PROTON MAGNETIC RESONANCE STUDIES OF RIBONUCLEIC ACID COMPLEXES

I. COMPLEXES OF BIOLOGICAL BASES AND OLIGONUCLEOTIDES WITH RNA

II. TEMPLATE RECOGNITION AND THE DEGENERACY OF THE GENETIC CODE

Thesis by

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ACKNOWLEDGMENTS

A doctoral thesis is not the accomplishment of one person, and due credit must be given to those people without whose help my personal goal would not have been achieved. My advisor, Dr. Sunney I. Chan, having great intellectual insight into the problems of chemistry and graduate students, was most instrumental in directing this work to completion.

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ABSTRACT

Part I. Complexes of Biological Bases and Oligonucleotides with RNA

The physical nature of complexes of several biological bases and oligonucleotides with single-stranded ribonucleic acids have been studied by high resolution proton magnetic resonance spectroscopy. The importance of various forces in the stabilization of these complexes is also discussed.

Previous work has shown that purine forms an intercalated complex with single-stranded nucleic acids. This complex formation led to severe and stereospecific broadening of the purine resonances. From the field dependence of the linewidths, T_1 measurements of the purine protons and nuclear Overhauser enhancement experiments, the mechanism for the line broadening was ascertained to be dipole-dipole interactions between the purine protons and the ribose protons of the nucleic acid.

The interactions of ethidium bromide (EB) with several RNA residues have been studied. EB forms vertically stacked aggregates with itself as well as with uridine, 3′-uridine monophosphate and 5′-uridine monophosphate and forms an intercalated complex with uridylyl (3′→5′) uridine and polyuridylic acid (poly U). The geometry of EB in the intercalated complex has also been determined.

The effect of chain length of oligo-A-nucleotides on their mode of interaction with poly U in D_2O at neutral pD have also been studied.

Below room temperatures, ApA and ApApA form a rigid triple-stranded complex involving a stoichiometry of one adenine to two uracil bases, presumably via specific adenine-uracil base pairing and cooperative base stacking of the adenine bases. While no evidence was obtained for the interaction of ApA with poly U above room temperature, ApApA exhibited complex formation of a 1:1 nature with poly U by forming Watson-Crick base pairs. The thermodynamics of these systems are discussed.

Part II. Template Recognition and the Degeneracy of the Genetic Code

The interaction of ApApG and poly U was studied as a model system for the codon-anticodon interaction of tRNA and mRNA in vivo. ApApG was shown to interact with poly U below ~20°C. The interaction was of a 1:1 nature which exhibited the Hoogsteen bonding scheme. The three bases of ApApG are in an anti conformation and the guanosine base appears to be in the lactim tautomeric form in the complex.

Due to the inadequacies of previous models for the degeneracy of the genetic code in explaining the observed interactions of ApApG with poly U, the "tautomeric doublet" model is proposed as a possible explanation of the degenerate interactions of tRNA with mRNA during protein synthesis in vivo.

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INTRODUCTION

The field of genetics has progressed from 1865 when Mendel first proposed that the various traits which define an individual are controlled by a pair of factors (genes), one derived from the male and the other from the female, [1] to today when the genetic process is being described on the molecular level. [2] The major breakthrough which allowed the molecular interpretation of the genetic transmission of data came in 1953 when the Watson-Crick double helix structure of deoxyribonucleic acid (DNA), the hereditary material, was proposed. [3-5] The structure of DNA had to answer two questions: (1) how does the molecule duplicate itself accurately, and (2) how is the genetic information coded on DNA translated into an amino acid sequence in enzymes? The Watson-Crick model met both of these requirements.

Their model consisted of two helical chains coiled around the same axis. Each chain is a polymeric nucleoside with the deoxyribofuranose ring of one nucleotide connected to its neighbor by 3′, 5′ linkages of a phosphate di-ester group. The bases of the nucleotide are perpendicular to the helix axis and are joined in pairs, with a single base from one chain hydrogen-bonded to a single base of the other chain. The base pairing is very specific, with each of the pyrimidine bases, thymine and cytosine, pairing only with one of the purine bases, adenine or guanosine, respectively. Because of this specific base pairing, the primary structure of one strand complements the primary structure of the other. [3-5]

The double helical structure of DNA is entirely compatible with the requirement of exact duplication of hereditary material during cellular mitosis. During duplication, the two strands of DNA separate and the helix unwinds. The single strands of DNA act as templates for the new DNA molecule. Monomeric precursors, deoxynucleoside-triphosphates, are enzymatically joined together in a complementary sequence to the single-stranded template as dictated by the specific base-pairing restrictions imposed between the purine and pyrimidine bases. The result of this process is the production of two identical DNA molecules.[2]

The genetic information of a given species is determined by the sequence of bases in the double helix. Each gene is a specific section of the DNA molecule. The implementation of this genetic information is complicated by the fact that the majority of the DNA is concentrated in the nucleus of the cell while the manufacture of enzymes takes place in the cytoplasm. The transmission of the genetic information from the nucleus to the cytoplasm is carried out by a short single-stranded nucleic acid called messenger ribonucleic acid (mRNA).[6] Each mRNA molecule corresponds to one gene for the production of one protein. The mRNA is transcribed from the DNA and its primary structure is defined by base pair complementation with that part of the DNA molecule corresponding to that particular gene. After transcription, the mRNA migrates from the nucleus to the cytoplasm[7] where it directs protein synthesis.

Protein synthesis occurs when the mRNA is bound to a ribosome where the nucleotide sequence is translated into the amino acid

sequence of the protein. This translation is carried out by another nucleic acid, transfer ribonucleic acid (tRNA). At least one tRNA molecule exists for each amino acid. The tRNA's translate the nucleic acid message by one part of the molecule, a three base sequence called the anticodon, interacting by complementary base pairing with a three base sequence of mRNA, the codon, while on another part of the tRNA, the specific amino acid asked for by the code is being positioned for incorporation into the protein being synthesized. Thus as the mRNA is being "read" in increments of three bases, the polypeptide chain grows in length and its sequence corresponds to that described by each gene on the DNA in the nucleus. (2)

The various nucleic acids play an important role in the life process. A better understanding of the chemical interactions of the nucleic acids with molecules of biological interest and the forces involved in these interactions will lead to a better understanding of the life process. In this manuscript, several experiments are described which elucidate the structure of complexes of biological bases and oligonucleotides with single-stranded ribonucleic acids and the types of forces involved in the stabilization of these complexes.

PART I

COMPLEXES OF BIOLOGICAL BASES AND OLIGONUCLEOTIDES WITH RNA

"It has often been commented that, while much is known about the structure of DNA and of its biological activity, almost nothing is known of its biochemical activity. This absence of knowledge, however, is only to be expected if the activity of DNA consists of transient hydrogen bonding to highly specific substances."(8)

M. H. F. Wilkins

1. INTRODUCTION

In retrospect, the structure of DNA almost appears to be intuitively obvious, but the intense effort by many research groups as well as the proposal of several wrong models for the structure clearly indicate that the solution required "a combination of genius and luck which seldom meet at the appropriate time."[9] The structure was determined with little knowledge of the nature of the chemical bonds involved, the most likely tautomeric state of the bases, or the nature of the secondary interactions involved in the structure. Since 1953, however, extensive studies in these fields have, to a considerable extent, revealed the nature of this vital information.

The nature of the forces which stabilize the helical structure of DNA is a combination of covalent bonds and both external and internal secondary bonds. The covalent bonds define the structure of the nucleotides as well as the nature of the polymeric chains. These interactions, however, have little to do with the actual stabilization of the double-helical structure since DNA has been shown to lose its helical structure in non-aqueous solvents.[10] The helical nature of DNA arises from the weaker secondary interactions of the various constituents with themselves and the solvent. The hydrogen bonds of the base pairs do contribute a negative enthalpy to the stabilization of the helix, but the magnitude of this enthalpy decrease is nevertheless not great enough to overcome the entropy decrease associated with the immobilization of the nucleotides in a rigid helix. This entropy

decrease, however, is compensated by the hydrophobic and hydrophylic interactions of the bases and the ribose-phosphate backbone, respectively. The hydrophobic purine-pyrimidine base pairs are stacked in the center of the DNA molecule which limits their contact with water. It has been argued that aromatic molecules form vertically stacked aggregates in aqueous solutions to minimize the extent of destruction of solvent structure by minimizing their exposed surface area. With the hydrophobic bases on the inside of the helix, virtually all of the surface atoms in the sugar and phosphate groups can form bonds to water molecules which stabilize the helix further. The high charge density of the phosphates does destabilize the helix to some extent but this interaction is minimized with increasing ionic strength of the solution. The double-stranded RNA helix is also stabilized by the same type of interactions. (2)

The single-stranded RNA molecule, however, lacks hydrogen bonding to the second strand and its resulting structure is of a more flexible nature. This is not to say the entire molecule is totally random, but rather that a statistical average of the conformation exists for sections of the molecule. The hydrophobic interactions of the bases do order the structure such that statistical segments exist along the chain which approximate the helix structure of one strand in a rigid helix. The length of the statistical segment varies with the aromaticity of the bases along the chain. This is reflected in the comparison of polyadenylic acid, which shows considerable base-stacking, with polyuridylic acid, which is relatively unstacked. (10-12)

The tRNA molecule exhibits both single-stranded as well as

double-stranded regions and both types of arguments have to be considered in a discussion of the stabilization forces in this molecule.[13]

The secondary forces, hydrogen bonding, hydrophobic and electrostatic interactions, are not only important in stabilizing the structure of nucleic acids, but are also dominant in defining the mode of interaction of nucleic acids and their derivatives with a wide variety of classes of the molecules of biological interest.

1.1. Nonspecific Base-Stacking and Specific Base-Pairing of Purine and Pyrimidine Residues.

Previous studies of the self-association of purine and pyrimidine bases as well as their interactions with monomeric and oligonucleotides have demonstrated that there can be two modes of interaction: nonspecific base-stacking and specific base-pairing. The mode of interaction is, however, strongly solvent dependent.

Infrared[14, 15] and magnetic resonance[16-18] spectroscopic studies of mixtures of purine and pyrimidine bases in nonaqueous solvents, DMSO and $CHCl_3$, have shown that base-pairing of complementary bases is the dominant mode of interaction. Normal Watson-Crick base-pairs were observed between adenine with uracil derivatives[14, 18] and guanine with cytosine derivatives.[14, 16, 18] Odd base-pairs were also observed between inosine and cytosine or adenine.[15, 17] Base-pairing has also been inferred from chromatographic studies where one base was bound to the resin and a mixture of the other bases was passed through the column. Only the base which was

complementary to the bound base showed an increase in the time required to pass through the column.[19]

In aqueous solutions, the mode of interaction differs from that in nonaqueous solvents. Osmotic studies revealed that the monomeric bases strongly self-associated and that the degree of self-association varied with the nature of the bases.[20, 21] The purines associated to a greater extent than the pyrimidines. The increase in association with higher concentrations is reflected in the proton magnetic resonance (pmr) spectra of these molecules by large upfield shifts of the resonances of the ring system.[22, 23] The upfield shifts clearly show that the mode of interaction is the formation of vertically stacked aggregates of the aromatic ring. This association resulted in increased secondary fields arising from the ring current magnetic anisotropy of the aromatic ring felt by protons in a given ring as the number of stacked molecules becomes greater with increasing concentration. It has been postulated that the main driving force of this type of interaction is the hydrophobic nature of the aromatic ring. This postulate is in good agreement with the observation that the degree of association increases with the aromaticity of the ring. This type of interaction is also nonspecific. A purine derivative, for example, will base-stack with any pyrimidine or purine base as well as with itself.

The hydrophobic nature of the purine molecule has been exploited for the characterization of the structural nature of small oligomers of nucleic acids. When purine is added to aqueous solutions

of single-stranded nucleic acids, dinucleoside monophosphates, or higher oligomers, [10-12, 24-26] it has been shown that purine will interact with the oligomer by external as well as internal stacking. Internal stacking or intercalation involves sandwiching of a purine molecule between adjacent bases of the oligonucleotide. The intercalated complex has been monitored by pmr through: (1) the effect of the intercalated purine on the chemical shifts of the base protons of the nucleic acid, [10-12, 24-26] and (2) conformational changes in the ribose-phosphate backbone reflected by changes in the vicinal coupling constant between the $H_{1'}$ and $H_{2'}$ ribose protons. [26] Due to the close proximity of the base protons to the strong shielding region of the purine ring in the intercalated complex, the purine induced chemical shifts of the base protons are upfield. The increase in the vicinal coupling constant has been postulated to be the result of the two bases moving apart upon purine intercalation and the ribose conformation tending to be restored to that of the mononucleotides.

In contrast to the studies of the interactions of purine with mononucleotides where the purine resonances were quite narrow, the purine resonances were appreciably broadened in the presence of dinucleotides or higher oligomers, particularly at low purine/nucleotide ratios where the fraction of incorporated to unbound purine was high. [24, 25] The three purine proton resonances were not equally broadened, the H_6 and H_8 resonances being affected to a considerably greater extent than the H_2 resonances. Chan et al. [24] have proposed that the purine protons experience a strong dipolar field when the purine base is incorporated between adjacent bases of the dinucleotide

segment. Line-broadening of the purine proton resonances thus results from nuclear spin relaxation induced by fluctuations of these local dipolar fields. In particular, it was proposed that the greater part of the dipolar field arises from the $H_{2'}$, $H_{3'}$, $H_{5'}$ and $H_{5''}$ ribose protons. These protons are situated around the bend of the "U" on the inner side of the cage when the conformation of the dinucleotide segment corresponds to that for maximum interaction of the nucleic acid bases with the incorporated purine base.

Several experiments, the field dependence of the purine linewidths, T_1 measurements of the purine protons, and nuclear Overhauser enhancement of the purine resonances upon strong irradiation of certain ribose protons, were implemented to study the nature of the purine line-broadening mechanism.

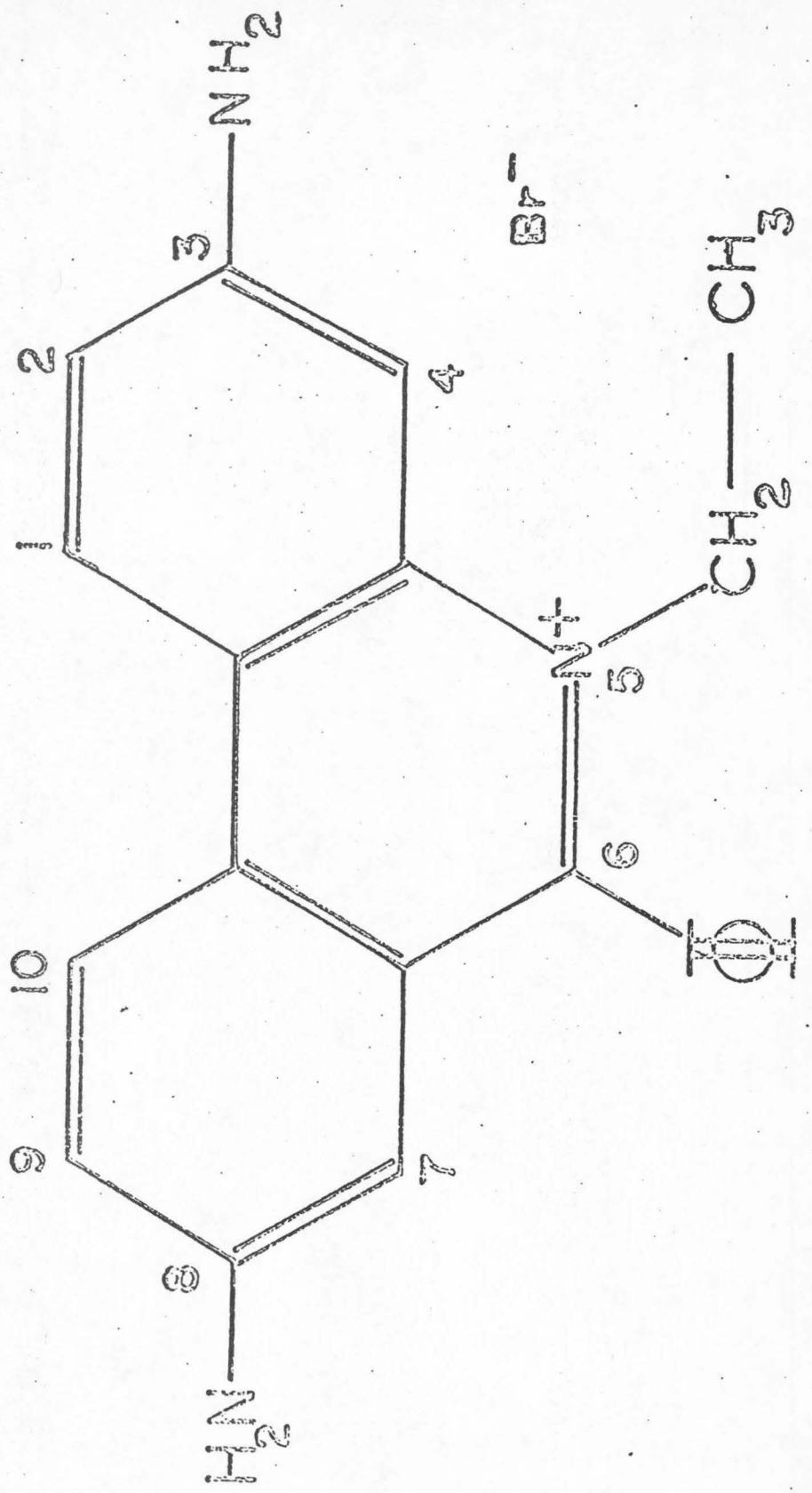
1.2. Interactions of Hydrophobic Dyes with Nucleic Acids.

In addition to the purine and pyrimidine bases, a wide variety of dyes and antibiotics are known to interact strongly with nucleic acids and polynucleotides. Representative ligands of these types include the aminoacridines,[27, 28] the actinomycins,[29] and the phenanthridinium derivative ethidium bromide,[30, 31] Fig. 1. This latter compound has been the subject of intense investigation in terms of both its chemical and biological effects upon nucleic acids.

Ethidium bromide (EB) is biologically active, possessing trypanocidal,[32] antiviral, and antibacterial properties.[33-35] EB inhibits DNA synthesis *in vivo* [36] and *in vitro*, and interferes

FIGURE 1

Ethidium Bromide.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with the DNA-dependent DNA and RNA polymerases of Escherichia coli.[37, 38] Chemical studies have shown that the dye exhibits two modes of interaction with DNA and RNA as well as transfer RNA.[39] These are generally referred to as strong and weak binding. The latter is presumably electrostatic in nature since it disappears at high ionic strength.

It has been generally accepted that EB binds to double-stranded nucleic acids by an intercalation mechanism in the strong binding region and by electrostatic interactions involving external stacking of dye molecules otherwise.[30, 31] The intercalation hypothesis was first proposed by Lerman[27] to explain the strong binding of the aminoacridines to DNA. In this mode of binding the planar dye is supposed to position itself between adjoining base pairs, which antecedently unwind with respect to each other to create the binding site. This type of binding involves some sort of stacking interaction between the dye and the surrounding base pairs. Lerman suggested that the unwinding angle is 45°, and the model building studies of Fuller and Waring[40] indicate that the minimum required angle is 12°.

EB-DNA complexes exhibit several properties which may be taken as indicative of intercalation. These include a diminished sedimentation coefficient,[31] enhanced intrinsic viscosity,[41] and demonstrable unwinding in studies with closed circular DNA.[42, 43] In addition, the EB moiety exhibits a greatly augmented fluorescence when bound to double-stranded DNA or to RNA.[31] More recently, V. W. F. Burns has shown from measurements of polarized fluorescence decay times of EB bound to both DNA and RNA that only the

strongly bound form participates in energy transfer from the nucleic acid.[44] These indices are highly convincing indications of the correctness of the intercalation model, but they do not represent complete proof of the hypothesis.

There has recently been much interest in EB because of the special features of its interaction with closed circular DNA. EB has become an extremely useful analytical and preparative reagent for closed DNA due to the large fluorescence enhancement upon binding, and also due to the retention of the strong binding at high salt concentrations.[43, 45, 56] Studies of the variations in sedmentation coefficient of a variety of closed circular DNA's in the presence of added EB have, in turn, demonstrated that an obligatory unwinding of the duplex occurs with EB strong binding.[42, 43, 47] A comparison of the dye-sedimentation velocity titration of SV4O DNA with the alkaline buoyant density titration of polyoma DNA has further indicated that the estimate of 12° for the unwinding angle is not greatly in error.[40, 48] The results obtained with closed circular DNA do not, however, bear directly upon the question of whether or not intercalation occurs.

In contrast to DNA and RNA systems, fluorescence studies revealed no fluorescent enhancement for solutions of EB containing poly A in acid or neutral solution, or poly I, poly C and poly U in neutral solutions.[41, 49] While these observations would tend to indicate the absence of formation of an intercalated complex, at least under the experimental conditions in which they were made, they should not be taken as evidence for no interaction between EB and the homopolyribonucleotides. Rather these results may reflect a

weaker binding constant for the formation of an intercalated complex in the single-stranded ribonucleic acid systems. Previous optical studies have indicated that EB does form metachromatic complexes with homopolyribonucleotides, although the nature of the interaction of EB with these homopolymers appears to be quite different from the binding of EB with DNA and RNA, and is as yet still unknown. (50)

Several experiments were undertaken to investigate the binding of EB to single-stranded ribonucleic acids by high resolution pmr spectroscopy. If EB does form an intercalated complex with single-stranded nucleic acids, this interaction should have profound effects on the pmr spectra of both the nucleic acid and EB. There are severe concentration limitations of the pmr method due to its inherent sensitivity. The principal limitation of the pmr method is that concentrations of nucleic acids of approximately 0.01 moles nucleotide/liter must be used, whereas optical and other experiments typically employ concentrations in the range 10^{-4} to 10^{-5} M. Although care is therefore indicated in comparing the results of this investigation with those at lower concentrations, pmr spectroscopy nonetheless provides detailed structural information concerning the mode of interaction of EB with nucleic acids.

1.3. Rigid Complementary Hydrogen Bonded Helixes.

In contrast to the non-specific hydrophobic interactions of aromatic molecules, rigid helical structures exhibiting complementary hydrogen bonding have been observed between various synthetic

homopolynucleotides as well as in certain monomer-polymer systems. The structure of these helixes varied from normal Watson-Crick double helixes for poly (A + U)[51] or poly C-GpGpG[52] systems, to abnormal helixes of: (1) a double-stranded nature of poly (A + I)[53, 54] or poly (C + C),[55] and (2) a triple-stranded nature of poly (A + 2U)[56, 57] and poly (A + 2 I).[54]

The double and triple-stranded complexes between U and A, however, have been the most extensively studied. Stevens and Felsenfeld have been able to determine a "phase diagram" for the poly U-poly A system which shows the conditions of stability of the two poly A-poly U structures as a function of salt and temperature.[58] At sodium ion concentrations of less than 0.2 M, only the double-stranded structure of poly (A + U) is formed. Moreover, the melting temperature of the complex increases with sodium ion concentration, presumably because of decreasing coulombic repulsion between the phosphate-ribose backbones. Above 0.2 M $[Na^+]$, there is a two phase transition. For an equimolar poly U-poly A system at temperatures lower than ~40°C, the double-stranded complex is favored but at higher temperature the triple-stranded complex of poly (A + 2 U) is in equilibrium with a free poly A strand. Since the enthalpy of formation for the two complexes is presumably comparable, this temperature behavior is the result of the entropy difference between the two structures. The requirement of high ionic strength for the formation of the triple-stranded structure clearly reflects the high negative charge density of the phosphate groups in a triple-helix. In the presence of magnesium ions, which are more effective than sodium

ions in neutralizing the charge of the phosphate group, only the poly (A + 2 U) structure is observed.

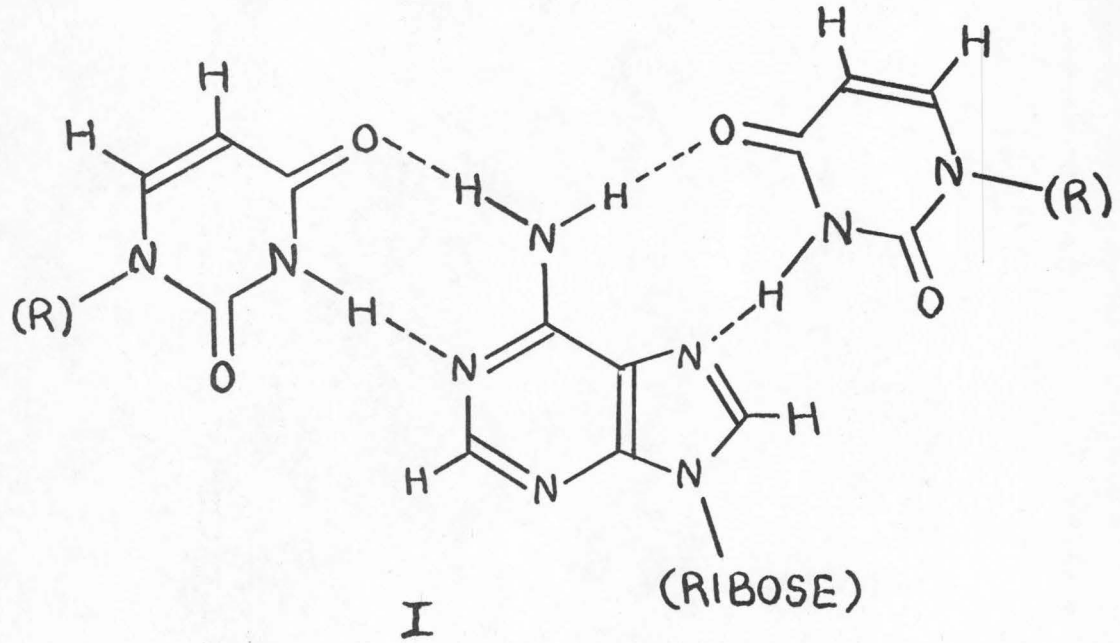
The hydrogen bonding schemes of the two types of structures have been tentatively assigned from infrared studies by H. T. Miles.[59] The poly (A + U) complex exhibits normal Watson-Crick bonding as is expected. The second poly U strand can bond in one of two schemes (Figure 2). Structure II appears to be the favored structure because in this scheme maximum separation of the ribose-phosphate backbones is achieved.

Although hydrogen bonding is important in ordering and stabilizing both types of complexes, a major contribution to their stability is the cooperative base-stacking interactions of the bases in the complex. Although the cooperative nature of the helix-random coil transition has been inferred by M. N. Lipsett et al. from the inverse dependence of the melting temperature with chain length,[60] clearer evidence for cooperativity can be obtained from monomer-polymer studies.

Pmr studies of the interaction of adenosine and poly U have shown that at temperatures above 26°C, adenosine forms an intercalated complex with poly U similar to that previously observed for purine with poly U; while below this temperature, a triple-stranded complex is formed, presumably involving both Watson-Crick and Hoogsteen hydrogen bonding.[61] Equilibrium dialysis measurements have shown that a threshold concentration of adenosine must be reached before complex formation takes place,[62] suggesting that the complex is stabilized by cooperative interactions between adjacent

FIGURE 2

Two possible hydrogen-bonding schemes for the triple-stranded poly (A + 2 U) complex.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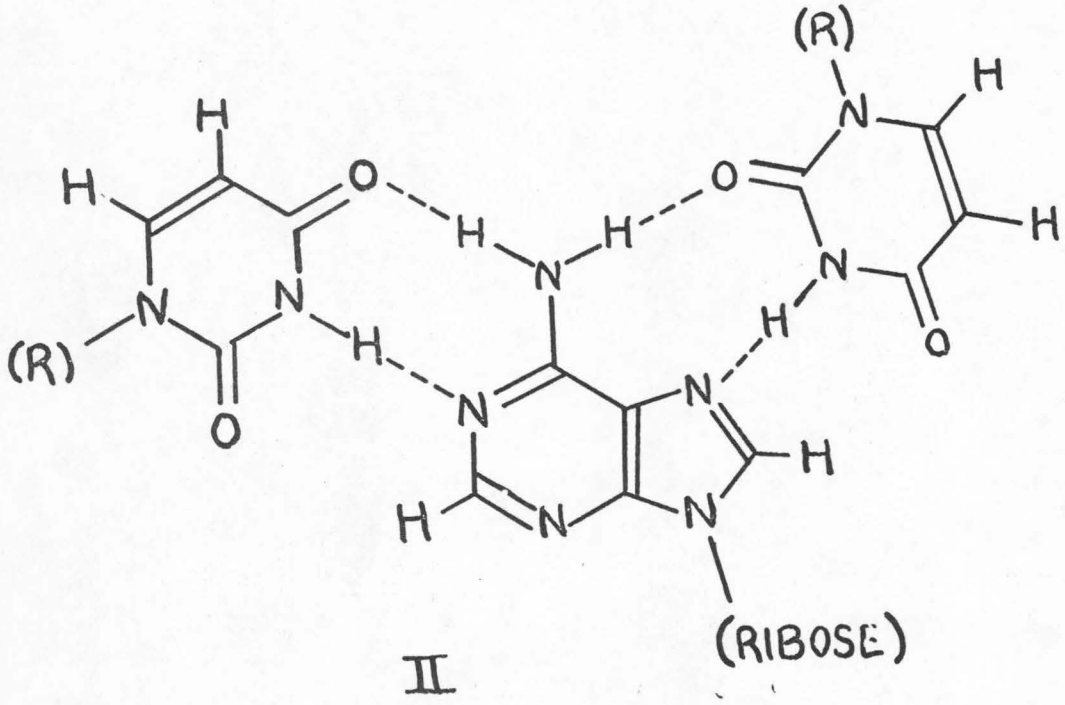
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adenosine molecules in the complex. This threshold base concentration should not be interpreted as evidence for the requirement of stacked aggregates of a minimum size to initiate complex formation.[62] Rather, it implies that cooperativity is governed by the magnitude of the vertical interactions in the complex.[63] Since uridine and poly A do not form a stable complex,[12] and it is known that the base-stacking interaction between uridine molecules is smaller than that for adenosine, the more favorable base-stacking interactions between adenosine molecules most likely provide the main driving force for the formation of the complex in the case of poly U and adenosine. It has been argued, however, that the additional enthalpy of formation resulting from the possible incorporation of two polynucleotide strands in the poly U + A complex plays an important role in stabilizing the triple-stranded structure by overcoming the large unfavorable entropy decrease accompanying the immobilization of the monomeric bases in these monomer-polymer interactions.[64]

The pmr study of adenosine and poly U has been extended to include the interactions of poly U with adenylyl (3′→5′) adenosine (ApA) and adenylyl adenylyl adenosine (ApApA). It is anticipated that both ApA and ApApA form a triple-stranded complex with poly U, as in the case of poly U and A. However, an intercalated complex is not expected here because ApA[26, 65-68] and ApApA[10, 11, 69] are themselves extensively stacked in solution and it would be sterically impossible for a stacked oligonucleotide to intercalate between adjacent uracil bases of poly U. Since it has been postulated that the nucleation process for the renaturation of double-stranded DNA is

the binding of a trinucleotide segment of one strand to the other, [70, 71] a double-stranded complex of ApApA and poly U may also be observed. Several experiments to detect these interactions were undertaken to confirm these expectations.

2. EXPERIMENTAL

2.1. Materials and Methods.

Polyuridylic acid, ammonium salt (poly U) of M. W. of ~100,000 was obtained from Sigma Chemical Company and Miles Laboratories. The poly U was converted to the sodium salt by passing the sample through a Dowex 50-W-X8 cation exchange resin in the sodium form and the resulting solution was lyophilized. All samples were prepared by dissolving the solid material in D_2O (99.5%) obtained from Columbia Organic Chemicals. One drop of $CHCl_3$ per 100 grams of D_2O was added to retard bacterial growth. A small amount of tetramethylammonium chloride (~0.01 M) was added to the solution to provide an internal reference for chemical shift measurements.

The various complexing agents were added to the poly U solutions. The purine (A grade) was obtained from Calbiochem and was sublimed in vacuo before use. The uridine, 3′-uridine monophosphate disodium salt (3′-UMP) and 5′-uridine monophosphate disodium salt (5′-UMP), all A grade, were obtained from Calbiochem and were used without further purification. The adenylyl (3′→5′) adenosine acid form (ApA) and uridylyl (3′→5′) uridine ammonium salt (UpU) were obtained from Sigma Chemical Co. and were converted to the sodium

salt in a manner similar to that used for poly U. Adenyl-adenyl-adenosine, acid form and lithium salt (ApApA), was obtained from Miles Laboratories. The acid form was converted to the sodium salt by the aforementioned method used for poly U. The ethidium bromide (EB) was kindly supplied by Jerome Vinograd of Caltech. The EB was produced by Boots Pure Drug Company, Ltd., but was given to Dr. Vinograd by J.-B. LePecq. The EB was used without further purification.

2.2. Instrumentation.

The pmr experiments were performed on a modified Varian HR-220 superconducting nmr spectrometer equipped with frequency sweep and multinuclei capabilities. A magnetic field of 52 kgauss and a resonance frequency of 220 MHz were used in the course of these experiments. The ambient probe temperature for the spectrometer is 17°C. Variable temperature work was accomplished with the aid of a Varian V-4343 variable temperature unit and was determined to ± 1°C using either the methanol or ethylene glycol sample and the appropriate calibration curve supplied by Varian. To improve the signal to noise ratio for some samples, a Varian C-1024 time-averaging computer, operating in either the field or frequency mode, was used. Chemical shifts were measured to ± 0.2 Hz by sideband modulation of the residual HOD resonance and are reported in Hz at 220 MHz.

The spectrum of purine with poly U was obtained on a Varian

HA-100 nmr spectrometer equipped with a Varian C-1024 time-average device. Studies of the interaction of EB with poly U were undertaken on the HA-100 which was equipped with a Varian V-4357 (FT-100) Fourier transform nmr accessory interfaced with a Varian 620i digital computer. These spectra were obtained with the spectrometer operating in the Fourier transform pulse mode. Both experiments on the HA-100 were run at the normal probe temperature of 30°C.

3. RESULTS AND DISCUSSION

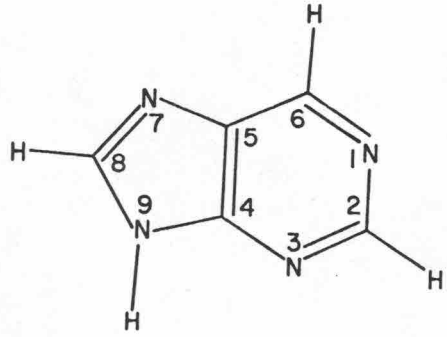
3.1. The Purine Line-Broadening Mechanism.

Several experiments have been performed which have led to further elucidation of the purine line-broadening mechanism. We have studied the purine line-broadening phenomenon at two different magnetic fields under otherwise identical experimental conditions, have measured the spin-lattice relaxation time (T_1) for the various purine protons, and have observed nuclear Overhauser enhancement of the purine resonances upon strong irradiation of certain ribose protons.

Linewidth measurements at two different magnetic fields (or nmr frequencies) for a 0.11 M purine solution containing poly U (0.10 M in uridine) at 30°C showed no magnetic field or frequency dependence of the purine linewidths. The observed linewidths of the H_6, H_2, and H_8 resonances are e.g. 16, 6.5, and 11 Hz respectively at 220 MHz, which can be compared with linewidths of 16, 6, and 11 Hz obtained by computer simulation of the observed spectrum at 100 MHz (Figure 3). The absence of an observable field dependence

FIGURE 3

The pmr spectrum of the purine resonances at 100 MHz and 220 MHz. Probe temperature at 30°C.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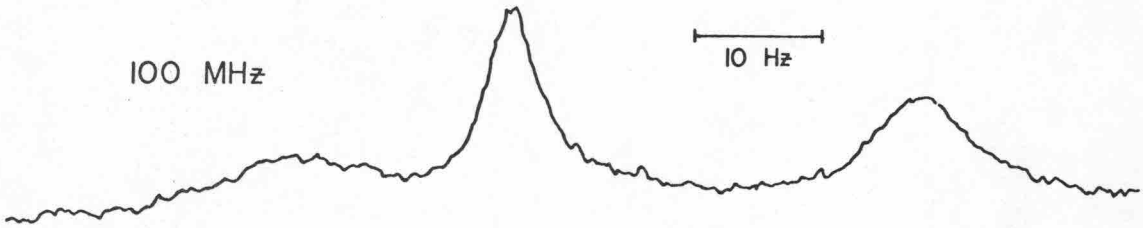
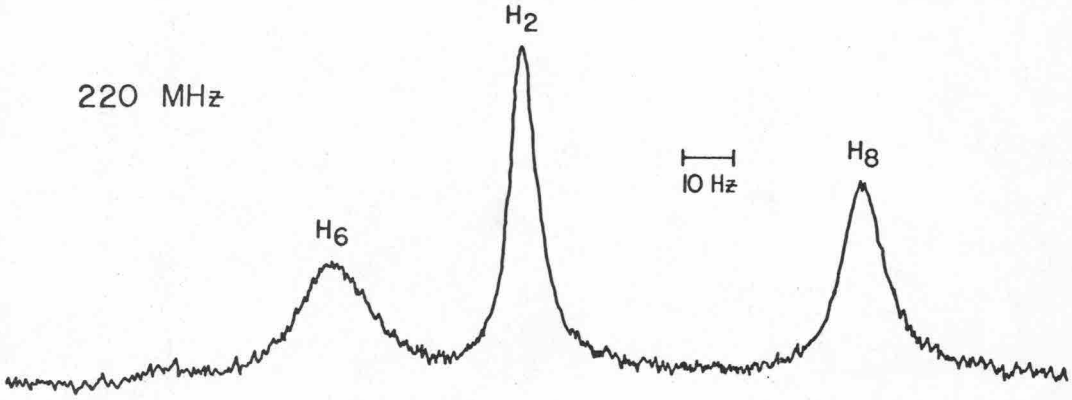

100 MHz
10 Hz


H2
220 MHz
10 Hz
H8
H6

would seem to rule out chemical exchange between chemically shifted purine species as a source of the purine line-broadening. Analysis of the linewidth data for solutions containing different purine/nucleotide ratios has previously indicated that the chemical exchange between bound and free purine is rapid on the nmr time scale.[12]

The T_1's of the various purine protons have been measured by the progressive saturation method.[72] These results are summarized in Table I and are compared with the T_2's calculated from the observed linewidths. In the case of the two purine resonances where T_1 was sufficiently long to be determined in this manner, the spin-lattice relaxation times were found to be extremely short, and insofar as we were able to ascertain, $T_1 \cong T_2$. This result, we feel, substantiates our contention that the purine resonances are relaxation broadened.

The nuclear Overhauser effect observed for the purine resonances upon strong irradiation of the ribose protons provides further insight into the mechanism of purine line-broadening. The observation of an intermolecular nuclear Overhauser effect is important confirmation that the purine protons are magnetically dipolar coupled to the ribose protons of the nucleic acid. Critical to the interpretation of these results is the assignment of the ribose proton resonances. The resonances of the ribose protons, $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$ and $H_{5''}$ at 17°C occur in the spectral region of 0.90 to 1.40 ppm downfield from the tetramethylammonium ion (Figure 4). This region of the spectrum is quite complex and is also complicated by spinning side bands arising from the residual HOD peak. These side bands, however, can be suppressed by the use of a teflon plug inserted into the sample tube.

TABLE I.

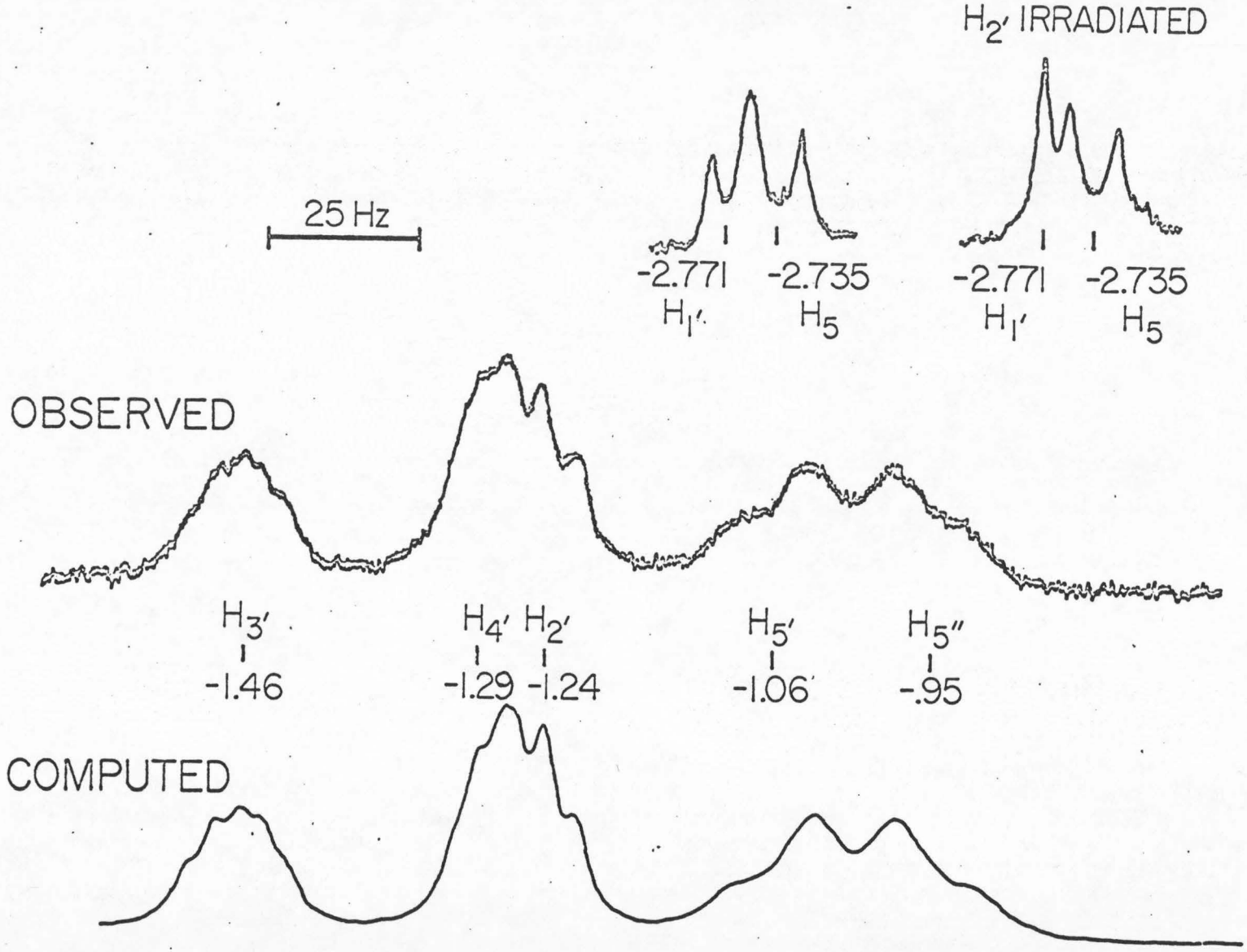
Observed T_1's and T_2's for the purine protons of a 0.11 M purine solution in the presence of poly U (0.10 M in uridine) at 17°C.

Proton	T_2, sec.	T_1, sec[a]
H_6	0.02	not saturable
H_8	0.032	0.038
H_2	0.064	0.060

[a] Precision of measurement: ± 10%.

FIGURE 4

220 MHz pmr spectrum of poly U (sodium salt, 0.078 M in uridine) in the region of the ribose proton resonances at 17°C. Chemical shifts indicated are in ppm downfield from internal $(CH_3)_4N^+$. See text for the discussion of the spectral assignment and the details of the computation of the theoretical spectrum.


H2' IRRADIATED
25 Hz
-2.771
H1'
-2.735
H5
-2.771
H1'
-2.735
H5
OBSERVED
H3'
-1.46
H4' H2'
-1.29 -1.24
H5'
-1.06
H5"
-.95
COMPUTED

The spectral assignment indicated in Figure 4 is based on spin-decoupling experiments, intensity correlations, comparison of spectral parameters with those of 3′-UMP and 5′-UMP, and computer simulation of the spectrum. The theoretical spectrum shown in Figure 4 was calculated using the LAOCOON III program[73, 74] and the coupling constants summarized in Table II. For sake of comparison, we have included a similar tabulation of the coupling constants for 3′-UMP and 5′-UMP. These values were deduced from a detailed analysis of the 220 MHz spectra of 0.1 M 3′-UMP and 0.1 M 5′-UMP solutions.

In order to fit the observed spectrum of the ribose region, it was necessary to use intrinsic linewidths of 5 Hz for $H_{2'}$, $H_{3'}$, and $H_{4'}$ and 8 Hz for the $H_{5'}$ and $H_{5''}$ resonances in composing the theoretical spectrum. These larger linewidths unquestionably reflect the increased rigidity of the ribose backbone when the nucleotide is incorporated in the polynucleotide. The difference linewidths for the $H_{5'}$ and $H_{5''}$ resonances compared with those of the other ribose proton resonances probably arise from slightly different dipolar fields experienced by the various protons. The relatively broad lines in this spectral region made it difficult to ascertain the coupling constants involving the $H_{5'}$ and $H_{5''}$ protons with great accuracy. For this reason, we have included in Table II the range over which these coupling constants could be varied with little change in the theoretical spectrum. Our value of $|J_{H_{3'}-P}|$ for poly U is in good agreement with that recently reported by Tsuboi et al.[75] for UpU on the basis of ^{31}P nmr studies. An attempt was made to simulate the spectrum using their published values of $|J_{H_{5'}-P}|$ and $|J_{H_{5''}-P}|$ for UpU,

TABLE II.

A comparison of the spin-spin coupling constants for the ribose protons of poly U with those for 3′-UMP and 5′-UMP.

Coupling Constant	Poly U	3′-UMP	5′-UMP
$\lvert J_{H_{1'}-H_{2'}} \rvert$	5.5	4.3	4.8
$\lvert J_{H_{2'}-H_{3'}} \rvert$	5.5	5.3	5.3
$\lvert J_{H_{3'}-H_{4'}} \rvert$	4.0	4.3	4.0
$\lvert J_{H_{3'}-P} \rvert$	8.2	8.0	---
$\lvert J_{H_{4'}-H_{5'}} + J_{H_{4'}-H_{5''}} \rvert$	7.5 ± 0.5	7.5	7.5
$\lvert J_{H_{4'}-H_{5'}} - J_{H_{4'}-H_{5''}} \rvert$	0.0 ± 1.0	2.8	2.8
$\lvert J_{H_{5'}-P} \rvert$	4.0 ± 0.5	---	4.0
$\lvert J_{H_{5''}-P} \rvert$	4.0 ± 0.5	---	4.0
$\lvert J_{H_{5'}-H_{5''}} \rvert$	12.5	12.5	12.5

3.4 Hz and 6.7 Hz respectively, but the computed spectrum was found to be in poor agreement with the observed spectrum.

As shown in Table III, a nuclear Overhauser enhancement of 11% is observed for the purine H_6 resonance when the $H_{5'}$ and $H_{5''}$ ribose protons are saturated, and a small enhancement of 6% is observed for the purine H_8 resonance when the $H_{3'}$ ribose proton is strongly irradiated. By contrast, a noticeable nuclear Overhauser enhancement was not observed for any of the purine resonances upon irradiation of the ribose $H_{1'}$, $H_{2'}$ or $H_{4'}$ protons. These observations suggest that the purine base in the purine-intercalated complex is preferentially orientated with either the H_6 or H_8 protons directed at the $H_{3'}$, $H_{5'}$ and $H_{5''}$ protons of the ribose moiety.

The lack of field dependence for the linewidths for the purine resonances appears to rule out chemical exchange between chemically shifted purine species as a mechanism. Furthermore, the T_1 measurements and the intermolecular nuclear Overhauser enhancements strongly imply that the purine resonances are relaxation broadened in the intercalated complex due to dipole-dipole interactions between the purine protons and the ribose moieties of poly U.

3.2. The Interaction of Ethidium Bromide and Single-Stranded Ribonucleic Acids.

Ethidium bromide (EB) has been shown to exhibit behavior similar to that of purine in D_2O. EB not only forms vertically stacked aggregates with itself but also with uridine, 3′-UMP and 5′-UMP. An

TABLE III.

Nuclear Overhauser effects observed for the purine proton resonances upon strong irradiation of the poly U ribose protons in a 0.4 M purine solution containing poly U (0.10 M in uridine)[a].

Ribose proton irradiated	Nuclear Overhauser enhancement observed for purine resonances, %		
	H_6	H_2	H_8
$H_{5'}$, $H_{5''}$	11 ± 3	0	0
$H_{2'}$, $H_{4'}$	0	0	0
$H_{3'}$	0	0	6 ± 3
$H_{1'}$	0	0	0

[a] Experiments were performed on a Varian HR-220 nmr spectrometer operating at a probe temperature of 17°C.

intercalated complex of EB with UpU and poly U has also been detected.

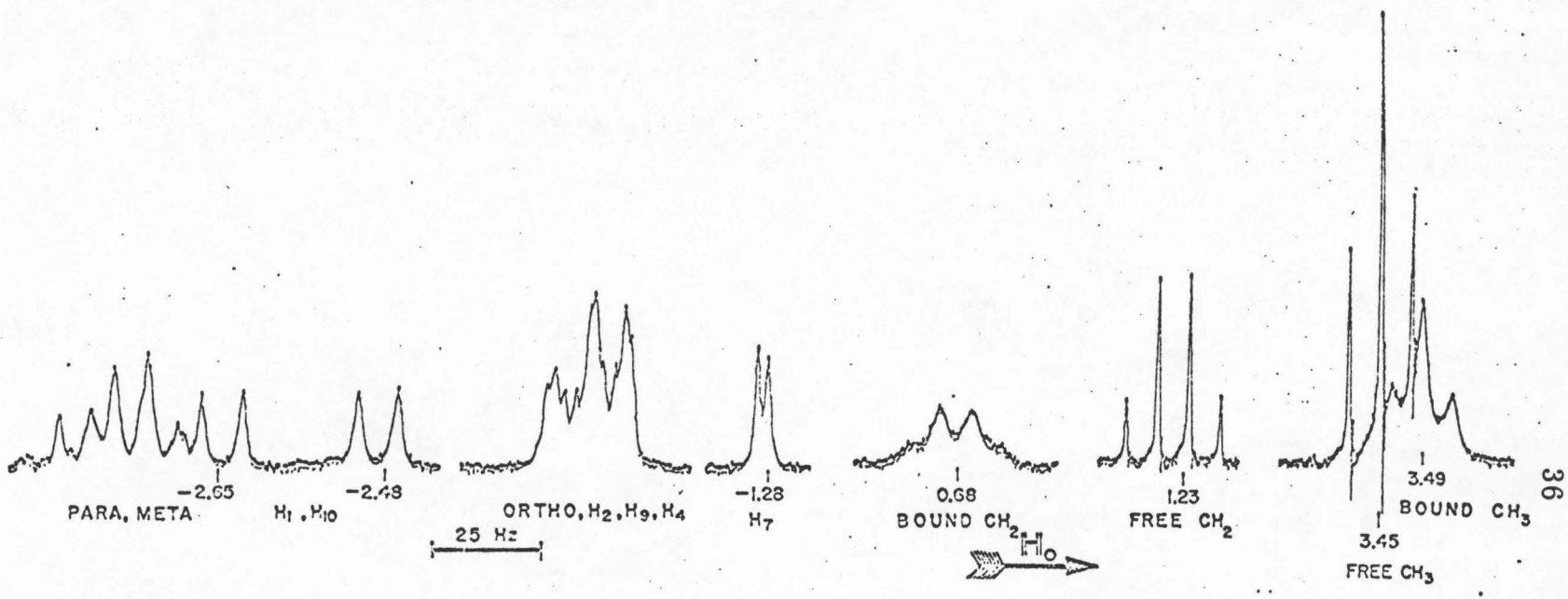
3.2.1. The pmr spectrum of ethidium bromide.

The expanded spectrum of a saturated solution of EB is shown in Figure 5. The assignment of the various spectral peaks was made in the following manner. The broad quartet centered at 0.68 ppm upfield from the HOD resonance and the broad triplet 3.49 ppm upfield from HOD can be readily assigned as the methylene and methyl protons of the ethyl group attached to the quaternary nitrogen. These protons are spin coupled to the quaternary nitrogen and the quadrupole relaxation of the quaternary nitrogen should broaden the resonances of these protons.[76] The sharp quartet and triplet, 1.23 ppm and 3.45 ppm, respectively, upfield from the HOD resonance have been tentatively assigned as ethanol impurity. The resonances downfield from the HOD resonance are due to the aromatic protons of the two ring systems. Examination of a CPK molecular model of EB reveals that the H_7 proton of the phenanthridinium ring is situated in the shielding region of the neighboring phenyl ring, and should therefore experience a magnetic anisotropy effect from the ring current of the phenyl ring, shifting this resonance upfield from the other aromatic resonances. The H_7 proton is also expected to be spin-spin coupled with H_9 with a small long range coupling constant. The doublet centered at 1.28 ppm downfield from the HOD resonance with a splitting of 2.3 Hz was thus assigned to the H_7 proton.

The spectral region corresponding to the remaining five phenanthridinium protons and the five phenyl protons is complicated

FIGURE 5

Expanded spectrum of a saturated solution of EB in D_2O at 17°C. Chemical shifts are in ppm relative to the residual HOD resonance.

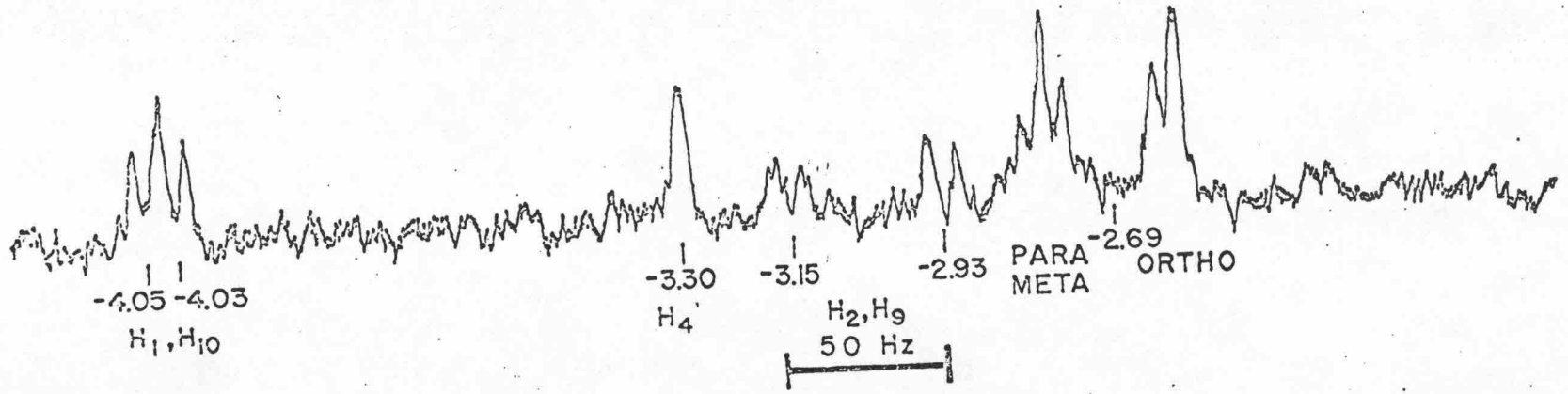

-2.65
PARA, META
H1 , H10
-2.48
25 Hz
ORTHO, H2 , H9 , H4
-1.28
H7
0.68
BOUND CH2
1.23
FREE CH2
3.49
BOUND CH3
3.45
FREE CH3
H0

by the mutual overlap of the various resonances. This complex region can, however, be analyzed by exploiting the pH dependence as well as the concentration dependence of these resonances. It is well known that aromatic systems tend to form vertically stacked aggregates in aqueous solutions. This stacking, which is presumably hydrophobic in origin, causes large upfield shifts of the ring proton resonances, as has been demonstrated for certain nucleic acid bases.[20, 23] EB is also expected to form vertically stacked aggregates. Evidence for this behavior is the change in color of the solution from red to orange upon dilution. This color change can also be observed when the pD of the solution is lowered to 1.9. Presumably protonation of the amino group breaks up the stacked aggregates as a result of electrostatic repulsion. We note that under these conditions the aromatic resonances are well resolved (Figure 6). The doublet at -3.30 ppm can be easily assigned as due to H_4, on the basis of its small long range coupling to H_2 of ~2 Hz. The resonances in the vicinity of -4.0 ppm, which can be resolved into two doublets with splittings of 9.0 Hz, can be unequivocally assigned to H_1 and H_{10} on the basis of known ortho coupling in aromatic systems. The sets of resonances centered at -2.93 and -3.15 ppm constitute the A part of an AMX multiplet with $|J_{AM}| = 9.0$ Hz and $|J_{AX}| = \sim 2$ Hz and can thus be assigned to H_2 and H_9. No attempt was made to distinguish between the H_1 and H_{10} resonances and between the H_2 and H_9 resonances. The multiplet centered at -2.69 ppm is a typical AB_2X_2 pattern characteristic of a monosubstituted phenyl ring.[77]

The various proton resonances can be followed as a function

FIGURE 6

220 MHz spectrum of the aromatic region of EB at pD 1.9 and 17°C. EB concentration: 0.032 M. Chemical shifts are in ppm relative to the residual HOD resonance.


-4.05
-4.03
H1, H10
-3.30
H4
-3.15
H2, H9
-2.93
PARA
META
-2.69
ORTHO
50 Hz

of pH and concentration and the spectra can be interpreted on the basis of the above assignment. The concentration dependence at neutral pH confirms the notion that EB is strongly intermolecularly stacked in aqueous solution. The pronounced upfield shifts of the phenanthridinium resonances depicted in Figure 7, and the extremely small concentration shifts observed for the phenyl resonances, suggest that the EB associates by forming vertical stacked aggregates of the phenanthridinium rings with the phenyl group on the periphery of the stack. The small concentration shifts observed for the phenyl resonances are to be expected if the phenyl ring is perpendicular to the phenanthridinium ring, as has been suggested by molecular model building and the chemical shift of the H_7 phenanthridinium proton.

3.2.2. The interaction of ethidium bromide with several uracil residues.

We have studied the interaction of EB with several uracil residues. The dependence of the chemical shifts of the H_6 and H_5 resonances of the uracil base and the $H_{1'}$ resonances of the ribose moiety of uridine, 3′-UMP and 5′-UMP on the EB concentration is shown in Figure 8. The uridine solution was studied at two different concentrations as well as in the presence of magnesium salt. The 3′-UMP and 5′-UMP samples were examined only in the presence of magnesium salt. In all five cases, the H_6, H_5 and $H_{1'}$ resonances of the uracil residues are shifted significantly upfield upon the addition of EB even at low concentration of the dye molecule. The EB induced shifts observed are greater for the uridine and 3′-UMP resonances

FIGURE 7

220 MHz pmr spectrum of the aromatic region of EB at various concentrations. Temperature: 17°C.

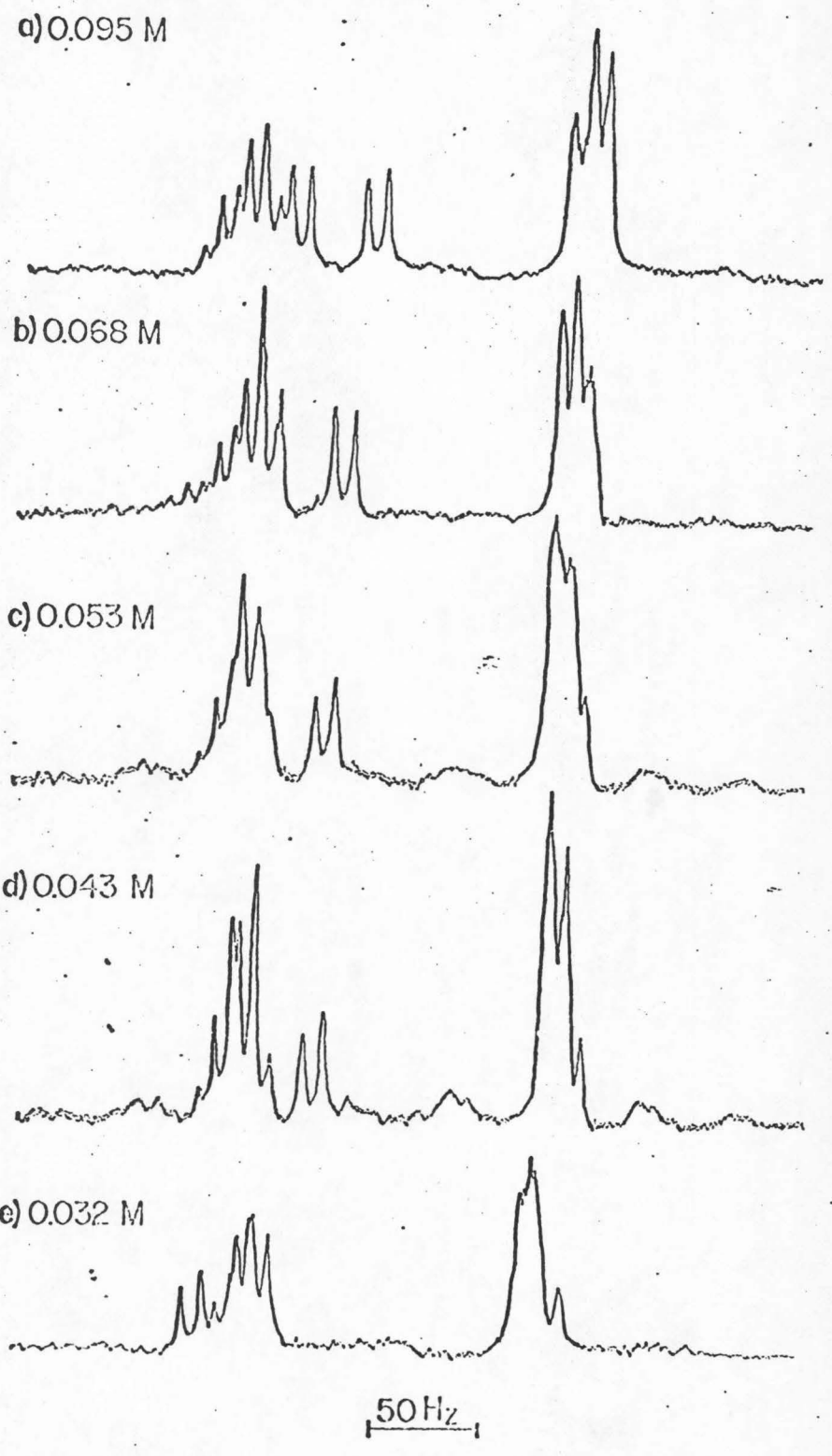
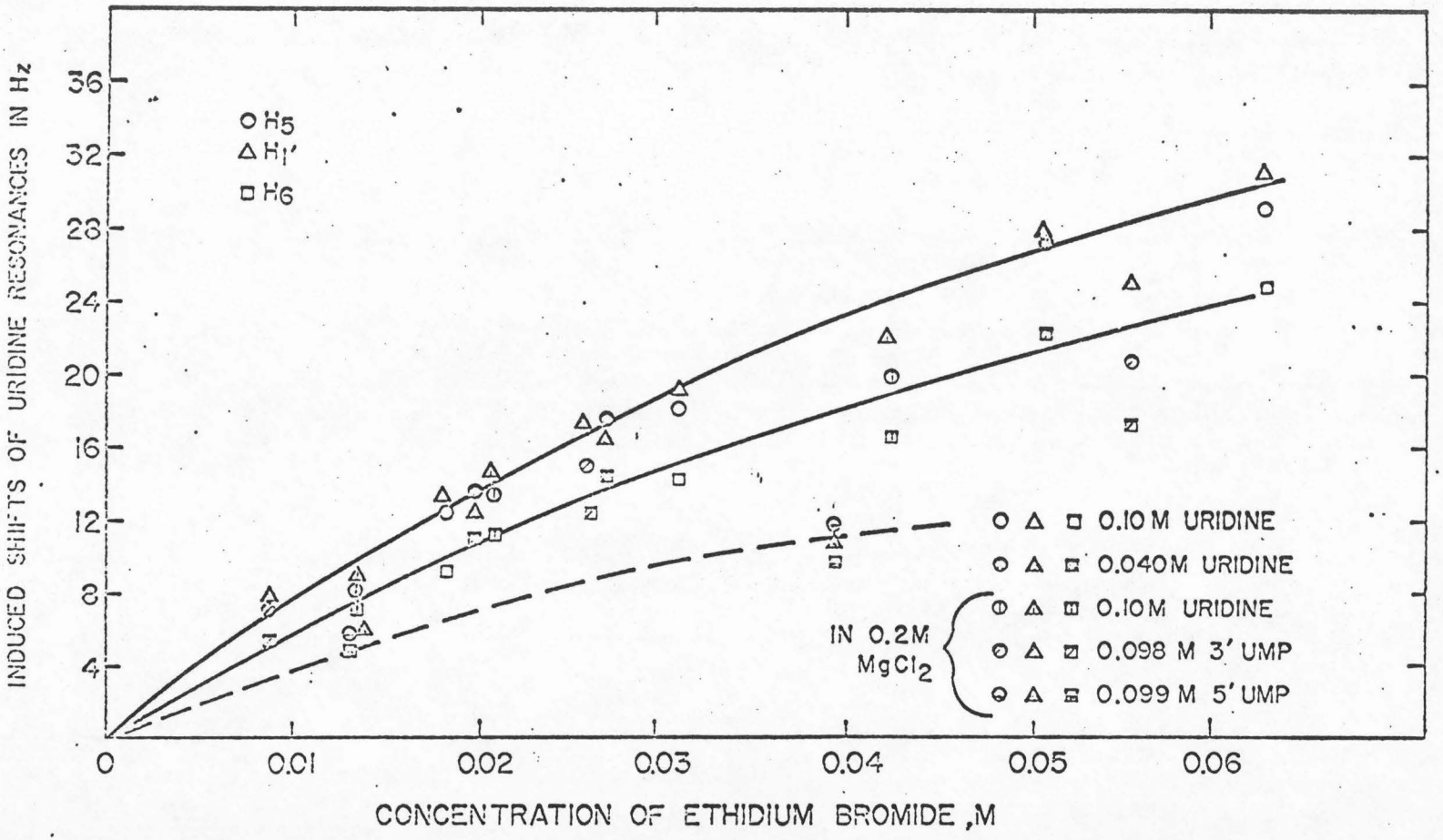

a) 0.095 M
b) 0.068 M
c) 0.053 M
d) 0.043 M
e) 0.032 M
50 Hz

FIGURE 8

EB induced shifts of the H_6, H_5 and $H_{1'}$ protons of uridine, 3′-UMP and 5′-UMP as a function of EB concentration. Temperature: 17°C.


INDUCED SHIFTS OF URIDINE RESONANCES IN Hz
36
32
28
24
20
16
12
8
4
0
0.01
0.02
0.03
0.04
0.05
0.06
CONCENTRATION OF ETHIDIUM BROMIDE, M
O H5
Δ H1'
□ H6
O Δ □ 0.10 M URIDINE
O Δ □ 0.040M URIDINE
IN 0.2M MgCl2
Θ Δ ⊞ 0.10M URIDINE
Θ Δ ⊠ 0.098 M 3' UMP
Θ Δ ⊟ 0.099 M 5' UMP

than for those of 5′-UMP. Whereas the $H_{1'}$ and H_5 resonances are shifted upfield more than the H_6 resonance in the cases of uridine and 3′-UMP, the shifts for all three resonances are nearly equivalent in the case of 5′-UMP.

We have interpreted the upfield shifts of the H_6, H_5 and $H_{1'}$ resonances observed upon the addition of EB in terms of costacking of EB with the pyrimidine base of the uracil residues. Similar results have previously been reported for the interaction between purine and uridine, [22] except here the induced chemical shifts are an order of magnitude larger for a given EB concentration, and there is a conspicuous lack of dependence of these shifts on the concentration of the uracil, even when the latter is greater than or comparable to the concentration of the dye molecules. These observations unquestionably reflect the stronger binding of these uracil residues to EB than to purine, a result which may be expected if the major driving force for the interaction is principally hydrophobic in nature.

The observed differences in the EB induced chemical shifts for 3′-UMP and 5′-UMP most probably arise from the effect of the negatively charged phosphate on the average orientation of EB in the complex. Other factors being equal, we expect the electrostatic interaction between the negatively charged phosphate group and positive quaternary nitrogen to favor stacked configurations in which these groups are in close proximity. Examination of CPK molecular models, however, reveals unfavorable steric interactions between the phenyl/alkyl groups of EB with the ribose moiety when the quaternary nitrogen is close to the phosphate group in the stacked

3′-UMP-EB complex. Thus, we suspect EB costacks with 3′-UMP with the bulky phenyl and alkyl groups attached to the phenanthridinium ring preferentially oriented away from the ribose moiety. We note that in this stacked configuration, the overlap of the phenanthridinium ring with the uracil base is such as to lead to maximum ring-current shifts for the H_5 and H_6 protons. The similarities in the EB induced shifts for uridine and 3′-UMP, we believe, reflect the controlling influence of the above-mentioned steric interactions. In the case of 5′-UMP, this steric interaction is either absent or less stringent because of the greater geometric flexibility of the exocyclic group to which the phosphate group is attached. When the EB is oriented with the quaternary nitrogen close to the 5′-phosphate in the stacked complex, the H_5 and H_6 uracil protons, however, tend to be on the periphery of the phenanthridinium ring and should experience smaller ring-current shifts.

Results of experiments with concentrations of EB greater than ~0.04 M are unreliable because of precipitation in the course of the experiment.

3.2.3. The interaction of ethidium bromide with UpU.

The success which we have had with the use of pmr spectroscopy in studying the intercalation of purine and other biological bases to dinucleotides prompted us to choose the dinucleotide UpU as a model system to investigate similar interactions of EB with single-stranded nucleic acids. As it turns out, the solubility of EB in aqueous solution was sufficiently high in the presence of UpU for

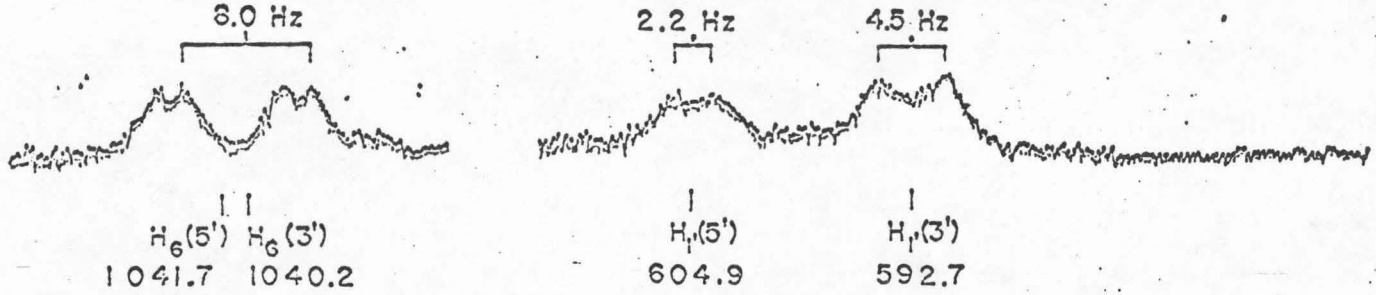
this study to be amenable to the pmr method.

The pmr spectrum of UpU in the region of the H_6, H_5 and $H_{1'}$ resonances is shown in Figure 9a and these resonances have been previously assigned.[12] The magnetic nonequivalence observed for the similar protons of the two uracil residues arises from asymmetric esterification of the phosphate in (3′→5′) dinucleoside monophosphate. The $H_6(3')$ and $H_6(5')$ resonances, which appear as doublets with splitting of 8.0 Hz because of spin-spin coupling between the uracil H_6 and H_5 protons, are found 1040.2 Hz and 1041.7 Hz downfield from $(CH_3)_4N^+$ respectively. The $H_{1'}(3')$ and $H_{1'}(5')$ resonances, which appear at 592.7 Hz and 604.9 Hz downfield from $(CH_3)_4N^+$ respectively, are also doublets due to spin-spin coupling with the $H_{2'}$ protons of their respective ribose moieties, and these coupling constants are 4.5 Hz and 2.2 Hz for the 3′- and 5′-esterified nucleosides respectively. The $H_5(3')$ and $H_5(5')$ resonances, which are normally expected to be in the region of the $H_{1'}$ ribose resonances, are extremely broad and are not readily observable. This broadening has been found to be dependent on the concentration of the dinucleotide and has accordingly been attributed to molecular association.[78] Bangerter and Chan, in particular, have suggested that UpU self-associates via the formation of intercalated dimers, where the uracil base of one UpU is intercalated between the uracil bases of a second dinucleotide molecule. Furthermore, they contend that the H_5 resonance broadening arises from the strong magnetic dipolar fields which this proton experiences from certain ribose protons of the cage dinucleotide, when the uracil base bearing the H_5 proton is sandwiched between the bases

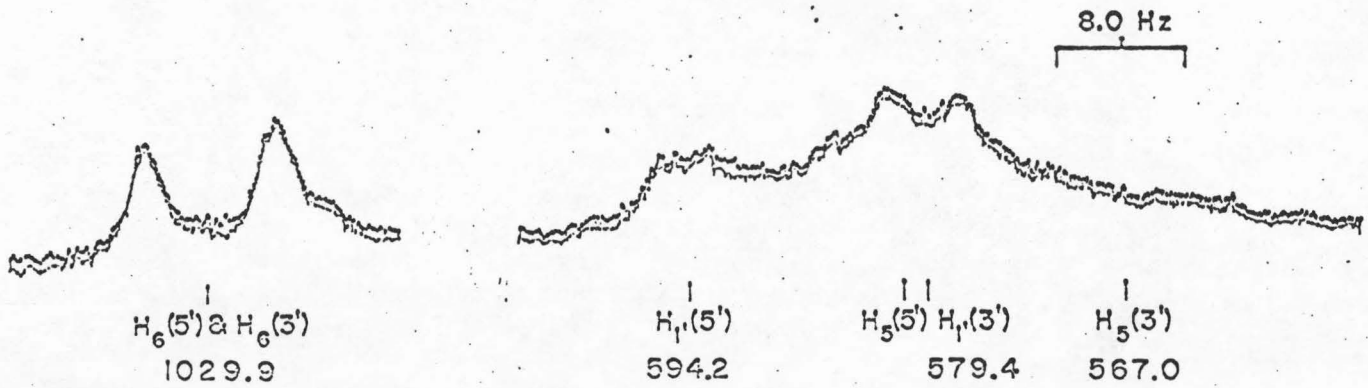
FIGURE 9

220 MHz pmr spectrum of UpU in the region of the H_6, H_5 and $H_{1'}$ resonances: (a) no EB; (b) in the presence of 0.0069 M EB. Chemical shifts are given in Hz downfield from internal $(CH_3)_4N^+$. Temperature: 17°C.

a) 0.021 M UpU



b) 0.021 M UpU , 0.0069 M E B U/E B = 6/1



of the cage dinucleotide.[78] This resonance broadening of the uracil H_5 resonance is analogous to the line-broadening observed for the purine proton resonances when purine is intercalated between the bases of dinucleotides and poly U.[24-26, 79]

Upon addition of EB to the UpU solution, several changes are immediately apparent. First, there is a change in the color of the EB solution from red to orange, indicating the destacking of the EB molecules. Pronounced changes also occur in both the EB and UpU resonances in the pmr spectrum. The H_6 and $H_{1'}$ UpU resonances are all shifted upfield 10 and 11 Hz, respectively, at an EB concentration of 0.0069 M, and these EB induced shifts are larger than those observed for the monomeric uracil residues (Figure 9b). In the presence of 0.0069 M EB, the $H_5(3')$ resonance is still very broad (~10 Hz), but it can now be observed. It is shifted upfield approximately 15 ± 3 Hz. This latter shift was determined by comparing the chemical shift difference of the $H_5(3')$ and the $H_{1'}(3')$ resonances in the presence of EB with the corresponding difference between these resonances in dilute UpU solutions, where the $H_5(3')$ resonances can be observed.[12] This comparison should be valid since the chemical shifts of the UpU resonances are almost independent of the concentration of the dinucleotide.

Previous work on the binding of purine to UpU also showed that at low purine concentrations, the UpU resonances are shifted in the order $H_5 > H_6 \cong H_{1'}$. These shifts are also accompanied by a concomitant sharpening of the H_5 resonances with increasing purine concentration attributable to the breakdown of the self-intercalated

complexes of UpU as a result of purine intercalation. One of the strongest evidences for purine intercalation in dinucleotides has, however, been the pronounced and specific broadening of the purine resonances,[24, 26, 78] a phenomenon not observed for purine-monomer solutions.[25] This broadening has been attributed to the strong magnetic dipolar interactions between the purine protons and certain ribose protons of the ribose-phosphate backbone in the intercalated complex.[79] The present study shows that whereas the EB resonances are sharp (~2Hz) in the presence of all three monomers (uridine, 3′-UMP and 5′-UMP), in the presence of UpU, several of the EB resonances are, however, significantly broadened, particularly H_1 and H_{10}, and have widths ~7 Hz in these experiments (Figure 10).

The EB induced upfield shifts of the UpU resonances, the sharpening of the UpU H_5 resonances, the specific line broadening of the EB resonances, and the destacking of the EB molecules in the presence of UpU, present strong evidence that EB forms an intercalated complex with UpU. The selective broadening of the H_1 and H_{10} resonances of EB suggests that in the complex the phenanthridinium ring is specifically oriented with these protons directed at the ribose moieties of the dinucleotide.

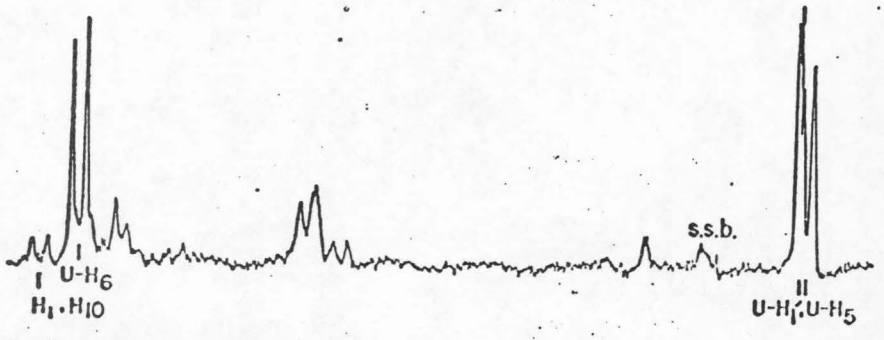
3.2.4. The interaction of ethidium bromide with poly U.

The interaction of EB with poly U was also studied. Because of the low solubility of poly U in the presence of EB, it was necessary to conduct this study using the Fourier transform technique.[80] For a sample of poly U, $\sim 3 \times 10^{-4}$ M in uridine and 0.20 M in $MgCl_2$,

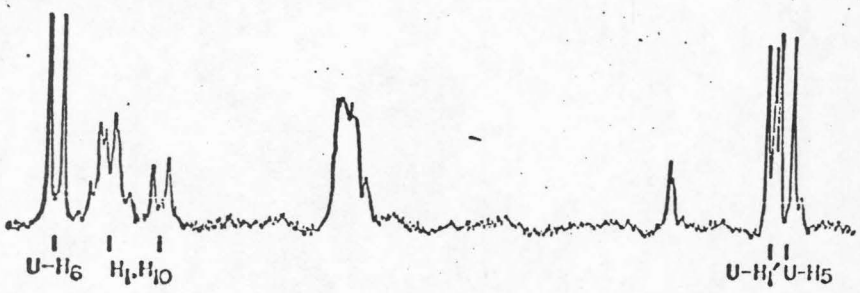
FIGURE 10

220 MHz pmr spectra, in the aromatic region, of solutions of (a) 0.10 M uridine and 0.018 M EB; U/EB = 6/1, (b) 0.098 M 3′-UMP and 0.026 M EB; U/EB = 4/1, (c) 0.099 M 5′-UMP and 0.013 M EB; U/EB = 7/1, and (d) 0.021 M UpU and 0.0069 M EB; U/EB = 6/1. Temperature: 17°C; abbreviation s.s.b. is spinning sideband.

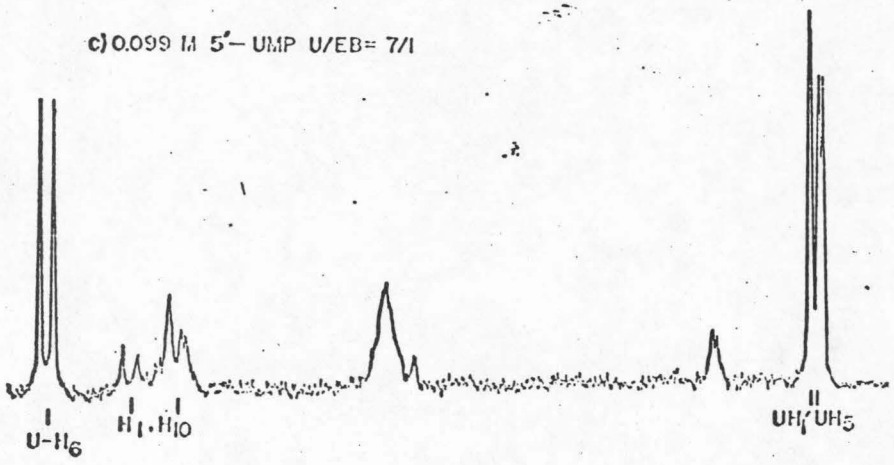
a) 0.10 M URIDINE U/EB= 6/1



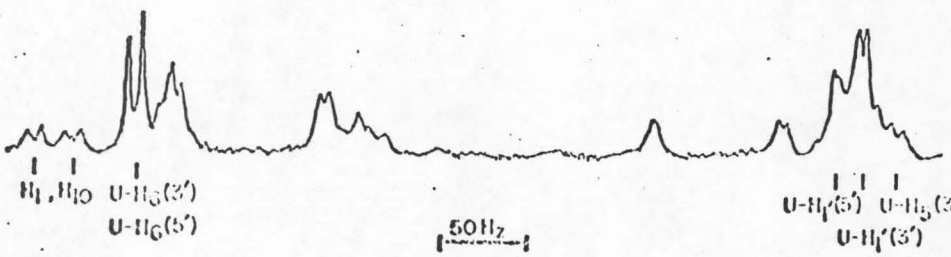
b) 0.098 M 3'-UMP U/EB= 4/1



c) 0.099 M 5'- UMP U/EB= 7/1



d) 0.021 M UpU U/EB= 6/1



small upfield shifts for the H_6, H_5 and $H_{1'}$ resonances were indeed observed upon the addition of $\sim 3 \times 10^{-4}$ M EB. Unfortunately these shifts were too small to enable us to draw quantitative conclusions. Intercalation of EB between adjacent uracil bases can nevertheless be inferred from the selective line-broadening of the EB resonances, as observed in the interaction of EB with UpU. Here, the H_1 and H_{10} resonances of EB were again found to be extremely broad, ~ 20 Hz, as compared to ~ 5 Hz for the phenyl resonances. This line-broadening, we feel, is indicative of intercalation of EB between adjacent bases of the poly U. Unfortunately, instrument sensitivity and the low solubility of this system did not permit these studies to be extended to higher EB concentrations.

3.2.5. The intercalated EB-RNA complex.

To the best of our knowledge, we have presented in this work the first direct structural evidence of the intercalation of EB in single-stranded nucleic acids. This conclusion was inferred from upfield shifts of the uracil proton resonances of UpU and poly U induced by the ring current magnetic anisotropy of the phenanthridinium ring of EB as well as specific line-broadening of the EB H_1 and H_{10} resonances as a consequence of the magnetic dipole-dipole interaction between these protons and certain protons of the ribose backbone. From the specificity of this line-broadening, we concluded that, in the intercalated complex, the phenanthridinium ring of the EB molecule is specifically oriented with the H_1 and H_{10} protons directed at the ribose-phosphate backbone, with the positively charged quaternary

nitrogen and the phenyl ring on the outside in contact with the solvent. This geometry of the EB molecule in the intercalated complex of single-stranded nucleic acids differs with that previously proposed for double-stranded nucleic acids, for which it has been suggested that the phenanthridinium ring is sandwiched between the base pairs with the two amino groups directed at the phosphate groups of the opposing strands and the alkyl/phenyl groups in the groove of the helix. (40)

That there should be conformational differences between the single-stranded and double-stranded complexes is not, however, surprising, since hydrophobic forces presumably play an important role in stabilizing the complex, particularly in the case of single-stranded nucleic acids. For the EB orientation which we have proposed for the UpU and poly U complexes, there would be maximum overlap of the hydrophobic surfaces of the phenanthridinium ring and the uracil bases. With double-stranded nucleic acids, maximum overlap of the hydrophobic surfaces can be attained by rotating the phenanthridinium ring by 90° about an axis perpendicular to the plane of this ring, although we expect that such factors as the free energy necessary to unwind the helix to create the binding site as well as interactions of the amino groups with the negatively charged phosphate may also need to be considered here.

3.3. The Interactions of ApA and ApApA with Poly U.

The results of several experiments which studied the interaction of ApA and ApApA with poly U show that below room temperature

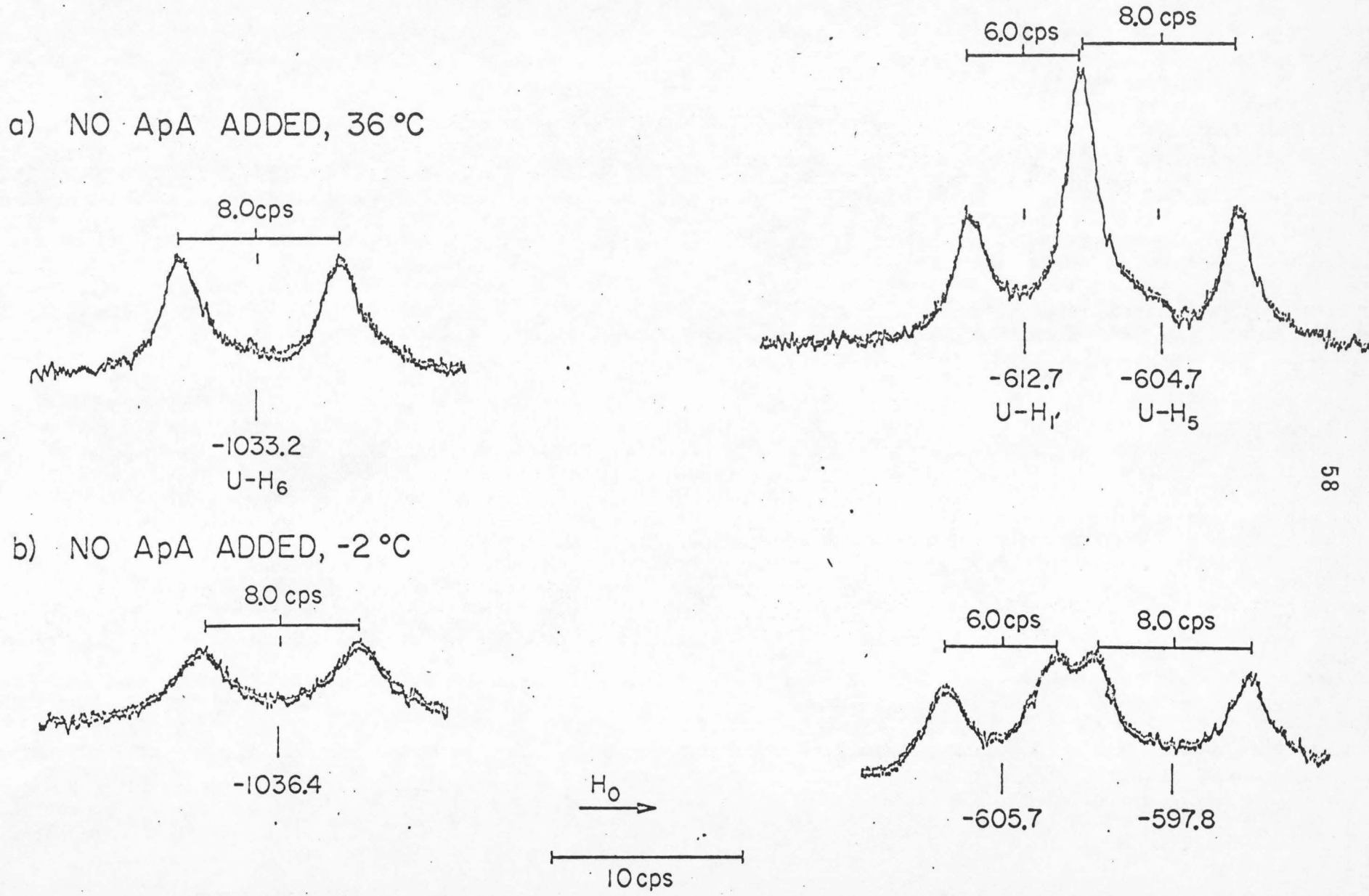
a rigid complex is formed, and that the stoichiometry of this complex involves two uracil bases per adenine base. On the basis of previous work, it is reasonable to conclude that the complex is a triple-stranded structure with stacked oligo-A-nucleotides forming horizontal adenine-uracil hydrogen bonds to two poly U strands. Presumably both Watson-Crick and Hoogsteen bonding schemes are involved as has been proposed for the triple-stranded complex formed between poly U and adenosine.[61] Above this temperature, ApA exhibits no detectable interactions by the pmr method with poly U. ApApA, on the other hand, exhibits complex formation of a 1:1 nature with poly U by forming Watson-Crick base pairs.

3.3.1. The pmr spectrum of free poly U, ApA, and ApApA in solution.

The 220 MHz pmr spectrum at 36°C of the H_5 and H_5 protons of the uracil base and the $H_{1'}$ proton of the ribose ring of poly U is shown in Figure 11a. The interpretation of the spectrum is straightforward and has been presented elsewhere.[25] The H_5 and H_6 resonances are doublets due to mutual spin-spin coupling with $|J_{H_5-H_6}| = 8.0$ Hz. The $H_{1'}$ resonance is also a doublet due to coupling with the $H_{2'}$ proton with $|J_{H_{1'}-H_{2'}}| = 6.0$ Hz. The linewidths of these resonances are typically 2 Hz which is somewhat broader than the linewidths in the monomer. At -2°C (Figure 11b), the linewidths of the H_6, H_5 and $H_{1'}$ resonances are slightly broader. The chemical shifts, however, change only slightly and the coupling constants remain unchanged over this temperature range.

FIGURE 11

220 MHz pmr spectrum of poly U (sodium salt, 0.078 M in uridine) in the region of the H_6, $H_{1'}$ and H_5 resonances at (a) 36°C and (b) -2°C. Chemical shifts are given in Hz downfield from internal $(CH_3)_4N^+$.


a) NO ApA ADDED, 36 °C
8.0cps
-1033.2
U-H6
6.0 cps
8.0 cps
-612.7
U-H1'
-604.7
U-H5
b) NO ApA ADDED, -2 °C
8.0 cps
-1036.4
6.0cps
8.0 cps
-605.7
-597.8
H0
10 cps

In the absence of complex formation, poly U is a random coil over the temperature range studied and under the stated conditions of ionic strength and pD. Although poly U is a large polymer with a long correlation time for the entire molecule, random motions along the length of the chain are sufficient to average the dipole-dipole interactions between nuclei yielding fairly long T_2's for each individual proton. As a result, the poly U resonances are fairly sharp. The widths should vary from proton to proton, since different protons experience somewhat different dipolar fields and the bases are also expected to have different motional correlation times relative to the ribose-phosphate back. If, however, a rigid hydrogen-bonded complex forms, the motions of the nucleotides are very slow and the T_2's for the protons are determined by the motional correlation time of the entire molecule which are very long. Thus, the T_2's of the complex are extremely short (on the order of msec or less) and as is well known, such a complex is not expected to give rise to a high resolution pmr spectrum.

In the pmr spectrum of ApA and ApApA, the resonances of the base proton occur approximately 4 to 5 ppm downfield from the tetramethylammonium resonance and are well resolved at 220 MHz. The assignment of the ApA and ApApA resonances has been previously reported.[26, 29] The linewidths of the adenine resonances vary slightly with temperature, but in contrast to the poly U resonances, the chemical shift of these resonances is strongly temperature dependent and this temperature behavior differs for the various esterified base protons (Figures 12-14). This temperature dependence has been attributed to the intramolecularly stacked nature of ApA and ApApA.[26, 29]

FIGURE 12

The temperature dependence of the chemical shift for the H_2 and H_8 resonances of ApA (0.078 M in adenosine). Chemical shifts are given in Hz downfield from internal $(CH_3)_4N^+$.

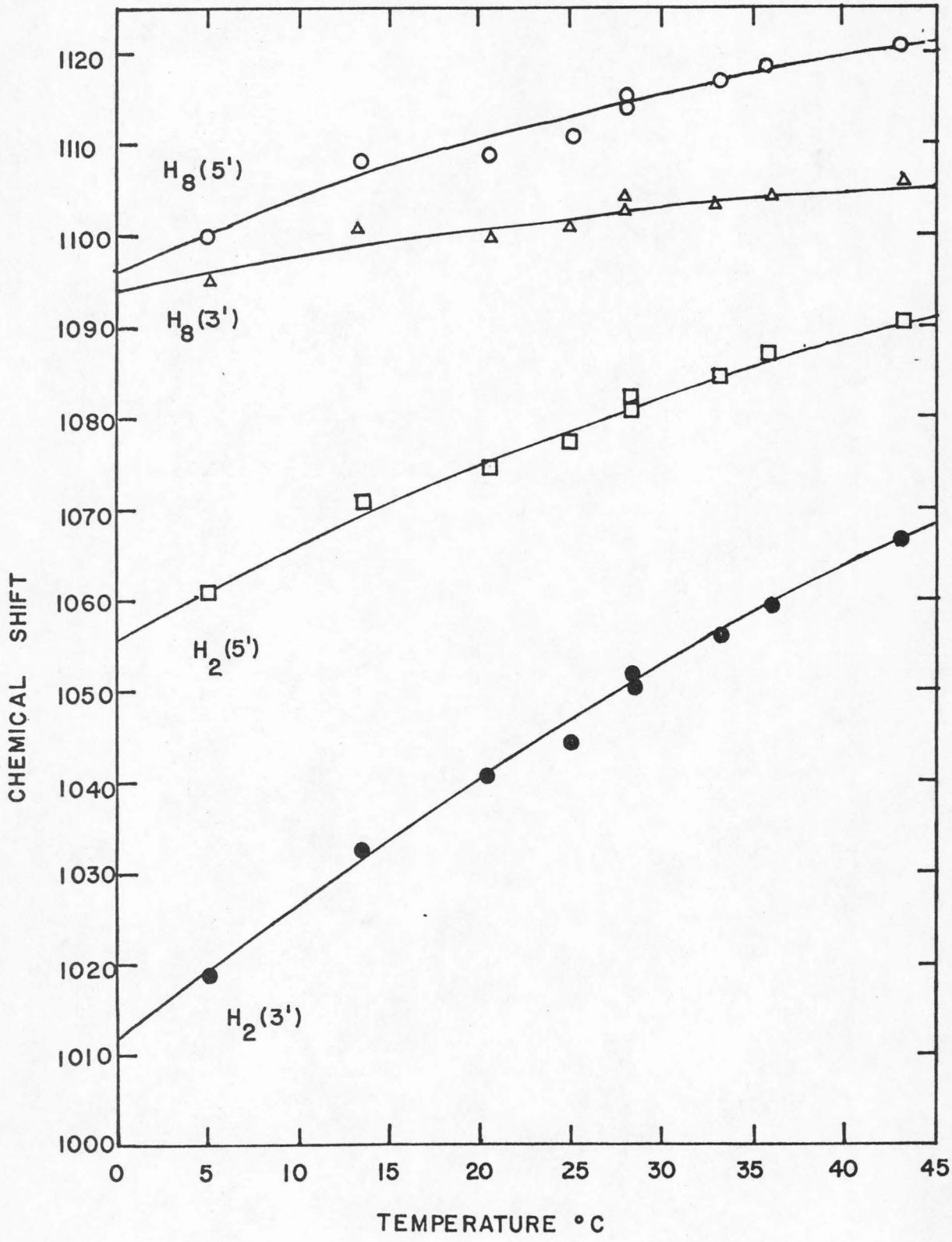

1120
1110
1100
1090
1080
1070
1060
1050
1040
1030
1020
1010
1000
CHEMICAL SHIFT
H8(5')
H8(3')
H2(5')
H2(3')
0
5
10
15
20
25
30
35
40
45
TEMPERATURE °C

FIGURE 13

The temperature dependence of the chemical shift for the H_2 resonances of ApApA (0.034 M in adenosine).

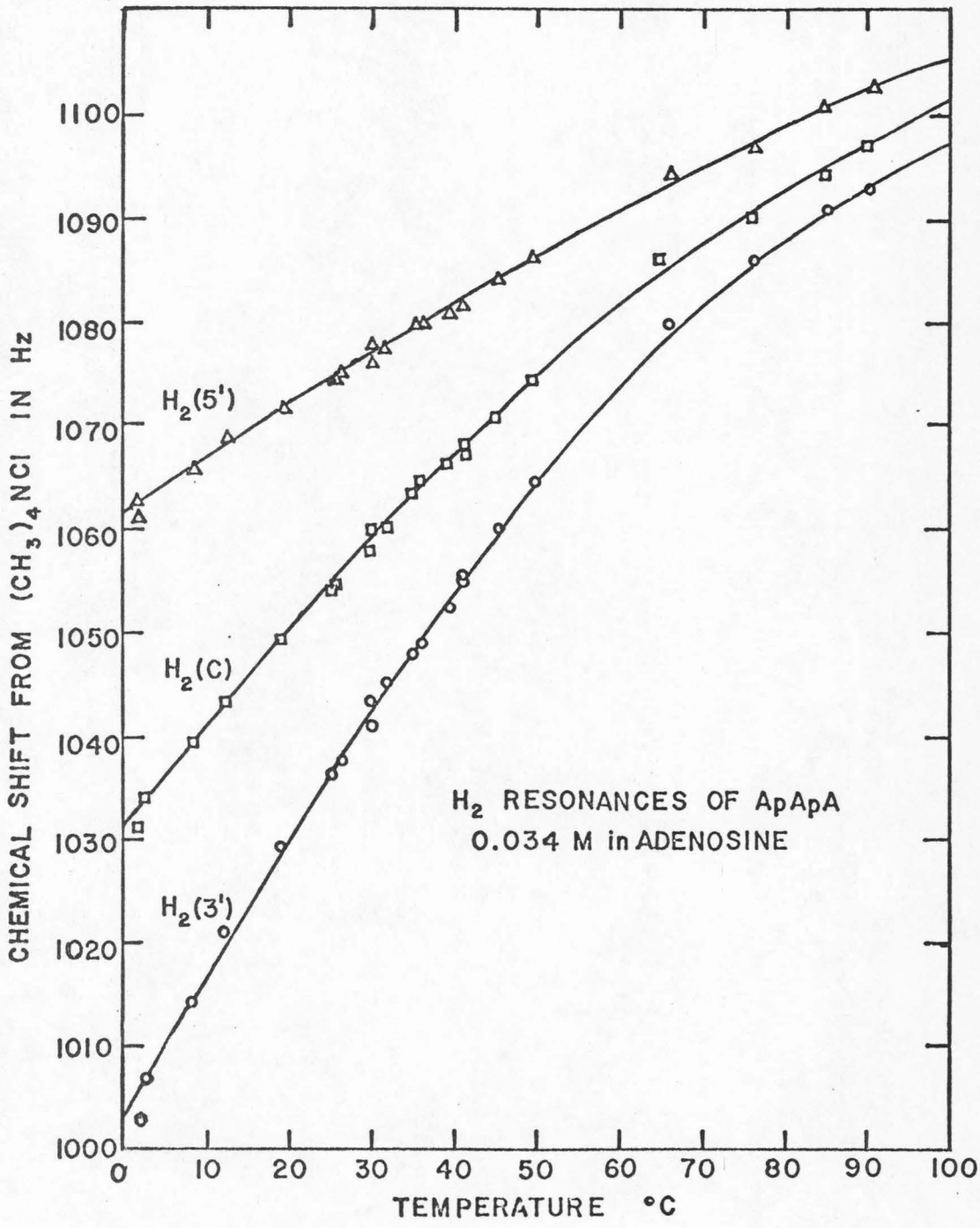
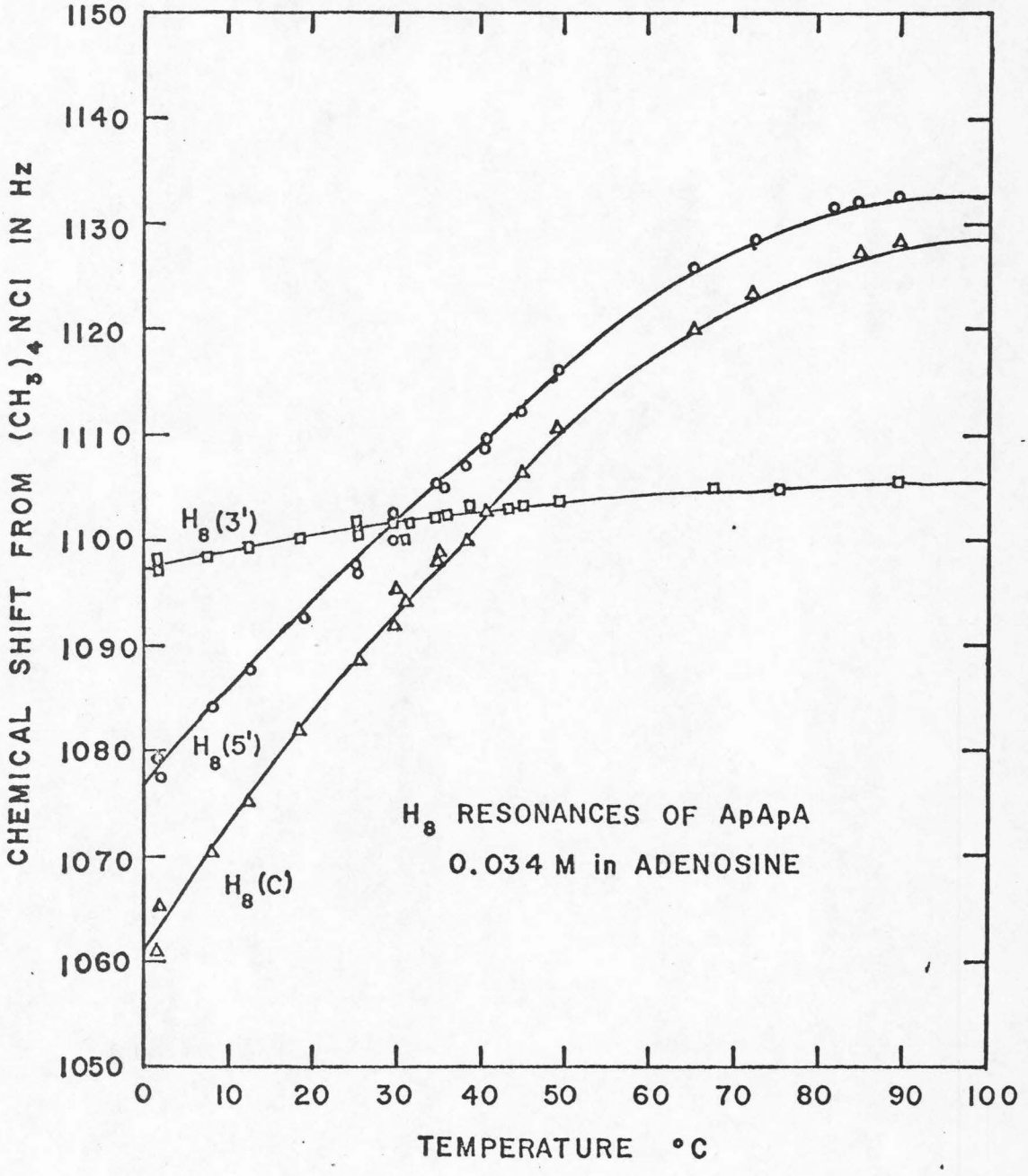

CHEMICAL SHIFT FROM $(CH_3)_4NCl$ IN Hz
1100
1090
1080
1070
1060
1050
1040
1030
1020
1010
1000
0
10
20
30
40
50
60
70
80
90
100
TEMPERATURE °C
$H_2(5')$
$H_2(C)$
$H_2(3')$
H_2 RESONANCES OF ApApA
0.034 M in ADENOSINE

FIGURE 14

The temperature dependence of the chemical shifts for the H_8 resonances of ApApA (0.034 M in adenosine).


1150
1140
1130
1120
1110
1100
1090
1080
1070
1060
1050
CHEMICAL SHIFT FROM $(CH_3)_4NCl$ IN Hz
0
10
20
30
40
50
60
70
80
90
100
TEMPERATURE °C
$H_8(3')$
$H_8(5')$
$H_8(C)$
H_8 RESONANCES OF ApApA
0.034 M in ADENOSINE

At a given temperature there is a distribution of conformations of the adenine bases, but on the average, ApA and ApApA are in a stacked conformation with the three adenine bases in an anti conformation. This stacked configuration leads to differences in the magnetic environments of the base protons of the three differently esterified nucleotides which is reflected in the pmr spectrum by the nonequivalence of the resonances of these protons. The average conformation changes with temperature leading to the marked and nonequivalent temperature dependence of the H_8 and H_2 resonances. The temperature dependence of the various resonances results from enhanced intermolecular and intramolecular stacking and conformational changes of the adenine bases about the $C_{1'}$-N_9 glycosidic bond with decreasing temperature. The details of this behavior are similar to those reported in a previous article on dinucleotides and will not be discussed here.[26]

3.3.2. Complex formation in ApA and ApApA plus poly U systems.

The addition of oligo-A-nucleotides to a solution of poly U has a profound effect on the pmr spectrum of both species. The results of a temperature study of the ApA and poly U system for different stoichiometric ratios of adenine bases to uracil bases are summarized in Figures 15 to 17. At a stoichiometry of one adenine base to four uracil bases, the added ApA was found to have little effect on the poly U above about 30°C. The chemical shifts as well as the linewidths of both the ApA and poly U resonances remained unchanged. At 26°C (Figure 15b), the ApA resonances become noticeably broader and

FIGURE 15

220 MHz pmr spectrum of a solution of poly U (0.078 M in uridine) and ApA (0.019 M in adenosine) at various temperatures. Base ratio A:U = 1:4.

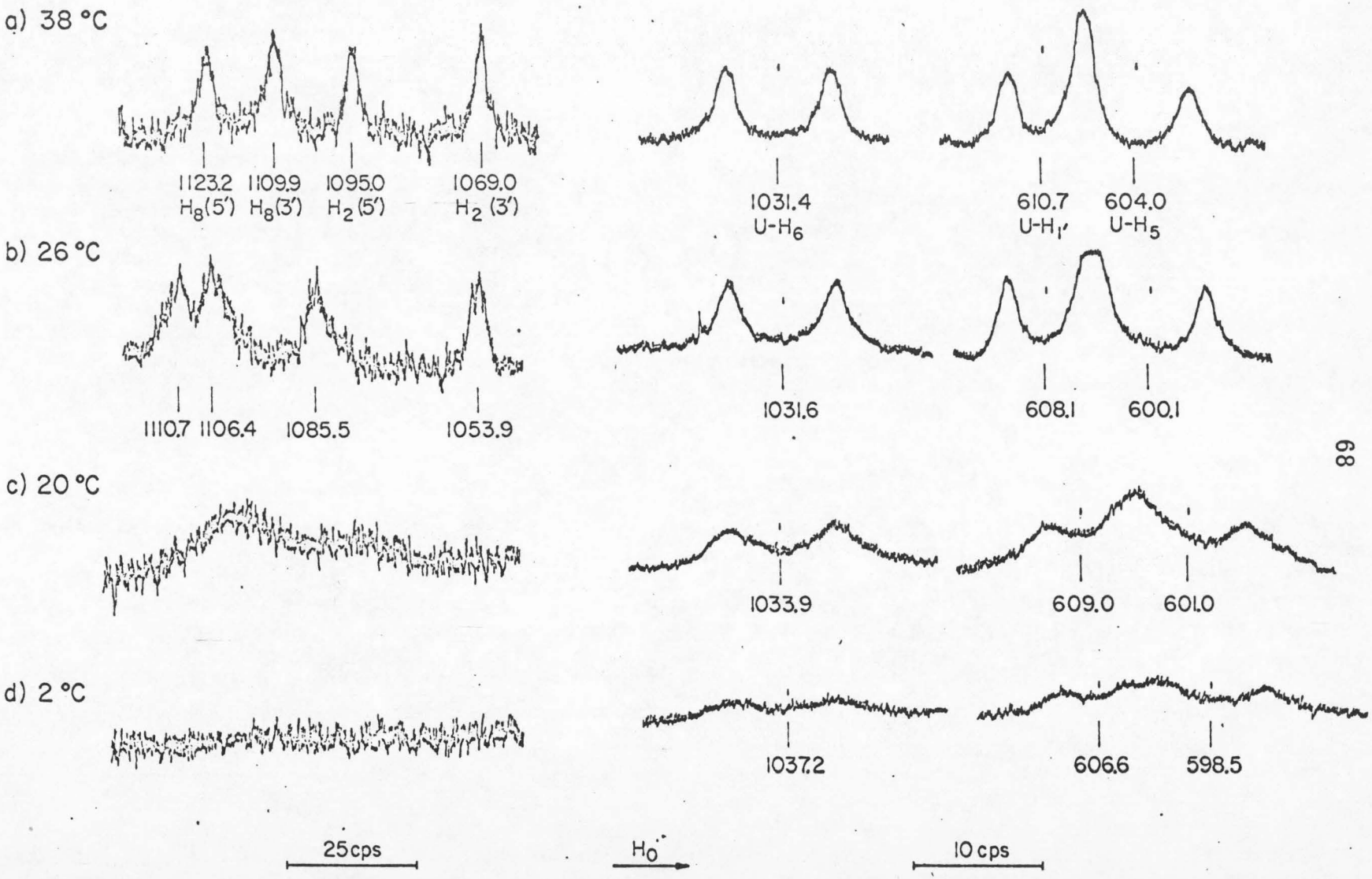

a) 38 °C
1123.2 H8(5')
1109.9 H8(3')
1095.0 H2(5')
1069.0 H2 (3')
1031.4 U-H6
610.7 U-H1'
604.0 U-H5
b) 26 °C
1110.7
1106.4
1085.5
1053.9
1031.6
608.1
600.1
c) 20 °C
1033.9
609.0
601.0
d) 2 °C
1037.2
606.6
598.5
25cps
H0
10 cps

FIGURE 16

220 MHz pmr spectrum of a solution of poly U (0.078 M in uridine) and ApA (0.039 M in adenosine) at various temperatures. Base ratio A:U = 1:2.

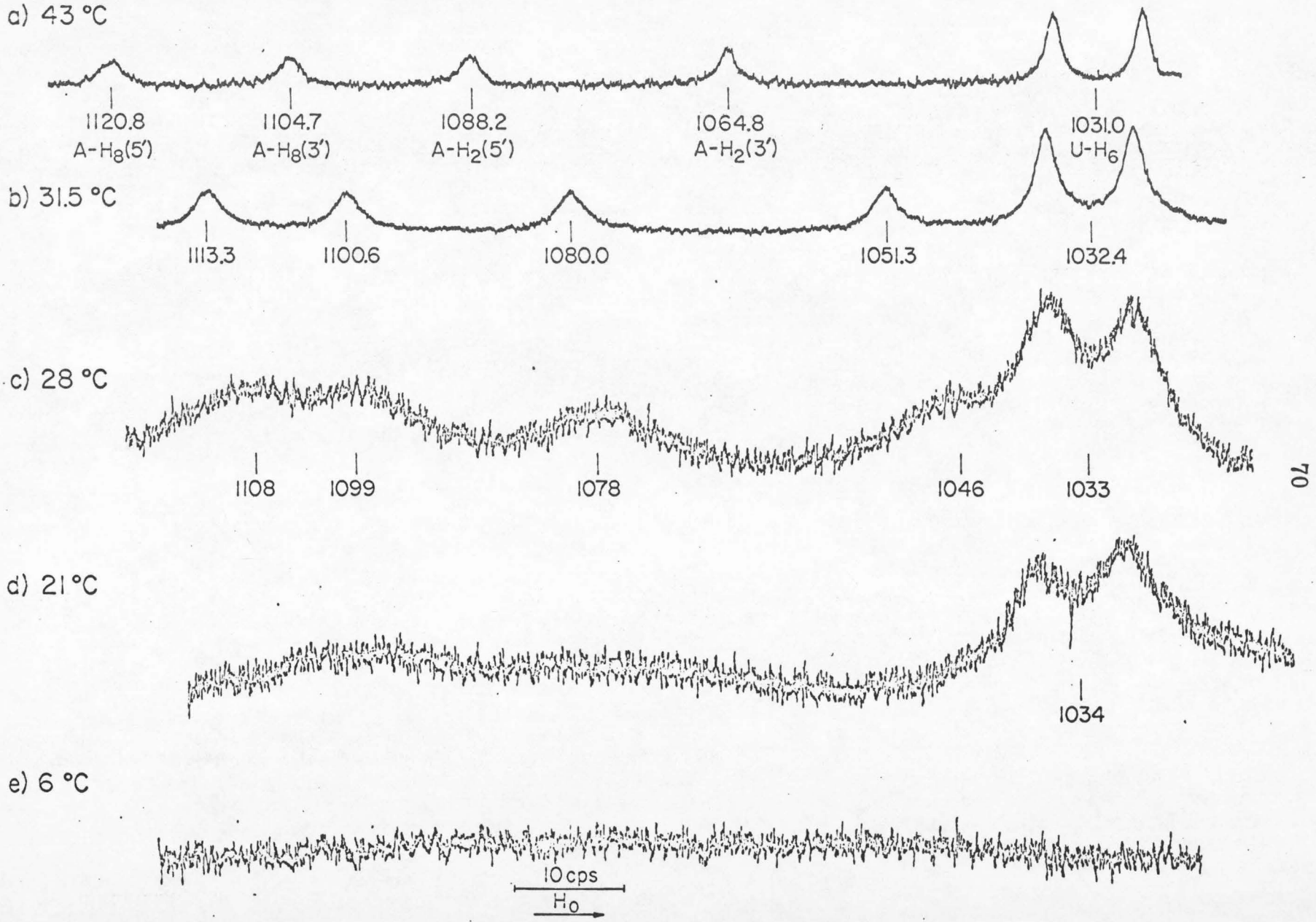
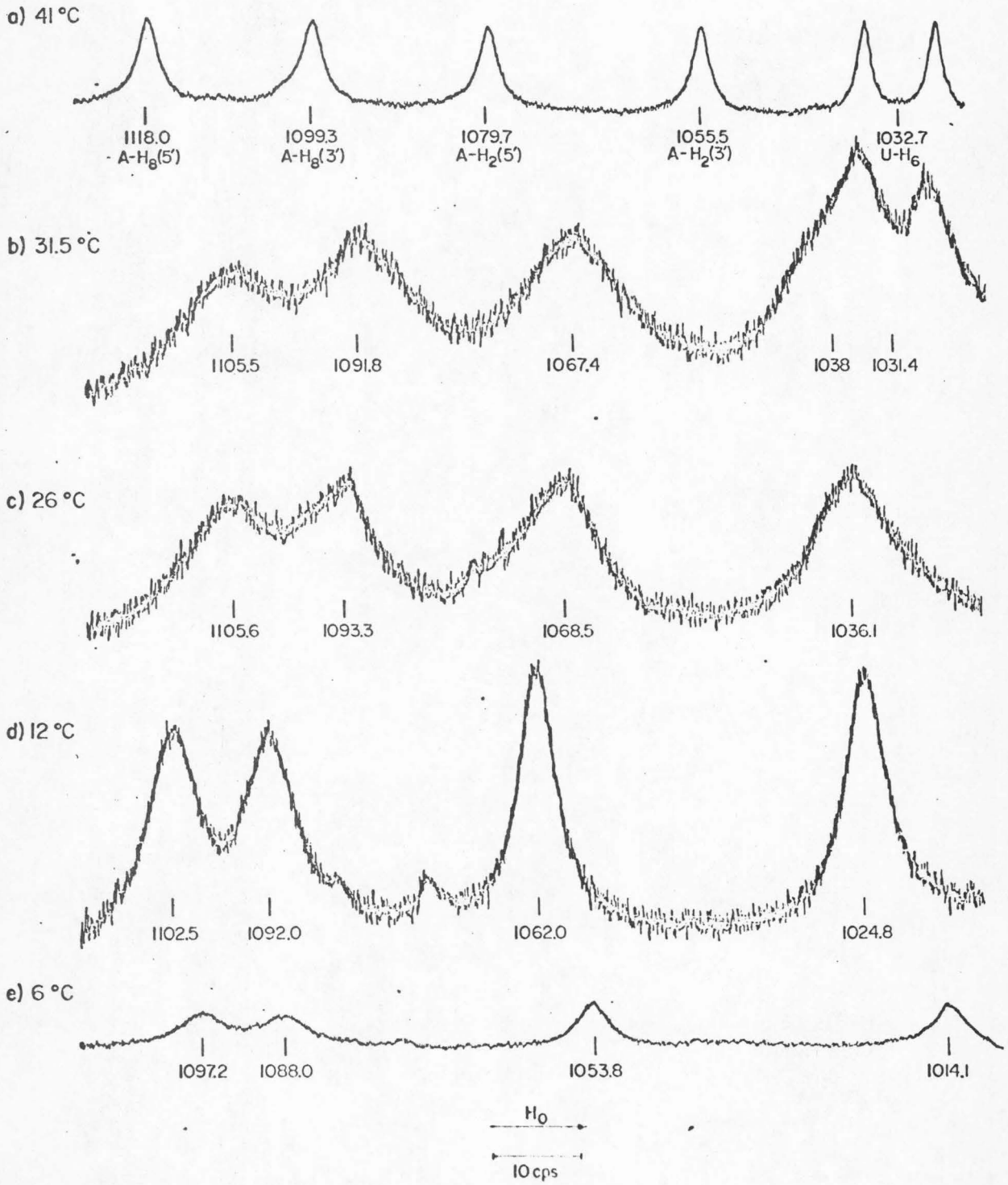

a) 43 °C
1120.8
A-H8(5')
1104.7
A-H8(3')
1088.2
A-H2(5')
1064.8
A-H2(3')
1031.0
U-H6
b) 31.5 °C
1113.3
1100.6
1080.0
1051.3
1032.4
c) 28 °C
1108
1099
1078
1046
1033
d) 21 °C
1034
e) 6 °C
10 cps
Ho

FIGURE 17

220 MHz pmr spectrum of a solution of poly U (0.078 M in uridine) and ApA (0.078 M in adenosine) at various temperatures. Base ratio A:U = 1:1.


a) 41 °C
1118.0
A-H8(5')
1099.3
A-H8(3')
1079.7
A-H2(5')
1055.5
A-H2(3')
1032.7
U-H6
b) 31.5 °C
1105.5
1091.8
1067.4
1038
1031.4
c) 26 °C
1105.6
1093.3
1068.5
1036.1
d) 12 °C
1102.5
1092.0
1062.0
1024.8
e) 6 °C
1097.2
1088.0
1053.8
1014.1
H0
10 cps

they are also shifted significantly upfield. As the temperature is lowered, further broadening of the ApA resonances is noted, as well as broadening of the poly U resonance. Below 20°C (Figure 15 c, d), the ApA resonances are broadened beyond detection; the poly U resonances are broad but can still be monitored. Integration of the uracil resonances indicated that the intensity has been reduced by a factor of two from that expected in the absence of complex formation.

Similar effects are observed for a solution with an adenine to uracil base ratio of one to two, except that both the uracil and adenine resonances begin to exhibit broadening at a slightly higher temperature and that both the ApA and uracil resonances are broadened beyond detection at low temperatures (Figure 16).

When the ApA concentration is increased to a base ratio of one to one, the onset of the broadening of the resonances occurs at a still higher temperature. At 41°C (Figure 17a), the resonances are still sharp and are near their normal spectral positions. As the temperature is lowered to 31.5°C (Figure 17b), both sets of resonances can been seen to become broader and the ApA resonances are again shifted significantly upfield. At 26°C (Figure 17c), the H_6 uracil resonances disappear completely. At this point, the ApA resonances begin to narrow and shift towards their normal spectral positions in the absence of poly U. At 6°C (Figure 17e), for example, the ApA resonances are almost as narrow and have nearly identical chemical shifts as free ApA under the same conditions. Integration of the ApA resonances indicates that only one-half of the original ApA is being monitored.

Below room temperature, the spectral behavior of the ApApA

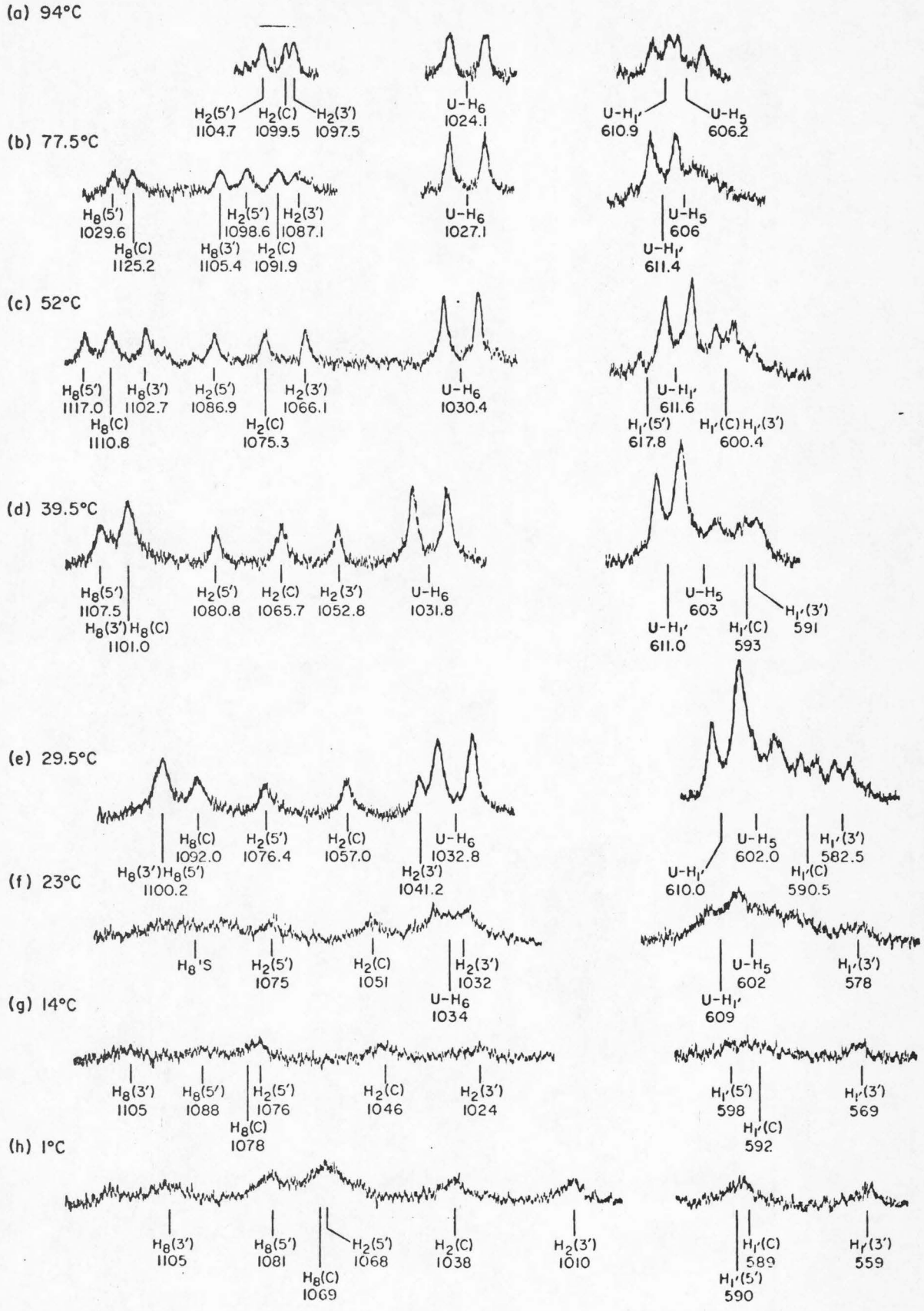
and poly U system is similar to that observed for ApA plus poly U, but above this temperature the two systems are different. The temperature dependence of the spectrum of a solution containing ApApA (0.034 M in adenosine) and poly U (0.034 M in uridine) (base ratio A:U = 1:1) is shown in Figure 18. At 94°C (Figure 18a), the resonances of ApApA and poly U are at their normal spectral positions and are of normal linewidth, as expected in the absence of complex formation. Below 90°C, the H_5 resonance of poly U markedly broadened and remains broad until ~50°C, where it begins to sharpen as the temperature is lowered to 30°C. The H_2 and H_8 resonances of ApApA exhibit the same linewidth behavior over this temperature range except to a lesser extent than that observed for the H_5 resonance of poly U. The H_6 resonance of poly U and the $H_{1'}$ resonances of both ApApA and poly U exhibit their normal linewidth within experimental error over this same temperature range (Figures 18 b, c, d, e). A dramatic change in the spectral behavior of all of the resonances occurs below room temperature similar to that which was observed in the A or ApA and poly U systems. In the present case, all the ApApA and poly U resonances are significantly broadened below ~29°C (Figure 18f). Below ~20°C (Figures 18 g, h), the H_6, H_5 and $H_{1'}$ resonances of poly U are broadened beyond detection while the H_2 and H_8 resonances of ApApA begin to sharpen with decreasing temperature. Integration of the H_2 and H_8 resonances of ApApA reveals that intensity of these resonances is one-half of that at high temperatures.

For a solution containing a different A to U ratio, such as a solution of ApApA (0.034 M in adenosine) and poly U (0.072 M in

FIGURE 18

220 MHz pmr spectrum of a solution of poly U (0.034 M in uridine) and ApApA (0.034 M in adenosine) at various temperatures. Base ratio A:U = 1:1.

POLY U (SODIUM SALT) + ApApA (SODIUM SALT); 0.034 M IN URIDINE, 0.034 M IN ADENOSINE



uridine) (base ratio A:U = 1:2.1), the resonances of ApApA and poly U exhibit the same temperature behavior as in the 1:1 solution, except at high temperatures the H_2 and H_8 resonances of ApApA are broadened to a greater extent and the H_5 resonance of poly U to a lesser extent than in the 1:1 case. Below ~25°C all the resonances, except for a small percentage of the poly U resonances, are broadened beyond detection (Figure 19).

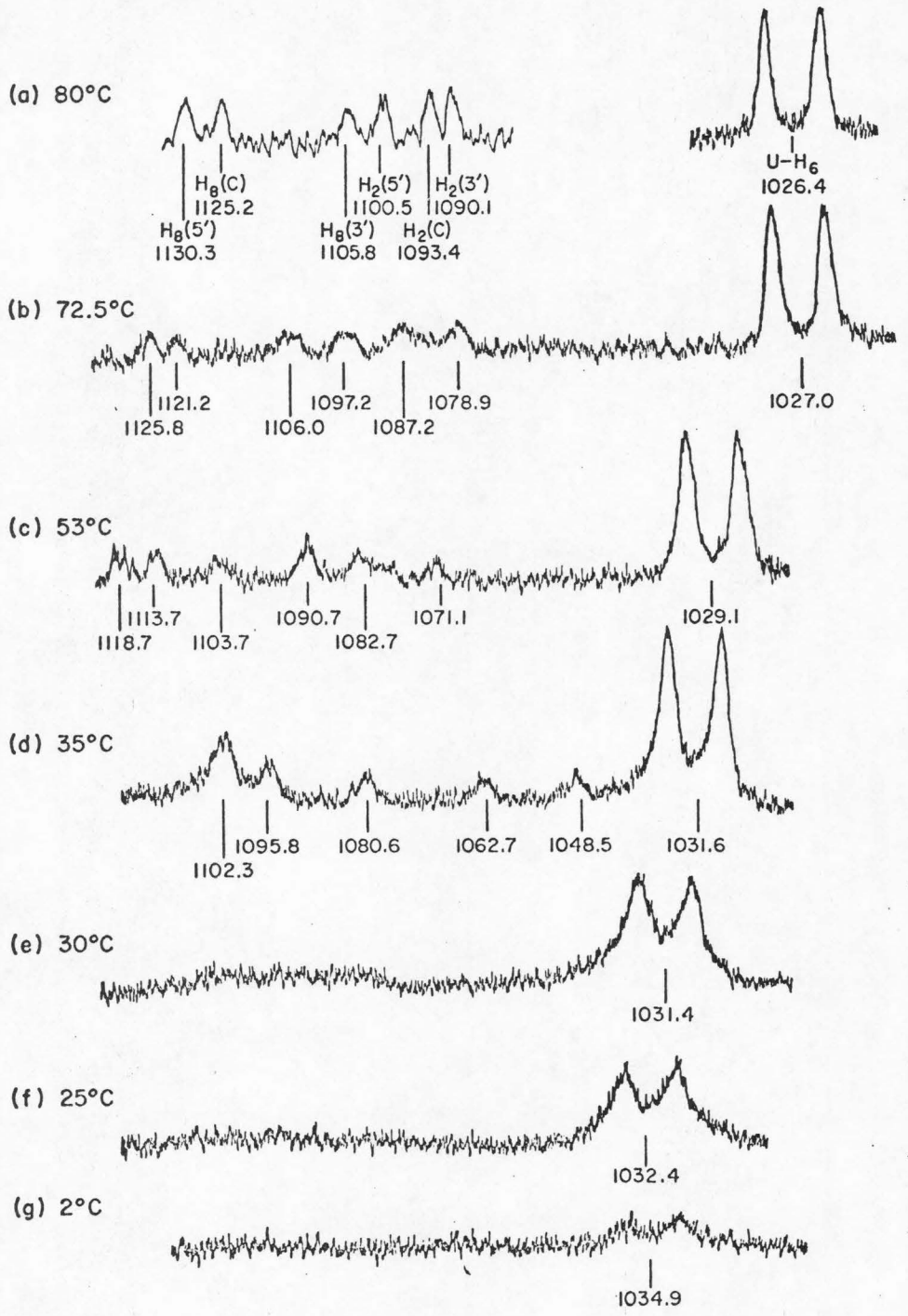
The temperature variation of the pmr spectrum of ApA or ApApA and poly U systems can only be interpreted as the variation in the rate of exchange of a given molecule between a free and complexed state with temperature. It is well known that spectral parameters of a pmr signal are sensitive to time-dependent processes with rates on the order of the pmr time scale of observation. This "critical rate" can be thought of as the inverse of the characteristic time in which the net magnetization in the transverse plane dephases after exchange from one site to the other. The dephasing is governed by two factors: (1) differences in Larmor frequencies of a given proton (i.e., chemical shift) in the two sites, and (2) differences in the transverse relaxation times of that proton in the two sites. Usually, the "critical rate" is governed by the chemical shift difference in cycles per second between the two sites because the inverses of the transverse relaxation times are of comparable magnitude in the two sites.

In the presence of a triple-stranded complex, however, such as that observed for A and poly U, the "critical rate" is determined by the inverse of the transverse relaxation time of the complex. In these systems, the molecules in the free state have fairly long T_2's

FIGURE 19

220 MHz pmr spectrum of a solution of poly U (0. 072 M in uridine) and ApApA (0. 034 M in adenosine) at various temperatures. Base ratio A:U = 1:2.

POLY U (SODIUM SALT) + ApApA (LITHIUM SALT); 0.072 M IN URIDINE, 0.034 M IN ADENOSINE



while the T_2 of a proton in the complex is extremely short. The exchange rate of a molecule between a free and bound site in the presence of such a complex is fast on the pmr time scale only when it is faster than the T_2 of the complex. If it is not, the net magnetization in the transverse plane would relax before the molecule is exchanged and only molecules in the free state would contribute to the high resolution spectrum.

At the onset of rigid complex formation, the kinetics of chemical exchange of an adenosine or a uridine residue between a bound and unbound state is expected to be fast on the pmr time scale. Under these conditions, the spectrum observed would be a weighted average of the molecules in the free and bound states. Both the bound and free molecules would contribute to the integrated intensity of the spectral peaks and the observed linewidths should be given by

$$\Delta\nu_{obs} = \Delta\nu_{free} + (\Delta\nu_{bound} - \Delta\nu_{free})\, f_{bound} ,$$

where $\Delta\nu_{obs}$ is the observed weighted averaged linewidth of a given spectral peak; $\Delta\nu_{free}$, the linewidth in the free state; $\Delta\nu_{bound}$, the linewidth in the complex; and f_{bound} corresponds to the fraction of adenosine or uridine residues in the bound state. Since the reciprocal of the transverse relaxation time of a proton in the rigid complex is expected to be greater than the chemical shifts changes of a proton upon complex formation, the observed chemical shifts in the limit of rapid exchange should also be a weighted average of the chemical shifts of the protons in the bound and unbound states, i. e.,

$$\delta_{obs} = \delta_{free} + (\delta_{bound} - \delta_{free}) f_{bound} ,$$

where δ_{obs} is the observed average chemical shift; δ_{free} is the chemical shift of a proton in an unbound molecule; and δ_{bound} is the chemical shift in the complex.

If the chemical exchange time between the bound and unbound sites is much slower than the transverse relaxation time of a proton in the rigid complex, the spectrum should correspond to that of the unbound molecules only. In this limit, the integration of the spectral intensities together with initial knowledge of the stoichiometry of the solution enables one to ascertain the stoichiometry of the complex unequivocally. This condition is satisfied in the poly U-ApA and poly U-ApApA systems at low temperatures. For example, below 20°C, for the case with A:U base ratio of 1:2, no detectable pmr signal was observed at low temperatures; and in the system containing one adenine base to one uracil base, the intensity of the adenine resonances corresponded to half of the intensity expected in the absence of complex formation. From these results, we unequivocally conclude that the stoichiometry of the complex involves one adenine base for every two uracil bases. The complex is therefore a triple-stranded complex similar to the adenosine-poly U complex previously reported,[61] with stacked oligo-A-nucleotides forming horizontal hydrogen bonds to two poly U strands. Presumably both Watson-Crick and Hoogsteen hydrogen bonding schemes are involved.

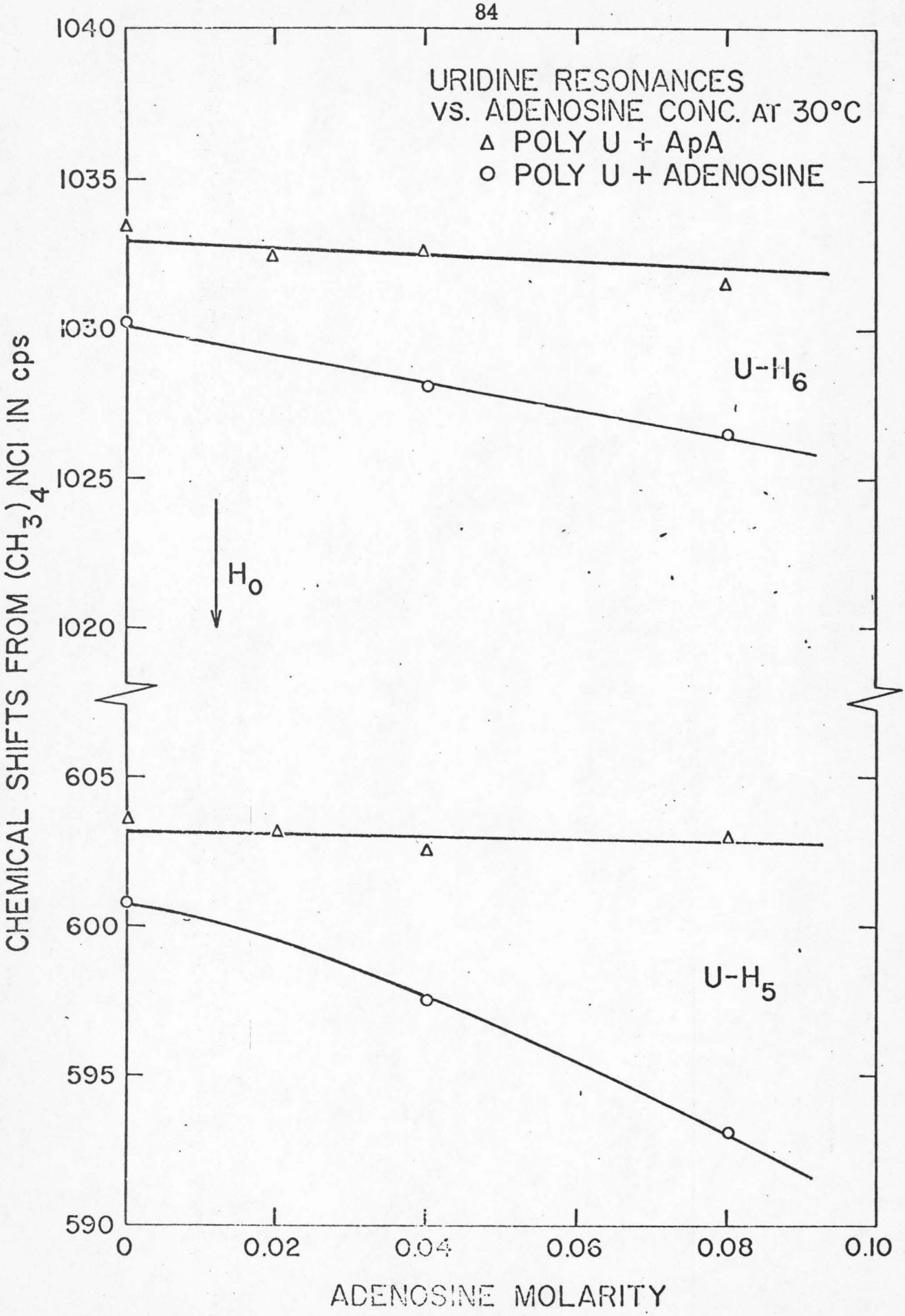
The poly U-ApA solution with an A:U base ratio of 1:4 was also carefully examined at low temperatures. Only half of the poly U in this

solution would be required to form the 1A:2U complex with ApA. The ApA adenine resonances were broadened beyond detection, as expected. However, the resonances due to the H_6, H_5 and $H_{1'}$ protons of the uncomplexed poly U residues were clearly discernible. These poly U resonances are quite broad, exhibiting linewidths which are even greater than those of the adenine resonances of the uncomplexed ApA in the A:U = 1:1 poly U-ApA system at low temperatures. These results would seem to indicate that there are no completely unbound poly U strands in solution. If there were, the resonances due to these free poly U's would be quite narrow. Thus all the poly U molecules are involved in complex formation, even though not all of the uracil residues are involved in adenine-uracil base-pairing at any one time. We suspect that these uncomplexed uridine residues are located in "looped out" regions of the complex and that the greater linewidths for the resonances from these residues are the consequence of a longer effective motional correlation time for the modulation of the magnetic dipolar interactions.

Below room temperature, the A, ApA or ApApA-poly U systems exhibit similar spectral behavior, while above this temperature, all three systems are different. It has been shown in previous work that adenosine intercalates between adjacent uracil bases of poly U at temperatures above ~26°C.[61] This led to upfield shifts of the H_5, H_1 and H_6 protons of the uracil bases and to some line-broadening of the H_2 and H_8 protons of adenosine due to magnetic dipole-dipole interactions of these protons with the ribose protons of poly U as was the case with purine and poly U.[79] Figure 20 clearly shows that

FIGURE 20

A comparison of the effects of adenosine and ApA on the H_5 and H_6 uracil resonances of poly U at various concentrations of the small molecule (expressed in monomeric units). Concentration of poly U: 0.078 M in uridine; temperature: ~30°C.


URIDINE RESONANCES
VS. ADENOSINE CONC. AT 30°C
Δ POLY U + ApA
○ POLY U + ADENOSINE
U-H6
U-H5
Ho
CHEMICAL SHIFTS FROM (CH3)4NCI IN cps
ADENOSINE MOLARITY
1040
1035
1030
1025
1020
605
600
595
590
0
0.02
0.04
0.06
0.08
0.10

the H_5 and H_6 uracil protons of poly U are not shifted upfield with increasing ApA concentration as they were with increasing adenosine concentration. Also no line broadening of the ApA resonances was noted above ~30°C. Therefore ApA does not interact with poly U by non-specific vertical base-stacking above ~30°C. This result is, however, expected since ApA is strongly self-stacked [26, 65-68] and it is sterically impossible for it to intercalate between two adjacent uracil bases.

Because of the lack of induced shifts of the H_5 and H_6 resonances of poly U in the presence of ApApA, intercalation does not occur. Complex formation, however, does occur because of the profound broadening of the H_5 resonance of poly U. With the breakdown of the rigid triple-stranded complex, the "critical rate" for an exchange process in this system is determined by the difference in chemical shift for a proton in the free and bound states. Under these conditions, only the resonances which have appreciably different chemical shifts in the two states should exhibit significant broadening.

In a Watson-Crick base pair of A and U (Figure 21), the only resonance of a non-bonding proton which is expected to be significantly shifted by hydrogen bonding is the H_5 resonance of the uracil base. Thus if a Watson-Crick base pair does form, over the temperature range where the chemical exchange rate varies from fast to slow exchange, the only resonance expected to exhibit broadening is the H_5 resonance of poly U. In a Hoogsteen hydrogen bonded complex (Figure 22), however, none of the resonances of either base are expected to have significantly different chemical shifts in the free and bound states

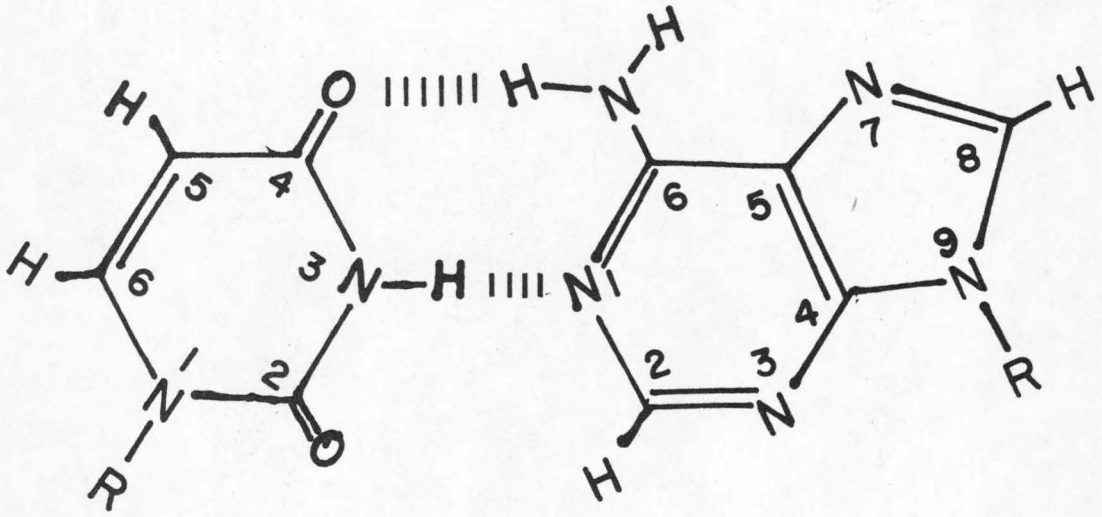


FIGURE 21

The Watson-Crick bonding scheme of a uracil-adenine base pair.

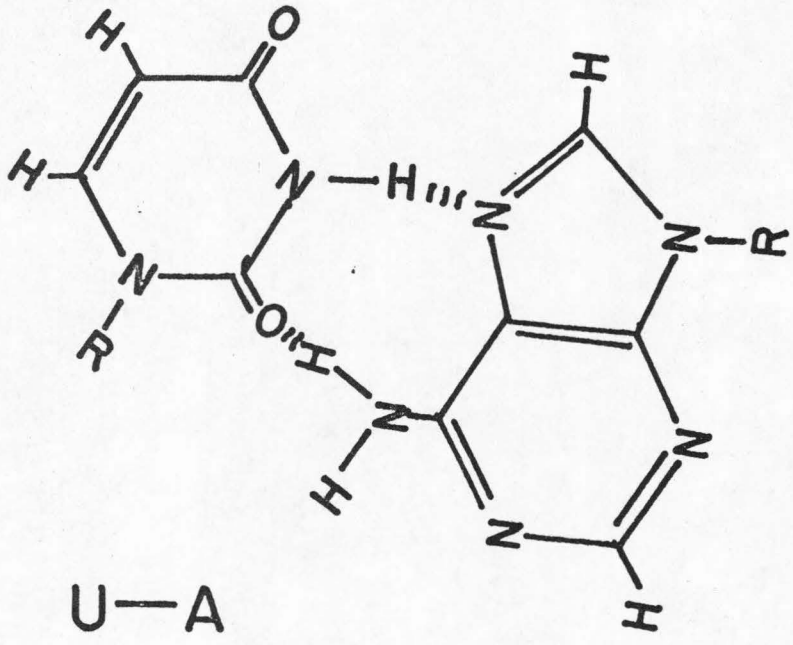


FIGURE 22

The Hoogsteen bonding scheme of a uracil-adenine base pair.

and thus none of the resonances are expected to be broadened. In Figure 23, the variation of the linewidth of a resonance as a function of the fraction bound is shown for various rate constants and appropriate chemical shift differences. From the correlation of this theoretical data and the expected chemical shift differences of the protons in the two schemes with that observed in Figure 18, it is concluded that ApApA forms Watson-Crick base pairs with poly U above room temperature.

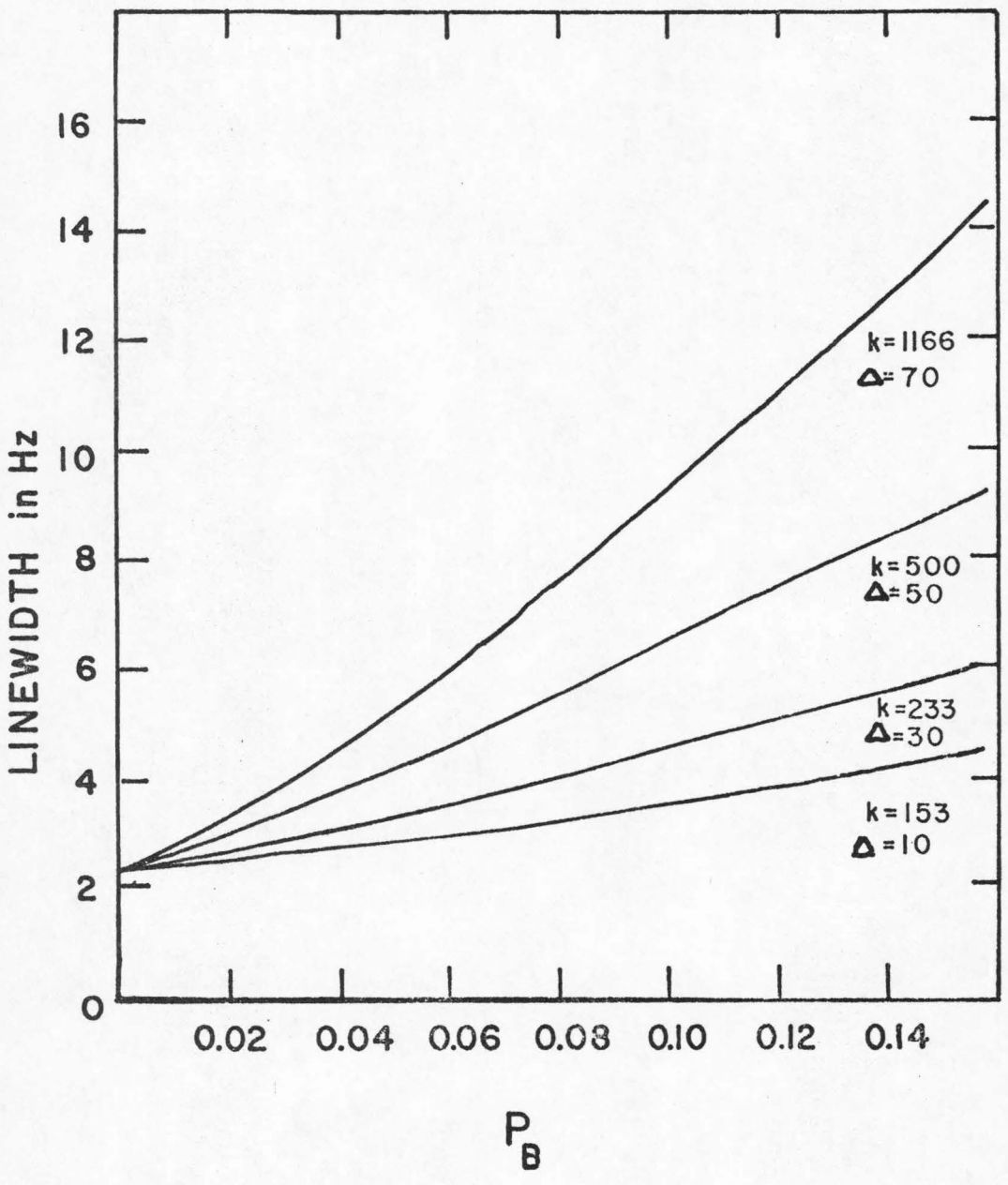
Over the temperature range of 90°C to 50°C, the exchange rate is fast on the pmr time scale and the H_5 resonance continues to broaden with decreasing temperature. Below 50°C, the exchange rate becomes slow and the H_5 resonance begins to narrow. In this temperature range, one would expect to observe both the free and complexed protons but since only a small percentage is bound, only the free state is observed. The slight line broadening of the H_2 and H_8 resonances of ApApA implies that they are near the site of interaction and helps substantiate the formation of some sort of hydrogen-bonded complex.

When the stoichiometry of the system is changed from 1:1 to 1:2 (A:U), the fraction of complexed A increases and U decreases. Thus we expect the line broadening of the H_5 resonance to decrease and the line broadening of the H_2 and H_8 resonances of ApApA to increase. This is concurrent with the observed data.

The spectral evidence clearly shows that ApApA forms Watson-Crick base pairs with poly U above room temperature. Complex formation does not occur along the entire length of the poly U strand but only short segments of the poly U are complexed while the remainder

FIGURE 23

The variation of the linewidth of a resonance with the fraction bound for various rate constants and the appropriate chemical shift differences.


LINEWIDTH in Hz
16
14
12
10
8
6
4
2
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
P_B
k=1166
Δ=70
k=500
Δ=50
k=233
Δ=30
k=153
Δ=10

of the strand is still random in nature. This type of structure leads to sharp lines for the poly U resonances even though parts are complexed.

3.3.3. Conformational analysis of the complexed oligo-A-nucleotide.

Although the rigid complex does not give rise to a high resolution pmr spectrum, the chemical shifts of protons in the complex can be ascertained from examination of the average chemical shifts under conditions of rapid to intermediate chemical exchange. Evidence of the changes of the ApA molecules upon complex formation is presented in Figures 24 and 25, where we have plotted the observed chemical shifts of the ApA resonances relative to the corresponding shifts of an equal concentration of ApA in the absence of poly U at the same temperature for the 1A:2U system. As expected, poly U "induced" shifts are observed for all the adenine resonances upon complex formation. It is noteworthy, however, that the poly U "induced" shifts are different for the four adenine protons. Specifically, the "induced" shifts of $H_2(3')$ are greater than those for $H_2(5')$, and those for $H_8(5')$ are greater than those for $H_8(3')$. Since these data are referenced to ApA in the absence of poly U at the same temperature, the shifts should only reflect intermolecular and intramolecular stacking effects on the magnetic environments of the ApA molecules as they are incorporated into the complex. The poly U "induced" intermolecular stacking of ApA molecules upon formation of the complex should result in upfield shifts of the ApA adenine resonances. Examination of CPK molecular

FIGURE 24

The effect of complex formation on the chemical shifts (at 220 MHz) of the H_2 resonances of ApA in the presence of poly U relative to an equal concentration of ApA at the same temperature in the absence of poly U. Base ratio A:U = 1:2.

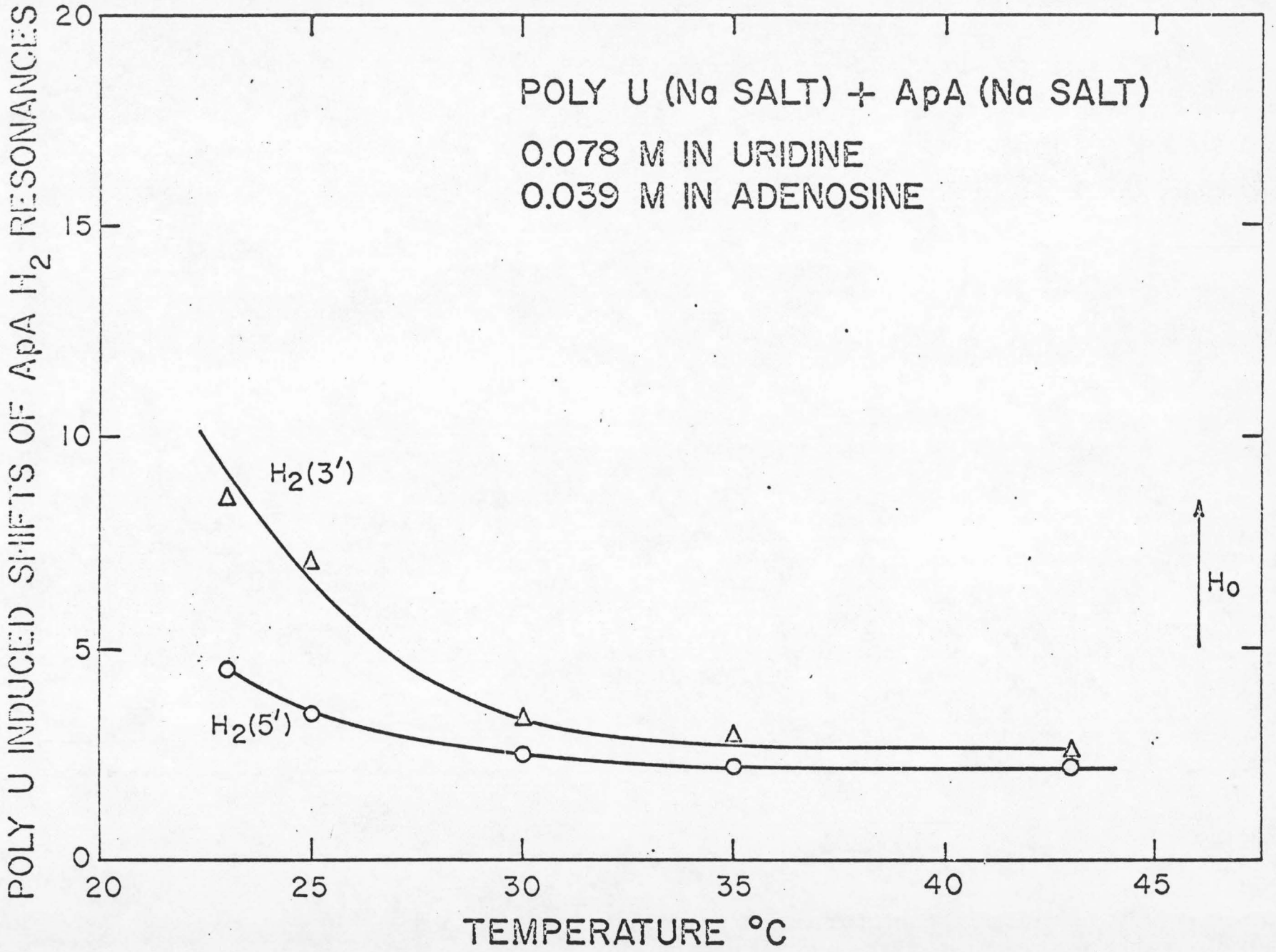
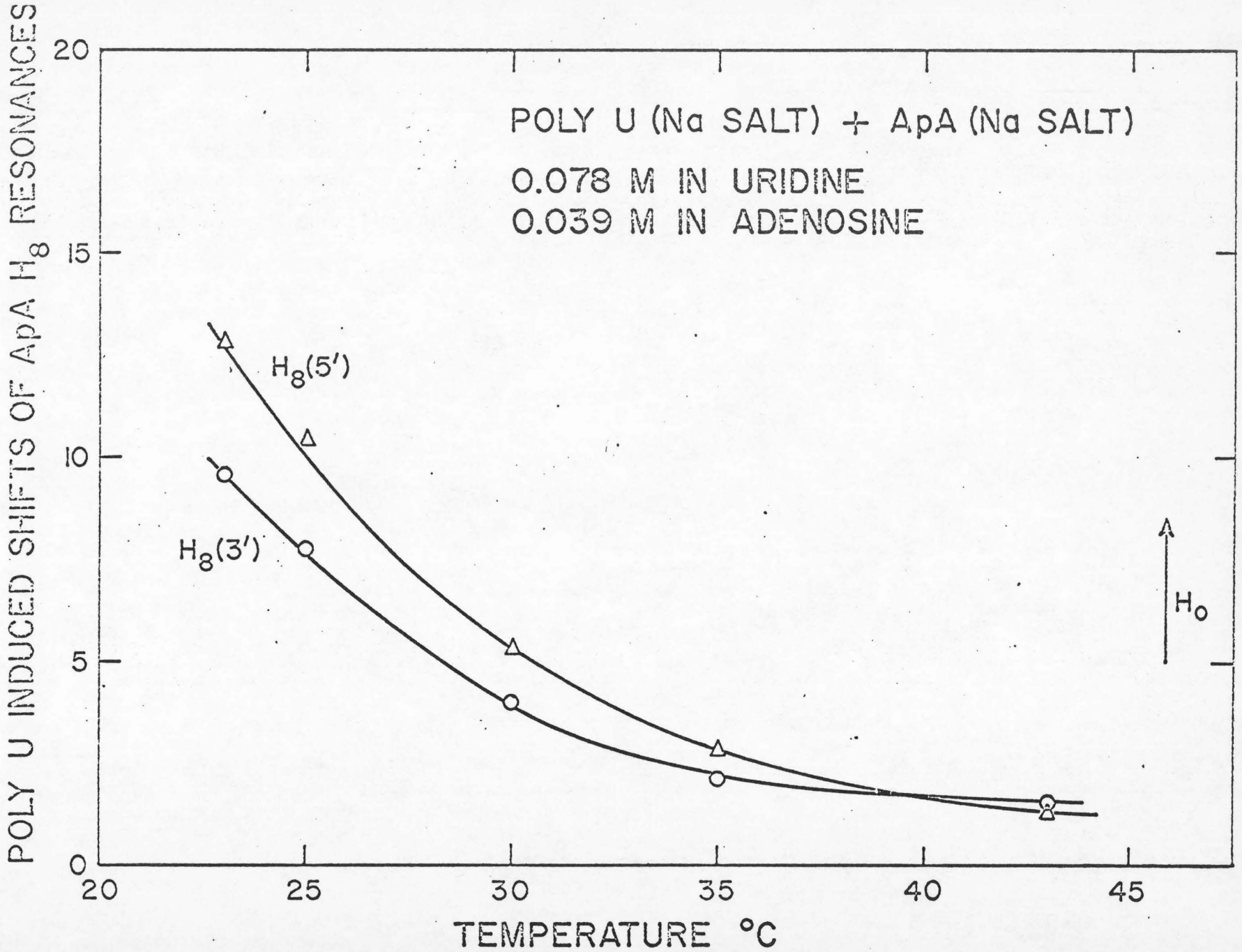

POLY U (Na SALT) + ApA (Na SALT)
0.078 M IN URIDINE
0.039 M IN ADENOSINE
POLY U INDUCED SHIFTS OF ApA H_2 RESONANCES
$H_2(3')$
$H_2(5')$
H_o
TEMPERATURE °C
0
5
10
15
20
20
25
30
35
40
45

FIGURE 25

The effect of complex formation on the chemical shifts (at 220 MHz) of the H_8 resonances of ApA in the presence of poly U relative to an equal concentration of ApA at the same temperature in the absence of poly U. Base ratio A:U = 1:2.


POLY U (Na SALT) + ApA (Na SALT)
0.078 M IN URIDINE
0.039 M IN ADENOSINE
POLY U INDUCED SHIFTS OF ApA H_8 RESONANCES
$H_8(5')$
$H_8(3')$
H_o
TEMPERATURE °C
0
5
10
15
20
20
25
30
35
40
45

models, however, reveals that if only the effects of intermolecular stacking were considered, one would expect the $H_2(5')$ and $H_8(3')$ resonances to be shifted upfield more than the $H_2(3')$ and $H_8(5')$ resonances, which is contrary to the observations depicted in Figures 24 and 25. On the other hand, changes in the extent and nature of the intramolecular stacking between the two adenine bases in an ApA molecule can also occur upon complex formation. It has been argued that in the free intramolecularly stacked ApA molecule, there is, at a given temperature, a distribution of distances between the planes of the two adenine bases. Since we expect the adenine bases to be essentially in van der Waals contact in the complex, incorporation of an ApA molecule in the complex will enhance the intramolecular base-stacking interaction and hence contribute to the poly U "induced" upfield shifts. For those ApA molecules with both bases in the anti conformation relative to their respective ribose moieties, the induced shifts should be larger for the $H_2(3')$ and $H_8(5')$ resonances than for the $H_2(5')$ and $H_8(3')$ resonances, as observed. However, if the adenine bases in some of the ApA molecules are in a syn conformation, the enhanced intramolecular stacking will preferentially shift the $H_2(5')$ and $H_8(3')$ resonances upfield if pairing of the adenine bases to the two poly U strands does not lead to conformational changes of the adenine bases about their respective $C_{1'}$-N_9 glycosidic bonds. Examination of CPK molecular models of the triple-stranded structure of poly U-ApA, however, suggests that ApA could pair to the poly U strands only if the conformation of both bases is anti. Since conformational changes about the glycosidic bond must then accompany complex formation for those ApA molecules with

one or both bases in the syn conformation, the poly U "induced" shifts would also reflect the effect of these conformational changes on the chemical shifts of the adenine resonances. For example, if the 5′-base were originally in the syn conformation, rotation of this base to an anti conformation would reduce the ring current magnetic anisotropy effect of the opposite adenine base on the $H_2(5')$ proton and increase the ring current effect for the $H_8(5')$ proton. This is true irrespective of whether the 3′-base is anti or syn. Similarly, flipping of the 3′-base from syn to anti will bring the 6-membered ring of the adenine base into the shielding region of the opposite 5′-base, thus shielding $H_2(3')$ and deshielding $H_8(3')$.[81] These considerations, therefore, indicate that for those ApA molecules with adenine bases oriented in the syn conformation, we also expect the poly U "induced" shifts to be greater for the $H_2(3')$ and $H_8(5')$ resonances than for the $H_2(5')$ and $H_8(3')$ resonances, because of the restrictions which base-pairing imposes on the conformation of the adenine bases in an incorporated ApA molecule. Clearly, the weighted averaged chemical shifts of the adenine resonances reflect both intermolecular and intramolecular stacking effects, although our observations together with the above considerations indicate that the effect of base-pairing on the intramolecular stacking interaction predominates at the onset of complex formation. This is not unexpected. Presumably, as a greater fraction of ApA molecules becomes incorporated, the intermolecular effects would prevail and at some low temperature, the curves of the poly U "induced" shifts for a given pair of protons (H_2 or H_8) would intersect, since we expect the magnetic environments of the

analogous protons of the two adenine bases of ApA to be rather similar in the rigid complex. Unfortunately, because of the large differences in the spectral linewidths of the poly U and ApA resonances in the free and complexed states and the profound effect of the dynamics of complex formation on the appearance of the spectrum, it was not possible to monitor the spectral changes much beyond the onset of complex formation in the 1A:2U system.

The results for the poly U-ApA system with a stoichiometry of one uridine base to one adenine base are presented in Figures 26 and 27. Here, we have plotted the chemical shifts of the ApA resonances as a function of temperature relative to internal $(CH_3)_4N^+$. The variations of the chemical shifts of these protons with temperature therefore arise from the aforementioned intermolecular and intramolecular base-stacking effects for the incorporated ApA molecules as well as the effect of temperature on the intermolecular and intramolecular base-stacking of uncomplexed ApA molecules. The abrupt change in the curvature of these chemical shift curves in the temperature range 20° to 30° C clearly shows the transition from fast to slow chemical exchange. As in the 1A:2U system, the pronounced upfield shifts of the adenine resonances above 30°C reflect the enhanced vertical base-stacking of the ApA molecules upon incorporation into the rigid complex. In this temperature range, the kinetics of exchange between bound and unbound ApA molecules or uridine residues is either rapid or intermediate as is evident from the intensities and the linewidths of the spectral peaks. Below 25°C, only the resonances from the free ApA molecules are observed and they have nearly the same chemical

FIGURE 26

The effect of complex formation on the chemical shifts (at 220 MHz) of the H_2 resonances of ApA as a function of temperature in the presence of poly U for a poly U-ApA solution with base ratio A:U = 1:1. Chemical shifts are given in Hz downfield from internal $(CH_3)_4N^+$.

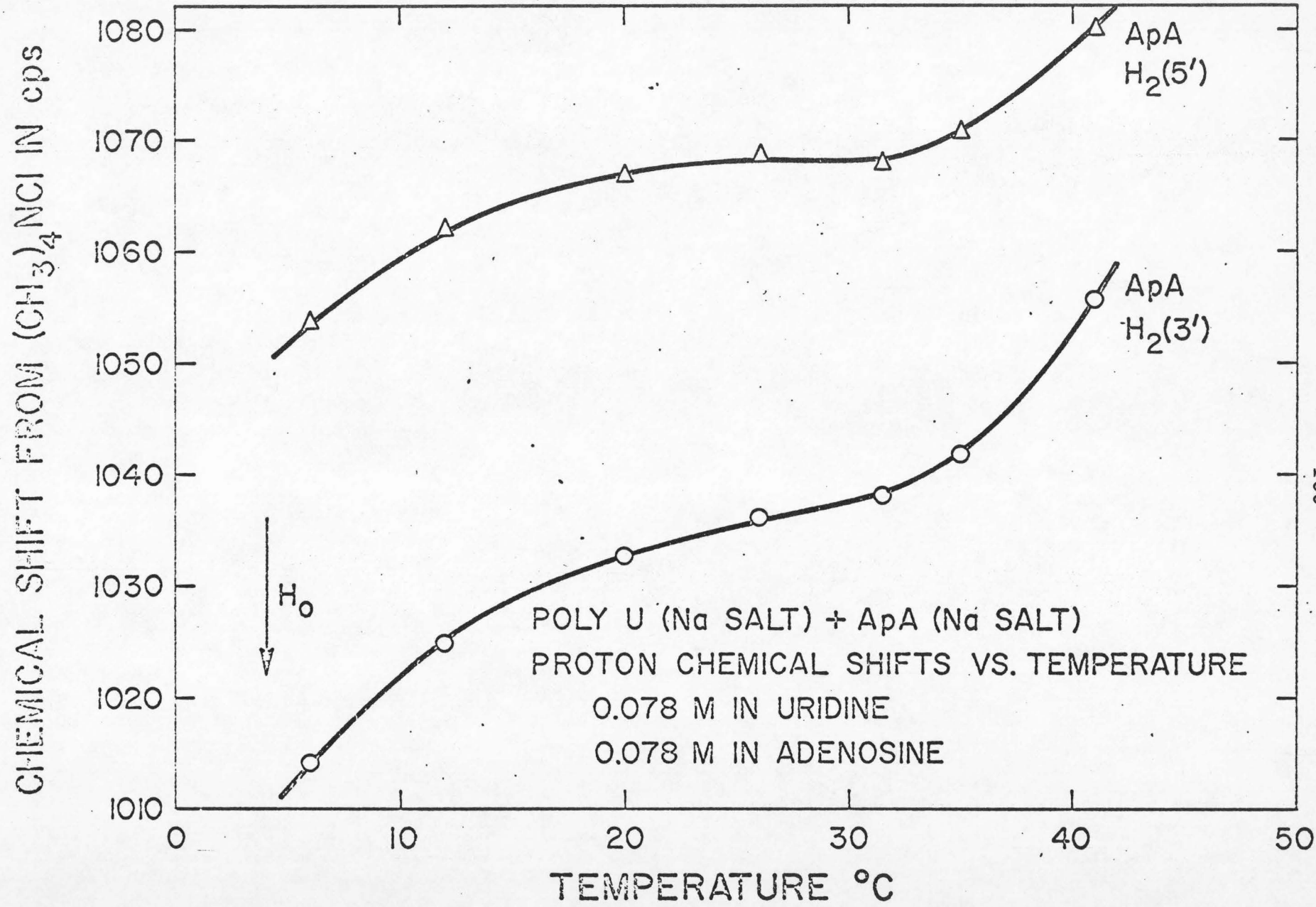
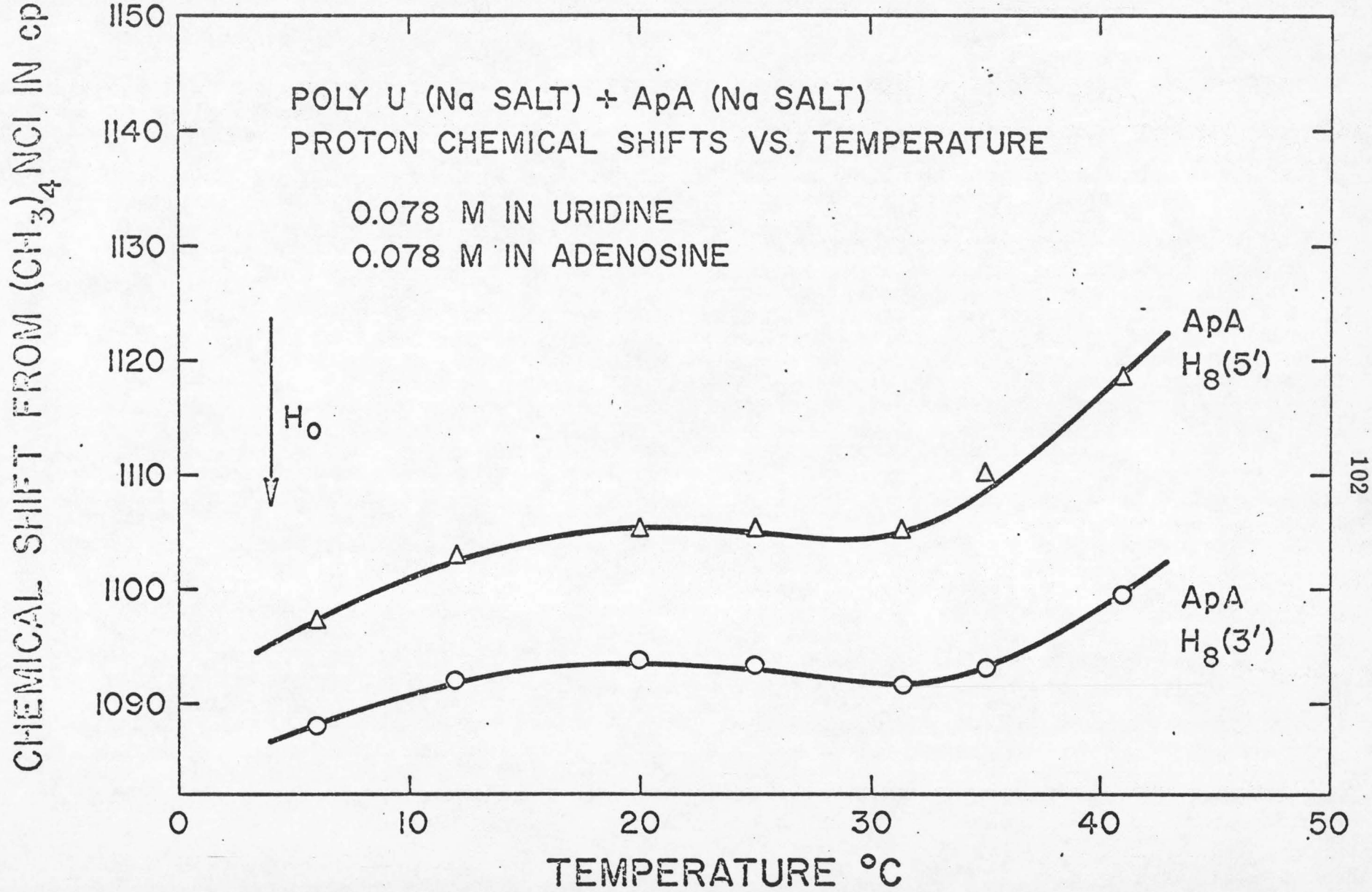

CHEMICAL SHIFT FROM $(CH_3)_4NCl$ IN cps
1080
1070
1060
1050
1040
1030
1020
1010
0
10
20
30
40
50
TEMPERATURE °C
ApA
$H_2(5')$
ApA
$H_2(3')$
H_o
POLY U (Na SALT) + ApA (Na SALT)
PROTON CHEMICAL SHIFTS VS. TEMPERATURE
0.078 M IN URIDINE
0.078 M IN ADENOSINE
100

FIGURE 27

The effect of complex formation on the chemical shifts (at 220 MHz) of the H_8 resonances of ApA as a function of temperature in the presence of poly U for a poly U-ApA solution with base ratio A:U = 1:1. Chemical shifts are given in Hz downfield from internal $(CH_3)_4N^+$.


POLY U (Na SALT) + ApA (Na SALT)
PROTON CHEMICAL SHIFTS VS. TEMPERATURE
0.078 M IN URIDINE
0.078 M IN ADENOSINE
H_o
ApA $H_8(5')$
ApA $H_8(3')$
CHEMICAL SHIFT FROM $(CH_3)_4NCl$ IN cps
1150
1140
1130
1120
1110
1100
1090
0
10
20
30
40
50
TEMPERATURE °C

shifts at each temperature as those of an ApA solution of the same concentration but without poly U. The ApA resonances, however, continue to shift upfield with decreasing temperature due to more extensive self-association of ApA molecules at this concentration of the dinucleotide at the lower temperatures.

For the ApApA-poly U system, however, no poly U "induced" shifts were observed for the fast exchange region of either complex (Figures 28 and 29). The lack of poly U "induced" shifts for each complex arises from two different factors. First, in the temperature range of 20° to 30°C, the ApApA molecule is intramolecularly stacked to a much greater extent than in ApA. Since the poly U "induced" shift is actually a reflection of the difference in stacking in a molecule between a free and bound state, this additional stacking of the ApApA in the free state makes the "induced" shift small enough to be within experimental error (~2 Hz) and thus unobservable. The $H_2(5')$ and the $H_8(3')$ resonances are shifted downfield below 20°C for the 1:1 case because below this temperature range the concentration of the ApApA is reduced by one half. The reduction in concentration leads to less intermolecular stacking and the only resonances which are sensitive to this are the $H_2(5')$ and $H_8(3')$ resonances. Secondly, in the high temperature range, 30° to 90°C, no "induced" shifts are observed even though the ApApA molecule is considerably less intramolecularly stacked, because the fraction of ApApA (~5%) is too low to make the "induced" shift greater than experimental error. We can, however, say that in the absence of poly U "induced" shifts, the conformation of the bases of ApApA is anti-anti-anti in complex since we

FIGURE 28

The effect of complex formation on the chemical shifts (at 220 MHz) of the H_2 resonances of ApApA as a function of temperature in the presence of poly U for poly U-ApApA solutions with base ratios A:U = 1:1 and 1:2. Chemical shifts are given in Hz downfield from internal $(CH_3)_4N^+$.

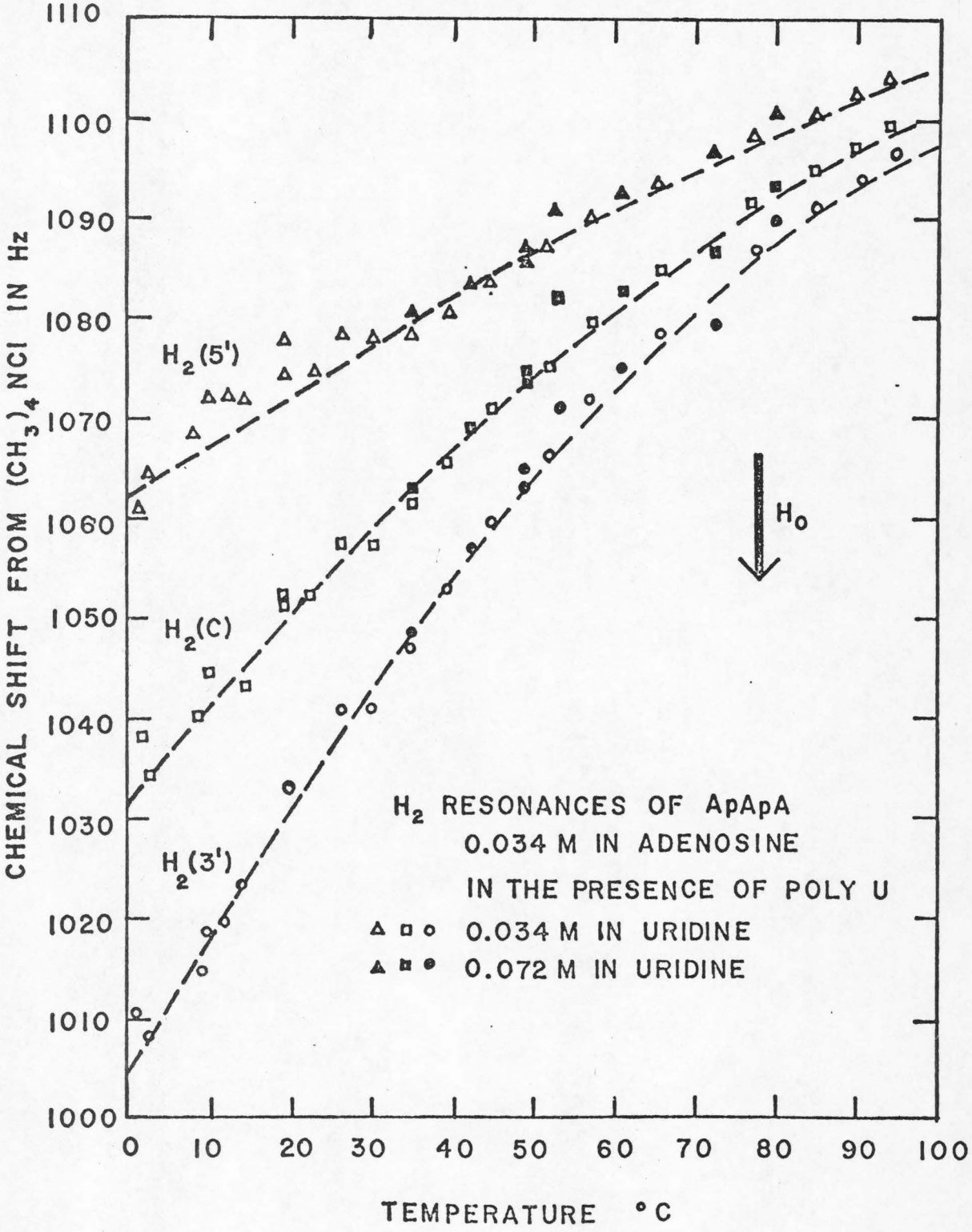
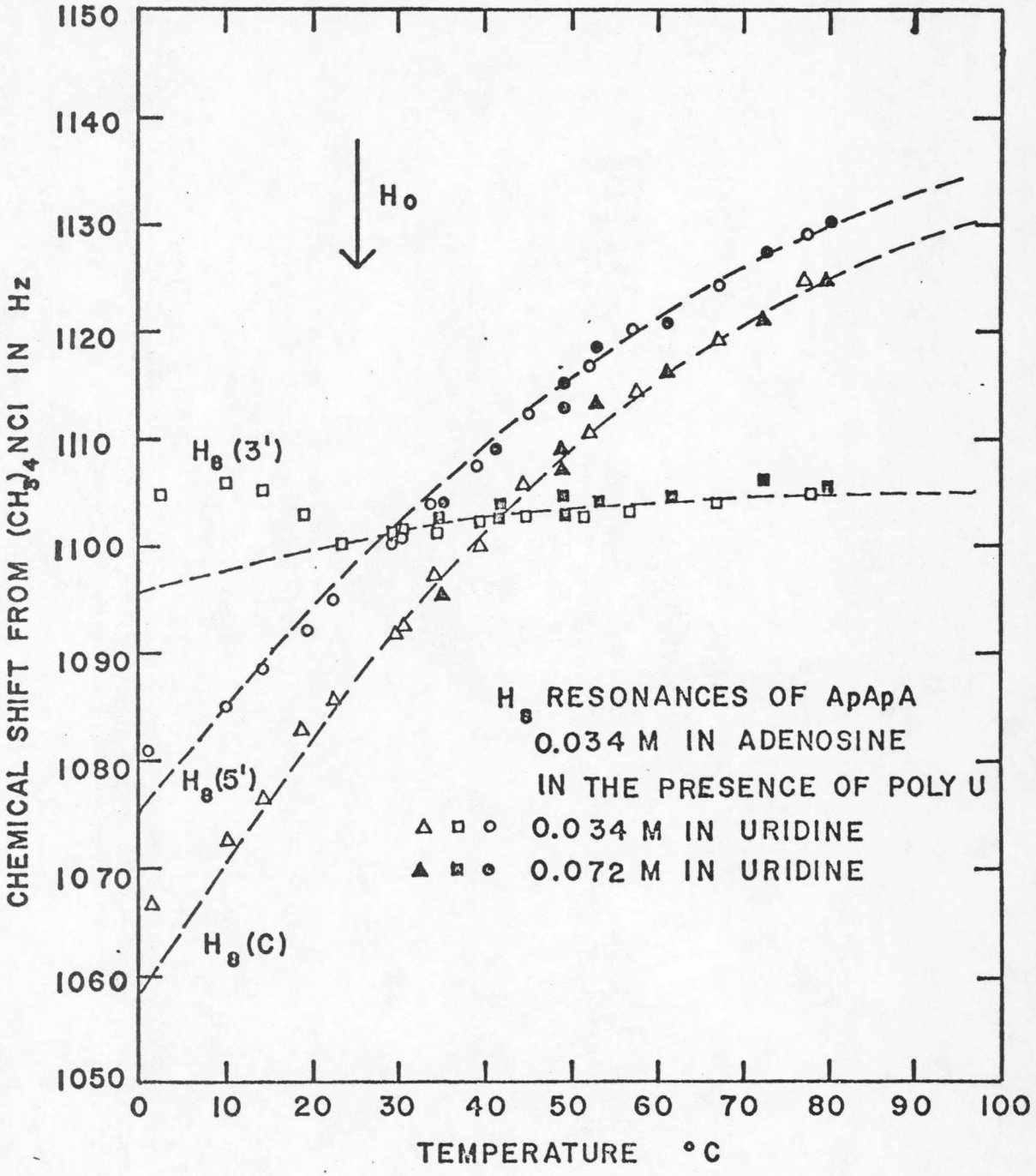

1110
1100
1090
1080
1070
1060
1050
1040
1030
1020
1010
1000
0
10
20
30
40
50
60
70
80
90
100
CHEMICAL SHIFT FROM $(CH_3)_4$NCl IN Hz
TEMPERATURE °C
H_2(5')
H_2(C)
H_2(3')
H_0
H_2 RESONANCES OF ApApA
0.034 M IN ADENOSINE
IN THE PRESENCE OF POLY U
△ □ ○ 0.034 M IN URIDINE
▲ ■ ● 0.072 M IN URIDINE

FIGURE 29

The effect of complex formation on the chemical shifts (at 220 MHz) of the H_8 resonances of ApApA as a function of temperature in the presence of poly U for poly U-ApApA solutions with base ratios A:U = 1:1 and 1:2. Chemical shifts are given in Hz downfield from internal $(CH_3)_4N^+$.


1150
1140
1130
1120
1110
1100
1090
1080
1070
1060
1050
0
10
20
30
40
50
60
70
80
90
100
CHEMICAL SHIFT FROM (CH3)4NCl IN Hz
TEMPERATURE °C
Ho
H8 (3')
H8 (5')
H8 (C)
H8 RESONANCES OF ApApA
0.034 M IN ADENOSINE
IN THE PRESENCE OF POLY U
△ □ ○ 0.034 M IN URIDINE
▲ ■ ● 0.072 M IN URIDINE

expect that a deviation from this conformation would lead to observable poly U "induced" shifts.

3.3.4. The thermodynamics and kinetics of complex formation.

It has been postulated that for the adenosine-poly U system, the second strand of poly U is required to compensate for the large unfavorable decrease in entropy resulting from the immobilization of the adenosine nucleotides in the complex.[64] This contention is substantiated by the increase in the "melting temperature" of the ApA-poly U system as compared to the A-poly U system. This apparent greater stability of the poly U-ApA complex is probably due to the somewhat less favorable entropy change in the interaction of the ApA molecule with poly U, since, in contrast to monomeric adenosine, two adenosine molecules are already connected by a phosphate group prior to incorporation in the complex. An increase in the "melting temperature" for the ApApA-poly U complex might also be expected for the same considerations. The increase in charge per nucleotide, however, causes a net destabilization of the complex due to coulombic repulsion of the phosphate groups despite the stabilization realized from the entropy decrease. This is not unexpected since it has been postulated that poly A does not form a triple-stranded complex with poly U under the ionic strength conditions used in this study because of coulombic repulsion of the phosphate groups.[58] The "melting temperature" must decrease at some point with increased oligonucleotide chain length in order to reach this polymeric limit.

For a trinucleotide, the entropy change upon complex formation

is reduced by partial immobilization of the adenine bases in the free state to the point where double-stranded helices are stable. This immobilization is due to an additional phosphate-ribose ester linkage as well as increased intramolecular stacking with larger chain lengths. There is, however, also a smaller but still significant decrease in entropy upon complex formation arising from the immobilization of the uracil residues accompanying the change from a random coil to a rigid rod. The magnitude of the entropy considerations are such that only short segments of double-stranded helices are formed between ApApA and poly U while the remainder of the poly U strand is still random in nature. As the temperature is reduced below 30°C, the free energy of a triple-stranded complex is less negative than the partially complex poly U and the rigid triple helix is the favored structure.

The mean lifetime of an ApA in the free state in the presence of the triple-stranded complex can be inferred from additional line broadening of the ApA resonances below ~26°C in the 1:1 ApA-poly U system as compared to their normal linewidths in the absence of poly U. Since the linewidths of the HOD resonance and the tetramethylammonium resonance are narrow (1 Hz) and change very little over the temperature range studied, the broadening is not due to changes in the viscosity of the solution, but instead, reflects the rate of chemical exchange of the ApA molecules between the two environments. In the limit of slow exchange, this leads to an additional broadening of the individual resonances by $\frac{1}{\pi}\frac{1}{\tau}$ (Hz) where τ is the mean lifetime of the ApA molecule in the uncomplexed

environment. At 6°C, the linewidth of the $H_2(5')$ is ~4Hz while its intrinsic linewidth is ~2 Hz. From the additional broadening, we thus obtain a pre-exchange lifetime of 0.159 sec. for the free ApA molecule in solution in the presence of the triple-stranded complex. Similar analysis yields lifetimes of 0.113 sec. at 12°C, 0.080 sec. at 20°C, and 0.042 sec. at 26°C. From the variation of these lifetimes with temperature we obtain an activation energy of 11 ± 1 kcal/mole for the incorporation of a free ApA molecule into the rigid complex. An activation energy of 8 kcal/mole was previously reported for the binding of adenosine to poly U.[25] Since we expect the rate of the overall process to be determined by the dissociation of an ApA molecule from the complex, the higher activation energy in the case of ApA probably reflects the increased number of hydrogen bonds formed per ApA molecule as compared to the adenosine case.

The kinetics of the formation of the 1:1 complex in the ApApA-poly U system should be in reasonable agreement with the renaturation of DNA where the rate determining step is believed to be nucleation by the formation of three base-pairs.[70, 71] The second order rate constant of ~10^3 liter/mole-sec inferred from our data is an order of magnitude greater than that previously reported for the renaturation of T4 DNA[82] but this discrepancy is not unreasonable since the trinucleotide is a much smaller molecule than a single-strand of T4 DNA.

4. CONCLUSION

This study clearly shows that the secondary forces, hydrophobic and hydrogen bonding interactions, are very important in defining the mode of interaction of small molecules with single-stranded ribonucleic acids. The hydrophobic forces are dominant in non-specific vertical base-stacking complexes of purine and ethidium bromide but also contribute to the stability of rigid complexes that are ordered by highly specific hydrogen bonding between bases.

The capabilities of pmr spectroscopy in probing the nature of either hydrogen-bonded or base-stacking interactions of small molecules with polynucleotides are clearly evident. In this work we have utilized the extreme sensitivity of the spectral parameters of a given proton to probe small structural and environmental effects and have exploited the dependence of the resonance linewidths for the detection of rigid secondary structure in the polymer as well as certain dynamical effects. These features of the NMR method make this spectroscopic tool uniquely valuable in the investigation of the structural and dynamical properties of biologically important systems.

PART II

TEMPLATE RECOGNITION AND THE DEGENERACY OF THE GENETIC CODE

"The so-called genetic code is one of the most popular facets of biology today. It is also perhaps the most important . . . If a biological system were incapable of this translation, or even if the process were less accurate than it is, . . . life, as defined by the biologist, could not exist."(83)

C. R. Woese

1. INTRODUCTION

The role of mRNA and tRNA during protein synthesis is a well understood biological process.[2, 13, 84] Imprinted on the mRNA molecule is a linear set of three letter "words," called codons, which define the specific primary structure of a given protein. The set of codons is read in a nonoverlapping manner with no "commas" and each codon is either specific for one amino acid or is a chain termination command.[84, 85]

The translation process of converting a nucleic acid sequence into the primary structure of a protein is carried out by the tRNA molecules. The generally accepted structure of tRNA is the cloverleaf model (Figure 1). The tRNA is charged with a specific amino acid at the 3′-terminal end by aminoacyl tRNA synthetase. Although several tRNA's which exhibit different coding properties have been found for one amino acid, one synthetase molecule will apparently charge all the tRNA's specific for that amino acid.[86, 87] The nature of the degenerate interactions of the synthetase molecule has been widely discussed but is still unknown. In the presence of the ribosome, the charged tRNA molecule interacts with the mRNA by "complementary" base-pairing between the three base anticodon region of the tRNA and the three base codon of mRNA. This interaction positions the amino acid, called for by the code, for incorporation into the growing peptide chain.[13]

Like the synthetase-tRNA interaction, the anticodon-codon

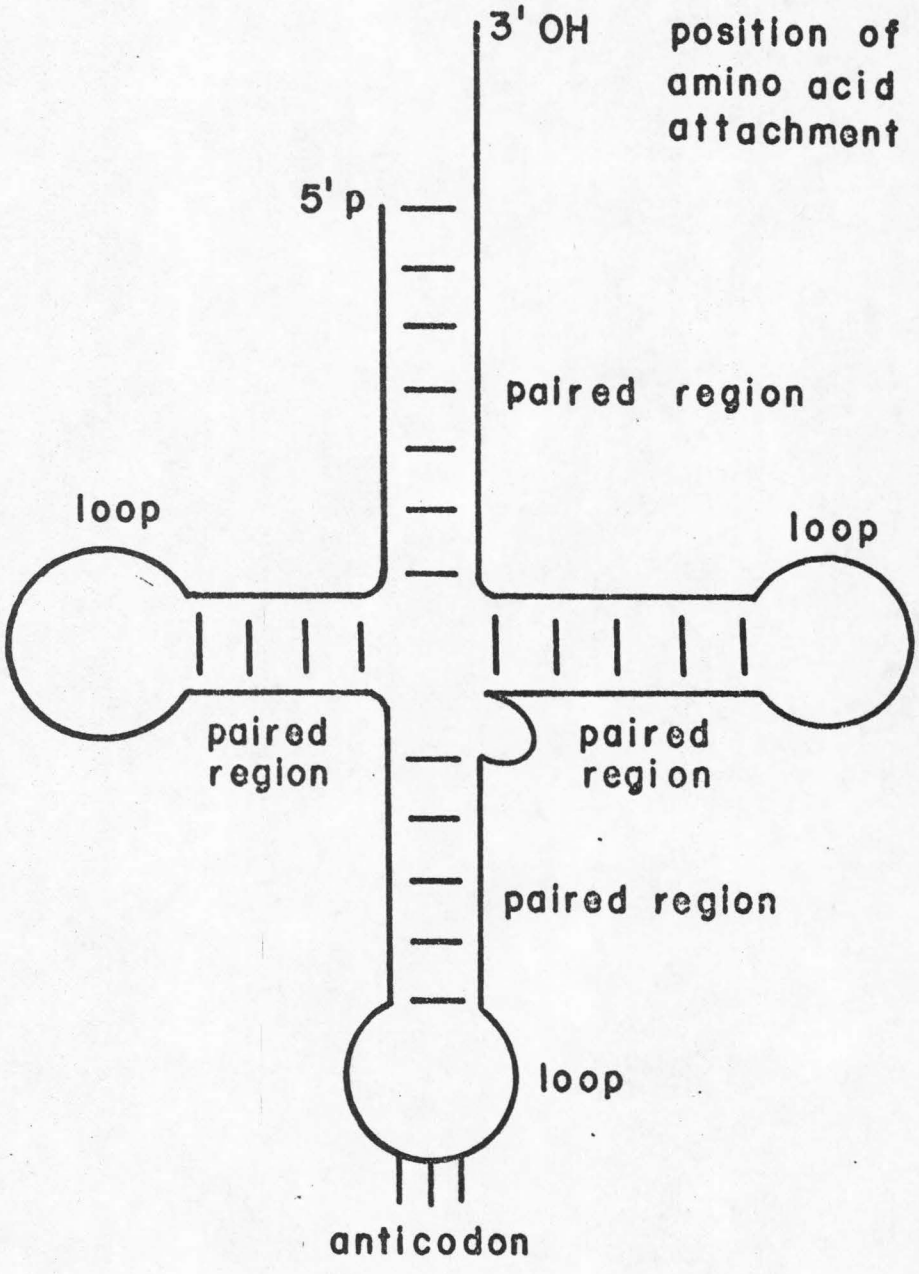


FIGURE 1

Generalized Cloverleaf Model of tRNA[10]

interaction is also degenerate. When the genetic code was deciphered employing three major lines of investigation, namely genetic, biochemical and amino acid replacement studies, the inherent degeneracy of the code showed marked regularity (Table I).[83] The first two letters are very specific. The third letter, however, is equivalent with U = C for 16 of the 16 possible cases of the first two letters and is also equivalent for A = G for 14 of the 16 possible cases. In 8 of the 16 cases, one amino acid is coded by four codons differing only in the third letter.

It should be emphasized that the degeneracy of the code lies in the codons and not in the anticodon since a single tRNA can interact with several codons, either in pairs, for example, XYU and XYC or XYA and XYG,[87-89] in triplets, XYU, XYC and XYA,[90, 91] or in quartets, XYU, XYC, XYA and XYG.[92] The mechanism of the interaction of a single tRNA molecule with several degenerate codons is still an unsolved problem. Several theories, however, have been proposed, most notable of which are the "wobble" hypothesis and the "base-flip" hypothesis. The "wobble" hypothesis proposes that the third base of the codon has a certain amount of play, or wobble, which allows this base to assume several positions enabling it to exhibit several hydrogen bonding schemes.[93] The "base-flip" hypothesis states that the third base can be in either a <u>syn</u> or <u>anti</u> conformation which allows this base to have two distinct hydrogen bonding schemes.[94] The bonding schemes for these two hypotheses for a G-U pair are shown in Figure 2. Recently, the missing triplet hypothesis has been proposed. This hypothesis states that ambiguity in the code arises from interactions

TABLE I

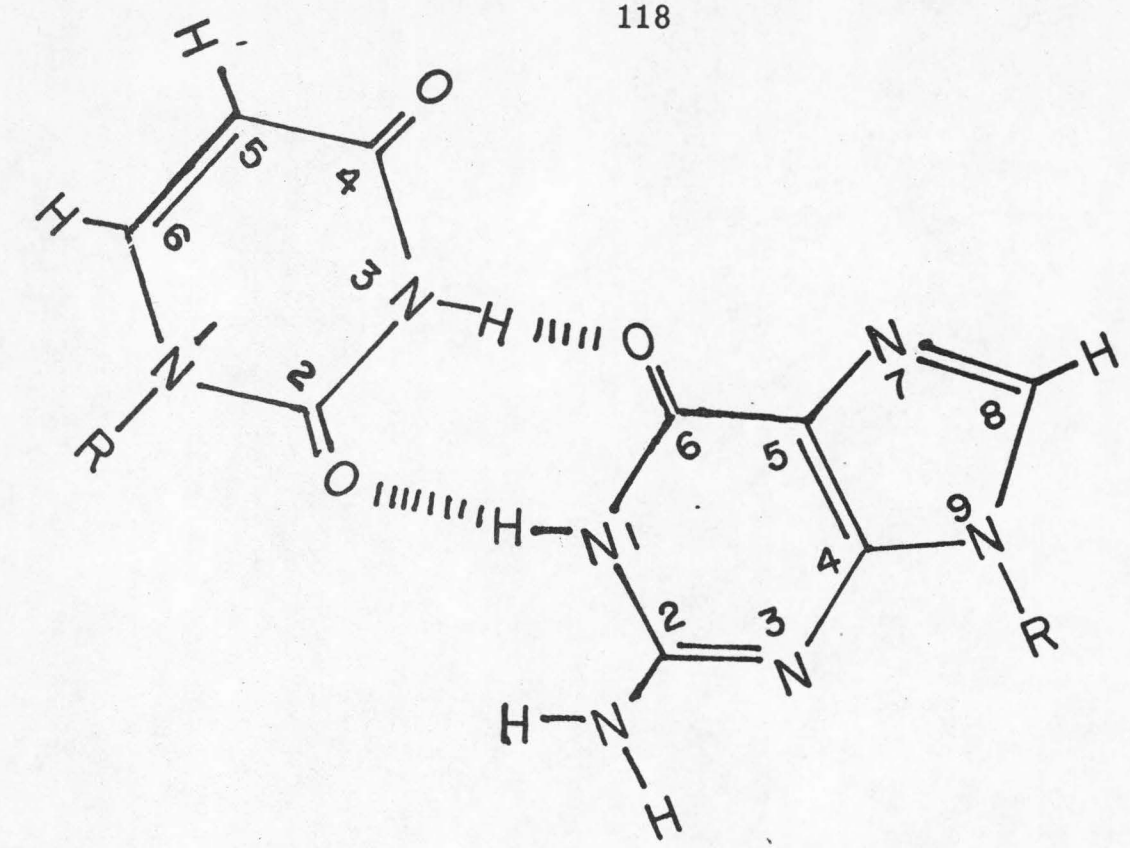
The Genetic Code

First position (5′end)	Second Position U	C	A	G	Third position (3′end)
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Term[a]	Term	A
	Leu	Ser	Term	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	GluN	Arg	A
	Leu	Pro	GluN	Arg	G
A	Ileu	Thr	AspN	Ser	U
	Ileu	Thr	AspN	Ser	C
	Ileu	Thr	Lys	Arg	A
	Meth	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

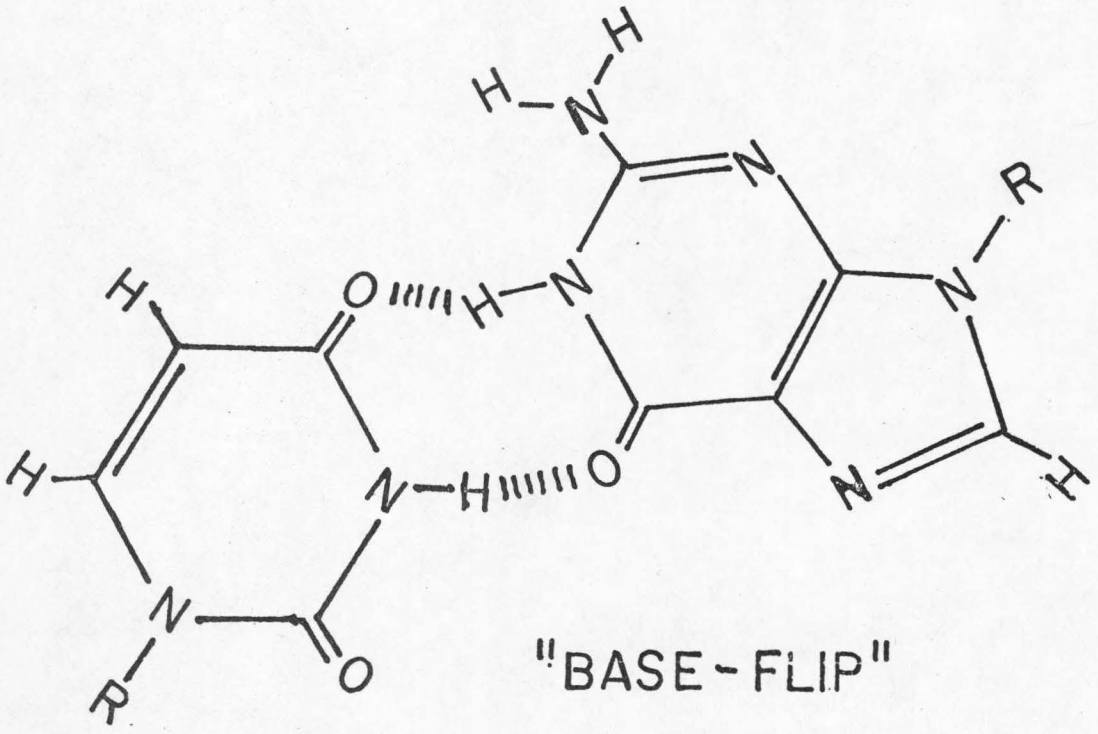
[a] Chain terminating (formerly called "nonsense").

FIGURE 2

The "wobble" and "base-flip" bonding schemes for the uridine-guanosine base-pair.



"WOBBLE"



"BASE-FLIP"

between non-hydrogen-bonded bases on opposite strands and for the organism to be viable, certain anticodons do not exist or are modified.[95] These hypotheses are ingenious but all have major drawbacks. In view of the recent findings that G and C (Lee and coworkers[96-99]) as well as I (Wolfenden[100, 101])* exist in solution with a significant portion of the molecules in their abnormal tautomeric form, the possibility that tautomeric exchange may be involved in degeneracy should also be considered. In fact, it has been proposed that tautomeric exchange of inosine may play a role in the degeneracy of the code.[100, 101, 103]

In order to determine the exact nature of the interactions of the anticodon with degenerate codons of single-stranded RNA, we have studied the interactions of the codon AAG, a trinucleotide ApApG, with poly U. It has been demonstrated that the G-base of the trinucleotide has more play or "wobble" than the A-bases[69] and it would be of interest to determine if the hydrogen-bonding scheme predicted by the "wobble" hypothesis does actually occur between ApApG and poly U. Since the pmr method is extremely sensitive to small environmental changes in molecules in similar systems, the results of this experiment might indicate the mode of binding of our model codon and may shed light on the mechanism of the degeneracy of the genetic code. Care, however, must be exercised in comparing the results of this work on trinucleotides to the behavior of a codon or anticodon <u>in vivo.</u>

---

* Another spectroscopic study of inosine reached the opposite conclusion that inosine is almost totally in its normal tautomeric form.[102]

2. EXPERIMENTAL

The poly U potassium salt was obtained from Calbiochem and was converted to the sodium salt by methods described in part I, section 2.1. The adenyly adenylyl guanosine, sodium salt (ApApG), was obtained from Miles Laboratories, Inc., and was used without further purification. The solutions were prepared in a manner similar to that previously described (part I, section 2.1.).

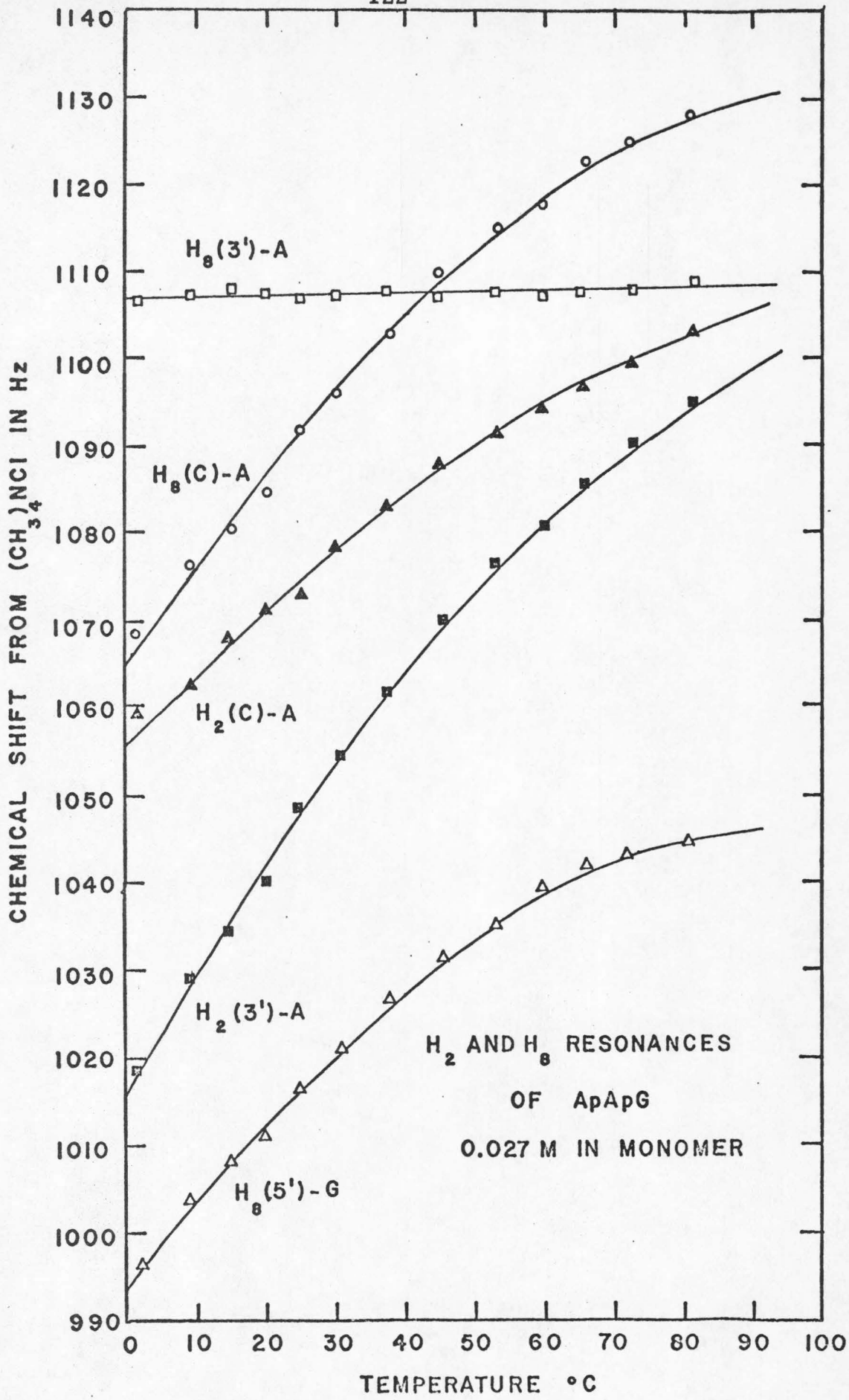
The 220 MHz pmr spectra were obtained on the instrumentation described in part I, section 2.2.

3. RESULTS AND DISCUSSION

The spectral behavior of free ApApG in solution is similar to that previously described for ApApA (part I, section 3.3.1.) and the spectrum has been previously assigned.[69] The chemical shifts of the resonances are strongly temperature dependent and the temperature variation for each resonance is different (Figure 3). This nonequivalent temperature dependence has been attributed to the variation of intermolecular and intramolecular base stacking and conformation about the C_{11}-N_9 glycosidic bond with temperature.[69] While the linewidths of the H_2 and H_8 resonances of the adenine bases change slightly with temperature, the H_8 resonance of guanine does exhibit broadening at low temperatures which has been attributed to tautomeric exchange in guanine.[69, 97, 99]

FIGURE 3

The temperature variation of the chemical shift for the H_2 and H_8 resonances of ApApG (0.027 M in monomer).


1140
1130
1120
1110
1100
1090
1080
1070
1060
1050
1040
1030
1020
1010
1000
990
0
10
20
30
40
50
60
70
80
90
100
CHEMICAL SHIFT FROM $(CH_3)_4NCl$ IN Hz
TEMPERATURE °C
$H_8(3')$-A
$H_8(C)$-A
$H_2(C)$-A
$H_2(3')$-A
$H_8(5')$-G
H_2 AND H_8 RESONANCES
OF ApApG
0.027 M IN MONOMER

The addition of poly U (0.013 M in uridine) to the solution of ApApG (0.009 M in codon) does affect the spectral behavior of both the poly U and ApApG resonances. Above room temperature (Figure 4a), the resonances are at their characteristic linewidth and chemical shift expected in the absence of complex formation. Below 20°C, all the resonances of poly U and ApApG exhibit broadening. The H_8 resonance of G is, however, broadened to the greatest extent (Figure 4b). Maximum broadening of all the resonances occurs at ~14°C. For the A resonances, the H_8 resonances are broader than the H_2 resonances (Figure 4c). Below ~14°C, the resonances begin to sharpen slightly and the intensities of all the resonances are somewhat reduced (~5%) (Figure 4d, e).

As was the case with the ApApA and poly U system above room temperature where a large rigid complex had not formed, the "critical rate" for an exchange process for the ApApG-poly U system is determined by the chemical shift difference of a given resonance between the free and bound states (part I, section 3.3.2.). The only resonance that exhibits extreme broadening in this system is the H_8 resonance of the guanosine base which strongly implies that the chemical shift of the H_8 proton is different in the free and bound states. The chemical shift difference can arise from two effects: either the conformation of the G base has changed with respect to the A bases, or the G base is in a lactim tautomeric form in the complex as compared to the predominate lactam form in solution.

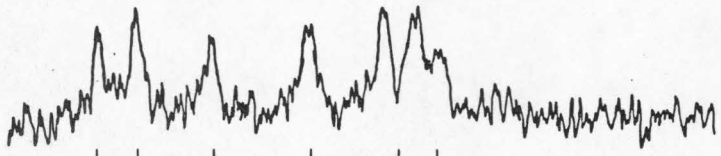
The first case would be predicted by the "wobble" or "base-flip" hypotheses. Both of these models also predict that the A-U

FIGURE 4

220 MHz pmr spectrum of a solution of poly U (0.013 M in uridine) and ApApG (0.009 M in codon) at various temperatures.

POLY U-Na^+ (0.013M IN URIDINE) + ApApG-Na^+ (0.009M IN CODON)

a) 31°C



$H_8(3')$ 1108.8 | $H_8(C)$ 1098.5 | $H_2(C)$ 1080.0 | $H_2(3')$ 1056.0 | U-H_6 1033.5 | $H_8(G)$ 1024.2

b) 18°C

$H_8(3')$ 1109.2 | $H_8(C)$ 1086.4 | $H_2(C)$ 1071.9 | $H_2(3')$ 1039 | U-H_6 1036 | $H_8(G)$ 1011

c) 14°C

$H_8(3')$ 1109 | $H_8(C)$ 1082 | $H_2(C)$ 1071 | $H_2(3')$ 1038 | U-H_6 1035.8 | $H_8(G)$ 1006

d) 8°C

$H_8(3')$ 1108.2 | $H_8(C)$ 1072.5 | $H_2(C)$ 1063.2 | U-H_6 1036.8 | $H_2(3')$ 1023.3 | $H_8(G)$ 1002.2

e) 2°C

$H_8(3')$ 1108.8 | $H_8(C)$ 1073.8 | $H_2(C)$ 1063.8 | U-H_6 1036.8 | $H_2(3')$ 1024.3 | $H_8(G)$ 1001.5

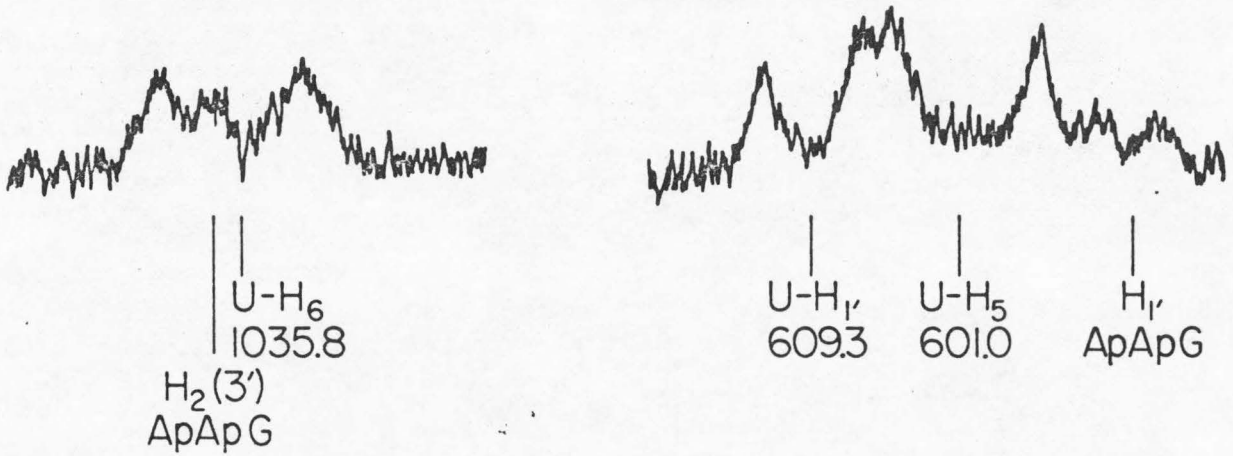
pairs should exhibit normal Watson-Crick bonding schemes.[93, 94] In Figure 5, the spectral behavior of the H_5, H_6 and $H_{1'}$ resonance of poly U in the ApApG-poly U system is compared to that in the ApApA-poly U system at the temperature where maximum line broadening occurs in the presence of the "labile" complex. In the ApApG-poly U system, the H_5 resonance does not exhibit the dramatic line broadening associated with the formation of a Watson-Crick base pair in the ApApA-poly U system. Since the rate of chemical exchange for any of the bases of the trinucleotide or the poly U is the same and the chemical shift difference of the H_8 resonance of G and the H_5 resonance of poly U between the free and bound states is expected to be approximately the same, the H_5 resonance of poly U should exhibit line broadening at approximately the same temperature as does the H_8 resonance of G if Watson-Crick base pairs do form. Due to the lack of line broadening of the H_5 resonance of poly U in the ApApG-poly U system, it is felt that normal Watson-Crick base pairs are not formed between any of the bases of ApApG and poly U. Even though the spectral behavior of the H_8 resonance is consistent with the "wobble" and "base-flip" hypotheses, both schemes can be eliminated for a ApApG-poly U complex because of the lack of line broadening of the H_5 resonance of poly U. From the line broadening of the H_8 resonance, the probable tautomeric form of the guanosine residue in the complex is the lactim form.

As was the case for the ApApA-poly U system, no poly U "induced" shifts were observed in the ApApG-poly U system upon complex formation (Figure 6). Since ApApG is extensively stacked

FIGURE 5

220 MHz pmr spectrum of poly U in the region of the H_6, $H_{1'}$ and H_5 resonances in the presence of (a) ApApG at 14°C and (b) ApApA at 77.5°C.

a) POLY U + ApApG AT 14°C



b) POLY U + ApApA AT 77.5°C

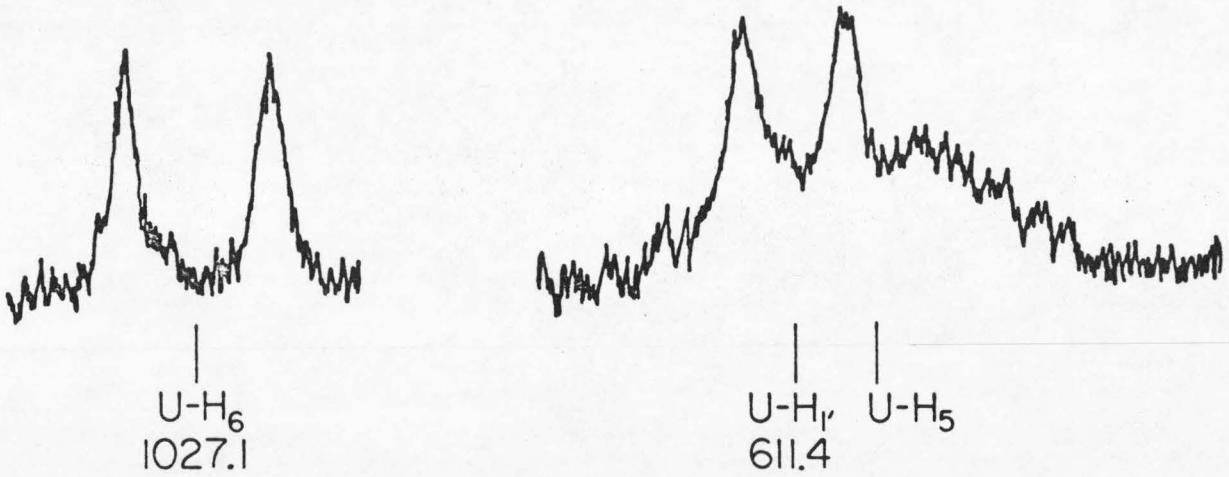
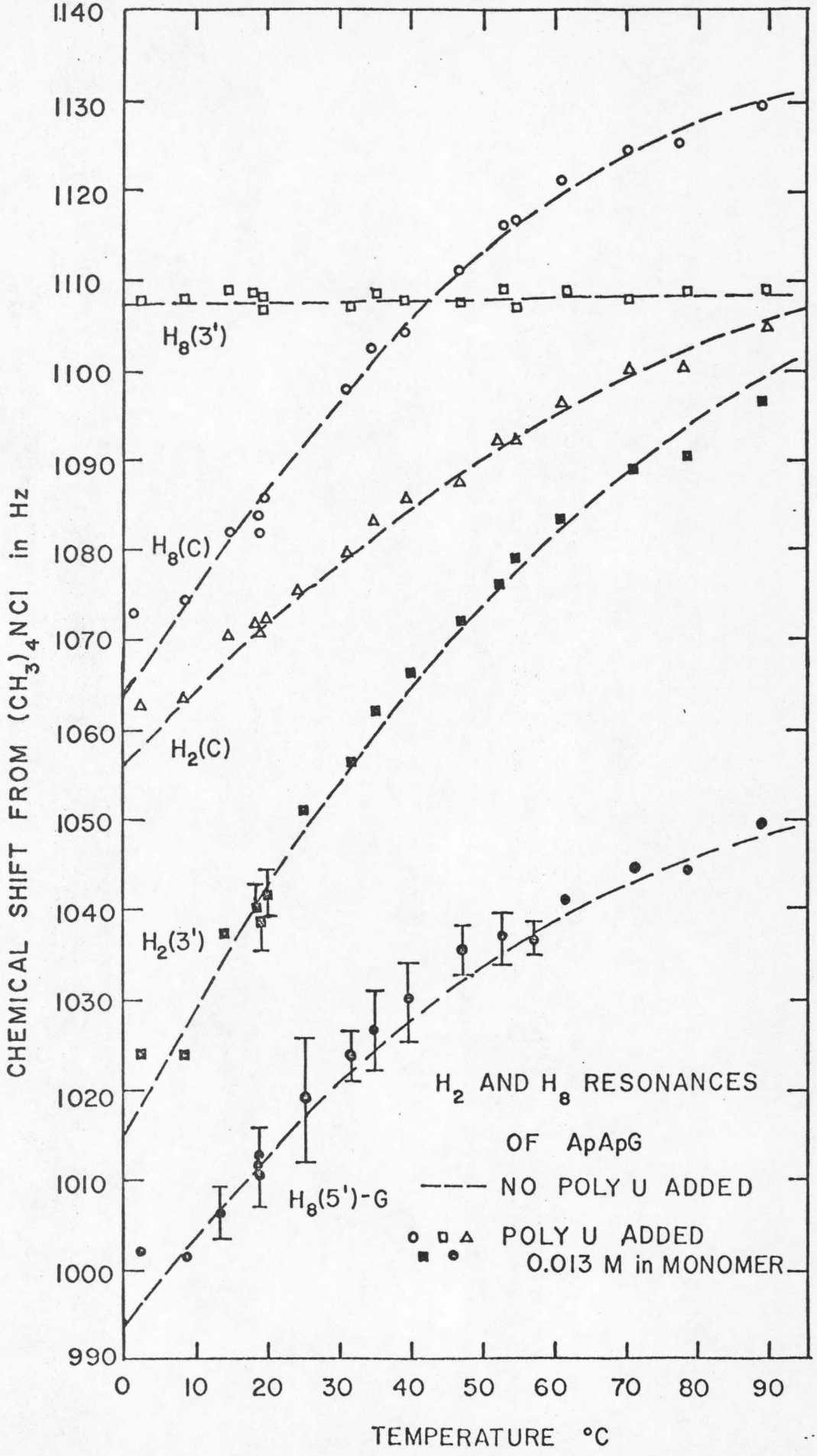


FIGURE 6

The temperature variation of the H_2 and H_8 resonances of ApApG (0.009 M in codon) in the presence of poly U (0.013 M in uridine).


CHEMICAL SHIFT FROM $(CH_3)_4NCl$ in Hz
TEMPERATURE °C
$H_8(3')$
$H_8(C)$
$H_2(C)$
$H_2(3')$
$H_8(5')$-G
H_2 AND H_8 RESONANCES
OF ApApG
NO POLY U ADDED
POLY U ADDED
0.013 M in MONOMER

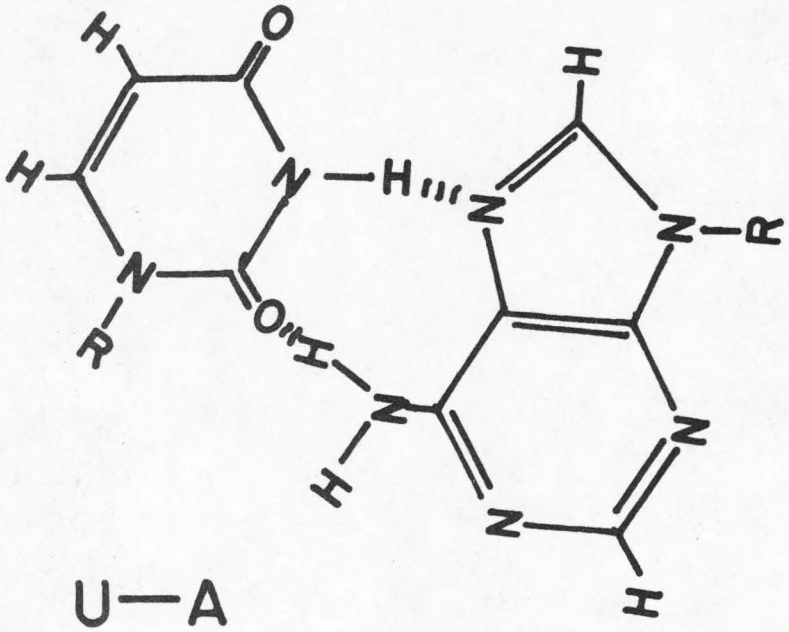
at low temperatures and the fraction bound is small, no poly U "induced" shifts are expected in the absence of conformational changes of A or G bases from their normal anti-anti-anti configuration.

With the ApApG molecule in the anti-anti-anti conformation, the G base in the lactim tautomeric form, and Watson-Crick base pairs absent, the only alternative bonding scheme for the ApApG-poly U complex that is consistent with the experimental data is the Hoogsteen scheme (Figure 7). In this scheme, the only resonance which would be expected to exhibit any broadening is the H_8 resonance of G. The H_8 resonance of A and the H_6 resonance of U might also be expected to exhibit slight broadening from small chemical shift differences in the free and bound states. Thus it is concluded that the ApApG molecule binds to poly U in the Hoogsteen hydrogen bonding scheme but only along short segments of the poly U strand, while the majority of the uracil residues are in a random coil.

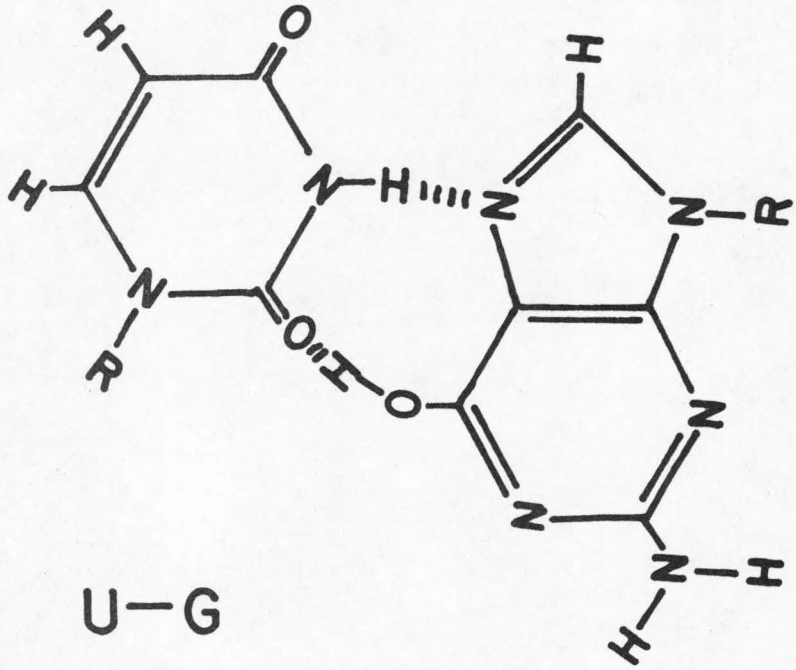
Although the reason for the greater stability of the Hoogsteen bonding scheme over the Watson-Crick bonding scheme is not entirely clear, several factors which favor the former scheme should be noted. First, there is one hydrogen bond more between the bases and the solvent and, second, the dipole moments of the U and G are in a more favorable alignment in the Hoogsteen scheme as compared to the Watson-Crick scheme.

FIGURE 7

The Hoogsteen bonding scheme for the uracil-adenine and uracil-guanosine base pairs.



U—A



U—G

4. A MODEL FOR THE DEGENERACY OF THE GENETIC CODE

The direct physical chemical evidence of the nature of the interaction of a trinucleotide and a single-stranded RNA has shown that "wobble" and "base-flip" hypotheses do not explain the mode of binding in this system. This work does bring out the inadequacies of these hypotheses and suggests that a new model, the "tautomeric doublet" hypothesis, is more consistent with the in vivo interactions of tRNA and mRNA.

The high degree of reversibility and extreme specificity of the process of template recognition by tRNA is indicative that the ΔG° of this process is very close to zero. Only in this way could the mispairing of one nucleotide pair in the first two positions lead to the rejection of a wrong tRNA. The mechanism for degeneracy must therefore be such that the energy difference between the multiple bonding schemes is very close to zero. Do the "wobble" or "base-flip" hypotheses meet this requirement?

The lack of "wobble" or "base-flip" in the ApApG-poly U complex strongly implies that a conformational change in the codon is energetically unfavorable. The "base-flip" method is presumably the least favorable of the two models from an energetic standpoint and can be ruled out as a mechanism for degeneracy at the outset. The free energy differences of the "wobble" schemes are probably closer to zero. In the studies of the binding properties of

trinucleotides with tRNA's, the thermodynamics of these systems were such that "wobble" interactions may have occurred. The majority of trinucleotides in these studies were either only partially stacked or exhibited no stacking at all.[104] If, however, a strongly stacked trinucleotide, GAA or AAG, or polynucleotide, poly (AG), was used in the studies, the "wobble" properties of the third base mysteriously disappeared.[105, 106] Recent studies of the effect of chain length in oligonucleotides on their conformational properties have revealed that the flexibility of the ribose-phosphate backbone in the middle of a single-stranded RNA chain decreases with increasing chain length.[10, 11, 69, 107] If the "wobble" interaction is not allowed for some trinucleotides, it would appear unlikely that the third base would exhibit the "needed play" in mRNA if it does not exhibit it in these more flexible trinucleotides.

A model for the degeneracy of the code must also explain the reason for the special nature of the third base and the mechanism must allow for the needed accuracy of the codon-anticodon interaction. It is the view of this author that the "wobble" properties of the third base were invented as a matter of convenience and not based on known chemical properties of nucleic acids. Also, the lines of reasoning used for the justification that the G = = = A pair is not allowable and that the G = = = U pair is allowable appear to be contradictory. Crick postulated that the G = = = A pair "will not occur, because the NH_2 group of guanine cannot make one of its hydrogen bonds, even to water," but did not apply this reasoning to the G = = = U pair where the NH_2 group also does not make one of its hydrogen bonds.[93]

Recently a tRNA has been found with no inosine present which has the binding properties of an IXY anticodon.[108] If in this case, a G-base can pair with an A-base by "wobble," then the maintenance of accurate recognition of the codons, $XY\begin{matrix}U\\C\end{matrix}$ and $XY\begin{matrix}A\\G\end{matrix}$, which code for different amino acids, becomes questionable.

The "wobble" hypothesis is reasonable in many aspects but it fails to meet several important requirements for the process of codon recognition if it is viewed from a physical chemical viewpoint.

A mechanism for the degeneracy of the code which meets all three requirements, namely small free energy differences of the degenerate forms, special nature of the third base and accuracy, can be deduced from the ApApG-poly U interaction by reversing the role of the ApApG as a model codon to that of the anticodon. Although ApApG binds to poly U in a Hoogsteen bonding scheme, this need not be the favored scheme for the AAG anticodon in tRNA. In a trinucleotide such as ApApG, the Watson-Crick and the Hoogsteen sites are almost equivalent and due to small energy differences of the two sites, the Hoogsteen scheme is favored. The Watson-Crick scheme, however, would be favored in vivo because of the steric restrictions of the tRNA-mRNA-ribosome interactions. The important factors in the ApApG-poly U complex which have a bearing on a mechanism for the degeneracy of the code are the guanosine base was in the lactim tautomeric form and no conformational changes in the guanosine base were observed.

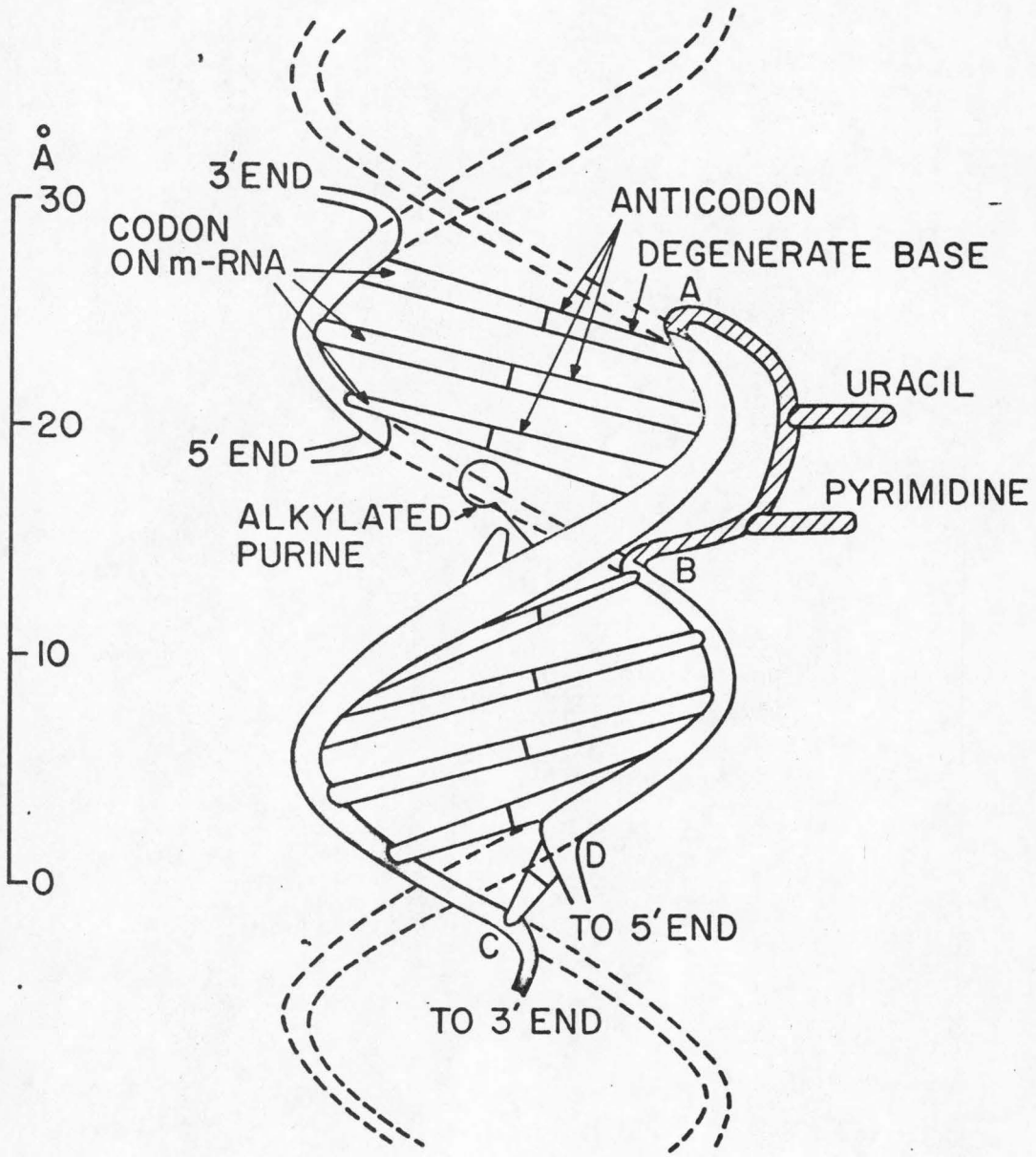
The anticodon, AAG, cannot interact with the UUU codon in vivo because UUU and CUU codons code for two different amino

acids.[84] The probable cause of this misreading in this system is the tautomeric exchange in the guanosine base of ApApG due to its exposure to the solvent. From the model building studies of Fuller and Hodgsen[109] (Figure 8), it is apparent that exposure of the first base to the solvent is minimized by the presence next to it of a hydrophobic alkylated purine, which is present in most tRNA's.[13] In fact, Gefter and Russell have shown that if the hydrophobic base is modified in $tRNA^{TRY}$, it will bind to the CAG codon by way of an abnormal A = = = C bond.[110] The degenerate base is, however, exposed to the solvent much like the guanosine in ApApG and thus could experience solvent enhanced tautomerization. In view of (a) the recent findings of Lee and coworkers that G and C exist in aqueous solution with a significant percentage of the bases in the abnormal tautomeric form, and (b) previous work of Wolfenden which demonstrated that either tautomeric form of inosine is equally probable in solution,[96-102] the possibility that tautomeric exchange may be involved in the degeneracy of the genetic code is a real possibility.

It is, therefore, proposed that the genetic code is degenerate in pairs and the anticodon is complementary to either one of the degenerate pair simply by being in either its normal or abnormal tautomeric form (Figures 9 and 10). The degenerate codons are pairs of the form XYU and XYC or XYA and XYG with complementary anticodons of the form $\begin{matrix} G \\ \text{or } I \end{matrix}$ YX or CYX, respectively. In the great majority of tRNA's sequenced, the third base has been either a normal or modified C, G or I.[13] In this scheme, the anticodon binds to the codon with the third base in its normal tautomeric form when a codon allows

FIGURE 8

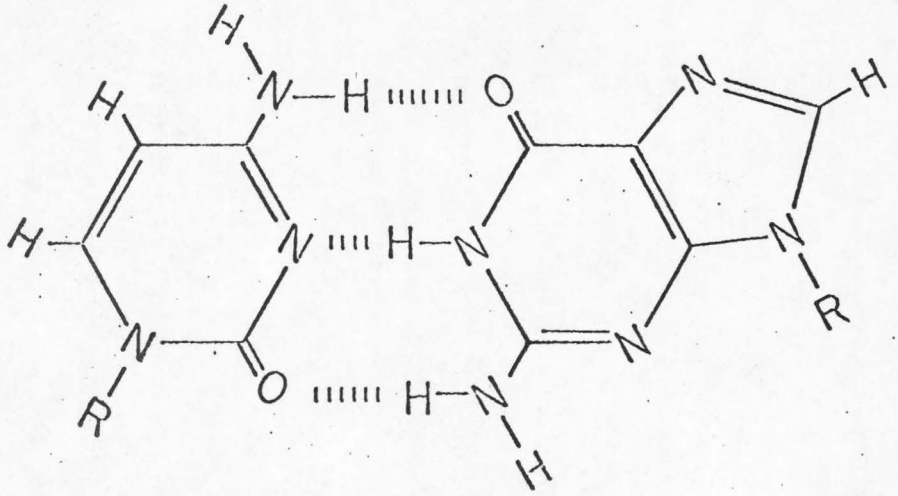
Schematic diagram of the anticodon loop of tRNA.



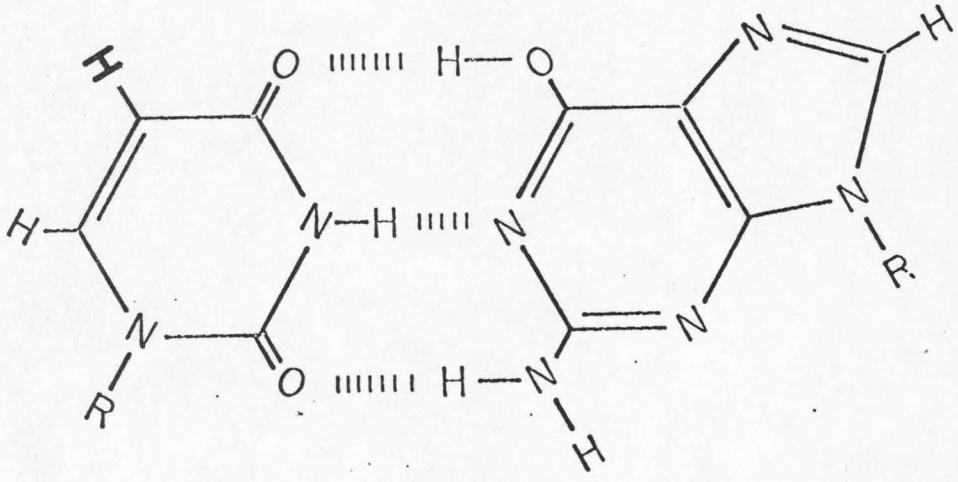
Reproduced from W. Fuller and A. Hodgson, Nature, 215, 817 (1967) with the permission of W. Fuller and Macmillan (Journals) Limited.

FIGURE 9

The hydrogen bonding schemes of the cytosine-guanine and uracil-guanine base pairs as predicted by the "tautomeric doublet" hypothesis.



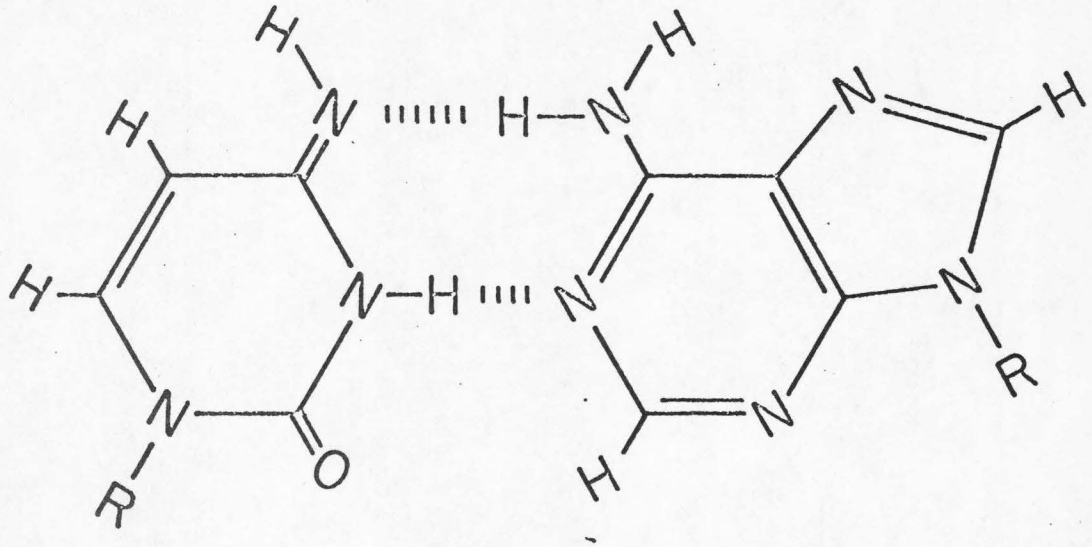
C — G



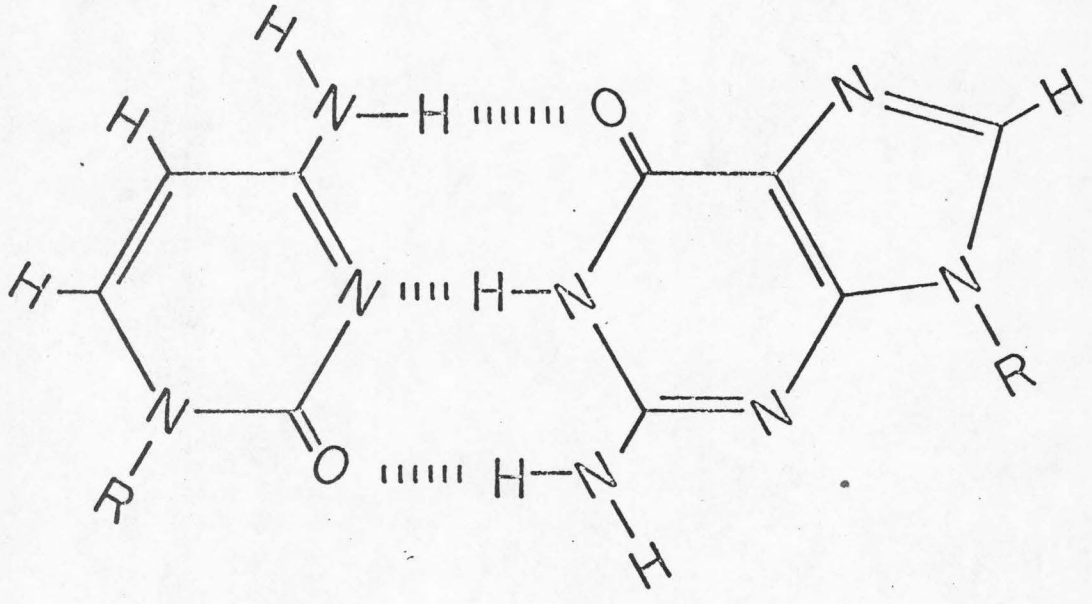
U — G

FIGURE 10

The hydrogen bonding schemes of the cytosine-adenine and cytosine-guanine base pairs as predicted by the "tautomeric doublet" hypothesis.



C — A



C — G

normal Watson-Crick hydrogen bonding (i. e., XYG and CYX) and in its abnormal tautomeric form when the anticodon is presented with the other codons (i. e., XYA and CYX). For this type of interaction, no "play" is required in the third base. There has been great speculation as to the reason why inosine is as prevalent as it is in tRNA. According to our scheme, the I-base is substituted for G because it is more susceptible to tautomeric exchange than G and would thus be more efficient as a degenerate base.

Our model also allows for special cases like $tRNA^{Meth}$ and $tRNA^{Trp}$ which are specific for only one codon. In these cases, the third base can be modified such that only one tautomeric form exists. The structure of the third base of $tRNA^{Meth}_{coli}$ has not been determined but it has been demonstrated that it is a modified C.

The three major requirements of a model for the degeneracy of the genetic code are met by the "tautomeric doublet" hypothesis. The third base is different because of its exposure to the solvent. The free energy difference of the two degenerate forms is close to zero with the ΔG° for tautomeric exchange equal to ~1 kcal/mole.[97] Finally the extreme accuracy of the code is maintained since our proposed degeneracy is in pairs, which matches the apparent pairwise degeneracy that exists in vivo.[111]

5. CONCLUSION

This study of the interaction of ApApG and poly U has brought out several interesting properties of trinucleotide-polymer interactions which could be applied to a mechanism for the degeneracy of the code. As previously mentioned, care must be exercised in applying the results of this experiment to the in vivo interactions of tRNA with mRNA. Several deficiencies of the "wobble" hypothesis have been brought out and a new model has been proposed which removes these ambiguities. Further research, however, must be done before the exact nature of the codon-anticodon interaction can be understood. Foremost in the substantiation of the "tautomeric doublet" model is the resolving of the controversy if inosine does exist in solution in two tautomeric forms.

The problem of codon recognition has been viewed as a purely physical chemical problem in this study. The study of the implications and the search for the actual existence of the "tautomeric doublet" model in real living systems will require a concerted effort of both chemists and biologists.

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PROPOSITIONS

PROPOSITION I

Magnetic Resonance Study of the Interactions of Gliotoxin and Polio Type 2 Virus RNA

Gliotoxin, in very small amounts (~7 μg/ml), has been shown to inhibit the growth of fungi, bacteria and transplantable neoplasma[1] and more recently it has been shown to exhibit antiviral activity.[2]

P. A. Miller and co-workers demonstrated that gliotoxin at low concentrations (~7 μg/ml) stopped the production of viral nucleic acid but did not affect viral protein synthesis or the DNA dependent synthesis of cellular RNA in the host $HeLaS_3$ cells. At significantly higher concentrations of gliotoxin, however, the production of cellular RNA was also stopped. Miller postulated that gliotoxin's specificity is on the molecular level and not due to topographical separation of the two sites of RNA synthesis.[3] Gliotoxin does not inactivate extracellular polio type 2 virus nor affect its absorption into monkey kidney cells but does, however, inhibit intracellular virus synthesis.[4] The antiviral activity of gliotoxin does not arise from any interaction of it with the viral protein covering it, but instead it must interact with some viral component after infection.

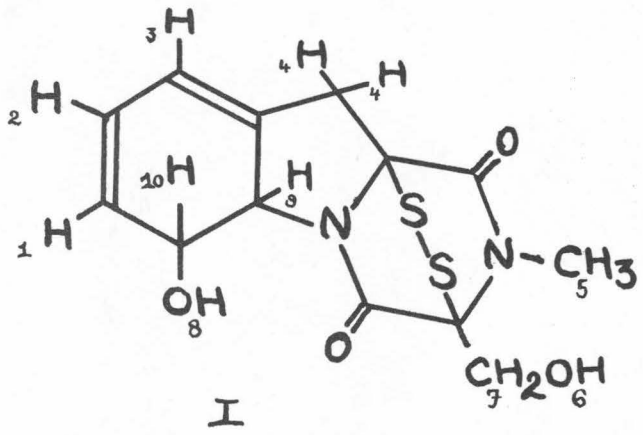
The poliovirus reproduction cycle has been determined. During the first one-half to one hour after the single strand of RNA has entered the host cell, the virus has directed the formation of a membrane bound structure in the cytoplasm of the host cell called the "replication complex" which contains viral polymerase, the replicative intermediate

and viral particles in various stages of assembly as well as whole ones. The replicative intermediate has, on the average, four molecules of viral RNA polymerase attached to it, implying that it consists of two complementary strands of RNA with an average of four partially completed viral RNA chains. The postulated purposes of the complex are efficiency, since all the viral synthesized products are compartmentalized for easy assembly, and protection of viral functions while the host cellular processes are being suppressed or destroyed. [5, 6]

In light of the known reproduction cycle of this type of virus, one possible mode of gliotoxin's antiviral activity is that it binds to the single-stranded RNA before it has been able to direct the formation of the "replication complex." It is therefore proposed that the interaction of gliotoxin with single-stranded RNA be studied by high resolution proton nuclear magnetic resonance spectroscopy.

The structure of gliotoxin (I) has been determined from its chemical and spectroscopic properties[7] and by x-ray crystallography. [8, 9] The expected chemical shifts of the resonances are given in Table I. [10] The insolubility of gliotoxin in water (0.07 mg/ml at 30°C)[11] could present some experimental problems but these probably could be overcome with the use of Fourier transform pmr spectroscopy.

If gliotoxin does interact with RNA by intercalation as might be expected, and the rate of chemical exchange is fast on the pmr time scale, changes in the spectral parameters of the resonances of both the gliotoxin and the RNA should indicate the nature of this complex, as was the case in the pmr studies of purine and ethidium



I

TABLE I

Proton	Expected Chemical Shift in δ
1	~5.3
2	~5.3
3	~5.3
4	3.2
5	2.3-3
6	exchanging with D_2O
7	3.6
8	exchanging with D_2O
9	~2.7
10	~4.1

bromide and single-stranded RNA.[12, 13] If, however, the rate of exchange is slow, the system would have to be simplified by using small oligonucleotides, such as dimers or trimers, in order to be able to resolve the gliotoxin resonances in the complex. Again, the spectral parameters should indicate the nature of this interaction.

If gliotoxin is shown not to interact with single-stranded RNA, another likely possibility for its antiviral activity is that it binds to the viral RNA polymerase.

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PROPOSITION II

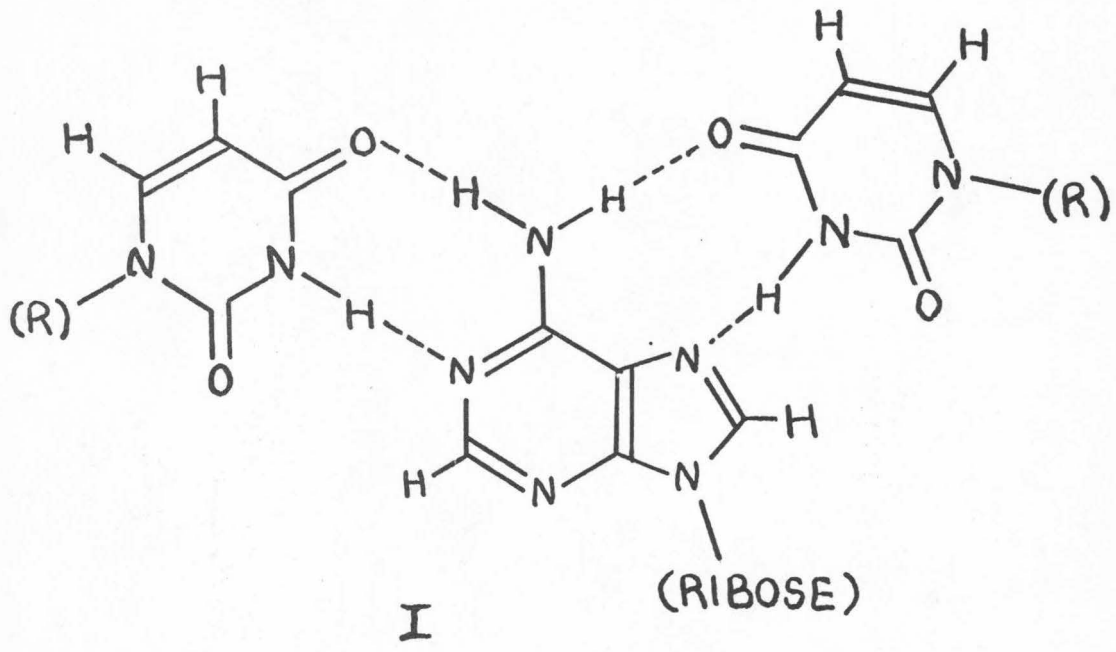
Characterization of Hydrogen-Bonded Complexes of Oligonucleotides and Polyuridylic Acid

Rigid hydrogen-bonded complexes between oligonucleotides and polyuridylic acid (poly U) have been deduced from optical and magnetic resonance studies. A rigid double helix presumably involved Watson-Crick base-pairs has been observed between poly A and poly U.[1, 2] Under appropriate conditions of ionic strength, a triple-stranded complex involving one poly A strand and two poly U strands has also been observed.[3, 4] A similar complex has been detected in adenosine, adenylyl (3′→5′) adenosine or adenylyl adenylyl adenosine-poly U systems.[5-7] Although two bonding schemes (I, II) for the triple-stranded structure, structure II has been tentatively assigned as the favored scheme from the infrared studies of H. T. Miles.[8]

Complexes of a 1:1 nature have been observed by pmr spectroscopy between adenylyl adenylyl adenosine and adenylyl adenylyl guanosine and poly U. The spectral data implies that the Watson-Crick bonding scheme is favored in the former complex while the Hoogsteen bonding scheme is predominant in the latter.[7]

It is proposed that O^{17} nuclear magnetic resonance spectroscopy be implemented to confirm the bonding schemes for the various complexes deduced from the optical and pmr studies.

O^{17} NMR should provide clear and conclusive proof as to


H
H
O
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(R)
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H
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H
O
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H
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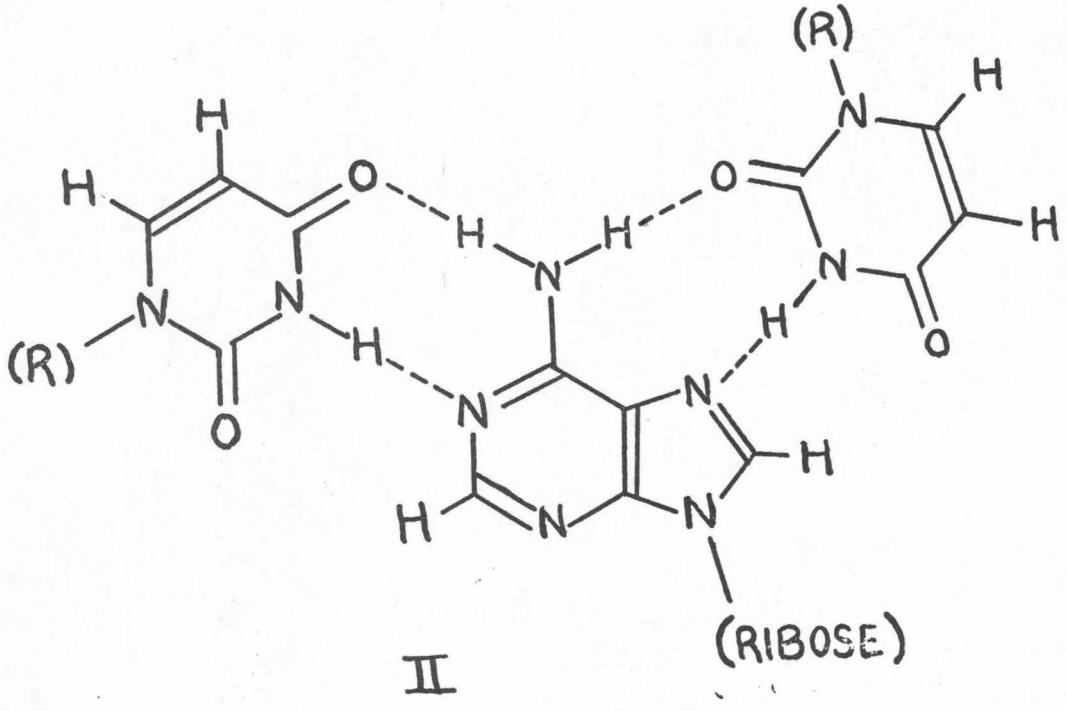
(RIBOSE)

I


(R)
H
H
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O
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H
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N
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(RIBOSE)

II

which of the two oxygens of a given uracil base are involved in hydrogen bonding. Over the temperature range where the rate of chemical exchange between a free and bound state is fast on the NMR time scale, the chemical shift of the oxygen resonance involved in hydrogen bonding will be the weighted average of the two states. Since an oxygen exhibiting hydrogen bonding is expected to be shifted upfield, the weighted average of the chemical shift of such an oxygen is also expected to be shifted upfield.

In a system where the triple-stranded complex forms, both oxygen resonances are expected to be shifted upfield if structure II is the favored scheme, while for structure I only the O_4 resonance is expected to be shifted. In the presence of complexes of a 1:1 nature, the O_4 resonance would be shifted if a Watson-Crick scheme is favored and the O_2 resonance will be affected if the Hoogsteen scheme predominates.

APPENDIX

Due to the low natural abundance and low sensitivity of the O^{17} nucleus, a uracil base must be synthesized that is enriched in O^{17} at the 2, 4-positions. The following synthesis can be implemented to produce this substituted compound. 2, 4-dichloropyrimidine is dissolved in absolute ethanol ($C_2H_5O^{17}H$) and the resulting solution is mixed with metallic sodium ion absolute ethanol ($C_2H_5O^{17}H$). The solution is concentrated under reduced pressure and the residue is then dissolved in 30% NaOH. Extraction with ether yields 2, 4-diethoxypyrimidine with O^{17} in the 2, 4-positions.[9] The addition of acetobromoribose to this compound followed by heat and alcoholic hydrochloric acid treatment yields the uridine nucleotide.[10] The uridine can then be converted to the 2′-phosphate[11] which in turn can be converted to the 2′ 3′ cyclic phosphates.[12, 13] Poly U with a (3′→5′) phosphate ester chain having an average length of 12 nucleotides can be synthesized from the tri-n-octylammonium salt of the cyclic phosphate in dioxane in the presence of tetraphenyl pyrophosphate and tri-n-butylamine.[14]

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PROPOSITION III

Determination of the Equilibrium Constant for the Nonactin-K^+ Complex

Nonactin, a macrotetrolide antibiotic, has been shown to bind selectively to potassium ions and as a consequence of this property has many effects on the metabolism of a cell. Its most important function in the cell is the facilitation of the transport of potassium ions across lipid bilayer membranes. It is postulated that the appropriate rings diameters of this type of antibiotics permit the carbonyl oxygens of the ring to substitute for the water molecules of the cation hydration sphere which allows the cation to pass through an organic membrane.[1, 2] Nonactin has also been shown to induce adenosine triphosphate activity in the presence of alkali metal ions other than lithium and its activity varied with the ion present (Na < Cs < K < Rb).[3] Rat mitochondria have shown induced swelling in the presence of nonactin and alkali metal ions.[4]

Although an approximate equilibrium constant for the complex has been determined in two solvents,[5, 6] a more accurate value of the binding constant and the diffusitivity of the complex in various organic solvents is needed. It is therefore proposed that these values be determined by electrochemical techniques. The structure of the nonactin-K^+ complex has been determined by X-ray crystallography (Figure 1).[7] The difference in size of this complex and that of the potassium ion in solution should be reflected in its mobility and thus

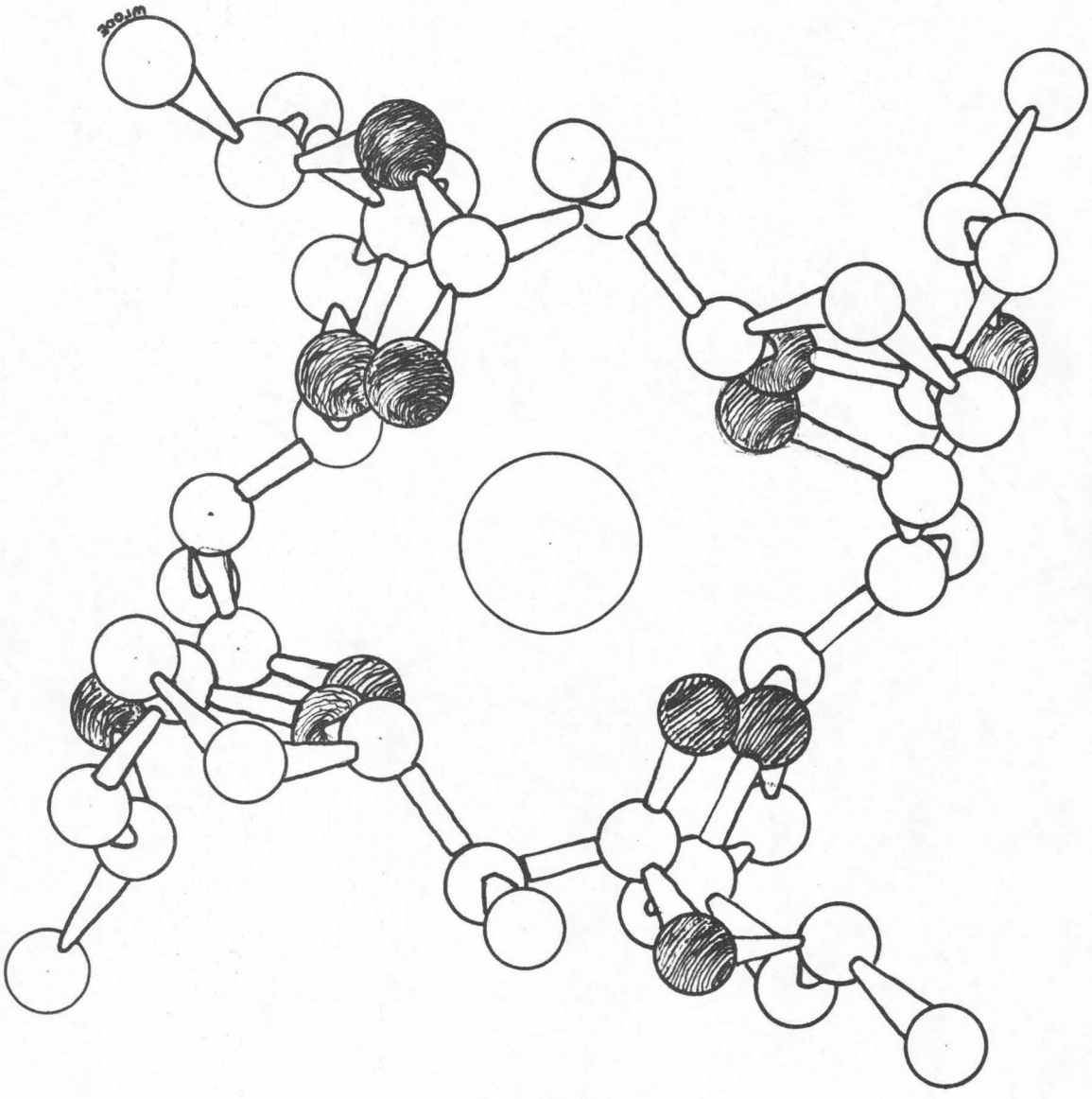

FIGURE 1

Nonactin

its transference number.

Experimentally, the equivalent conductance is given by[8]

$$\Lambda = \frac{1000}{C} \kappa$$

where C = concentration in equivalents per liter

κ = specific conductance κ = (cell constant) $1/R$.

R = resistance of the cell.

The determination of the equivalent conductance at infinite dilution for organic solvents with a low dielectric constant is complicated by the formation of ion pairs. The ion pairs are relatively stable to thermal dissociation because the coulombic attractive forces between two oppositely charged ions are large compared to kT. In an external field, the ion pair acts as a dipole and experiences a torque causing it to rotate but neither ion of the pair can contribute to the transport of charge. Thus the ionic association reduces the number of charge carriers and consequently the equivalent conductance.[9]

Fuoss and Kraus have developed a method of successive approximations which takes into account the formation of ion pairs. For a completely dissociated electrolyte, the equivalent conductance would be

$$\Lambda = \Lambda_0 - S_\Lambda \sqrt{C} .$$

If only a fraction (α) of the electrolyte is present as ions, the equation becomes

$$\Lambda = \alpha (\Lambda_0 - S_\Lambda \sqrt{\alpha C}) .$$

Letting

$$F(Z) = 1 - Z\{1 - Z[1 - Z(\text{etc.})^{-\frac{1}{2}}]^{-\frac{1}{2}}\}^{-\frac{1}{2}}$$

in which $Z = S_\Lambda \sqrt{\Lambda C}\,(\Lambda_0)^{-\frac{3}{2}}$, the equation for the degree of dissociation becomes

$$\alpha = \frac{\Lambda}{\Lambda_0 F(Z)} \tag{1}$$

A rough value of Λ_0 can be obtained by free-hand extrapolation of the experimental data. This value is used to calculate $F(Z)$ and α. Combining the thermodynamic ionization constant

$$K = \frac{C \alpha^2 y_\pm^2}{1 - \alpha}$$

where $\log y_\pm = -S_{(f)} \sqrt{\alpha C}$ with equation 1, one obtains the equation

$$\frac{F(Z)}{\Lambda} = \frac{1}{\Lambda_0} + \frac{[C \Lambda y_\pm^2 / F(Z)]}{K(\Lambda_0)^2} .$$

By plotting $F(Z)$ versus $C \Lambda y_\pm^2 / F(Z)$, a better value of Λ_0 than the original guess is obtained. The process is redone until a satisfactory Λ_0 is obtained.[10] Kohlrauch's law of independent migration of ions states that

$$\Lambda_0 = \sum_{i=1}^{n} \lambda_{0n}$$

where λ_{0n} is the equivalent ionic conductance of the n^{th} ion and that at infinite dilution, the contribution of an individual ion is independent of the others.[11] In a system such as potassium picrate (KPi) and nonactin in acetone, $\lambda_{0(K^+)}$ and $\lambda_{0(Pi)}$ can be determined from equivalent

conductance and transference number studies of KPi alone in acetone which automatically gives $\lambda_{o\,(K^+_{NON})}$.

The transference number (n_+) for all potassium ions in the presence of nonactin can be determined by the Hittorf method where[12]

$$n_+ = \frac{\Delta n_a}{Q\mathcal{F}}$$

Δn_a = change in equivalent weights of K^+ in the anode compartment

Q = current passed through

$\mathcal{F}$ = Faraday's constant.

Another expression for the transference number is[13]

$$n_+ = \frac{\lambda_K + [K^+] + \lambda_{K^+_{NON}} [K^+_{NON}]}{1000\,\kappa} \qquad (2)$$

The equivalent conductance of each ion at concentrations less than 0.05 M can be determined by the Onsager equation[14]

$$\lambda_i = \lambda_{i\,o} - S\sqrt{C} \ .$$

Since some of the potassium ions are complexed, the free potassium ion concentration is given by

$$[K^+] = C - [K^+_{NON}] \ .$$

Using this relationship, equation 2 becomes

$$n_+ = \frac{\lambda_{K^+}(C - [K^+_{NON}]) + \lambda_{K^+_{NON}}[K^+_{NON}]}{1000\,\kappa}$$

which can be solved for $[K^+_{NON}]$, thereby giving $[K^+]$ and $[NON]$. With these values, one can determine the equilibrium constant for the nonactin-K^+ complex.

The ionic mobility is given by[15]

$$\nu_+ = \frac{(\lambda_+)_0}{\mathcal{F}} \quad .$$

The diffusivity of the K^+_{NON} ion in the solvent can be determined from the Einstein relation[16]

$$D = kT \cdot \nu_+$$

The same procedure can be used for solutions of other alkali metal ions and nonactin in various organic solvents.

APPENDIX

An approximate idea of the change in the transference number of K^+ in the presence of nonactin can be obtained from examination of the system which is 10^{-4} M in KPi and 10^{-3} M in nonactin in acetone. Since the binding constant for the nonactin-K^+ complex is relatively high in acetone,[7] the effects of free K^+ ions can be neglected in this calculation. Equation 2 then reduces to

$$n_+ = \frac{\lambda_{K^+_{NON}} [K^+_{NON}]}{\lambda_{K^+_{NON}} [K^+_{NON}] + \lambda_{Pi} - [Pi^-]} \qquad (3)$$

Approximating the K^+_{NON} complex by the $(C_4H_9)_4N^+$ ion which is of comparable size and charge, values for $\lambda_{0\,K^+}$, $\lambda_{0\,Pi^-}$ and $\lambda_{0\,K^+_{NON}}$ of 108, 58 and 96, respectively, can be determined from the known values of Λ_0 for KPi and $(C_4H_9)_4NPi$ and n_+ for K^+ in acetone.[17] Using these values in equation 3, an approximate value of 0.63 for the transference number of all potassium ions in solution is obtained. The difference of this number and n_+ for K^+ alone in solution of 0.65 is greater than experimental error.

Although the values for n_+ for the two systems are fairly close together, the difference between n_+ for K^+_{NON} and K^+ in an actual solution will probably be greater than this approximation. Assuming that organic solvents do not interact strongly with ions and since the nonactin complex is slightly larger than the $(C_4H_9)_4N^+$ ion and a smaller value for $\lambda_{0\,K^+_{NON}}$ might be expected. A 10% decrease in $\lambda_{0\,K^+_{NON}}$ will reduce n_+ for the nonactin system to 0.58 which would make this equipment more feasible.

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PROPOSITION IV

Characterization of the Elusive Uracil-Inosine Base-Pair

In the degenerate interactions of tRNA and mRNA *in vivo*, it has been postulated that a uracil-inosine base-pair exists as one element of the degeneracy.[1] The exact bonding scheme for this base-pair, however, has not been determined nor has a reasonably stable base-pair been observed in mononucleotide systems.[2, 3] Several bonding schemes have been proposed. The "wobble" hypothesis predicts the uracil base moves from its normal position and binds to the inosine with its O_2 group and the N_3-H hydrogen bonding to the N_1-H and O_6 group, respectively, of the inosine.[4] Recently, it has been proposed that tautomeric exchange in the inosine is the mechanism that allows the formation of this pair In this scheme, the inosine is in its *lactim* tautomeric form and exhibits hydrogen bonding similar to that of adenosine.[5, 6]

The sequence of the $tRNA^{Glu}_{III}$ of yeast with SUC as the anticodon, where S is 2-thiouridine, has been determined. Yoshida *et al.* contend that S in the third position blocks the "wobble" scheme and this tRNA binds only to the codon GAA and not GAG.[7, 8] The tautomeric hypothesis claims that S exists in only one tautomeric form and can only bind to an adenosine in the codon.[5, 6]

To help resolve this controversy, it is proposed that the hydrogen-bonding properties of inosine be studied by column

chromatography.

Tuppey and Küchler have demonstrated that a nucleoside can be convalently bonded to a resin and still retain its complementary hydrogen bonding properties. In their system, a purine base, adenosine or guanosine, was bound to the resin and a mixture of the pyrimidine bases, uridine and cytosine, was passed through the column. The complementary base required the longest time to be eluted from the column. The other base, however, was also appreciably slowed in its passage through the column. They postulated that the "non-complementary" base forms abnormal base-pairs with the bound base, which are slightly less energetically favorable than the normal Watson-Crick pairs and are therefore eluted faster. They also contend that these base-pairs are such that the dipole moments of the bases are favorably aligned.[9-11] This interpretation, however, is dubious since these "non-complementary" base-pairs are not observed in nonaqueous solvents.[12-14] The formation of abnormal base-pairs, however, is consistent with the tautomeric hypothesis.[5, 6]

The various proposals can be differentiated in the following system. With inosine bound to the resin, a mixture of uridine, 2-thiouridine and cytosine in aqueous and nonaqueous (i. e. DMSO) solutions is passed through the column. Under both conditions, the cytosine should remain on the column the longest. If the "wobble" hypothesis is correct, then the 2-thiouridine would be eluted much faster than uridine in both solvents. If Tuppey and Küchler's hypothesis is correct, then both the uridine and 2-thiouridine should be

eluted in approximately equal time in both solvents. Since inosine might be expected to be in its normal tautomeric form in the non-aqueous solvent, the uridine and 2-thiouridine should exhibit only slight retention on the column in this system if the tautomeric hypothesis is correct. In aqueous solution, both uridine and 2-thiouridine should be retained for approximately equal times.

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PROPOSITION V

Dissociative Recombination of CO_2^+ Ions With Electrons in the Upper Atmosphere of Mars

Recently the ultraviolet spectra of the upper atmosphere of Mars have been returned by the Mariner 6 and 7 spacecraft. Barth et al. have attributed the spectral lines to various electronic transitions that accompany the reactions of CO_2, CO_2^+, CO and O between themselves and the photon and electron dissociative excitation of the various constituents. They did, however, rule out the dissociative recombination of CO_2^+ ions with electrons as one contribution to the Cameron bands of the spectra. Even though it is energetically capable of producing these bands, the laboratory measured recombination rate was too small to produce sufficient recombinations.[1]

Weller and Biondi measured the value of the dissociative recombination coefficient, $\alpha(CO_2^+)$, for the reaction

$$CO_2^+ + e^- \longrightarrow CO + O$$

by using microwave-afterglow differentially pumped quadrupole mass spectroscopy.[2] Their value for $\alpha(CO_2^+)$ of $3.8 \pm 0.5 \times 10^{-7}$ cm^3/sec was an order of magnitude less than the required coefficient of $\sim 10^{-6}$ cm^3/sec for one model of the Martian atmosphere[3] and for recombination to have an effect on the UV spectrum.[1] They generated a quasi-neutral plasma of CO_2^+ and electrons by ionizing CO_2 in a microwave

cavity by means of a pulsed magnetron. The decay of the CO_2^+ population was monitored by the quadrupole mass spectrometer and the electron density was determined by the standard cavity frequency shift technique.[4] Subsequent analysis of the decay of each constituent yielded the rate constant for the reaction.

Although the ratio of electrons to molecules used in this study approximated the predicted ratio in the upper atmosphere of Mars,[5] the laboratory rate constant may be different from the actual one for the reaction in the Martian atmosphere. Under the conditions used in the laboratory, the various components of the plasma are basically in thermal equilibrium with each other. This need not be the case in the Martian atmosphere. One of the proposed mechanisms for the formation of CO_2^+ is the electron impact excitation of neutral CO_2 by photoelectrons,

$$CO_2 + e^- \longrightarrow CO_2^+ + 2\,e^- \qquad E > 17.3 \text{ ev}$$

Since one source of high energy electrons is the electron flux of the solar wind and the energy of these electrons is much greater than electrons in thermal equilibriumin the Martian atmospheric plasma,[6] this reaction will produce electrons with sufficiently greater energy than the thermal electrons. After subsequent reactions an equilibrium will be reached and there will be a given distribution of energies for the electrons in the atmosphere. This distribution can be represented by

$$[e^-]_{total} = [e^-]_{thermal} + \sum_{n=1}^{N} [e^-]_i$$

where $[e^-]_i$ is the number of electrons with energies in the range $E_i + \Delta E$. Assuming that all of the CO_2^+ ions are close to thermal equilibrium, the rate constant of the reaction of the CO_2^+ ion and an electron will vary with the energy of the electrons. The new rate equation will be

$$\frac{d[CO]}{dt} = [CO_2^+] \left\{ \alpha_{lab} [e^-]_{thermal} + \sum_{i=1}^{N} \alpha_i [e^-]_i \right\}$$

Thus the effective rate constant for the reaction under the conditions of the Martian atmosphere may be totally different from that measured in the laboratory under near thermal conditions.

It is therefore proposed that the rate constant for the dissociative recombination of CO_2^+ ions with electrons be measured under conditions that better approximate those in the Martian atmosphere. The same experimental apparatus of Weller and Biondi with one modification can be used.[2, 7] Instead of producing the ionized plasma by microwave excitation, the CO_2 in the microwave cavity can be excited by ultraviolet light and high energy electrons as they would be on Mars. After a sufficient time of excitation, the loss of CO_2^+ and electrons can be monitored. The apparent rate constant of the dissociative recombination can be followed as a function of energy of the impact electrons and a more realistic rate constant for this reaction in the Martian upper atmosphere can be determined.

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