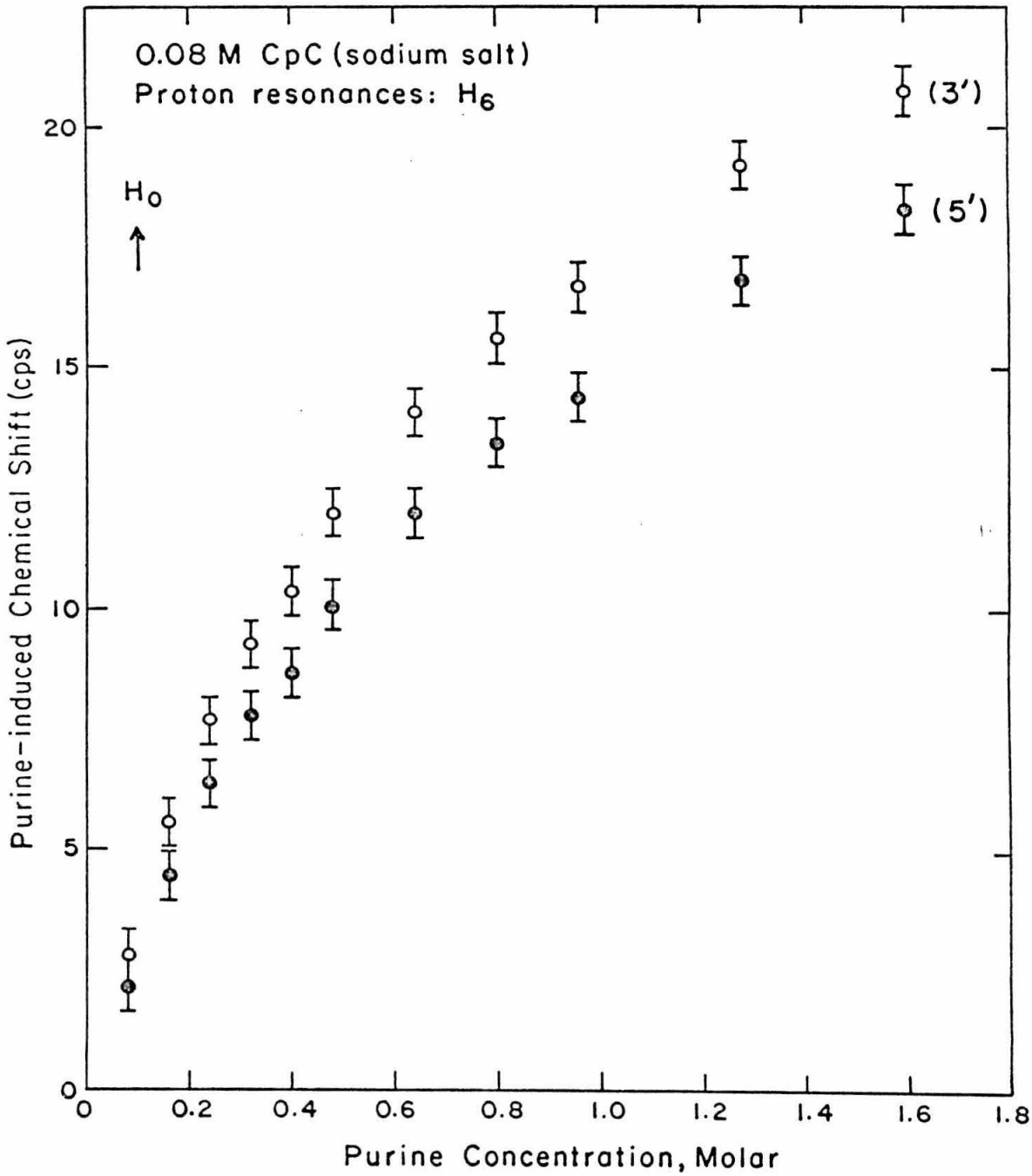
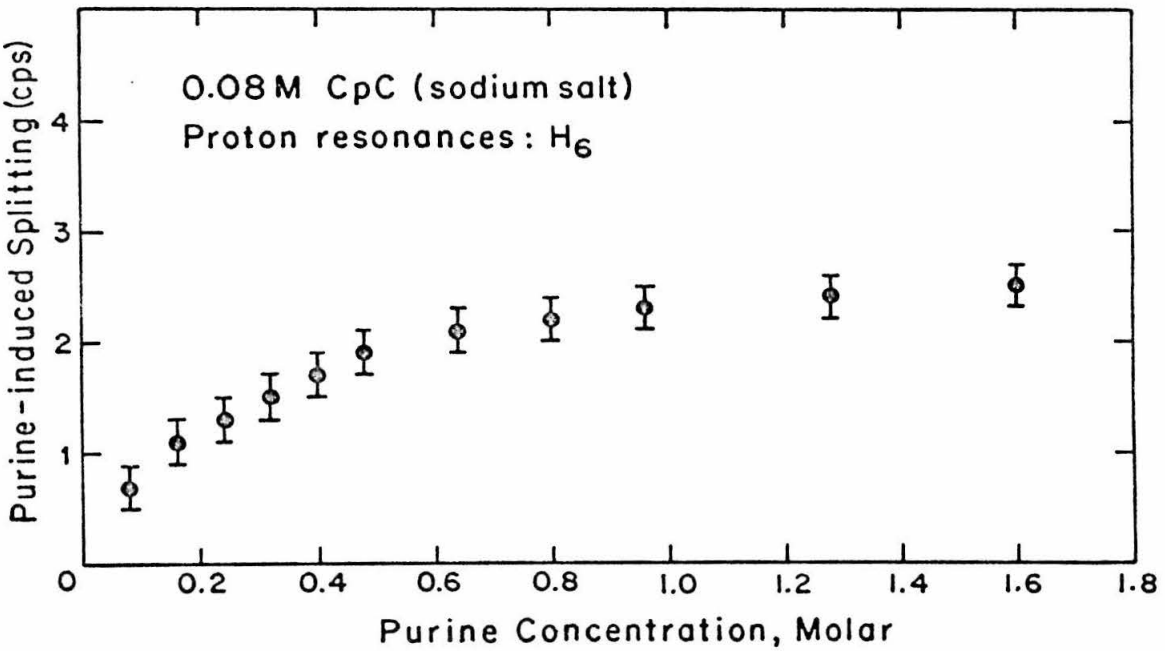

0.08 M CpC (sodium salt)
Proton resonances: H6
(3')
(5')
H0
Purine-induced Chemical Shift (cps)
Purine Concentration, Molar
0
5
10
15
20
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8

FIGURE 38

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


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

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

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

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

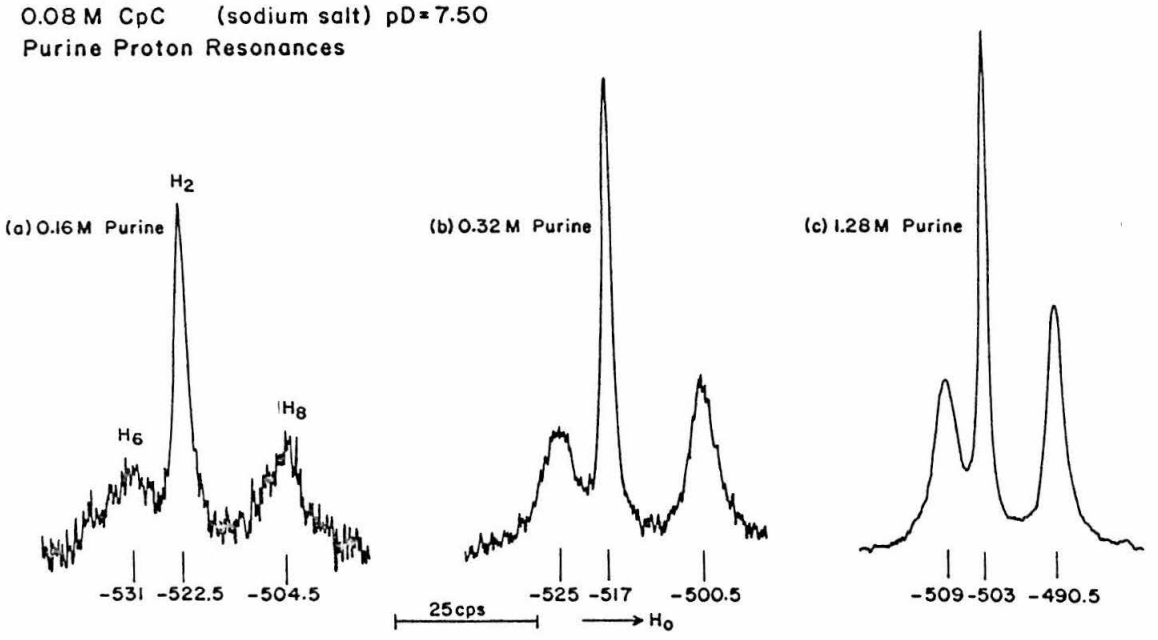
TABLE XIX. Purine proton resonance linewidths in the presence of 0.08 M CpC (sodium salt, pD = 7.50). (Full linewidth at half-height in cps, measured at 60 Mcps).

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

[a]Too broad to measure accurately.

FIGURE 39

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


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

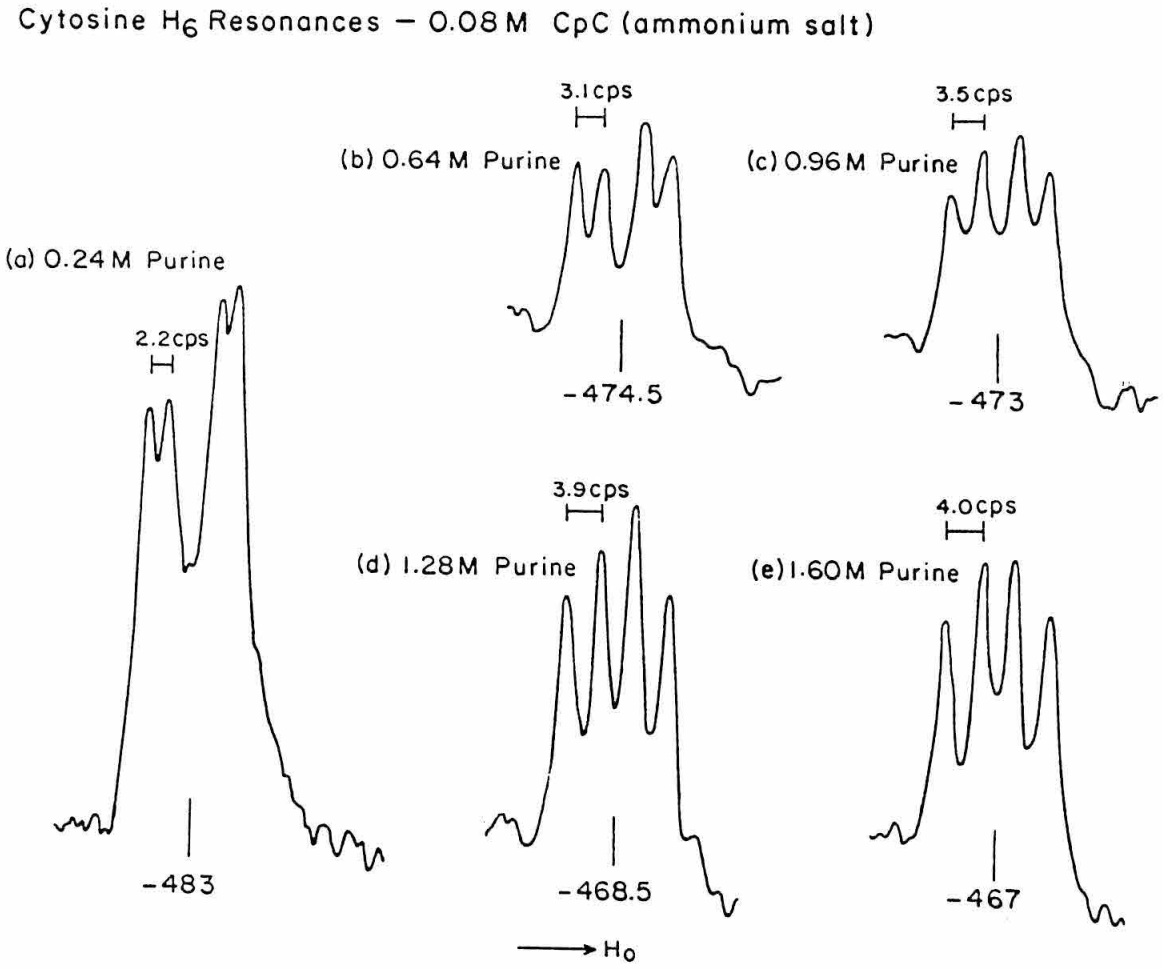
of the ammonium salt of a dinucleoside monophosphate has a pD of about 5 as a result of the acidic hydrolysis of the NH_4^+ ion. Since the cytosine base has a pK of about 4. 3, [98] a fraction of the bases of CpC will be protonated in a solution of the ammonium salt of this dinucleotide in the absence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

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

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

FIGURE 40

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


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

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

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

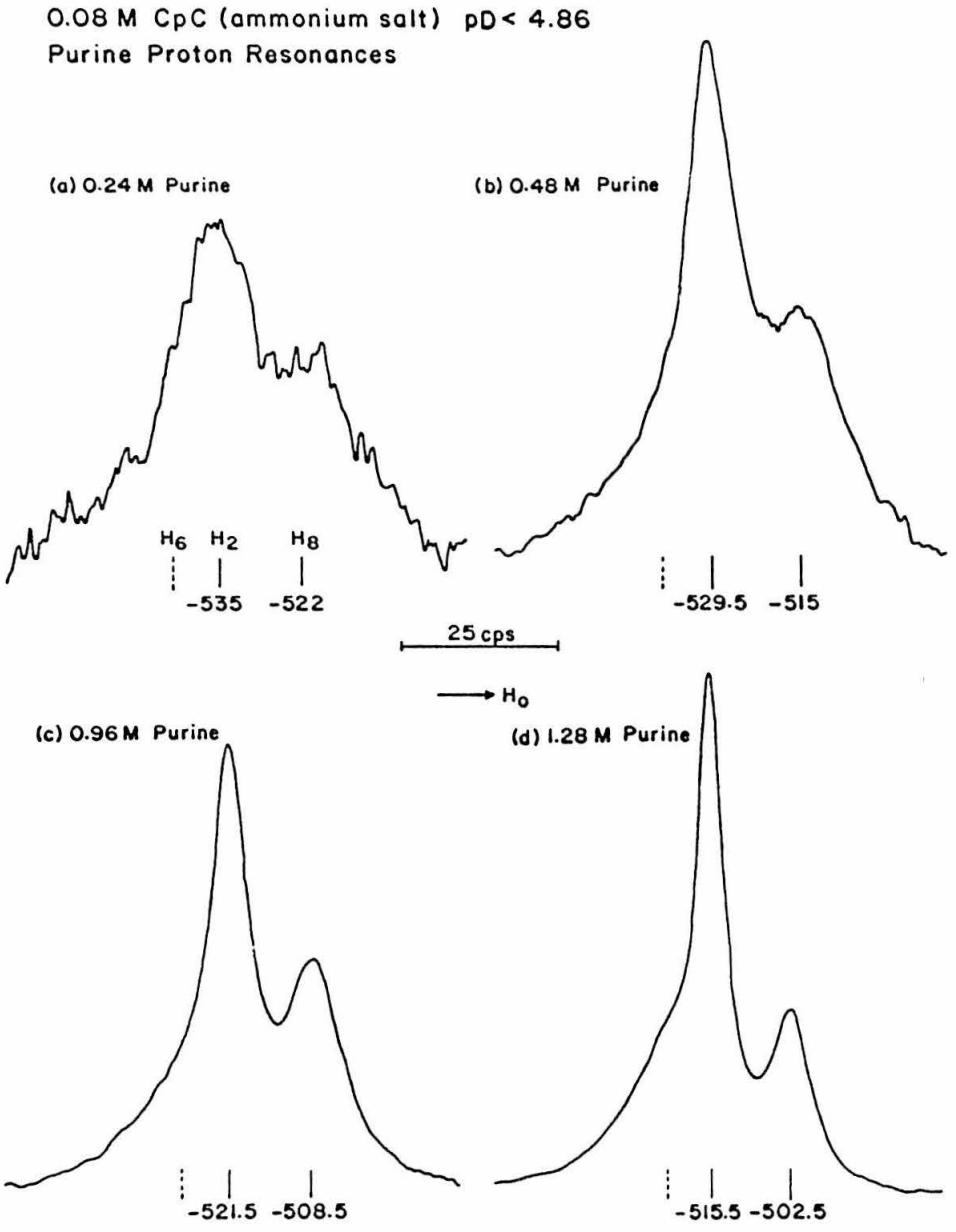
TABLE XX. Purine proton resonance linewidths in the presence of 0.08 M CpC (ammonium salt, pD < 4.86). (Full linewidth at half-height in cps, measured at 60 Mcps).

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

[a]Too broad to even be estimated.

FIGURE 41

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


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

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

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

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

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

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

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

FIGURE 42

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

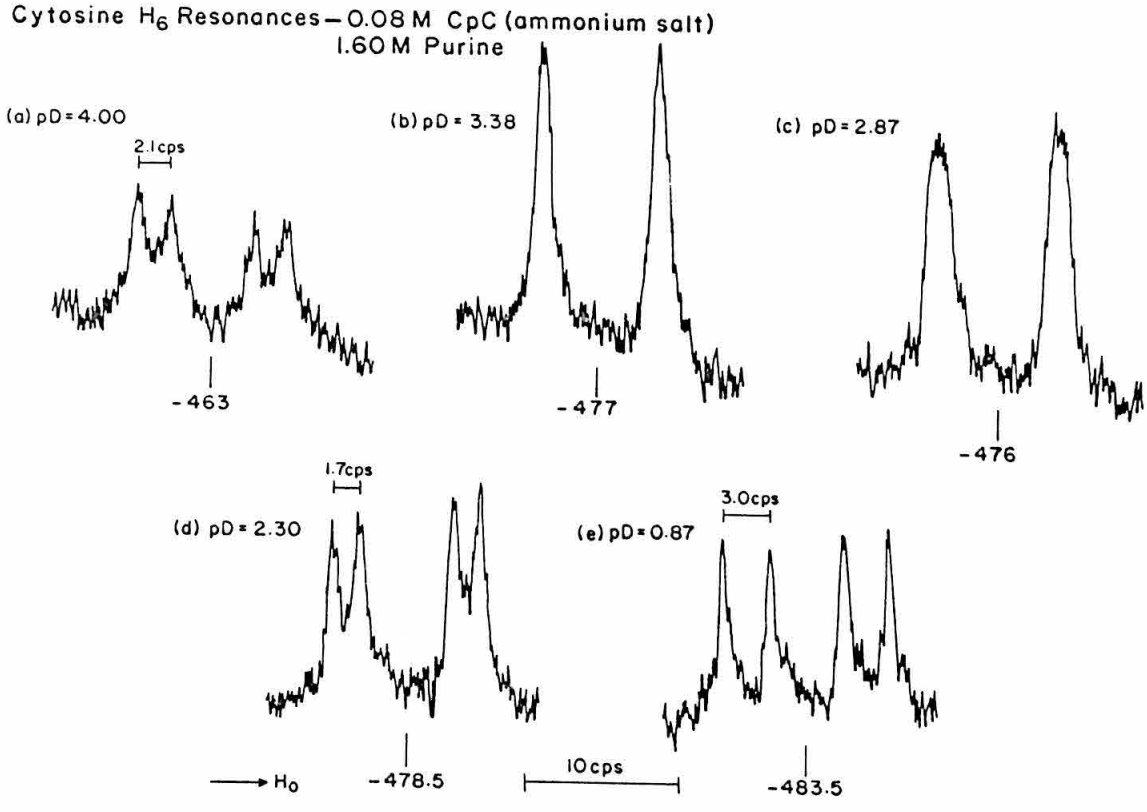

Cytosine H_6 Resonances — 0.08 M CpC (ammonium salt)
1.60 M Purine
(a) pD = 4.00
2.1cps
-463
(b) pD = 3.38
-477
(c) pD = 2.87
-476
(d) pD = 2.30
1.7cps
-478.5
(e) pD = 0.87
3.0cps
-483.5
H_o
10cps

FIGURE 43

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

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

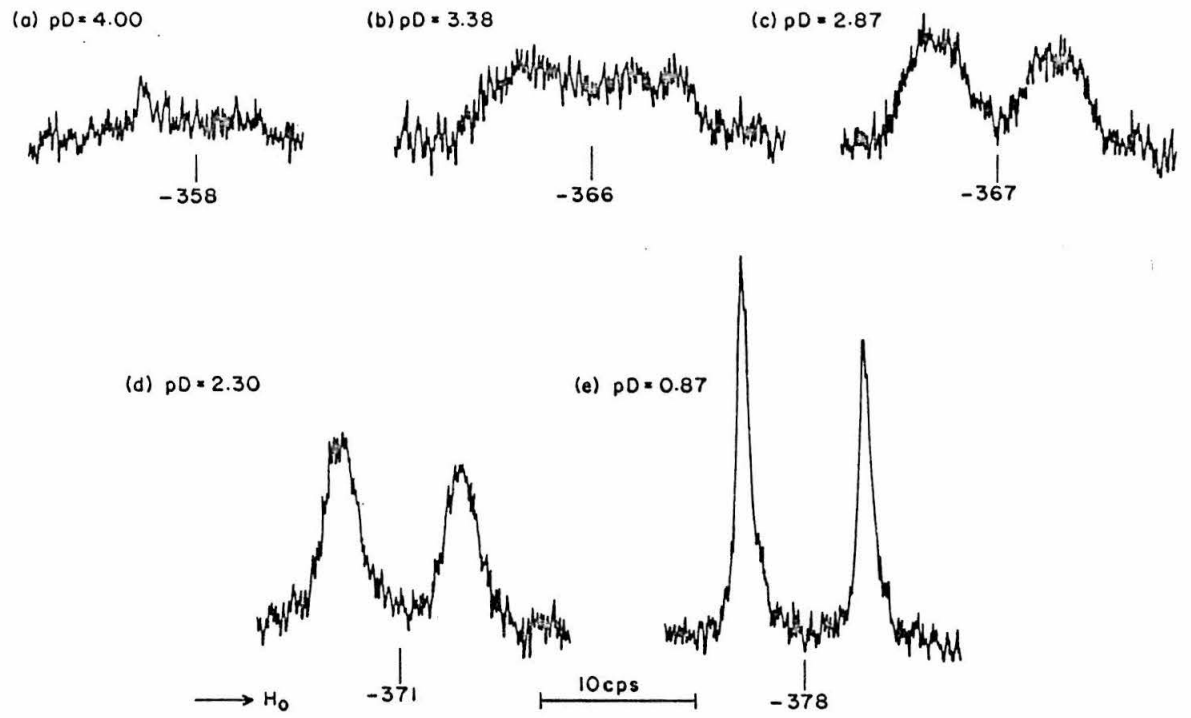


FIGURE 44

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

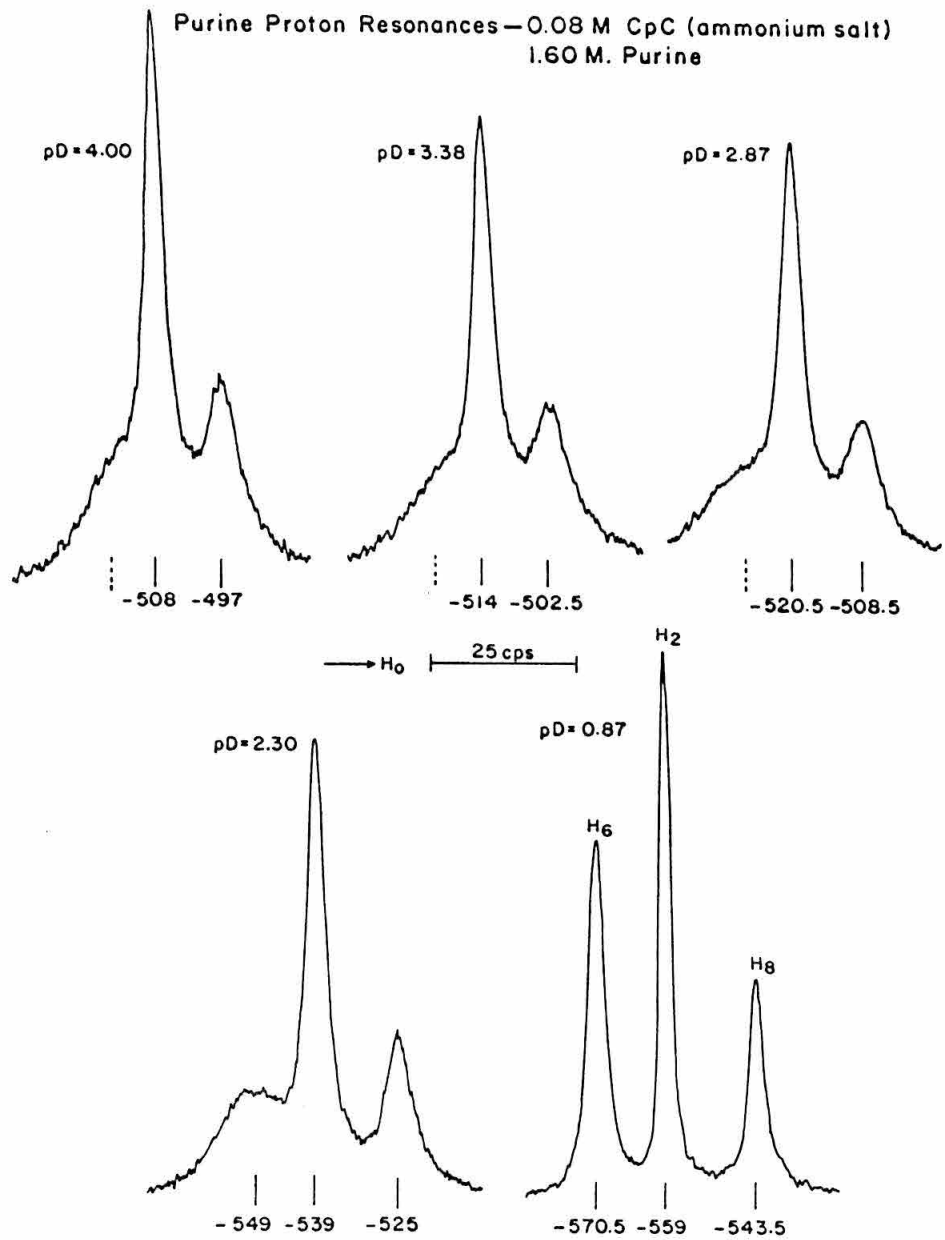

Purine Proton Resonances—0.08 M CpC (ammonium salt)
1.60 M. Purine
pD = 4.00
-508 -497
pD = 3.38
-514 -502.5
pD = 2.87
-520.5 -508.5
→ H₀
25 cps
pD = 2.30
-549 -539 -525
pD = 0.87
H₂
H₆
H₈
-570.5 -559 -543.5

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

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

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

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

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

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

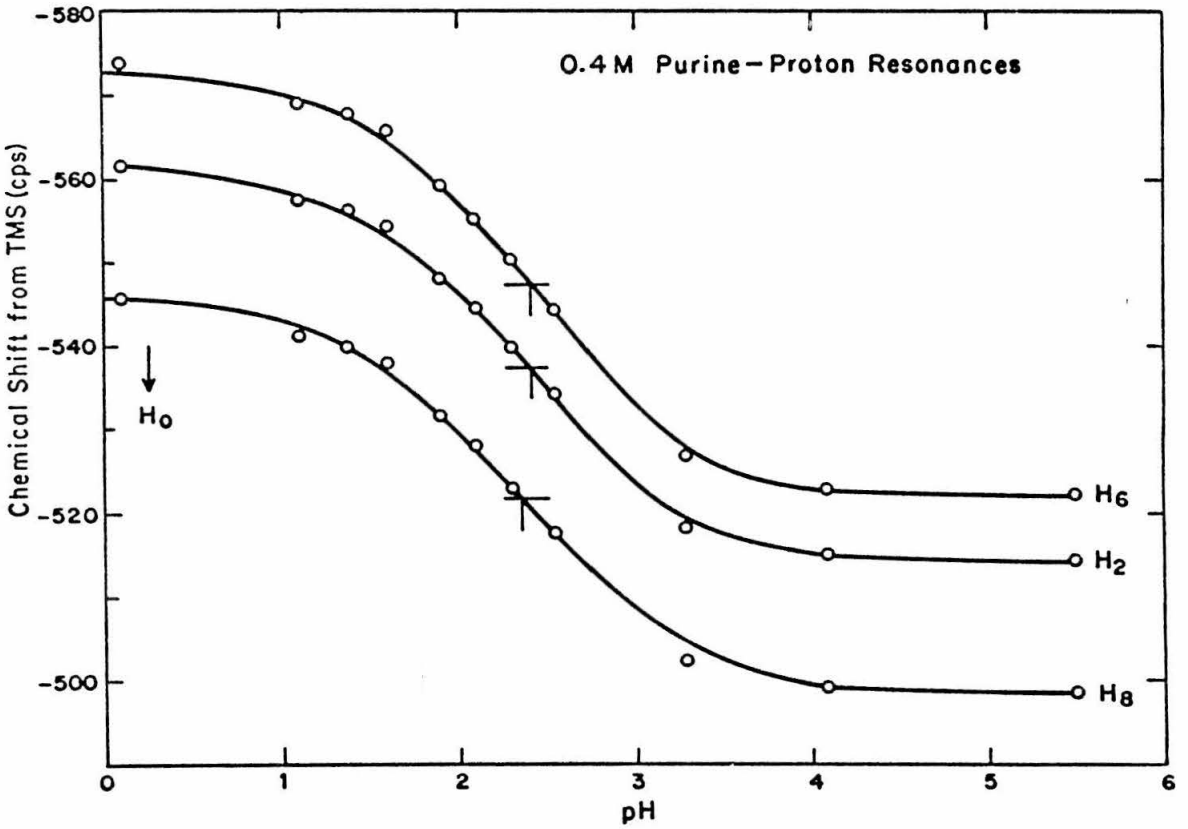
agree well with the pH behavior of a 0.4 M purine solution in the absence of the dinucleotide, shown in Figure 45. The purine resonances begin to narrow appreciably only below pD 2.30, where all of the cytosine bases and more than half of the purine molecules are protonated.

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

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

FIGURE 45

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


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

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

6. Uridylyl (3′→5′) Uridine

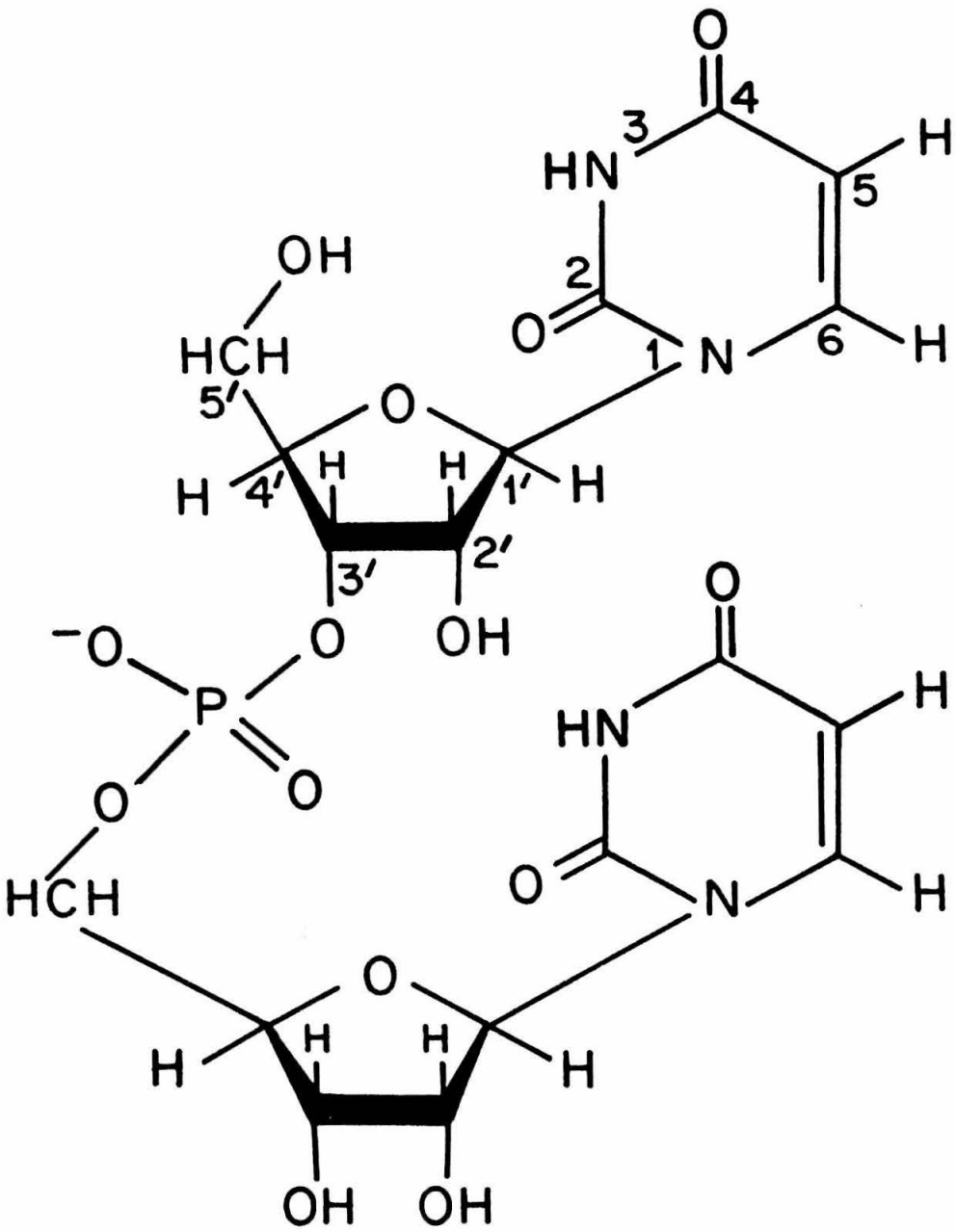
The ribose dinucleoside monophosphate UpU, shown in Figure 46, was investigated at several concentrations and temperatures, and the interaction of this dinucleotide with purine was examined at both high and low UpU concentrations.

6.1. Pmr Spectrum of UpU

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

FIGURE 46

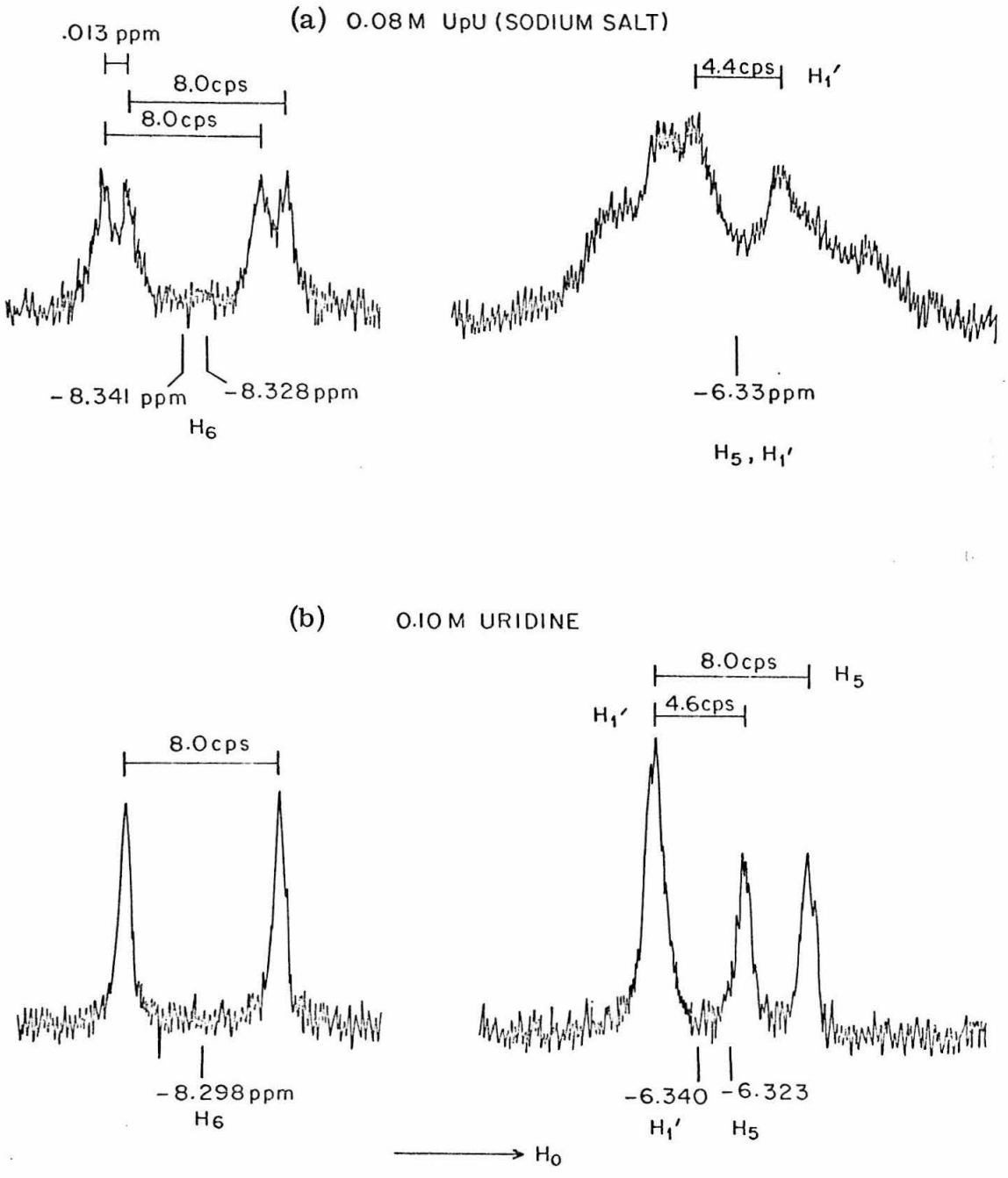
Structural diagram of uridylyl (3′→ 5′) uridine, UpU.



UpU

FIGURE 47

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


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

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

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

6. 1. 1. Assignment of resonances

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

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

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

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

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

6. 1. 2. Discussion of chemical shifts

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

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

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

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

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

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

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

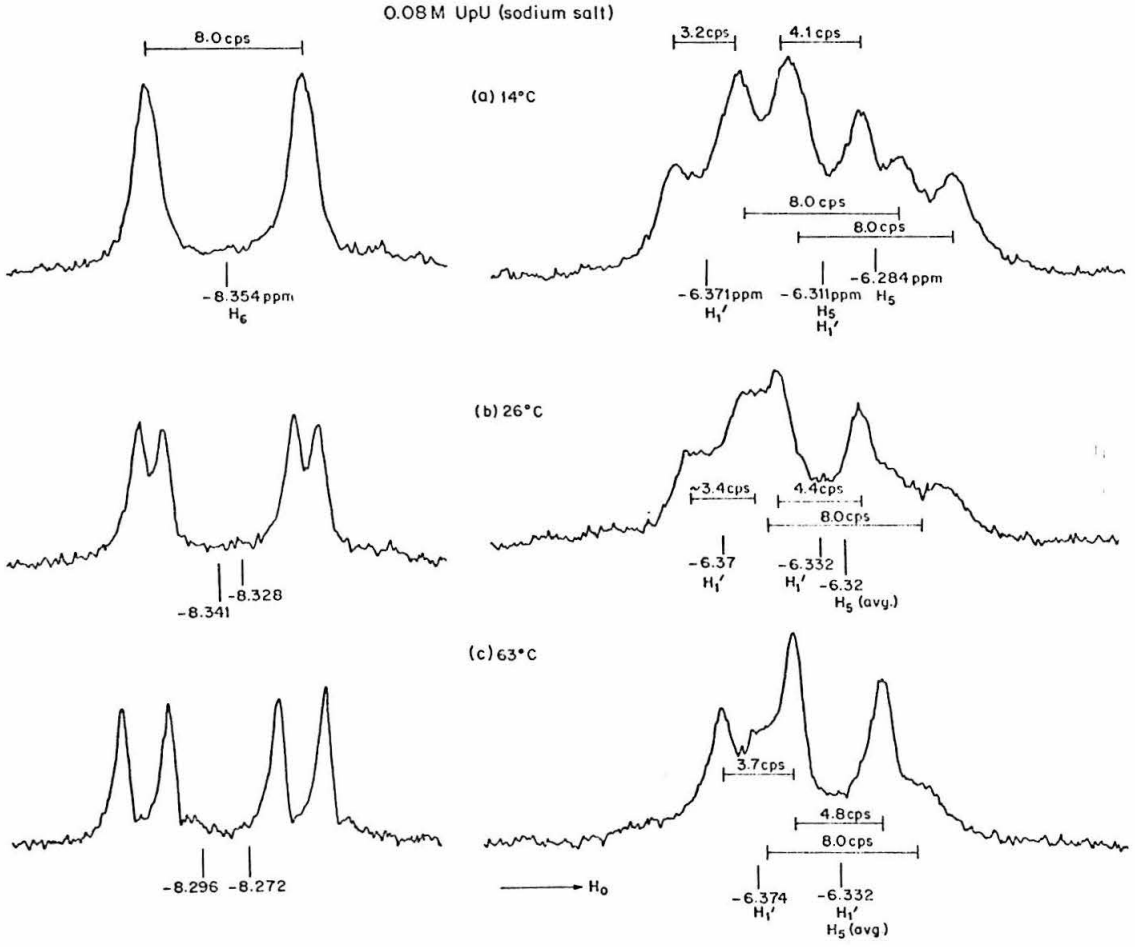
6. 2. Temperature Dependence

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

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

FIGURE 48

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


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

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

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

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

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

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

6. 3. Purine Binding Studies

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

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

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

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

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

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

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

FIGURE 49

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

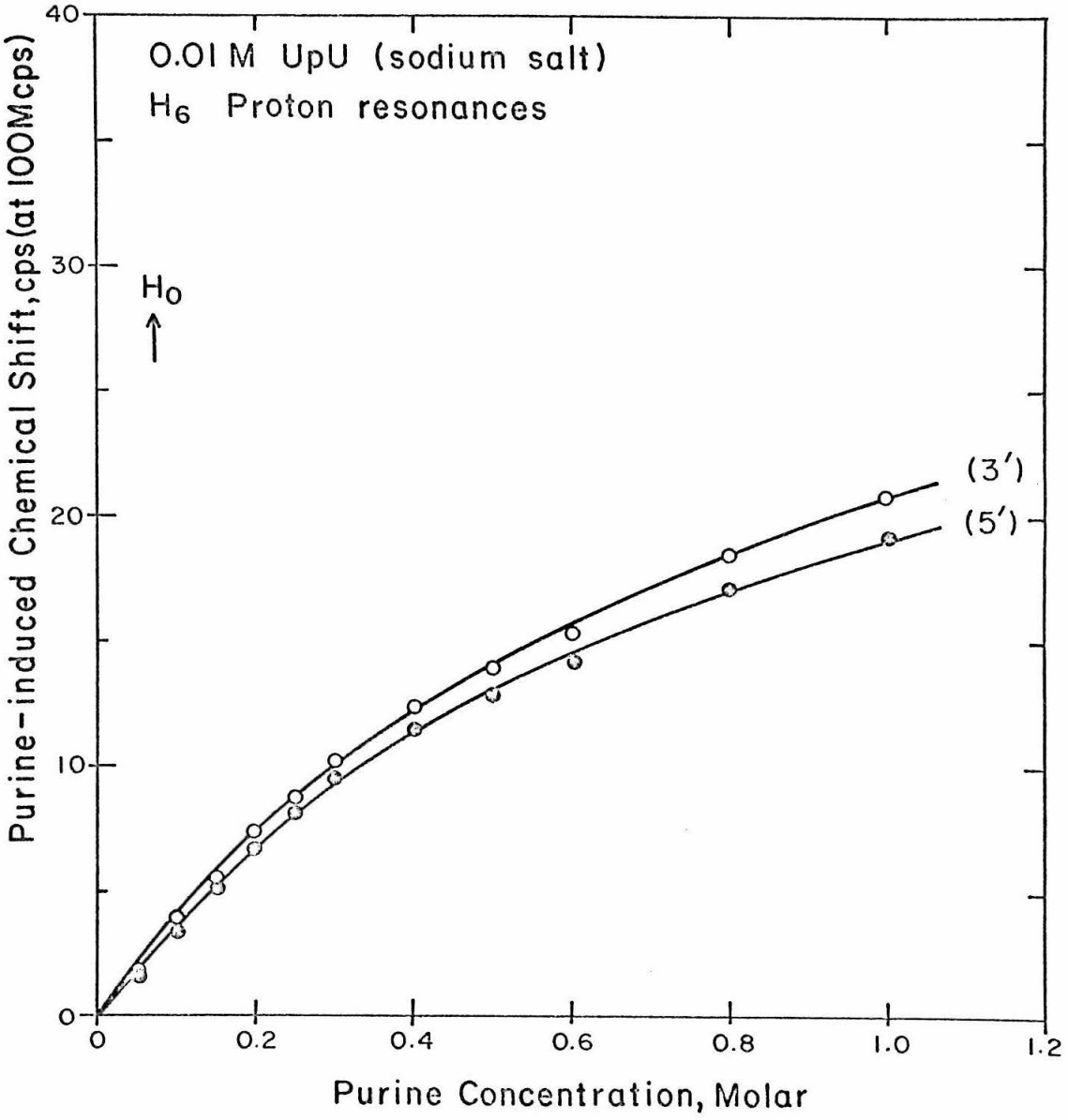

0.01 M UpU (sodium salt)
H6 Proton resonances
Ho
(3′)
(5′)
Purine-induced Chemical Shift, cps (at 100Mcps)
Purine Concentration, Molar
0
10
20
30
40
0
0.2
0.4
0.6
0.8
1.0
1.2

FIGURE 50

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

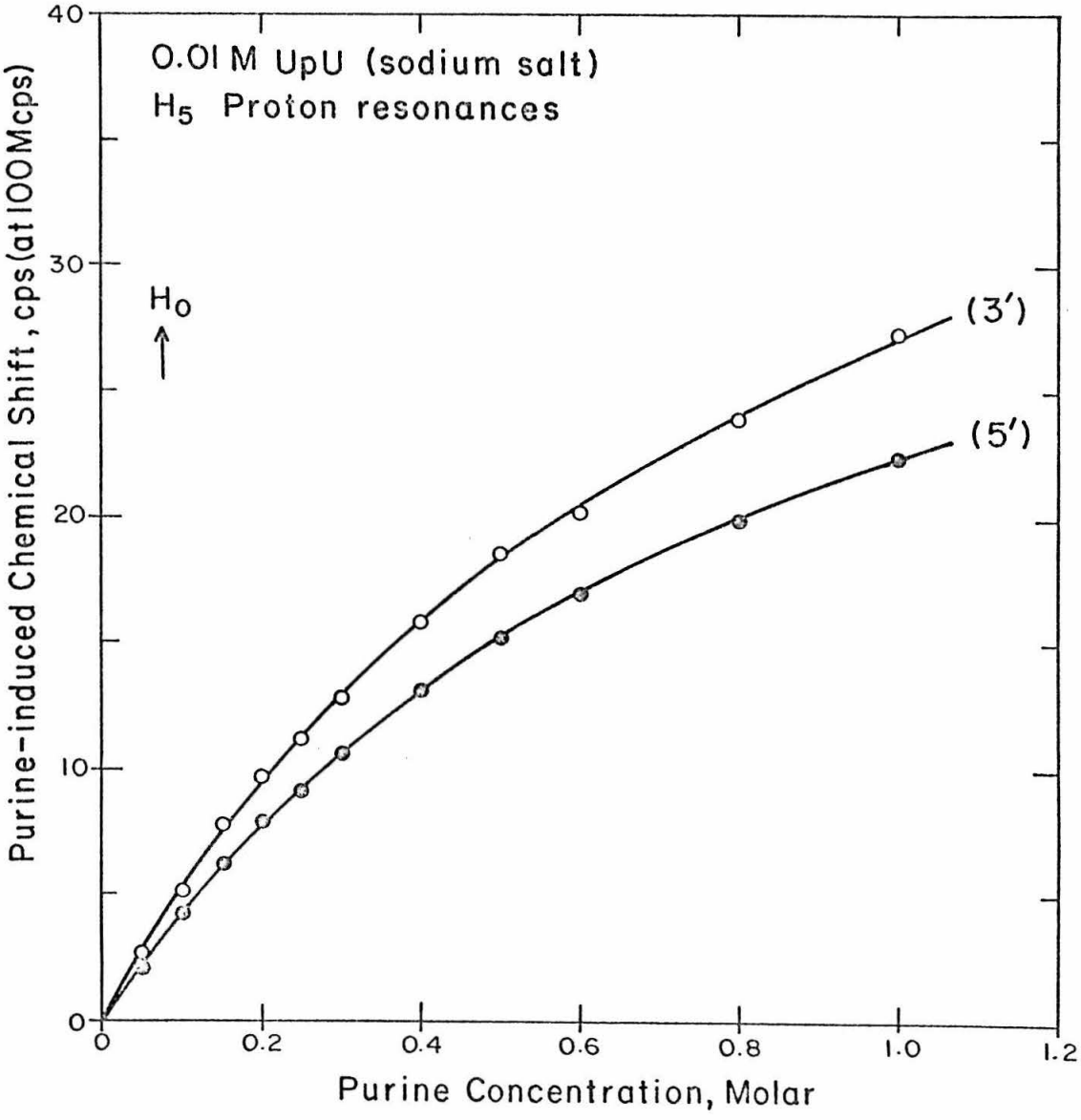
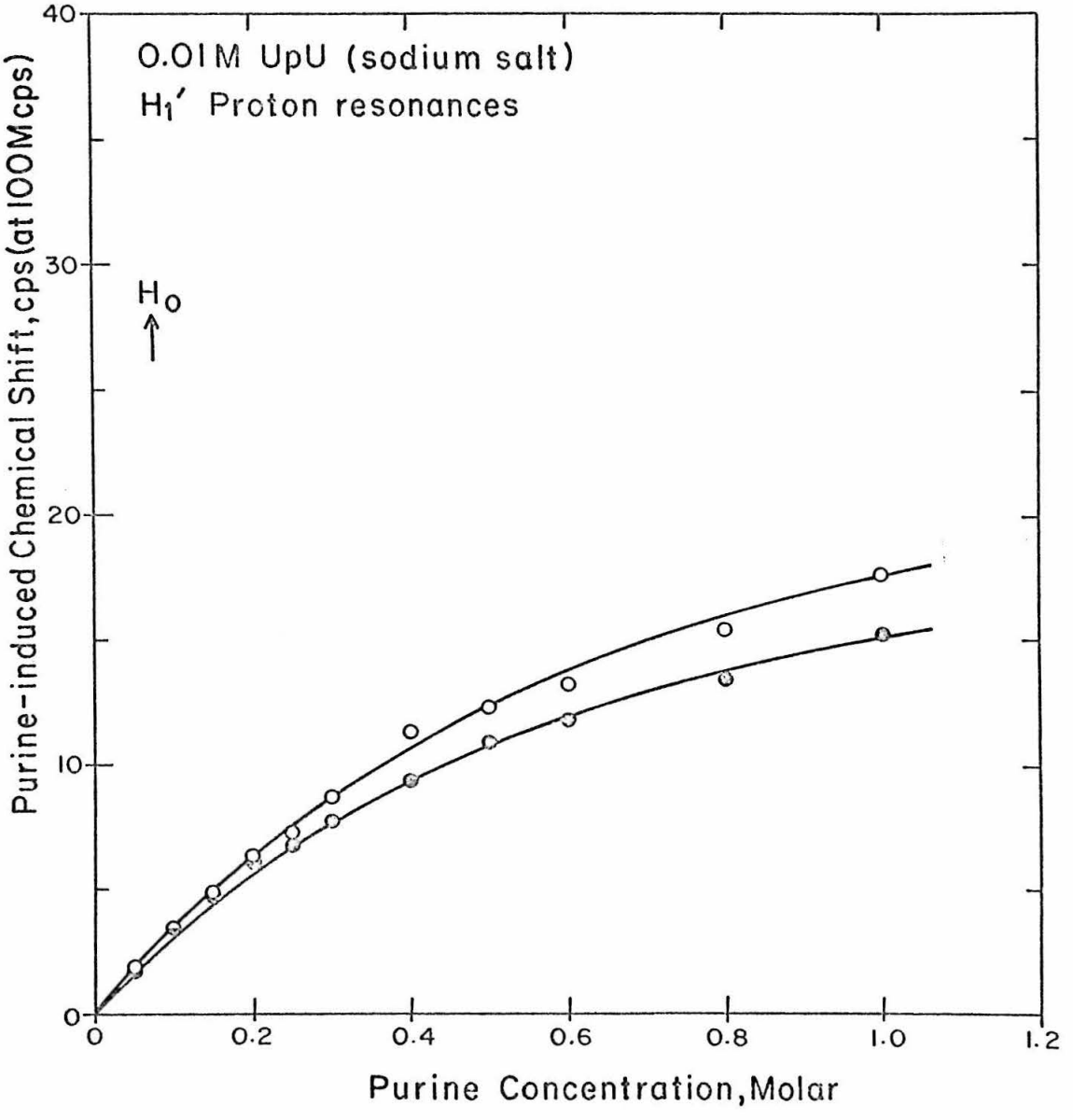

0.01 M UpU (sodium salt)
H5 Proton resonances
Ho
(3′)
(5′)
Purine-induced Chemical Shift, cps (at 100 Mcps)
Purine Concentration, Molar
0
10
20
30
40
0
0.2
0.4
0.6
0.8
1.0
1.2

FIGURE 51

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


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

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

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

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

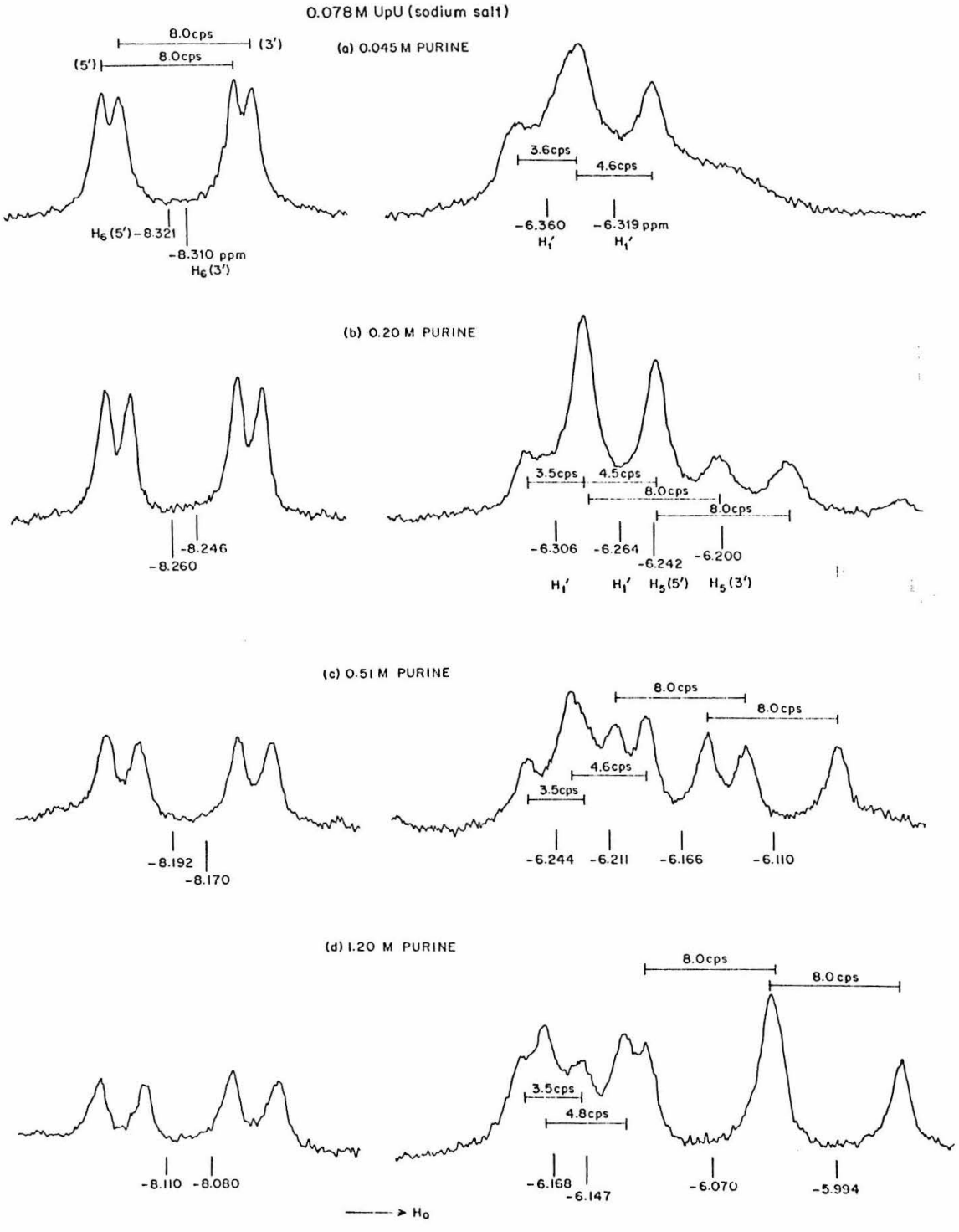
and become narrower as the purine concentration is increased. At 0. 05 M purine concentration, the linewidths are as follows: H_6, 4. 6 cps; H_2, 1. 8 cps; H_8, 3. 6 cps. This suggests that an intercalated complex is formed between purine and UpU, as with the other dinucleoside monophosphates whose interactions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

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

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

FIGURE 52

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


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

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

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

As mentioned previously, the nucleosides have no apparent

FIGURE 53

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

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

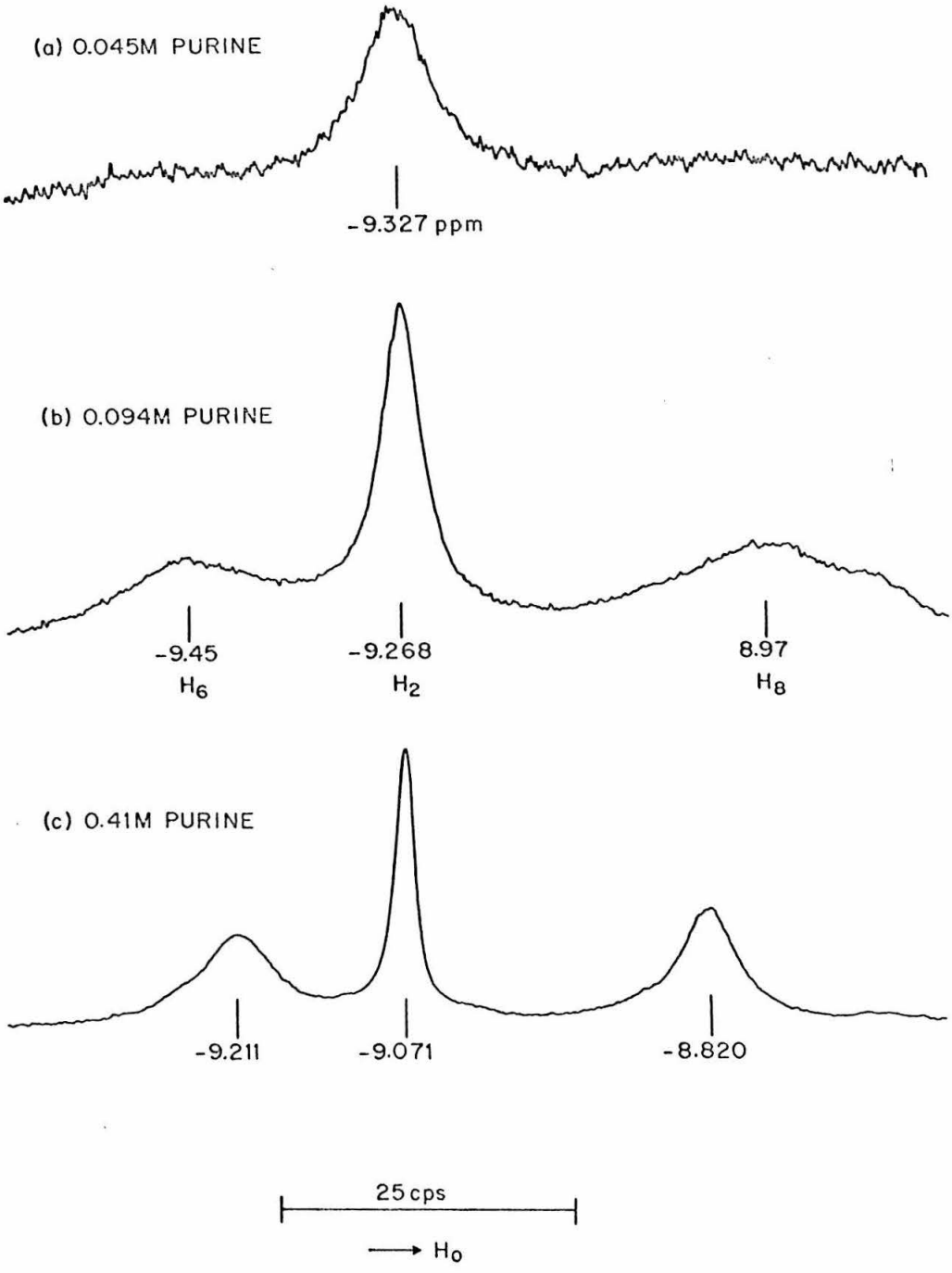


TABLE XXVI. Purine proton resonance linewidths in the presence of 0.08 M UpU (sodium salt). (Full linewidth at half-height in cps, measured at 100 Mcps.)

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

[a]Broad beyond detection.

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

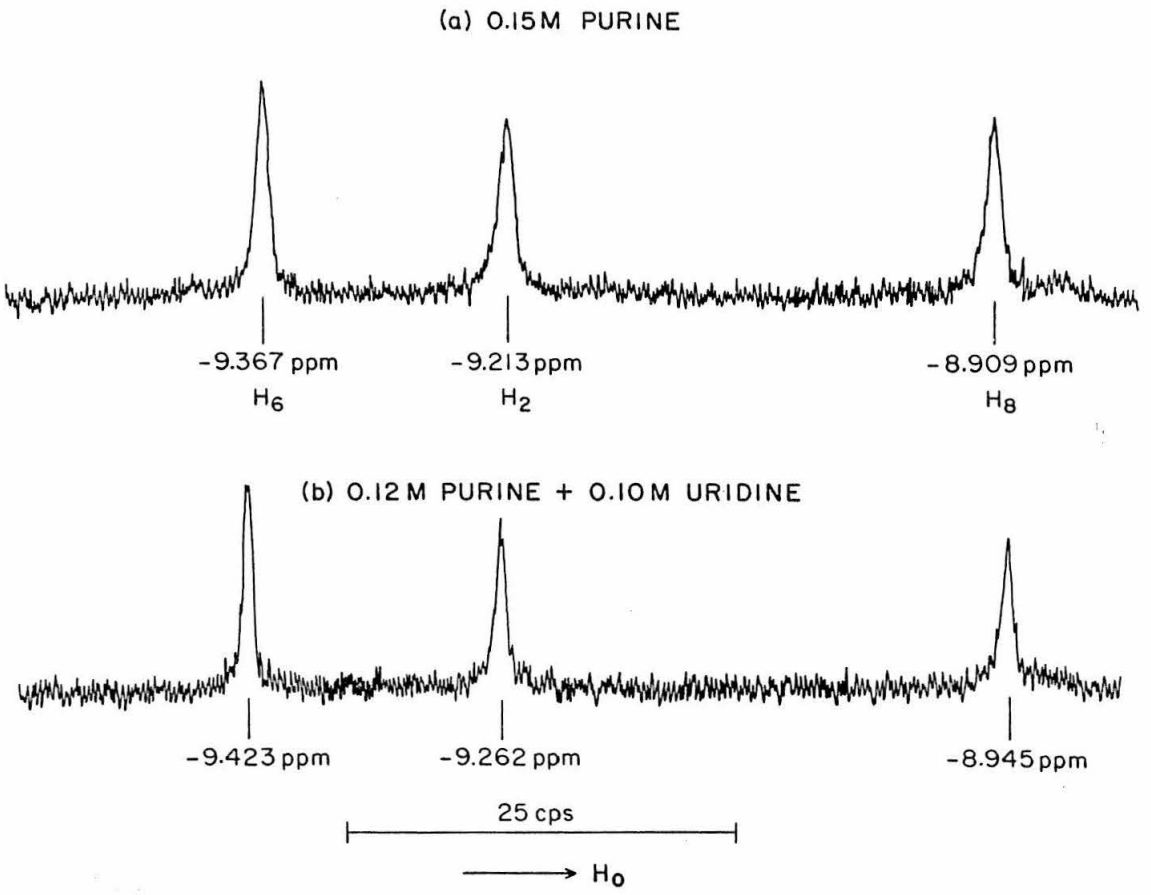
Although the effect of solution pD on the purine-UpU interaction (as reported for the purine-CpC system in Section 5. 3. 3) was not investigated, it might be expected that the purine binding would persist to very low pD, since the uracil bases are not protonated.

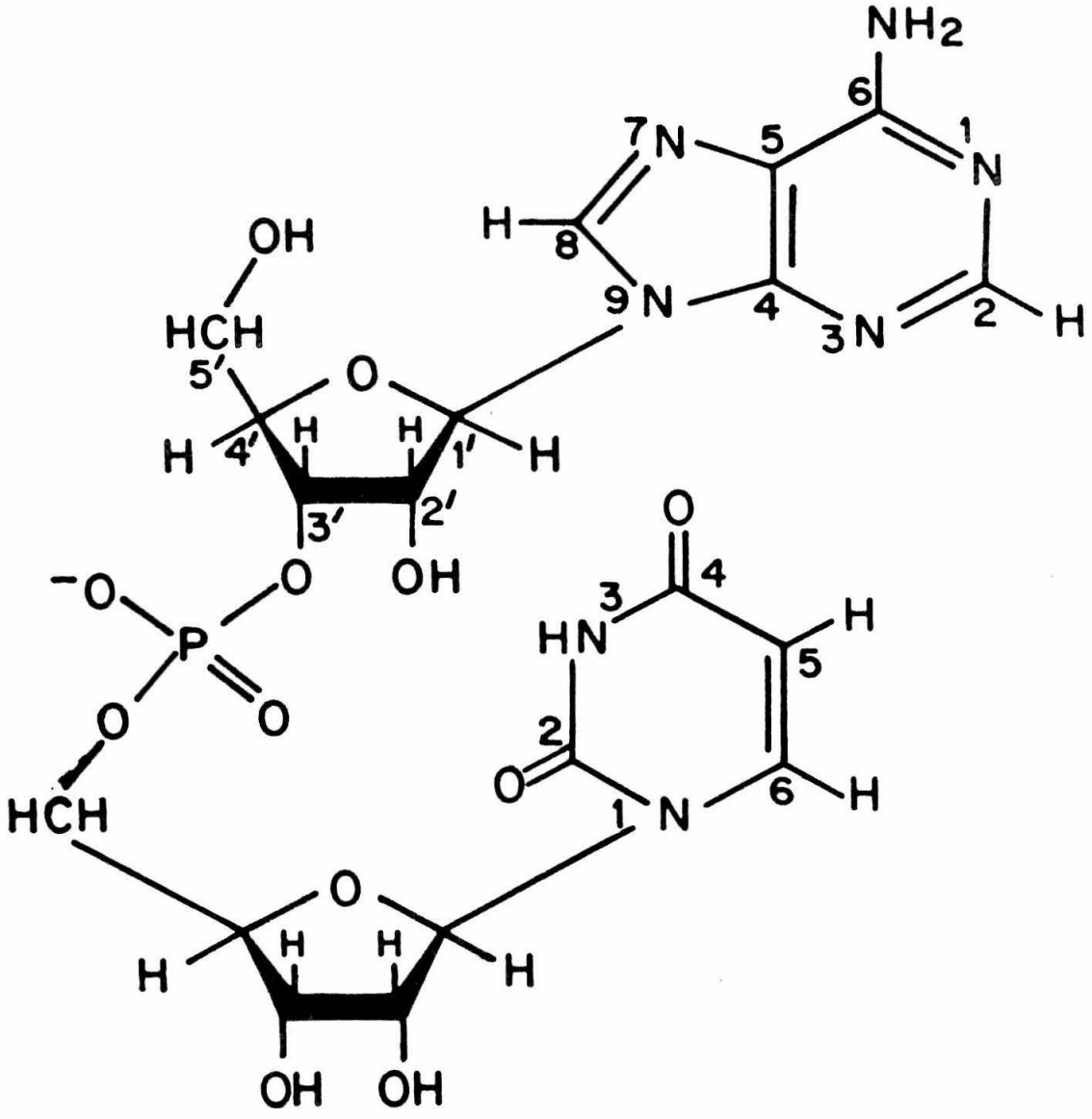
7. Adenylyl (3′→5′) uridine and Uridylyl (3′→5′) adenosine

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

FIGURE 54

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


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



ApU

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

7. 1. Pmr Spectra and Assignment of Resonances

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

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

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

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

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

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

7. 2. Purine Binding Studies

The sequence isomers ApU and UpA were selected to serve as

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

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

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

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

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

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

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

FIGURE 56

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

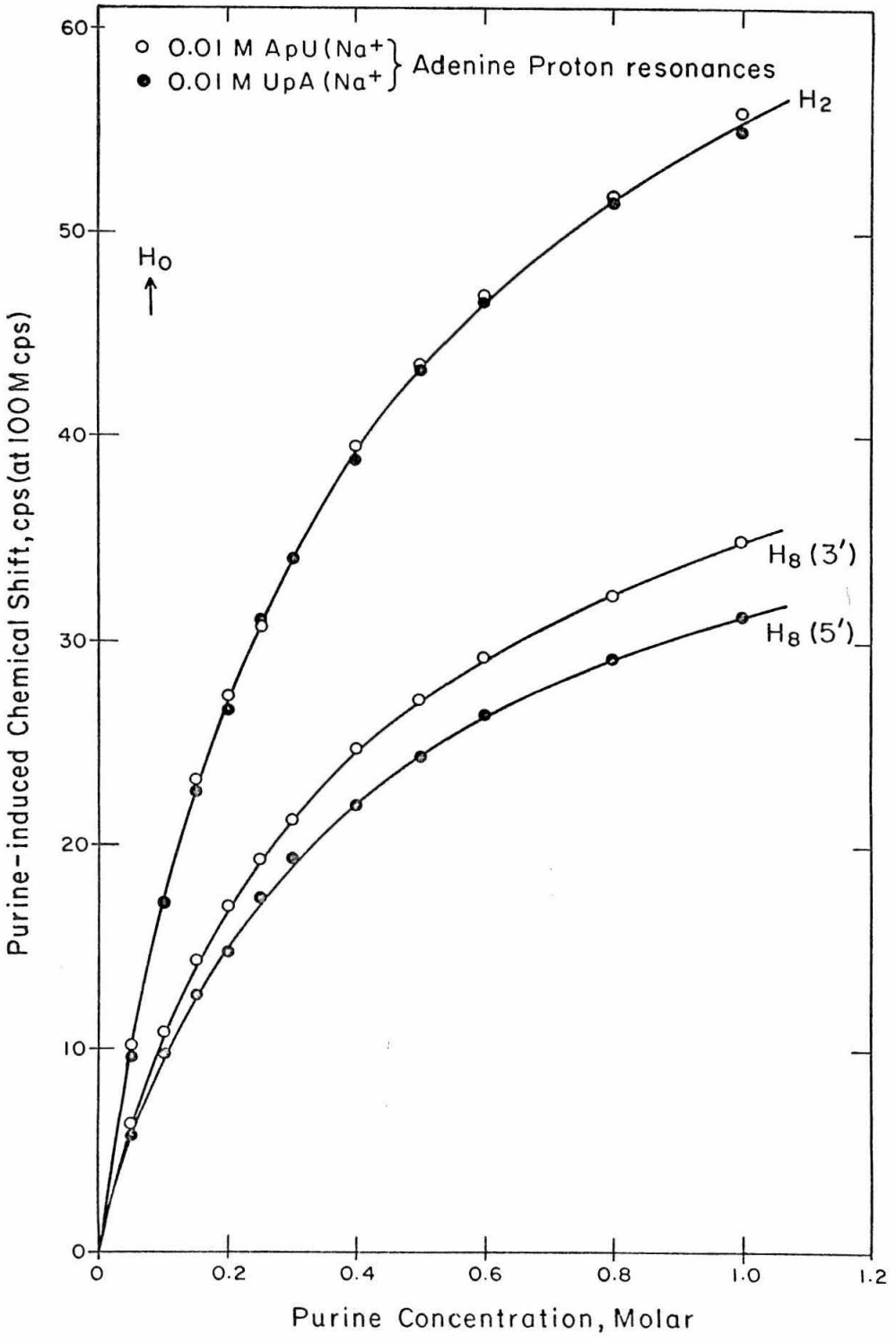

○ 0.01 M ApU (Na+)
● 0.01 M UpA (Na+)
Adenine Proton resonances
H2
H8 (3′)
H8 (5′)
H0
Purine-induced Chemical Shift, cps (at 100 M cps)
Purine Concentration, Molar
0
10
20
30
40
50
60
0
0.2
0.4
0.6
0.8
1.0
1.2

FIGURE 57

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

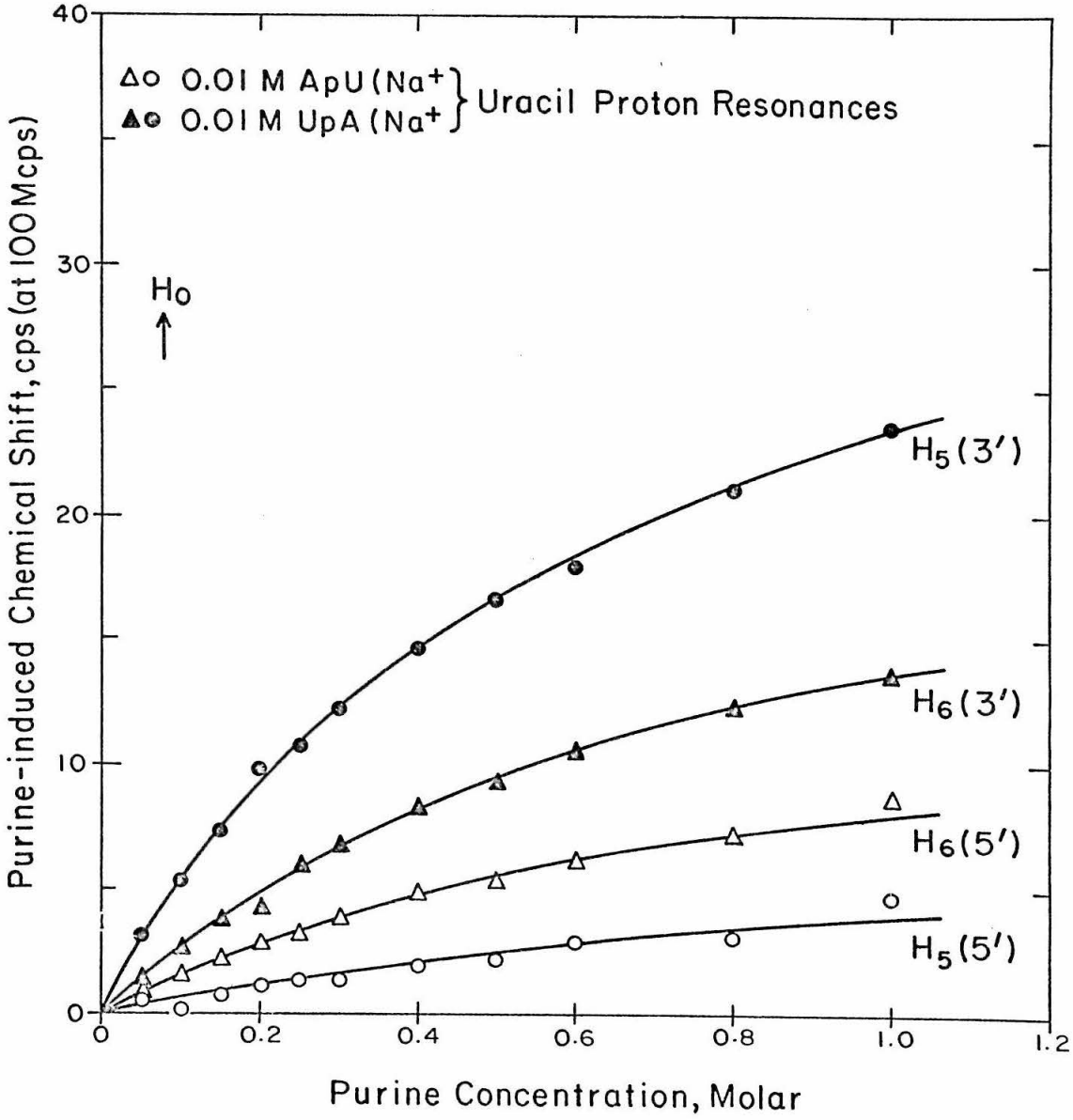
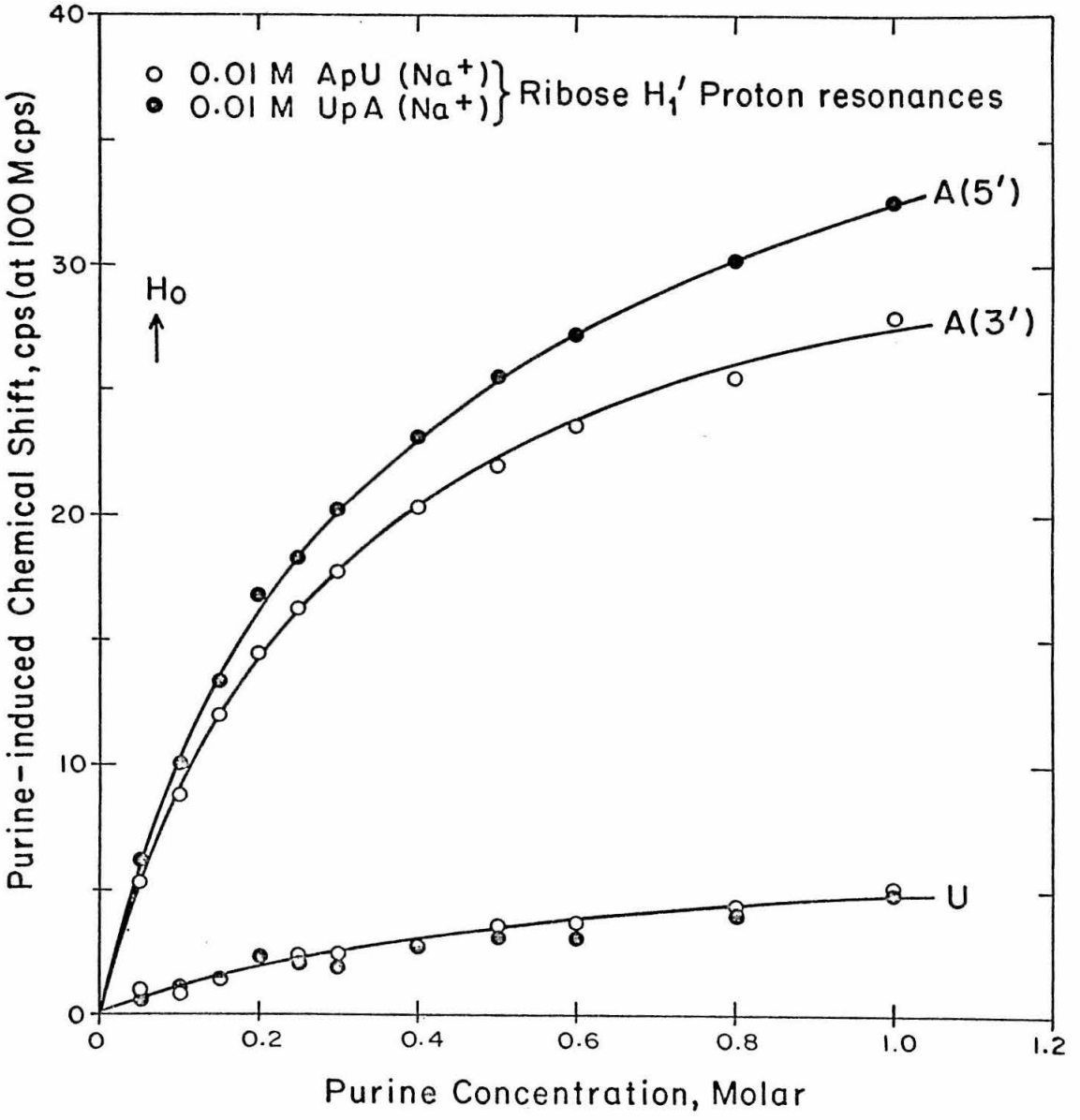

40
30
20
10
0
0.2
0.4
0.6
0.8
1.0
1.2
Δ○ 0.01 M ApU (Na+)
▲● 0.01 M UpA (Na+)
Uracil Proton Resonances
H0
H5 (3′)
H6 (3′)
H6 (5′)
H5 (5′)
Purine-induced Chemical Shift, cps (at 100 Mcps)
Purine Concentration, Molar

FIGURE 58

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


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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

8. Nature of the Purine-Dinucleotide Intercalated Complex

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

8. 1. Mode of Purine-Dinucleotide Interaction

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

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

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

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

FIGURE 59

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

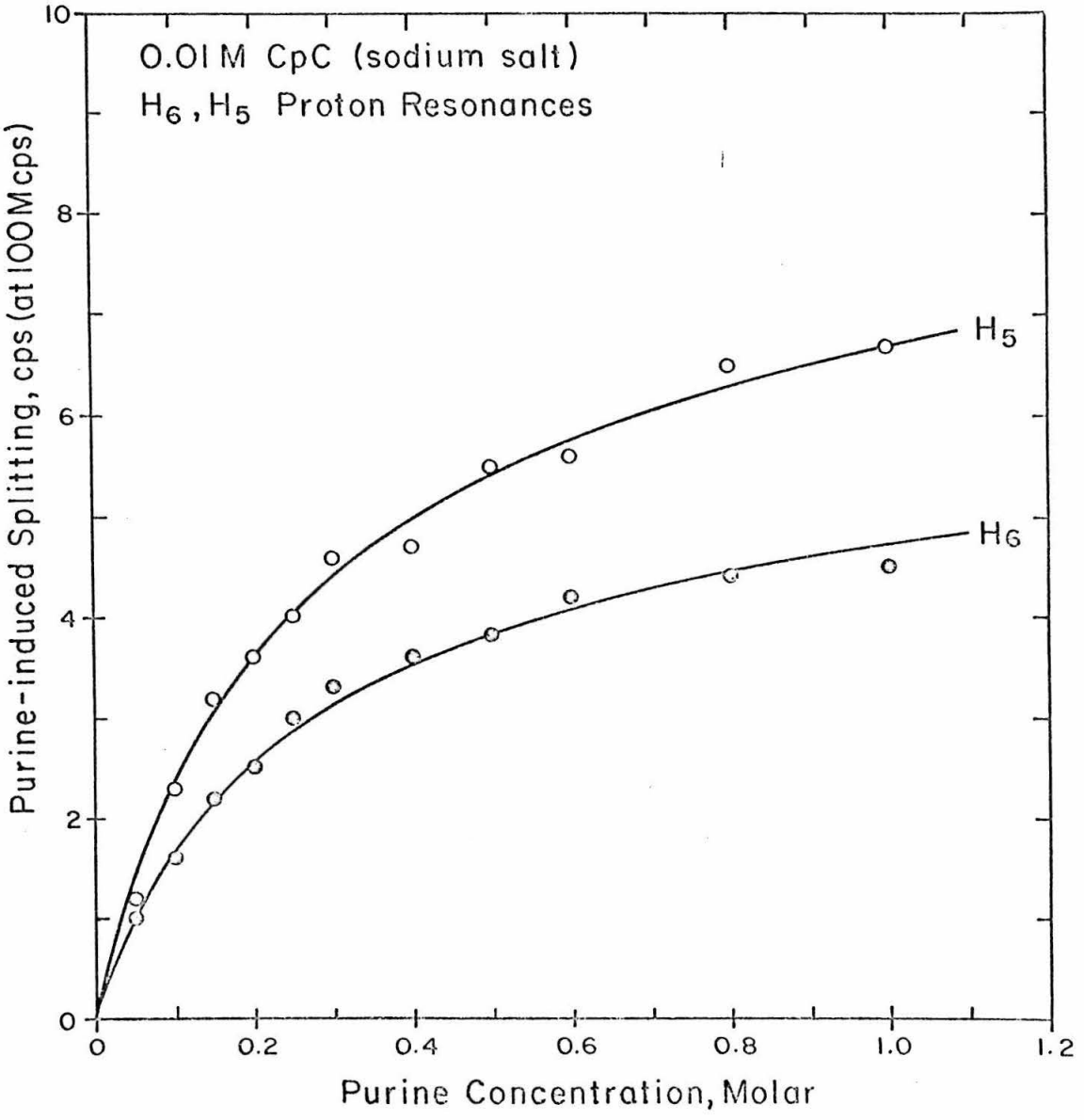
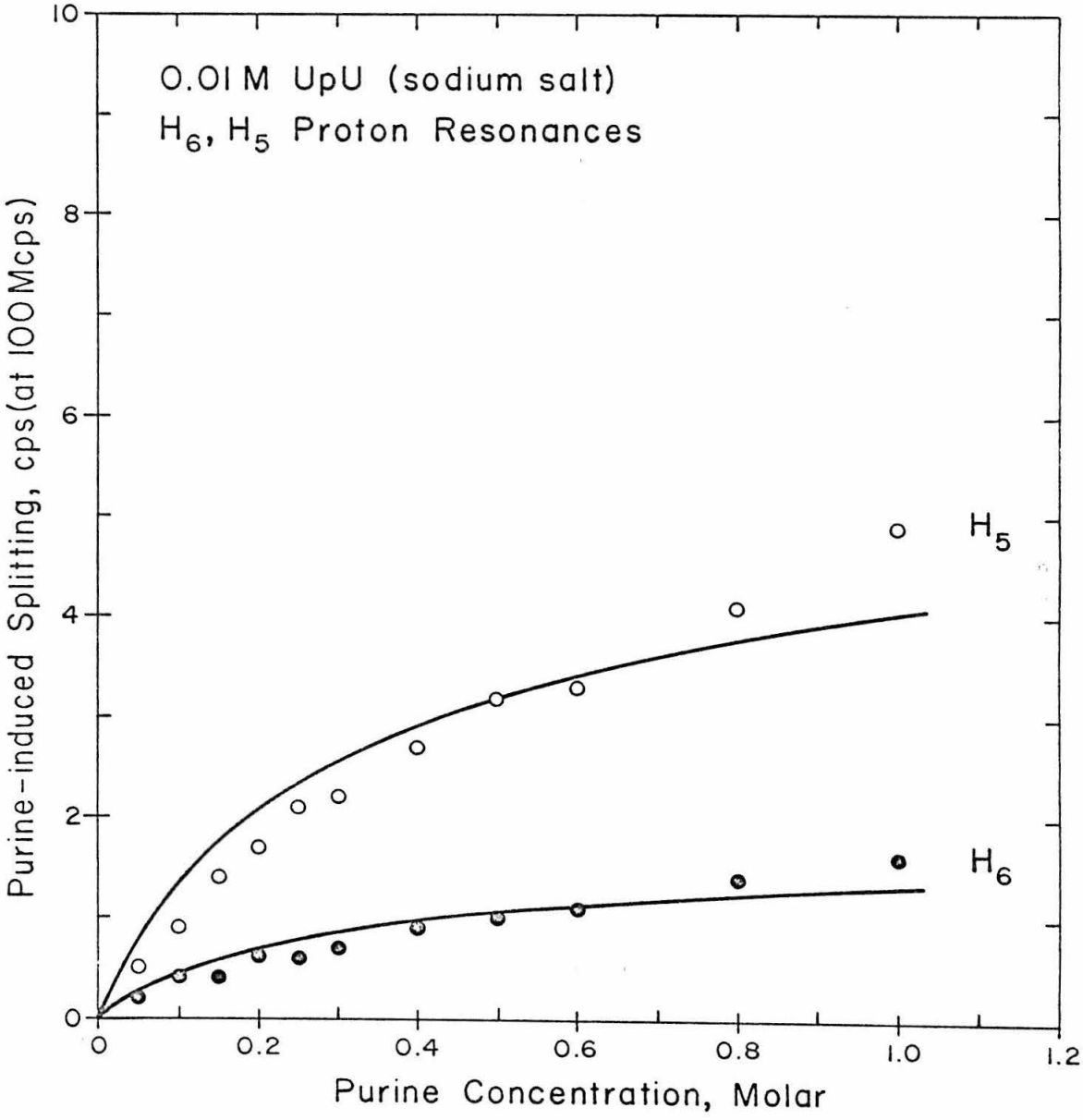

0.01 M CpC (sodium salt)
H6, H5 Proton Resonances
H5
H6
Purine-induced Splitting, cps (at 100 Mcps)
Purine Concentration, Molar
0
2
4
6
8
10
0
0.2
0.4
0.6
0.8
1.0
1.2

FIGURE 60

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


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

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

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

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

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

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

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

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

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

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

8. 3. Consideration of the Purine Intercalation Equilibrium

The equilibrium for the interaction of a monomeric purine

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

First of all, the following quantities are defined:

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

m = concentration of purine monomer, moles/l.

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

D = total stoichiometric dinucleotide concentration, moles/l.

and

$$D = d + C.$$

The intercalation equilibrium, involving intercalation constant K_I, is simply

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

8.4. Purine Resonance Linewidths in the Intercalated Complexes

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

The relationship giving the observed resonance linewidth is as follows:

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

where:

W_{obs} = observed resonance linewidth, cps.

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

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

f_B = mole fraction of purine bound.

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

Expression (11) is rearranged to give:

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

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

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

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

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

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

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

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

FIGURE 61

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

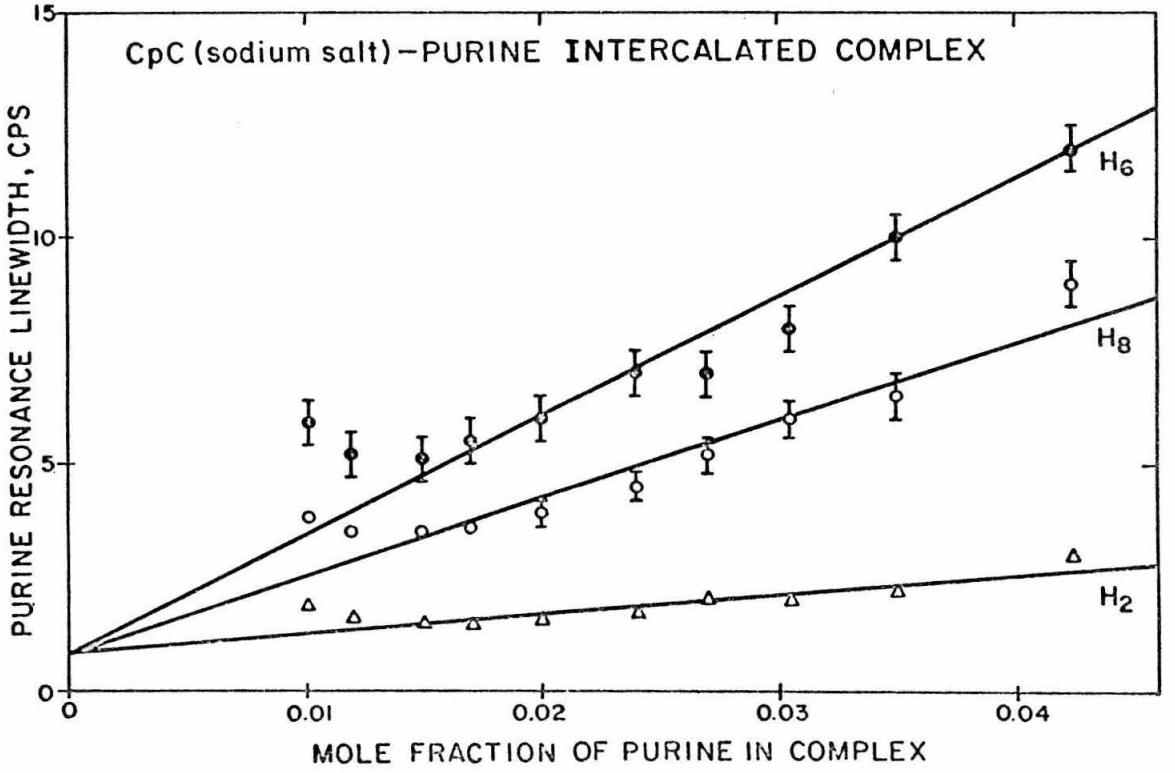

CpC (sodium salt)–PURINE INTERCALATED COMPLEX
PURINE RESONANCE LINEWIDTH, CPS
MOLE FRACTION OF PURINE IN COMPLEX
H6
H8
H2
0
5
10
15
0
0.01
0.02
0.03
0.04

FIGURE 62

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

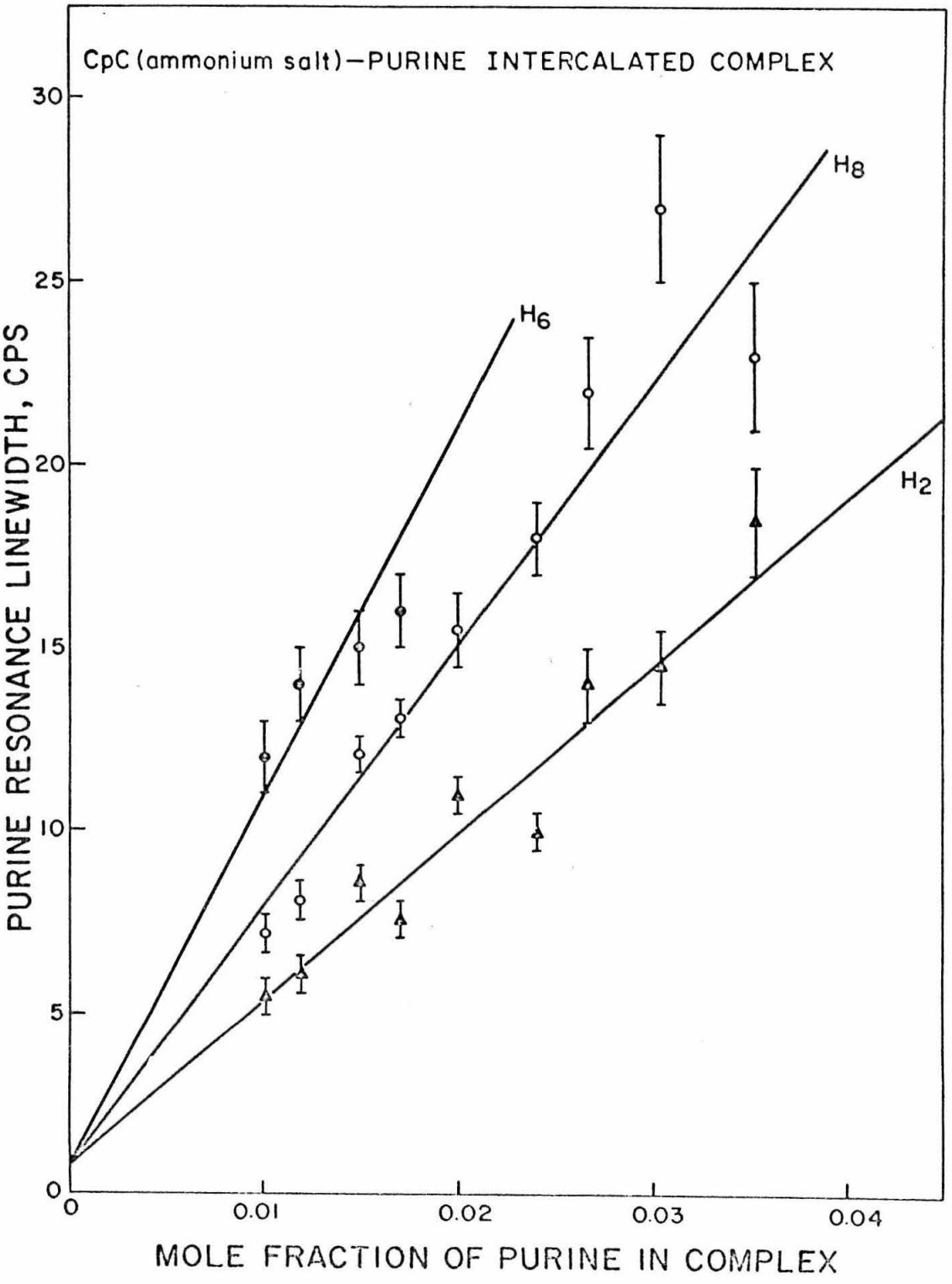

CpC (ammonium salt)—PURINE INTERCALATED COMPLEX
PURINE RESONANCE LINEWIDTH, CPS
MOLE FRACTION OF PURINE IN COMPLEX
H6
H8
H2
0
5
10
15
20
25
30
0
0.01
0.02
0.03
0.04

FIGURE 63

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

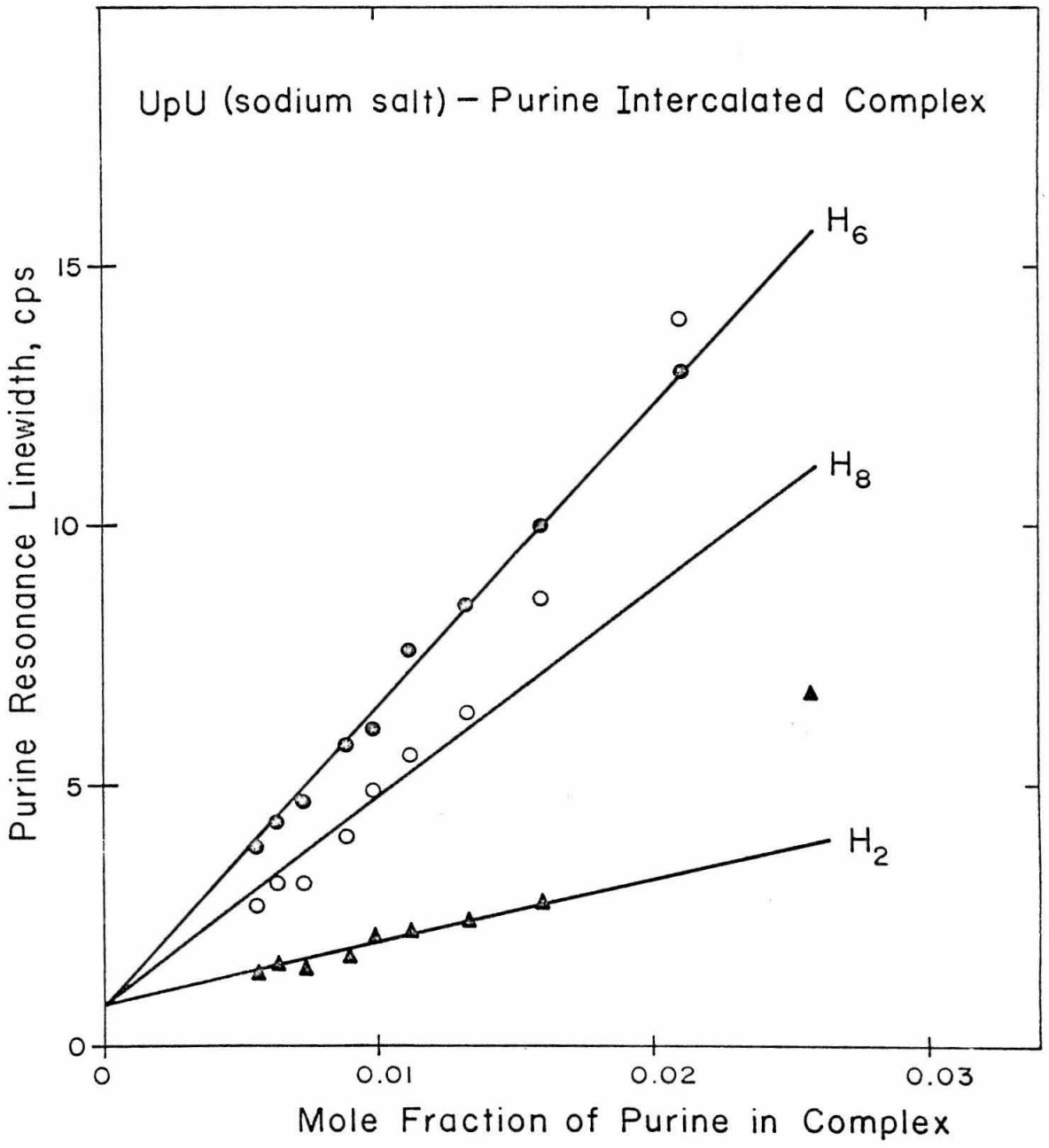

UpU (sodium salt) – Purine Intercalated Complex
H6
H8
H2
Purine Resonance Linewidth, cps
0
5
10
15
0
0.01
0.02
0.03
Mole Fraction of Purine in Complex

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

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

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

9. Summary

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

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

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

IV. PMR STUDIES OF MONOMER-POLYNUCLEOTIDE INTERACTIONS

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

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

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

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

1. Binding of Purine to Polyuridylic Acid

Purine was chosen to serve as the monomer component in this

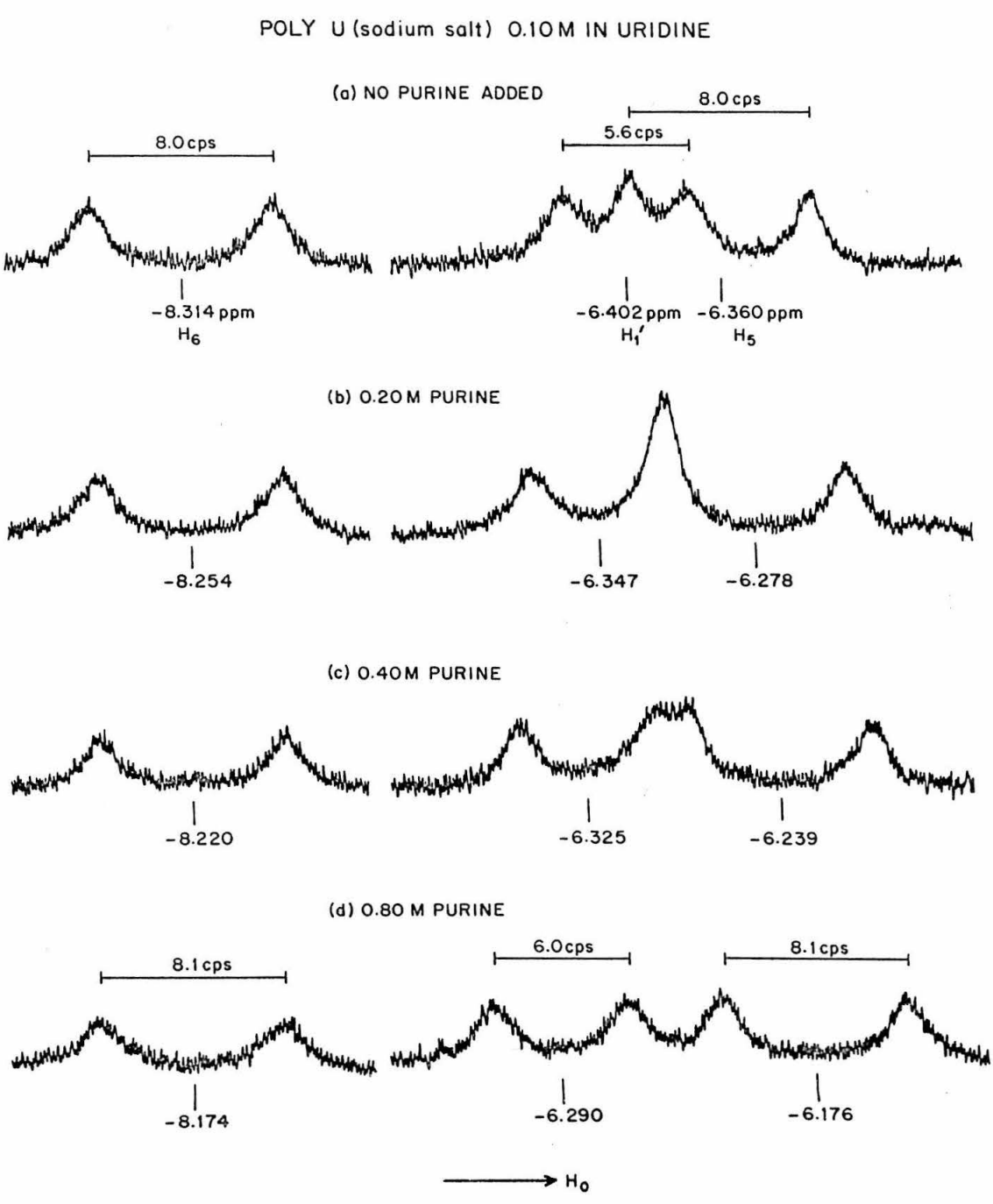
preliminary study, since the interaction of purine with bases, nucleosides, and dinucleoside monophosphates by base-stacking is well understood, and the mode of the purine-poly U interaction should be readily indicated by pmr spectroscopy. This investigation is of particular interest because of the possibility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

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

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

FIGURE 64

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


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

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

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

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

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

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

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

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

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

FIGURE 65

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

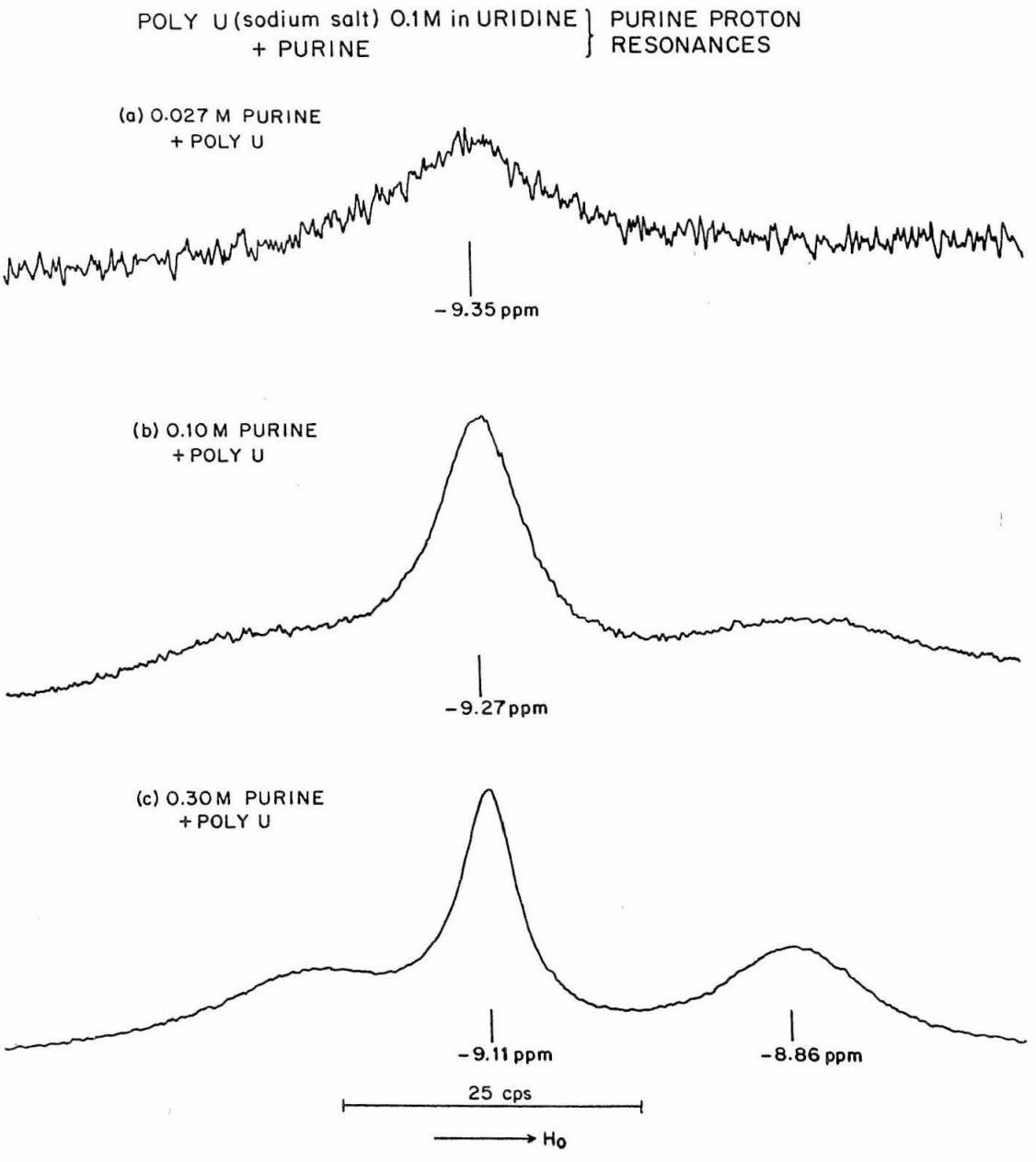

POLY U (sodium salt) 0.1M in URIDINE + PURINE
PURINE PROTON RESONANCES
(a) 0.027 M PURINE + POLY U
-9.35 ppm
(b) 0.10 M PURINE + POLY U
-9.27 ppm
(c) 0.30 M PURINE + POLY U
-9.11 ppm
-8.86 ppm
25 cps
Ho

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

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

[a]Broadened beyond detection.

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

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

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

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

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

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

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

2. Interaction of Adenosine with Polyuridylic Acid

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

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

FIGURE 66

A possible hydrogen-bonded purine-uracil complex.

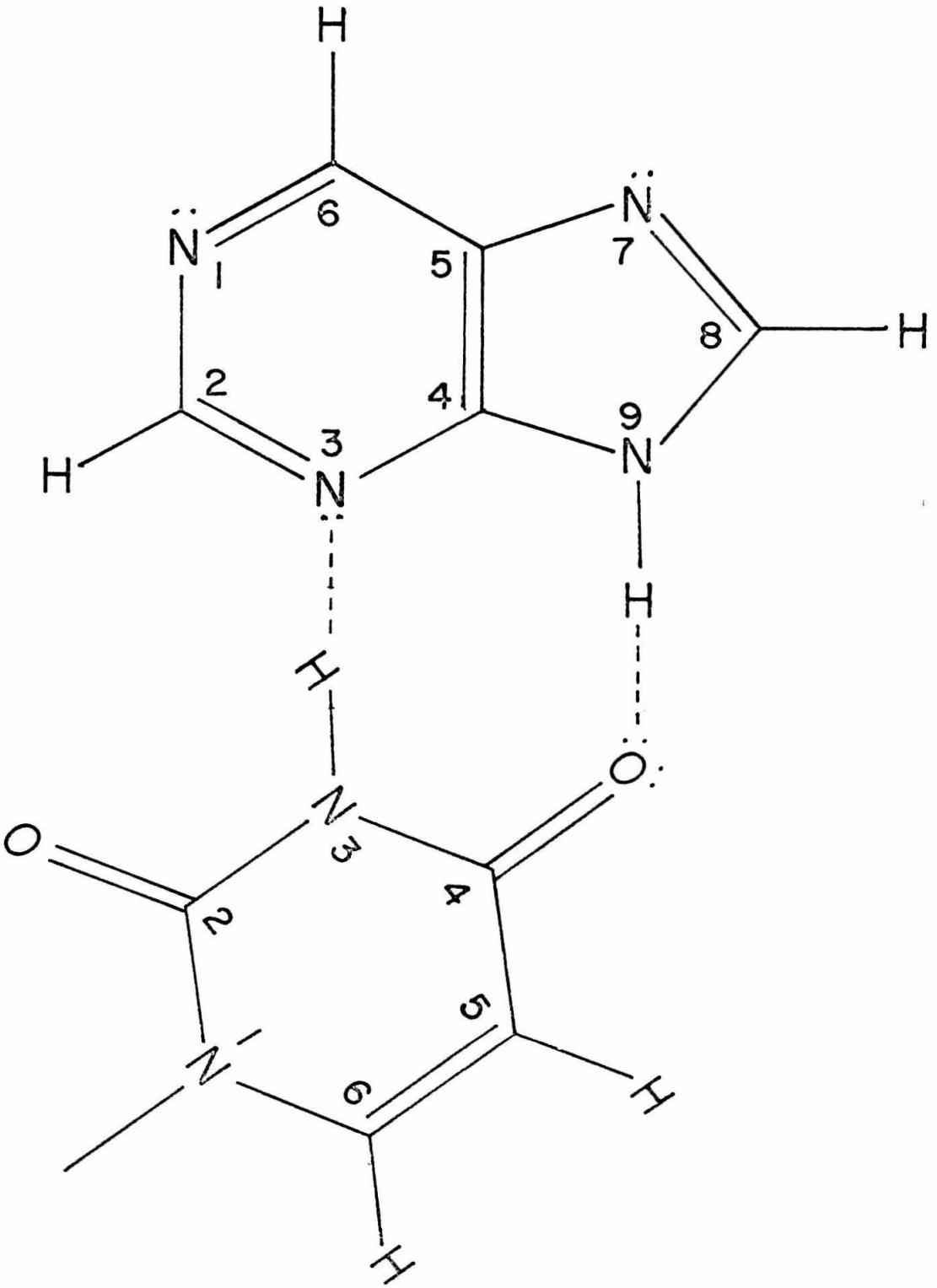
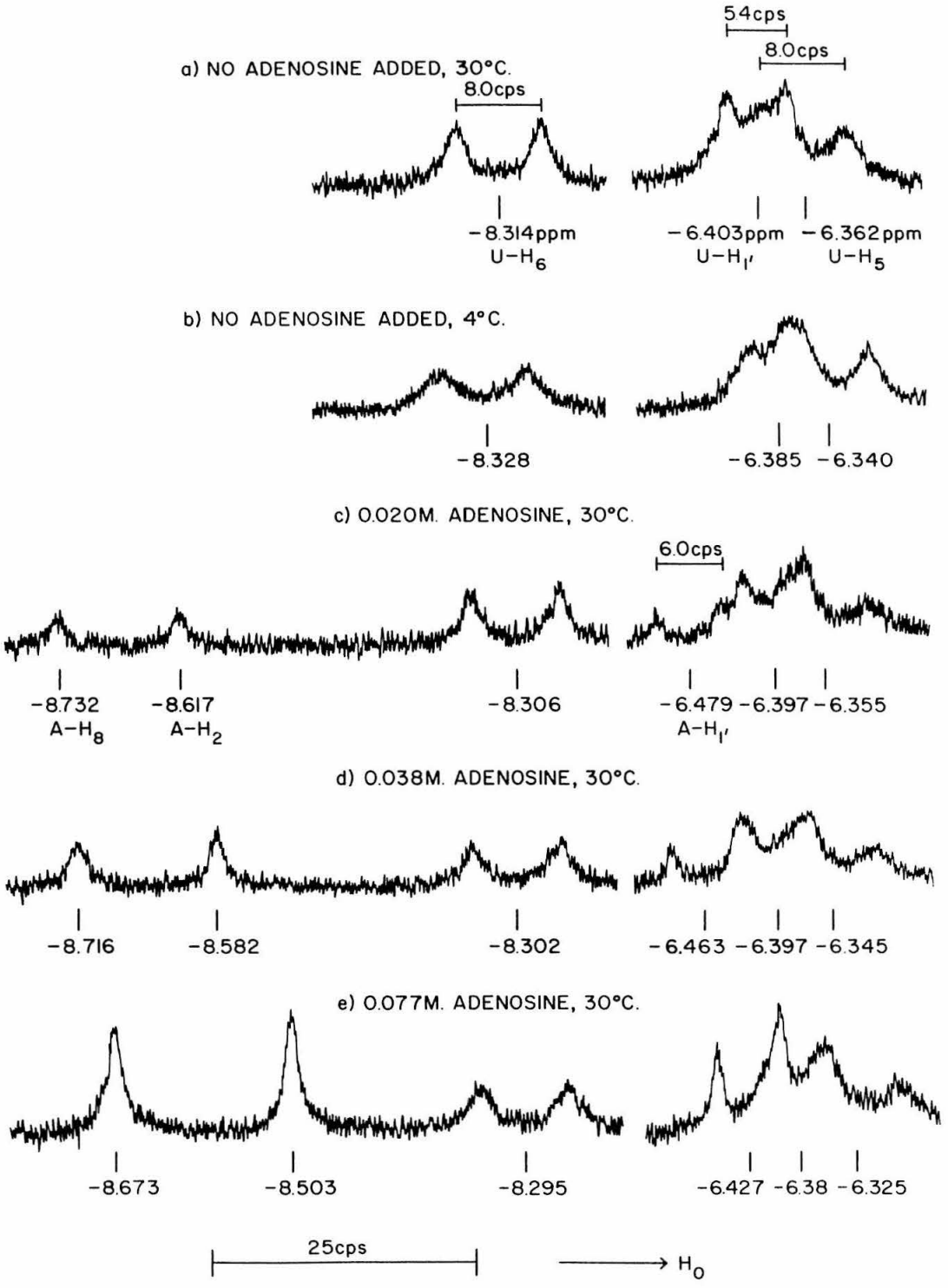

H
N
1
6
5
N
7
8
H
2
4
9
H
3
N
N
H
H
O
O
N
3
2
4
5
N
6
H
H

FIGURE 67

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

POLY U(sodium salt) 0.078M IN URIDINE



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

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

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

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

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

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

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

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

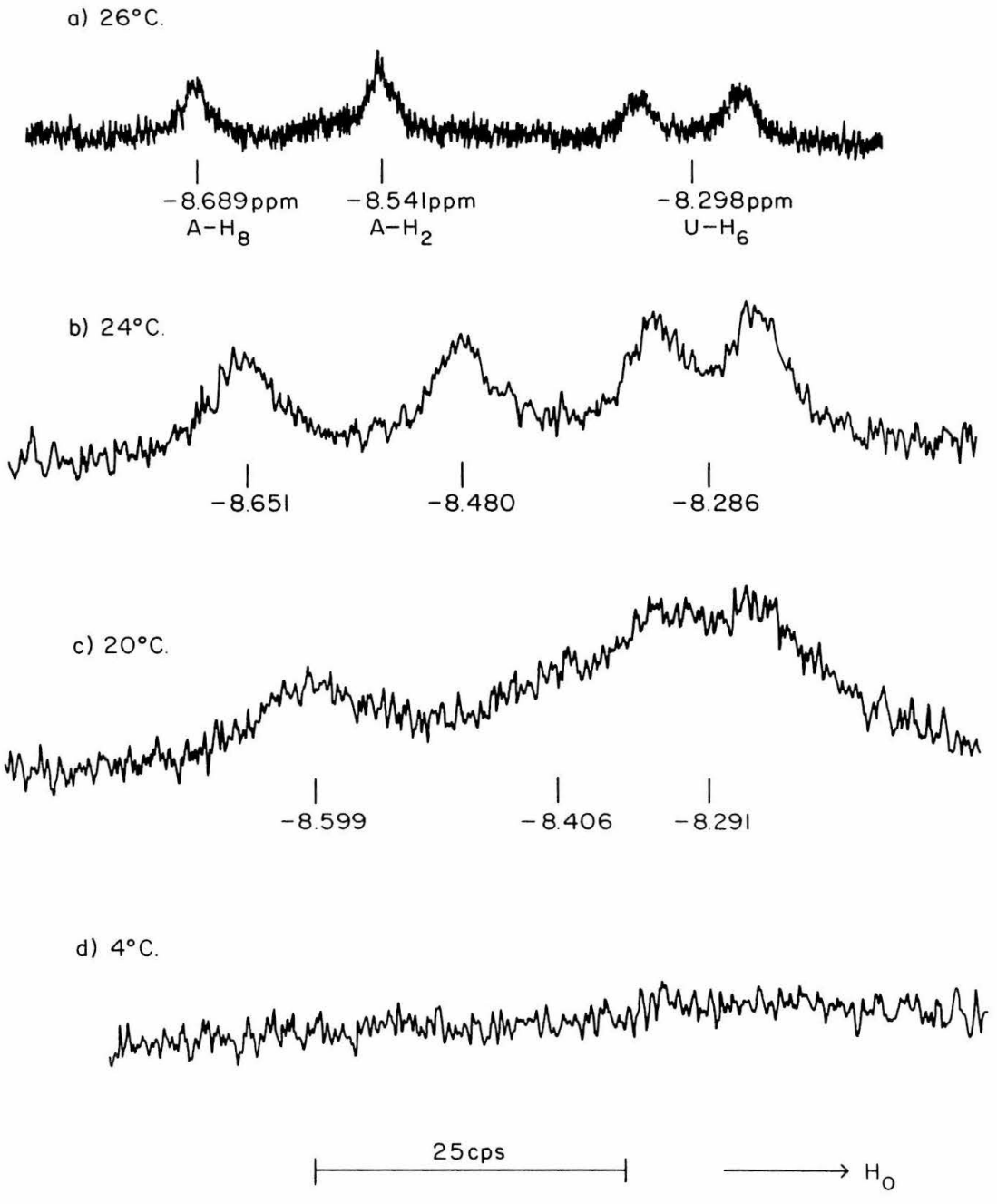
Since previous studies of this system[109, 110] indicated an adenosine-poly U interaction only below ~25°C, the effect of temperature on several adenosine-poly U solutions was studied. The results for a solution 0.078 M in poly U and 0.038 M in adenosine (base ratio A:U = 1:2) are shown in Figure 68.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

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

FIGURE 68

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

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



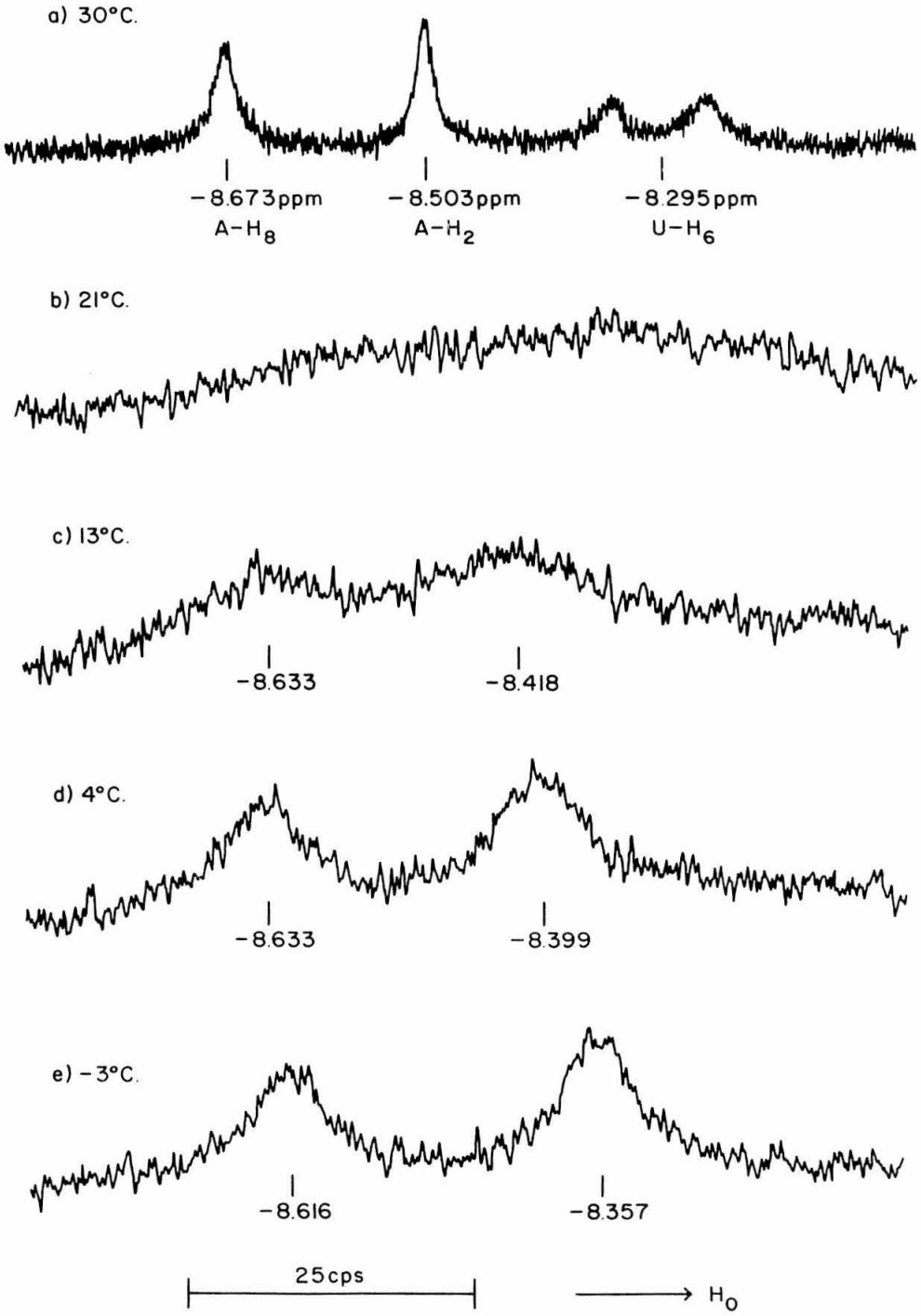
the formation of a rigid, ordered complex through a cooperative interaction of adenosine with poly U. The absence of detectable resonances at 4° suggests that either all of the reactants present are involved in complex formation or there is rapid exchange of the adenosine and poly U molecules between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

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

FIGURE 69

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

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



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

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

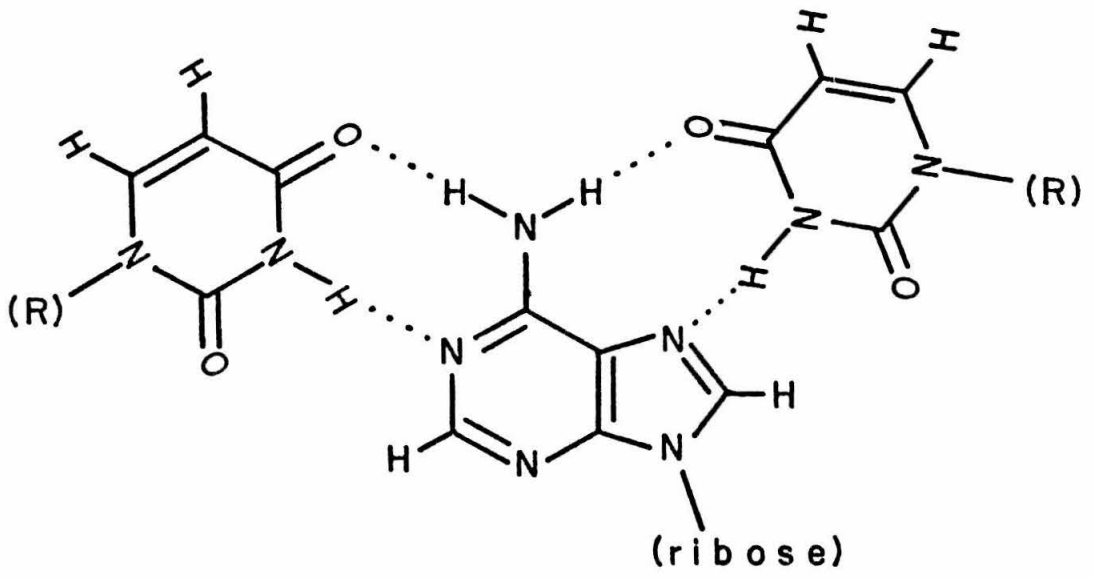


FIGURE 70. A possible base-pairing scheme for the 1 A : 2 U adenosine-poly U complex.

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

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

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

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

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

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

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

3. Summary

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

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

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

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PROPOSITION I

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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PROPOSITION II

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

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

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

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

resonances which could not be resolved further.

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

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

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

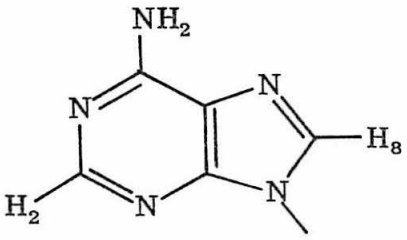
The field positions of the adenine H_8 or H_2 proton resonances are also sensitive to the presence of an adjacent adenine base. Appropriate comparisons among the dinucleoside monophosphates ApC, CpA, and ApA are summarized in Table II. From these results, the adenine H_8 proton would be expected to resonate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

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

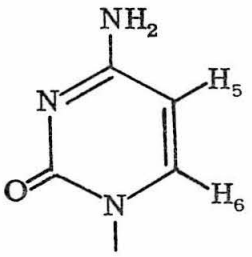
	H_6		H_5	
	(3′)	(5′)	(3′)	(5′)
	ppm		ppm	
ApC		-8.163		-6.151
CpA	-8.149		-6.281	
CpC	-8.308	-8.358	-6.400	-6.408
Shift	+0.159	+0.195	+0.119	+0.257

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

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



adenine



cytosine

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

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

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

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

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PROPOSITION III

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

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

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

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

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

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

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

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

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PROPOSITION IV

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

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

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

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

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

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

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

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

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

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

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

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PROPOSITION V

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

ion show little activity with regard to influencing membrane conductances.

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

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

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

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

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

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