previously.[19]

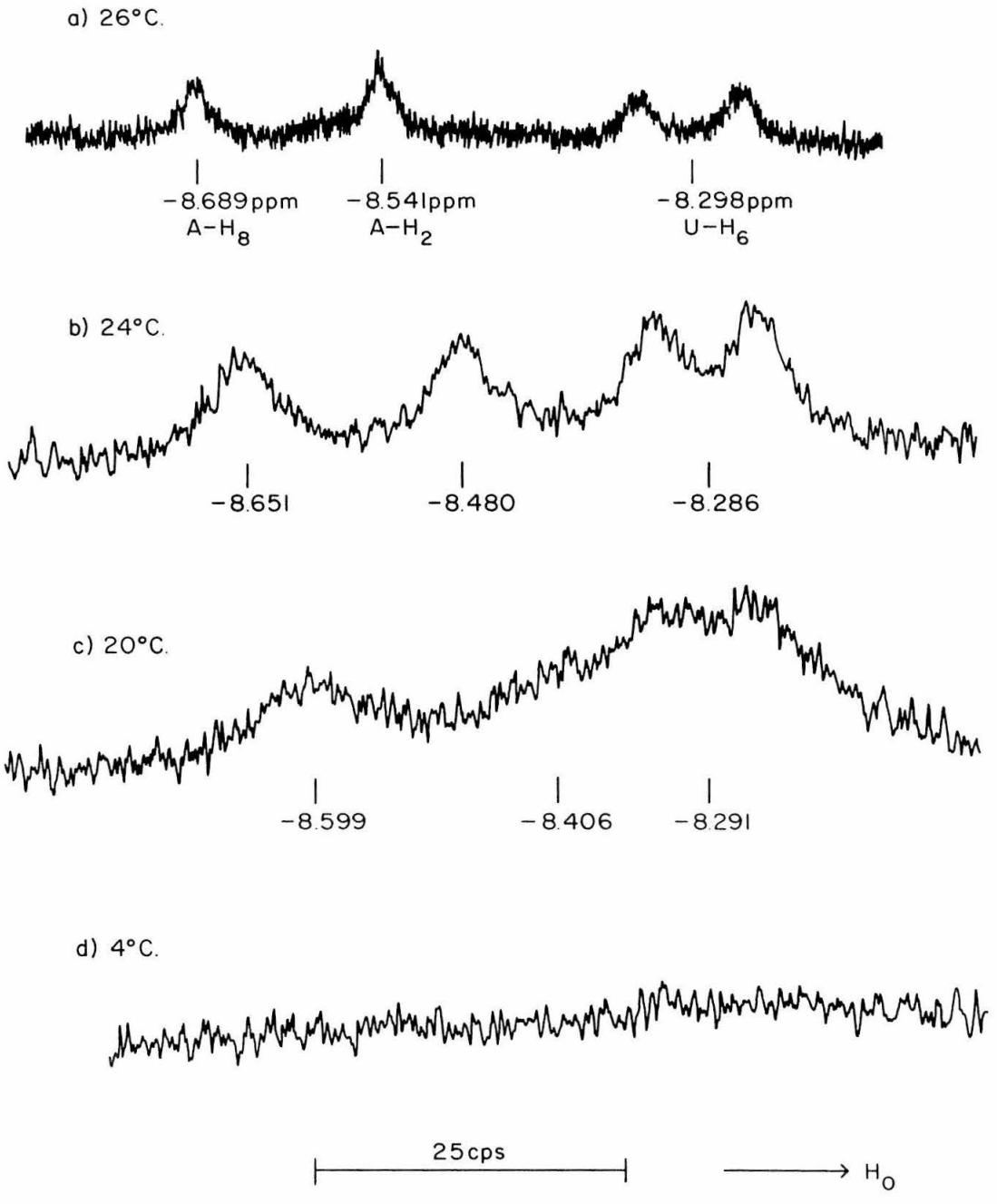
Since previous studies of this system[109, 110] indicated an adenosine-poly U interaction only below ~25°C, the effect of temperature on several adenosine-poly U solutions was studied. The results for a solution 0.078 M in poly U and 0.038 M in adenosine (base ratio A:U = 1:2) are shown in Figure 68. Only the spectral regions involving the adenosine H_2, H_8 and uridine H_6 protons are presented here. At temperatures from 37° to 26°, the linewidths of the monitored resonances are constant and the chemical shifts change only slightly. Below 26° however, all the resonances broaden markedly with decreasing temperature. This line broadening is of approximately the same magnitude for all the adenosine and uridine resonances. In addition, the adenosine resonances shift abruptly to higher fields with decreasing temperature below 26°, paralleling the linewidth behavior. The resonances of the uridine protons shift little with temperature between 37° and 20°. All the monitored resonances are broadened beyond detection at 4°C.

The narrow temperature range over which the pmr spectral behavior of this system changes, particularly the onset of the extreme line broadening of the proton resonances observed, suggests

FIGURE 68

Poly U H_6 and adenosine H_8 and H_2 resonances at 100 Mcps. Base ratio A:U = 1:2. (a) 26°C, single scan; (b) 24°C, 17 scans; (c) 20°C, 26 scans; (d) 4°C, 20 scans.

POLY U(sodium salt) 0.078M IN URIDINE
+ 0.038M ADENOSINE



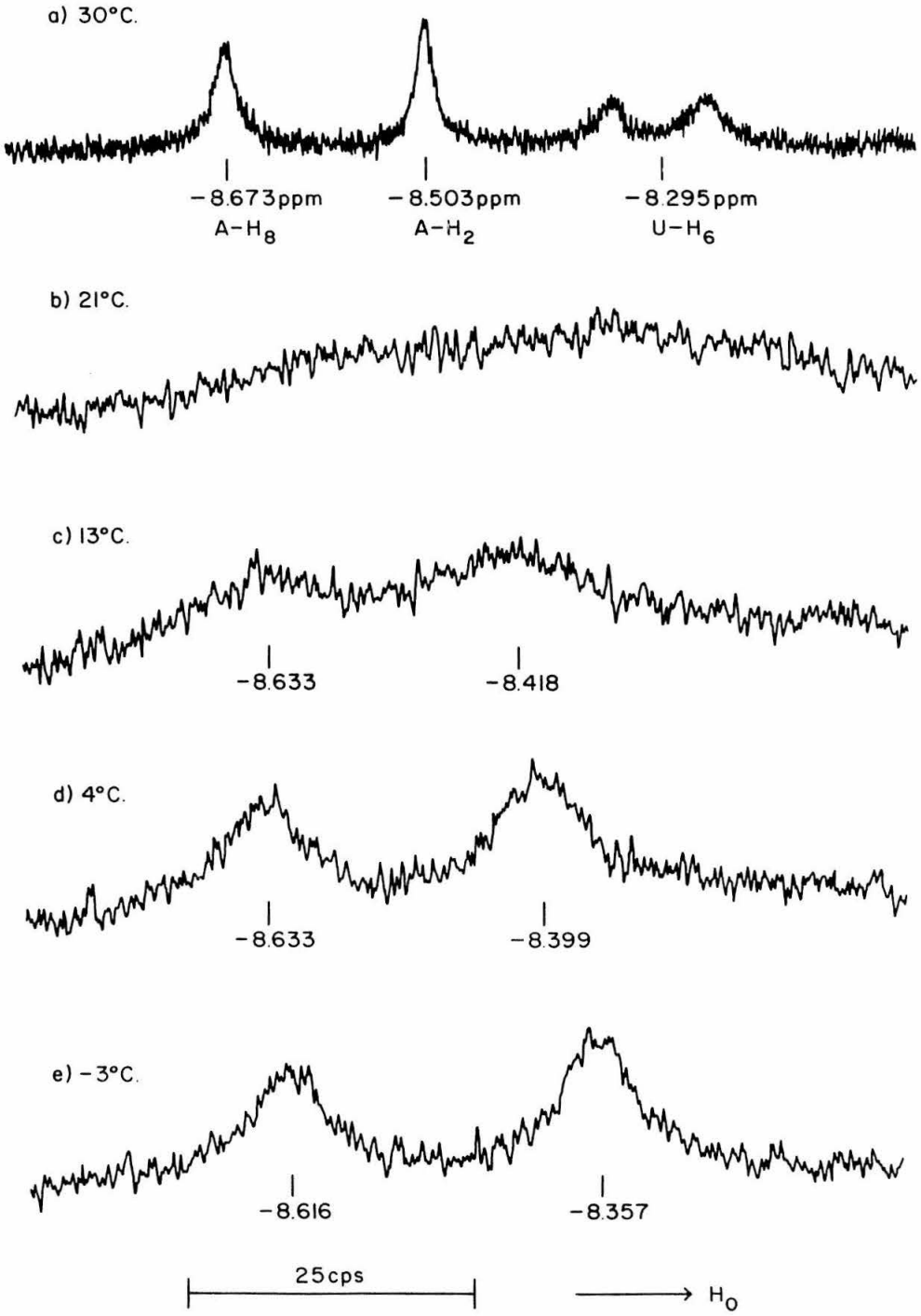
the formation of a rigid, ordered complex through a cooperative interaction of adenosine with poly U. The absence of detectable resonances at 4° suggests that either all of the reactants present are involved in complex formation or there is rapid exchange of the adenosine and poly U molecules between the free and complexed environments. The upfield shifts of the adenine proton resonances observed as the adenosine-poly U complex is formed indicate a high degree of adenine-adenine base-stacking in the complex. The absence of any appreciable shifts of the uracil base proton resonances with complex formation shows that adenine-uracil base-stacking is not involved. As discussed in Section 6. 2, uracil-uracil base-stacking cannot be detected by pmr, since the uracil base does not exhibit measurable ring-current magnetic anisotropy.

The nature of the poly U-adenosine complex below 26°C was further investigated by examining the proton magnetic resonance spectral behavior of a 0. 078 M poly U solution containing 0. 077 M adenosine (base ratio A:U = 1:1) over the same temperature range. Above 20°C, the behavior of the base proton resonances in this solution is similar to that for the A:U = 1:2 solution. The adenine H_8 and H_2 and uracil H_6 resonances broaden, and the adenine resonances shift upfield as the temperature is lowered. Below 20°C, however, the adenine resonances begin to narrow with decreasing temperature as shown in Figure 69. The uracil H_6 doublet, though, remains broad beyond detection. From the intensities of the adenine resonances at -3°C and + 30°C, it is estimated that the low temperature absorptions

FIGURE 69

Poly U H_6 and adenosine H_8 and H_2 resonances at 100 Mcps. Base ratio A:U = 1:1. (a) 30°C, single scan; (b) 21°C, 20 scans; (c) 13°C, 20 scans; (d) 4°C, 15 scans; (e) -3°C, 15 scans.

POLY U(sodium salt) 0.078M IN URIDINE
+ 0.077M. ADENOSINE



of the adenosine proton resonances account for approximately half the total adenosine in solution. These results clearly indicate that the chemical exchange of adenosine between the free and complexed environments is slow on the NMR time scale below ~20°C, and that the stoichiometry of the adenosine-poly U complex formed below 26°C involves 2 uracil bases per adenosine. Thus, the adenosine-poly U complex formed below 26°C is a triple-stranded structure, with stacked adenosine molecules forming horizontal adenine-uracil hydrogen-bonds with two poly U strands. Presumably Watson-Crick and Hoogsteen[37] hydrogen-bonding schemes are both involved. A possible hydrogen-bonded structure involving one adenine base and two uracil bases is shown in Figure 70. However, in view of the strong tendency for monomeric adenosine to associate in aqueous solution,[19] and the high degree of adenine-adenine base-stacking in the 1 A : 2 U adenosine-poly U complex demonstrated here, it is felt that the vertical interactions between adjacent bound adenosine molecules provide an important part of the driving force toward the formation of the complex.

The variation of the spectral positions of the adenosine H_2 and H_8 resonances with temperature for the A:U = 1:1 adenosine-poly U system sheds additional light on the dynamics of the system. At 21°C, where the spectrum for these protons consists of one broad envelope centered at ~ - 8.36 ppm, the resonances for these protons are at appreciably higher fields than their normal spectral positions in the absence of poly U. Since the adenosine concentration is in excess of the stoichiometric concentration required for the formation of the

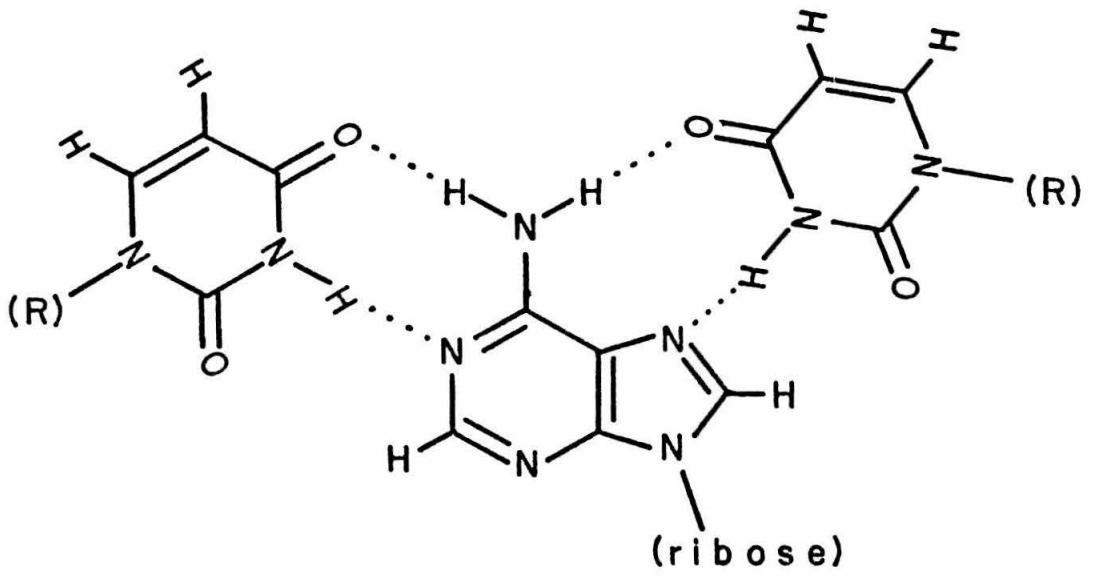


FIGURE 70. A possible base-pairing scheme for the 1 A : 2 U adenosine-poly U complex.

poly-U adenosine complex, it is clearly evident that the chemical exchange of the adenosine molecules between the bound and uncomplexed environments is fairly rapid, approaching conditions where the resonances would collapse into an averaged resonance whose position is determined by the weighted mean of the chemical shifts for the two adenosine environments. The resonances of the adenosine protons are at appreciably higher fields in the complex than in bulk solution because of the enhanced adenine-adenine base-stacking in the triple stranded complex. At 13°C, the onset of slow chemical exchange between the two adenosine environments is apparent. Not only are the H_2 and H_8 resonances noticeably narrower, but they also appear at considerably lower fields, more characteristic of their positions in the absence of poly U at this temperature. Below 13°C, the spectrum thus consists of relatively narrow resonances of uncomplexed adenosine superimposed on the very broad resonances of the complex. Note that as the adenosine H_2 and H_8 resonances narrow with decreasing temperature, they are also shifted to higher fields. These upfield shifts, it is felt, arise from the increasing self-association of unbound adenosine in bulk solution with decreasing temperature.

Finally, it is noted that even at -3°C the linewidths of the resonances due to the excess adenosine in the poly U solution are significantly broader than those for free adenosine in the absence of the adenosine-poly U complex. Since the HOD resonance and that of the tetramethylammonium standard are still extremely narrow (~1 cps), these widths cannot be interpreted on the basis of viscosity

effects, but instead, reflect the rate of chemical exchange of adenosine molecules between the two environments. In the limit of slow exchange, this leads to an additional broadening of the individual resonances by

$$\frac{1}{\pi} \quad \frac{1}{\tau} \quad \text{(cps)}$$

where τ is the mean lifetime of an adenosine molecule in the uncomplexed environment.[111] At -3°C, the linewidths of the adenosine H_8 and H_2 resonances (Figure 69e) are ~7.5 cps. The intrinsic linewidths for the free adenosine in the absence of chemical exchange are ~1 cps. From the additional broadening, a lifetime of 5×10^{-2} sec is thus obtained for the free adenosine molecule in solution in the presence of the triple-stranded complex. A similar analysis can be carried out for the adenosine resonances observed at 4°C and 13°C, although the linewidths are less reliable because of weak intensities and significant overlap between the H_2 and H_8 resonances. Lifetimes of $\sim 4 \times 10^{-2}$ sec and $\sim 2 \times 10^{-2}$ sec can be obtained for these temperatures. From the variation of the lifetimes with temperature, an apparent activation energy of 8 kcal/mole for the exchange process is obtained.

The 0.078 M poly U solution containing 0.20 M adenosine (base ratio A:U = 1:4) was also examined at +3°C. Only half the poly U in this solution would be required to form the 1 A : 2 U complex with adenosine. The pmr spectrum in the -9.00 to -8.00 ppm region showed a broad absorption at -8.24 ppm due to the uracil H_6 doublet. The adenine resonances were broad beyond detection, as expected.

These results would seem to indicate that there are no completely unbound poly U strands in solution under these conditions. If there were, the resonances due to these free poly U's would be quite narrow in view of the time scale of the dynamics indicated by the results for the A:U = 1:1 solution. Thus, all the poly U molecules are involved in complexation, even though not all of the uracil residues are involved in adenine-uracil base-pairing at any one time. The fact that the H_6 resonances were observed at all suggests that the time scale of exchange of uridine residues in the polymer between free and bound environments must also on the average approach that of slow chemical exchange. The H_6 doublet here is not as narrow as the resonances of the uncomplexed adenosine in the A:U = 1:1 solution at the same temperature (3°C). This suggests that the mean lifetime of a free uridine residue in the A:U = 1:4 solution is shorter than that of a free adenosine molecule in the A:U = 1:1 solution at this temperature. However, it is possible that this difference in resonance widths may also be due in part to a larger intrinsic linewidth for the H_6 resonance of an unbound uridine residue in a complexed poly U molecule compared to that in the uncomplexed poly U random coil.

3. Summary

The investigation of the purine-poly U system at 29°C demonstrates that purine binds to the polymer by base-stacking and intercalation. The adenosine-poly U study reveals two modes of interaction between the monomer and the polymer. At temperatures above

26°C or so, adenosine binds to poly U by non-cooperative A-U base stacking. Below this temperature, a rigid triple-stranded 1 A : 2 U complex is formed, presumably via cooperative hydrogen-bonding, as has previously been reported. These results not only clearly illustrate the importance of base-stacking in non-specific interactions between bases, nucleosides, and nucleotides; they also reveal the important role of the base-stacking interactions in cooperatively formed structures involving specific base-pairing, where both types of interaction can occur.

The capabilities of pmr spectroscopy in probing the structure of such complex systems as adenosine and poly U is clearly evident. The extreme sensitivity of the pmr spectrum to small structural and environmental effects is evident here, and the dependence of the resonance linewidths on the presence of rigid secondary structure, as well as certain dynamical effects, has been exploited. These features of the NMR method promise to make this spectroscopic tool uniquely valuable in the investigation of the structural and dynamical properties of similar monomer-polymer systems.

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PROPOSITION I

The first compounds of xenon, the fluorides, were prepared in 1962, and studies of the hydrolysis products of these compounds followed shortly thereafter. During the past few years, much work has been done to characterize the xenon species existing in aqueous solution, and the solution chemistry of this element is both interesting and complex.

Hydrolysis of XeF_2 results in the oxidation of water, with xenon quantitatively reduced to the Xe(0) state[1]:

$$XeF_2 + H_2O = Xe(0) + \tfrac{1}{2}O_2 + 2HF$$

This reaction occurs in acid, neutral, or basic solution.

The hydrolysis of XeF_4 is more complicated. The tetrafluoride reacts with water to yield an Xe(VI) species through a disproportionation process.[1] The yield in acid or neutral solution is about 30%, suggesting:

$$3XeF_4 + 6H_2O = 2Xe(0) + 1\tfrac{1}{2}O_2 + XeO_3 + 12HF$$

Hydrolysis in basic solution results in yields of ~5-10% of Xe(VI).[2] The Xe(VI) species is stable in acid or neutral solution; it decomposes slowly in basic solution by oxidizing H_2O. Evaporation of the solution resulting from XeF_4 hydrolysis in water yields crystalline XeO_3. Xenon

trioxide is a very weak acid, probably existing in solution as XeO_3 (or H_2XeO_4, etc.) except at high pH where an OH^- ion is accepted to give $HXeO_4^-$. (3)

XeF_6 hydrolyzes in neutral or acid solution to give Xe(VI) in solution quantitatively. (1) Hydrolysis in strongly basic solution leads first to Xe(VI), which then undergoes disproportionation to precipitate the sparingly soluble perxenate salt, Xe(VIII). Perxenate decomposes slowly in basic solution, liberating Xe(0) and O_2, and decomposes rapidly in acid solution to give Xe(VI) quantitatively. Solid sodium perxenate has the stoichiometry $Na_4XeO_6 \cdot xH_2O$, and the perxenate species in solution probably include $HXeO_6^{-3}$, $H_2XeO_6^{-2}$, and $H_3XeO_6^{-1}$, depending on pH. (4)

Both Xe(VI) and Xe(VIII) species are strong oxidizing agents, oxidizing IO_3^- to IO_4^-, Mn^{++} to MnO_4^-, etc. It seems obvious that a knowledge of the oxidation potentials of the various xenon solution species would be useful in aiding understanding of the chemistry of this element.

Appelman(4) has estimated the oxidation potential of Xe(VIII) to be between 0. 7v (iodate-periodate) and 1. 24v (oxygen-ozone) in basic solution, since perxenate oxidizes IO_3^- to IO_4^- and O_3 oxidizes Xe(VI) to Xe(VIII) in basic solution.

Gunn(5) has found the heat of formation of XeO_3 to be +96 kcal/mole. Ignoring the entropy contribution and the free energy of solution, considering ΔF_f^0 = +96 kcal/mole, the reaction:

$$XeO_3 + 6H^+ + 6e^- = Xe(0) + 3H_2O$$

has a calculated ΔF^0 = -266 kcal/mole. This leads to a standard oxidation potential of -1.9v for XeO_3 in acid solution, according to Gunn.

These estimates are obviously very approximate, yet no attempt to directly measure the various oxidation potentials has been reported. It is therefore proposed that an attempt be made to measure the EMF's of the following three electrode reactions by the potentiometric method: Xe(0)|Xe(VI); Xe(0)|Xe(VIII); and Xe(VI), Xe(VIII).

It is necessary to consider the stabilities of the xenon solution species at various pH. A 0.003 M solution of Xe(VIII) has been found to have a half-life of 36 hours at pH 12-13, and only one hour at pH 8.5.[4] Therefore, a pH > 10 or so would be required for the determination of the Xe(0)|Xe(VIII) potential with a reasonably stable Xe(VIII) concentration. According to Appelman,[4] the perxenate species at pH 10 is $H_2XeO_6^{-2}$. The corresponding half-cell reactions would be:

pH 10: $Xe(0) + 10\ OH^- = H_2XeO_6^{-2} + 4\ H_2O + 8\ e^-$

pH 12: $Xe(0) + 11\ OH^- = HXeO_6^{-3} + 5\ H_2O + 8\ e^-$

Xe(VI) is stable in acid and neutral solution, and decomposes very slowly in dilute base (pH < 10). The Xe(VI) species is XeO_3 below pH 10-11.[6] Appropriate half-cell reactions are:

acid solution: $Xe(0) + 3\ H_2O = XeO_3 + 6\ H^+ + 6\ e^-$

basic solution: $Xe(0) + 6\ OH^- = XeO_3 + 3\ H_2O + 6\ e^-$

It has been found[7] that a 0.03 M solution of Xe(VI) in 0.03 M KOH loses 23% of the xenon (as Xe(0)) in 21.6 hours, due to a complex disproportionation reaction. Thus at pH 12, where Xe(VIII) is reasonably stable, and at dilute concentrations ($< \sim 0.03$ M), Xe(VI) should also be fairly stable. The Xe(VI) species at this pH is $HXeO_4^-$.[6] The appropriate half-cell reaction would be:

$$HXeO_4^- + 4\ OH^- = HXeO_6^{-3} + 2\ H_2O + 2\,e^-$$

The desired Xe(VI) solutions could be prepared by dissolving solid XeO_3 in a solution of the desired pH. The Xe(VIII) solutions could be prepared by dissolving $K_4XeO_6 \cdot 9\,H_2O$[7] in H_2O and adjusting to the desired pH with acid. The solutions could be analyzed for the xenon species iodometrically.

A platinum electrode should be suitable for the Xe(VI), Xe(VIII) couple, and a platinum-black surfaced Pt electrode would be appropriate for the Xe(0)|Xe(VI) and Xe(0)|Xe(VIII) half-cells. A calomel reference electrode with a KCl bridge could be used, since Cl^- is oxidized at a very slow rate by the xenon solution species.[1] The Xe(0) electrode would have to be of such a design as to permit the recovery of the xenon, since this element is very expensive.

It seems likely that the Xe(0)|Xe(VI) and Xe(0)|Xe(VIII) electrode reactions might be highly irreversible, in which case no measurement of these potentials would be possible. This has not been shown conclusively, however, and the experiment seems warranted. The fact that Xe(VI) and Xe(VIII) can be interconverted chemically:

$$Xe(VI) + O_3 = Xe(VIII) + 1\tfrac{1}{2}\, O_2$$

$$Xe(VIII) + H_2O = Xe(VI) + \tfrac{1}{2}\, O_2$$

encourages one to expect that the Xe(VI), Xe(VIII) electrode reaction might be reversible and thus capable of measurement.

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PROPOSITION II

Knowledge of the local environment of a particular base in a polynucleotide is important with regard to determination of both the primary structure (or sequence) and secondary structure of a polynucleotide strand. Although chemical methods of sequence determination have been successfully applied in elucidating the primary structures of several transfer RNA molecules,[1-3] these methods have yielded scant information about the secondary structures of these molecules. The use of spectroscopic methods to study both the sequence and the secondary structure of polynucleotides is currently of considerable interest. Several optical methods (optical rotatory dispersion, circular dichroism, and hypochromicity measurements) have been rather extensively applied to these problems.[4-6] Gross structural features of the polynucleotides have been revealed by such studies, but little detailed information regarding sequence has been obtained. The extensive overlapping of the electronic absorption bands of the various bases constitutes a drawback for the optical methods, severely limiting the resolution of the various local environments for a particular base in a polynucleotide strand.

The application of proton magnetic resonance (pmr) spectroscopy to such studies is also being explored. The chemical shift of a particular proton of a given base in a polynucleotide strand is dependent upon the local environment of the base in the strand: i. e., the nature of

the neighboring bases and the degree of secondary structure. This characteristic makes the pmr method potentially useful for sequence determination. However, there is also a resolution problem inherent in the study of naturally-occurring polynucleotides by pmr spectroscopy. Although the base proton resonances of the various monomeric nucleosides do not overlap one another significantly and are quite narrow, the sixteen possible combinations of nearest-neighbor bases for a given base in a single polynucleotide strand can lead to as many as sixteen chemically shifted resonances for a particular base proton. Furthermore, the possibility that bases other than the nearest-neighbors can affect the chemical shift of a given proton compounds the problem. Thus the pmr spectrum of a polynucleotide consists of envelopes containing unresolved, overlapping resonances from protons of bases in different magnetic environments. (This discussion assumes the absence of a rigid secondary structure, which would make the problem even more difficult because of severe line-broadening.)

The work of McDonald and Phillips[7] provides the clearest demonstration of the dependence of base proton chemical shifts on the nature of the neighboring bases in a polynucleotide. These workers showed that the thymine methyl proton resonances in melted DNA's consist of two peaks, one arising from thymine bases with a purine base (adenine or guanine) as the nearest-neighbor esterified to the 5′-position of the thymine residue, the other resulting from thymine bases with a pyrimidine base (cytosine or thymine) as the 5′ nearest-neighbor. Each of these peaks constitutes an envelope of several

resonances which could not be resolved further.

It is proposed that examination of an adenine-containing synthetic binary copolymer, such as that between adenylic and cytidylic acids (polyAC), by high-resolution pmr spectroscopy should reveal distinct resonances for a given base proton in each of the four possible local environments for the base (considering nearest-neighbors only). The four adenine environments are: (a)... CpApC..., (b)... CpApA..., (c)... ApApC..., and (d)... ApApA... (reading in the direction of 3′ to 5′ phosphate esterification). Similarly, the four cytosine environments are: (e)... CpCpC..., (f)... CpCpA..., (g)... ApCpC..., and (h)... ApCpA... . A given adenine base proton (H_8 or H_2) would be expected to have a different chemical shift in each local environment, as a consequence of the magnetic influences of the neighboring bases. Similarly, the cytosine H_6 (or H_5) proton should have a different chemical shift in each of the four local environments for the cytosine base. These chemical shift differences are expected to result primarily as a consequence of the ring-current magnetic anisotropy of the adenine base.[8] This effect caused the proton resonances of bases adjacent to adenine to be shifted to higher fields, relative to their positions in the absence of a neighboring adenine base. The cytosine base appears to have a much smaller magnetic anisotropy than adenine.[9]

The influence of the adenine ring-current on the proton chemical shifts of an adjacent base is highly sequence-dependent. Considering first of all the cytosine base protons, a comparison of the

chemical shifts of the H_5 protons of the 3′-esterified cytidine nucleosides in the (3′→5′) dinucleoside monophosphates CpC and CpA (at 30°C) indicates that the substitution of adenine for cytosine as the adjacent base results in a shift of the H_5 resonance ~0.12 ppm to higher field. A similar comparison for CpC and ApC indicates that substitution of adenine for cytosine shifts the H_5 resonance of the 5′-esterified cytidine nucleoside upfield ~0.26 ppm. Extrapolating these results for the dinucleoside monophosphates to the copolymer polyAC, one would expect the cytosine H_5 proton in environment (f) to resonate ~0.12 ppm upfield and the H_5 proton in environment (g) to resonate ~0.26 ppm upfield from the H_5 proton in environment (e). Since the effects of adenine bases in both nearest-neighbor positions should be essentially additive, the cytosine H_5 proton in position (h) should resonate at highest field, ~0.38 ppm upfield from the H_5 resonance in environment (e). The cytosine base proton chemical shifts in the dinucleoside monophosphates are listed in Table I. Similar comparisons of H_6 of cytosine lead to the prediction that this proton should resonate ~0.16 ppm, ~0.20 ppm, and ~0.36 ppm to higher field in environments (f), (g), and (h) respectively, compared to the resonance position in environment (e).

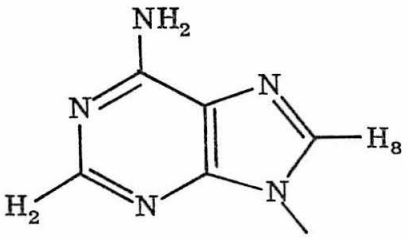
The field positions of the adenine H_8 or H_2 proton resonances are also sensitive to the presence of an adjacent adenine base. Appropriate comparisons among the dinucleoside monophosphates ApC, CpA, and ApA are summarized in Table II. From these results, the adenine H_8 proton would be expected to resonate ~0.12 ppm, ~0.16 ppm,

TABLE I. Chemical shifts of cytosine protons in the dinucleoside monophosphates ApC, CpA, and CpC at 30°C (concentration 0.010 M).[9] Reference: TMS.

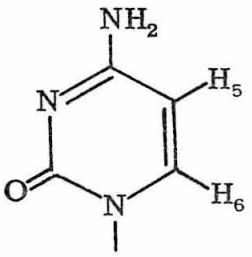
	H_6		H_5	
	(3′)	(5′)	(3′)	(5′)
	ppm		ppm	
ApC		-8.163		-6.151
CpA	-8.149		-6.281	
CpC	-8.308	-8.358	-6.400	-6.408
Shift	+0.159	+0.195	+0.119	+0.257

TABLE II. Chemical shifts of adenine protons in the dinucleoside monophosphates ApC,[9] CpA,[9] and ApA[10] at 30°C (at infinite dilution). Reference: TMS.

	H_8		H_2	
	(3′)	(5′)	(3′)	(5′)
	ppm		ppm	
ApC	-8.80		-8.62	
CpA		-8.87		-8.71
ApA	-8.68	-8.71	-8.48	-8.61
Shift	+0.12	+0.16	+0.14	+0.10



adenine



cytosine

and ~0.28 ppm to higher field in environments (b), (c), and (d) respectively, compared to the resonance position in environment (a). Also, the H_2 protons in environments (b), (c), and (d) should resonate ~0.14 ppm, ~0.10 ppm, and ~0.24 ppm, respectively, upfield from the H_2 protons in environment (a).

These predictions are based on the assumption that the geometries and the magnitudes of the pairwise base-stacking interactions between adjacent adenine and cytosine bases in the polynucleotide are about the same as in the corresponding dinucleoside monophosphates, and are unaffected by the other bases in the molecule. This discussion also requires that the chemical shifts of protons of a given base be influenced by only the immediate nearest-neighbors. How well these assumptions correspond to the actual situation in polyAC should be evident from the pmr spectrum of the copolymer. For example, the occurrence of more than four resonances for each

type of proton would indicate that the specification of the nearest-neighbor bases is not adequate to define the local environment for a given base. However, should four distinct resonances be observed for each type of base proton in polyAC, it would be possible to assess the degree of randomness of the copolymer by comparing the intensities of the resonances for the various local environments.

Finally, it is necessary to consider the probable magnitudes of the base proton resonance linewidths in polyAC. Assuming that this single-stranded polynucleotide is not highly structured at 30°C (or somewhat above this temperature) it is reasonable to expect resonance linewidths of 2-4 cps. If the pmr spectrum were determined at 220 Mcps, the overlapping of the predicted chemically shifted resonances should not present a serious problem.

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PROPOSITION III

Hydrolysis of xenon fluorides has been shown to lead to stable xenon containing species in solution. Over the past few years, several studies have been aimed at elucidating the structures of the various xenon solution species. The Xe(VI) species has been obtained in crystalline form as XeO_3, but the degree of hydration of XeO_3 in solution is not known with certainty.

Dudley, Gard and Cady[1] have suggested the formula $Xe(OH)_6$ for the Xe(VI) species. Raman and infrared spectroscopy by Claassen and Knapp[2] gives evidence for a molecule of C_{3v} symmetry for XeO_3 in solution, the same symmetry as for XeO_3 in the crystalline state.[3] This would rule out the $Xe(OH)_6$ structure, or an intermediate degree of hydration such as H_2XeO_4. The Xe(VI) species is a weak acid, accepting an OH^- ion to give $HXeO_4^-$ or $XeO_3 \cdot OH^-$, according to Appelman and Malm.[4]

It is proposed that nuclear magnetic resonance of xenon could be useful in studying the xenon solution species. Two xenon isotopes are of interest. ^{129}Xe has a spin of 1/2 and an abundance of 26%, and ^{131}Xe has a spin of 3/2, a quadrupole moment of -0.12, and an abundance of 21%.[5] Both these nuclei have been studied by NMR,[6, 7] but only in the free element and, indirectly by double irradiation, in the xenon fluorides. These isotopes possess rather small magnetic moments (^{129}Xe: -0.77 n. m., ^{131}Xe: 0.69 n. m., compared to 2.79 n. m. for H^1) and observation of their spectra requires sensitivity much

greater than is required for H^1 or F^{19}. Nevertheless, usable spectra could probably be obtained.

It appears that ^{131}Xe spectra might help define the structure of the Xe(VI) species in solution. If the structure is XeO_3, the low symmetry of the molecule will give rise to an electric field gradient at the xenon nucleus. The nuclear quadrupole moment of ^{131}Xe will interact with this field gradient to produce a rapid spin relaxation, resulting in a broadened ^{131}Xe resonance compared to, say, perxenate, which has octahedral symmetry.[8] If this is found to be the case, the ^{131}Xe O_3 spectrum could be studied as a function of pH. A narrowing of the resonance at pH 10-11 would indicate that a more symmetrical conjugate base is formed, perhaps $HXeO_4^-$. The persistence of a broadened resonance at high pH would tend to confirm the $XeO_3 \cdot OH^-$ structure proposed by Appelman and Malm.

Study of the chemical shift of ^{129}Xe (or ^{131}Xe) as a function of pH could be helpful in determining the pK_a's of the perxenate species ($HXeO_6^{-3}$, $H_2XeO_6^{-2}$, $H_3XeO_6^{-1}$) as well as of XeO_3. This method depends on the chemical shift δ being a linear function of the mole fractions of the acid and of its conjugate base. Potentiometric titrations of perxenate solutions have yielded approximate pK_a's for the several steps,[4, 9] but have been plagued by decomposition of the xenon species. This would be a less important cause of error with the NMR method.

Xe(VIII) decomposes in neutral or acid solution to give Xe(VI), and the rate of this reaction could be studied by measuring the rate of disappearance of the ^{129}Xe(VIII) resonance and the rate of appearance

of the ^{129}Xe(VI) resonance. In addition, other applications of Xe NMR seem possible, if the studies proposed above produce results.

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PROPOSITION IV

Recent investigations of the interaction of purine with several dinucleoside monophosphates[1-3] and with polyuridylic acid[4] using proton magnetic resonance spectroscopy have shown the purine proton resonances to be markedly broadened in the presence of a di- or polynucleotide. This purine line broadening is believed to be the result of magnetic dipolar coupling between the purine protons and protons of the ribose groups of the di- or polynucleotide, in a molecular complex involving the intercalation of a purine molecule between two adjacent bases of the oligonucleotide. Motion of the purine molecule relative to the ribose-phosphate backbone of the larger molecule is believed to produce the fluctuations in this dipolar interaction which are responsible for nuclear magnetic spin relaxation of the purine protons.

This interpretation of the purine line broadening phenomenon, observed for purine in the presence of molecules containing at least two bases connected by a ribose-phosphate backbone, seems rather reasonable in view of the magnitude of the observed effect. Furthermore, the possibility of a slow or intermediate rate of chemical exchange (on the NMR timescale) between bound and free purine molecules can be largely eliminated as a major cause of the purine line broadening. However, there exists no direct evidence for the involvement of the magnetic dipolar coupling mechanism in the purine proton spin relaxation phenomenon. It is noted that the protons of the ribose groups would

experience spin relaxation effects due to the magnetic fields produced by the purine protons, by this mechanism. However these effects are difficult to verify because of the complexity of the proton magnetic resonance spectrum of the di- or polynucleotide in the ribose region and the fact that, whereas a given purine proton can be brought into close proximity to three or four ribose protons at once, a given ribose proton could be close to only one purine proton at any particular time. Thus the magnetic dipolar coupling between the purine and ribose protons would be expected to have a rather small effect on the line-widths of the ribose proton resonances.

It is proposed that this tentative explanation of the source of the purine line broadening phenomenon in the complexes of purine with a di- or polynucleotide might be further investigated by means of the nuclear magnetic double resonance experiment to be described. In this experiment, the ^{2}H NMR spectrum of deuterated purine* in the presence of a dinucleoside monophosphate [for example cytidylyl (3′→ 5′) cytidine, CpC] would be observed both in the presence and in the absence of a radio-frequency magnetic field applied at the resonance frequency for protons.

Consider first of all the deuterium resonance spectrum of purine in the absence of the r. f. field at the proton frequency. The deuterium nuclei should experience a contribution to their spin relaxation from the dipolar magnetic coupling to the ribose protons of the

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*Procedures for preparing purine deuterated at the C-6 and C-8 positions[5] are quite straightforward.

dinucleoside monophosphate in the intercalated complex. This contribution would be smaller than that for a purine proton by a factor of the square of the ratio of the gyromagnetic ratios for protons and deuterons: $\left(\frac{\gamma_D}{\gamma_H}\right)^2 \sim 0.02$.[6] Despite the smaller effect of the magnetic dipolar coupling on the relaxation rate of ^2H compared to ^1H, and the occurrence of broader resonances for deuterium than for protons due to electric quadrupolar relaxation of the deuteron,[7] the magnetic dipolar relaxation effect should still be observable as a line broadening in the deuteron magnetic resonance spectrum under the appropriate conditions of concentration of interacting molecules and temperature.

The application of a strong radio-frequency magnetic field at the proton resonance frequency would significantly shorten the lifetime of a proton in a particular spin state. This would cause the magnetic fields produced by the ribose protons to be less effective in controlling the relaxation rates of deuterium nuclei on purine molecules in the purine-dinucleoside monophosphate complex.[8] Consequently, this would be observed in the deuteron magnetic resonance spectrum of purine as a narrowing of the resonances. (Because of the necessity of saturating the several chemically shifted ribose proton resonances simultaneously, it would be necessary to modulate the proton radio-frequency with audio-frequency noise to produce an adequate power bandwidth--effectively saturating the resonances of all the protons in the sample.) Any purine nuclear spin relaxation process which does not involve the protons of the dinucleoside monophosphate should be

unaffected by the application of r.f. power at the proton resonance frequency.

Thus a narrowing of the deuteron resonances of deuterated purine in this system accompanying the application of r.f. power at the ribose proton resonance frequency would indicate the involvement of the ribose protons in the purine deuteron (or proton) relaxation process, supporting the proposed dipolar relaxation mechanism. A negative result for the double-irradiation experiment (provided the addition of the dinucleoside monophosphate to deuterated purine does result in a broadening of the deuterium resonances) would be less clear-cut, however it would tend to suggest that some other mechanism for the purine deuteron (or proton) spin relaxation process must be dominant.

It might be mentioned that the best way to investigate the involvement of the ribose protons of the dinucleoside monophosphate in the purine line broadening mechanism would be to examine the proton magnetic resonance spectrum of purine in the presence of a deuterated dinucleoside monophosphate. However since there are no procedures available for exchanging the ribose protons of a nucleoside with deuterium, the synthesis of the deuterated dinucleoside monophosphate would present a formidable task.

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PROPOSITION V

In recent years, it has been established that macrocyclic antibiotics of the valinomycin, nactin, and enniatin groups are capable of affecting alkali metal ion transport through biological (mitochondrial) membranes[1, 2] as well as through synthetic bimolecular lipid membranes.[3, 4] It has also been shown that the effect of these compounds on the ion permeability of the membrane correlates well with their antimicrobial activity in many cases. The high specificity with respect to the metal ion shown by these compounds in increasing membrane conductance indicates that they interact with the ion as well as with the membrane. In a recent study on the effects of several of these macrocyclic antibiotics on the ion conductivity of lipid membranes,[4] it was concluded that appropriate ring diameters permit carbonyl oxygens of the ring to substitute for water molecules of the hydration shells of the cations. There were shown to be marked differences in the membrane conductivity enhancement between the various compounds studied depending on the size of the ring. It would appear that in the larger rings, the ion and its first hydration shell would fit into the center of the ring, with the water molecules forming hydrogen bonds with carbonyl oxygen atoms. In the smaller rings, it was concluded that the carbonyl oxygens of the ring could coordinate with a fully dehydrated cation, replacing the first hydration shell. Compounds in which the ring is too small to accommodate the ion and its first hydration shell and too large to coordinate with the dehydrated

ion show little activity with regard to influencing membrane conductances.

The nature of the complexes formed between these cyclic antibiotics and the alkali metal ions has not been studied in great detail, and most of the conclusions stated above are based on possibilities suggested by consideration of molecular models of the macrocyclic compounds. Shemyakin and co-workers[5] have investigated the formation of complexes between the enniatins and alkali metal ions in ethanol by optical rotatory dispersion and by the electro-conductivity of these solutions, and have found stability constants for these complexes to be $\sim 10^3$ l/mole. The ORD curves of the enniatins change greatly with complex formation, indicative of large conformational changes in the molecule. Infrared spectra of the molecules and their metal ion complexes show a sizable red shift (~ 30 cm^{-1}) for the ester carbonyl band, evidence of a strong ion-dipole interaction with the ester groups. This suggests that complex formation involves participation by these carbonyl groups. It was observed that the enniatin-alkali metal complexes are most stable in non-polar media, and do not occur at all in water. Thus it may be assumed that these complexes (in aqueous media) form only after binding to the membrane.

Proton magnetic resonance spectroscopy is a method which is particularly sensitive to environmental effects and conformational changes, and it is proposed that an investigation of the interaction of the macrocyclic antibiotics with alkali metal ions in solution would provide rather detailed information regarding the nature of the

complexes formed. Changes in the pmr spectrum of the molecule resulting from complex formation could result from several causes. The electric field produced by the ion would be expected to have a marked effect on the proton chemical shifts in the molecule. These effects are rather well understood, [6] and should give some indication of distances and geometries of the various protons with respect to the incorporated cation. In addition, spin-spin coupling constants between vicinal protons are sensitive to the dihedral angle between these protons, [7] and changes in the conformation of the macrocyclic molecule with complex formation would be reflected in changes in these coupling constants.

These studies of the enniatins would have to be made in non-aqueous solution, in view of the lack of complex formation in water. However a small amount of water could be added to these solutions, and the effects of complex formation on the H_2O proton resonance could be observed. Since the size of the enniatin ring could not accommodate the hydration shell of the ion, the addition of the antibiotic to a non-aqueous solution of an alkali metal salt containing a small amount of H_2O should result in a stripping of the water molecules from the cation as the complex is formed. This process would be reflected in the chemical shift of the H_2O protons.

An investigation by pmr of the interaction of nonactin (a cyclic macrotetrolide antibiotic)with alkali metal salts has recently been initiated, and preliminary results[8] indicate that large changes in proton chemical shifts and coupling constants do occur with complex formation.

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