Studies of Inter- and Intramolecular Interaction in Mononucleotides by Proton Magnetic Resonance[1]

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Abstract: The interaction and conformation of 5'-, 3'-, and 2'-nucleoside monophosphates in aqueous solutions have been studied mainly by pmr at varying concentrations and pH. The chemical shifts of the base protons and H-1' protons of the adenine nucleotides were found to shift upfield at increasing concentration in a manner analogous to adenosine. This result indicates that the AMP molecules also associate to form vertical stacks with similar geometry in solution, a conclusion also supported by the vapor pressure osmometry data. The phosphoryl substitution at the 5' or 3' position reduces the tendency of association to about 30–40% as compared to the corresponding adenine nucleosides. Substitution of the phosphoryl group at the 2' position has a greater influence in reducing the association, especially in the dianionic form. The 5'-phosphoryl group (not the 3'- or 2'-phosphate) was found to have a specific deshielding effect on the H-8 proton (and not H-2) of the 5'-purine nucleotides and on the H-6 proton (and not H-5) of the 5'-pyrimidine nucleotides. This deshielding effect is largest (~0.2 ppm) when the phosphate is in the dianion form (above pD 7.4), less when in the monoanion form (~0.12 ppm, below pD 5.9), and least as the monomethyl ester (~0.07 ppm). These data indicate that the 5'-nucleotides must be in the "*anti*" conformation. The mechanism of this phosphate deshielding effect is discussed based on the observation that this effect appears to be dependent on the "acidity" of the sensitive protons. Comparison of the pmr data on the ribose and deoxyribose 5'-nucleotides suggests the presence of intramolecular hydrogen bonding between the 2'-OH group and the N-3 of the purine or the 2-keto of the pyrimidine.

For the past few years the properties of various purine bases and purine and pyrimidine nucleosides in aqueous solutions have been studied extensively by vapor pressure osmometry and by proton magnetic resonance.[2-7] The results conclusively show that these compounds, especially the purines and the purine nucleosides, associate extensively in water primarily by way of vertical stacking of base rings. In the studies of cooperative binding of adenosine to polyuridylic acid,[8] the stacking energy has been shown to be the major factor contributing to the conformational stability of nucleic acid.

In the continuation of our recent research on the purine and pyrimidine nucleosides by pmr,[5,7] we report here the investigation on the solution properties of purine and pyrimidine mononucleotides. We have studied the influence of the charged phosphate group upon the stacking interactions of these mononucleotides. In addition, we have found a specific intramolecular deshielding of the H-6 proton of pyrimidine nucleotides and the H-8 proton of purine nucleotides by the 5'-phosphate group. This finding indicates that in aqueous solution the 5'-nucleotides are in "*anti*" conformation. Also, comparison of the data on the deoxyribonucleotides and the ribonucleotides suggests the possibility of intramolecular hydrogen bonding of the 2'-hydroxyl group to the bases in these compounds.

Experimental Section

Instrumentation. Pmr spectra were recorded with Varian Associates A-60 and HA-100 spectrometers. Probe temperatures were 28–33 and 28–30°, respectively. Chemical shifts were measured from an external TMS capillary. These shifts were measured to better than ±0.005 ppm at 100 Mc and ±0.009 ppm at 60 Mc. Extrapolated infinite dilution values are measured with an accuracy of about 0.02 ppm. No bulk susceptibility corrections have been made. However, it is confidently estimated these corrections would be less than 0.01 ppm for the 0.02–0.09 *M* solutions and 0.02–0.03 ppm for the concentration studies where solute concentrations were up to 0.9 *m*.

Jardetzky's assignments of nucleotide proton resonances were used.[9] These assignments were verified in the case of 5'-AMP by incorporating deuterium into the adenine ring at C-8.[10,11]

Paper electrophoreses were run using Whatman 3MM paper, a Savant power supply, and a unit built to specifications of Savant Model HV 5000-3. Determination of apparent pH values in D_2O solvent were made with a Model 22 pH meter from Radiometer, Copenhagen. Microcalomel and microglass electrodes were used on 0.5-ml volumes. To obtain pD values, the equation pD = meter reading + 0.4 of Glasoe and Long[12] was used.

Thin layer chromatography (tlc) was employed using Eastman "Chromagram" silica gel sheets or cellulose-coated glass plates; solvent A, 2-propanol–concentrated ammonium hydroxide–H_2O (7:1:2); B, 2-propanol–0.5 *M* ammonium acetate (5:2), pH 6; C, ethanol–0.5 *M* ammonium acetate (5:2), pH 3.8; D, isobutyric acid–H_2O–concentrated ammonium hydroxide (4:2:0.004); E, 1-butanol (saturated with H_2O).

Vapor pressure osmometry measurements were made as previously described using a Mechrolab 301A vapor pressure osmometer.[2] Elemental analyses were obtained from Galbraith Laboratories, Knoxville, Tenn.

Optical rotatory dispersion measurement (ORD) was done in a Cary Model 60 spectropolarimeter. The sample concentrations were between 1 and 5 OD at various wavelength regions.

Materials. When possible, commercially available compounds of the highest degree of purity were used without further purification. The following compounds were purchased from Sigma Chemical Co., St. Louis, Mo.: 5'-AMP, 5'-GMP, 5'-CMP, 5'-

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(2) P. O. P. Ts'o, I. S. Melvin, and A. C. Olson, *J. Amer. Chem. Soc.*, **85**, 1289 (1963).
(3) P. O. P. Ts'o and S. I. Chan, *ibid.*, **86**, 4176 (1964).
(4) S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, *ibid.*, **86**, 4182 (1964).
(5) M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, *ibid.*, **87**, 5241 (1965).
(6) O. Jardetzky, *Biopolym. Symp.*, **1**, 501 (1964).
(7) A. D. Broom, M. P. Schweizer, and P. O. P. Ts'o, *J. Amer. Chem. Soc.*, **89**, 3612 (1967).
(8) W. M. Huang and P. O. P. Ts'o, *J. Mol. Biol.*, **16**, 523 (1966).
(9) C. D. Jardetzky and O. Jardetzky, *J. Amer. Chem. Soc.*, **82**, 222 (1960).
(10) F. J. Bullock and O. Jardetzky, *J. Org. Chem.*, **29**, 1988 (1964).
(11) J. R. Fox, Ph.D. Dissertation, University of Illinois, 1965.
(12) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).

dCMP, 5′-dAMP, 5′-TMP. California Corporation for Biochemical Research, Los Angeles, Calif., supplied: 5′-UMP, 5′-dUMP, 3′(2′)-CMP and 5′-AMP. 5′-IMP, 2′-AMP, and 3′-AMP were from Schwarz. Deuterium oxide, 99.5 mol %, was obtained from Matheson Coleman and Bell. 5′-Fluorouridine 5′-phosphate[13] and 4-amino-7-β-D-ribofuranosyl 5′-phosphate-7H-pyrrolo[2,3-*d*]-pyrimidine (tubercidin 5′-phosphate)[14] were gifts and are gratefully acknowledged. The 5′-monomethyl phosphates of adenosine, cytidine, guanosine, and uridine were prepared according to the procedure of Smith, *et al.*[15] The 5′-monomethyl phosphate of adenosine was found to be anhydrous (*Anal.* Calcd: C, 34.47; H, 3.94; N, 18.27. Found: C, 34.70; H, 4.06; N, 18.11). The 5′-disodium phosphate of adenosine was found to contain 2.5 mol of water by analyses (*Anal.* Calcd for 2.5 mol of water/mol of nucleotide: C, 27.5; H, 3.89; N, 16.05. Found: C, 27.31; H, 4.32; N, 15.95).

Poly-L-lysine was purchased from Pilot Chemicals, Inc., Watertown, Mass. The hydrobromide salt was converted to the free base by passing through Dowex 1 (OH^-) resin.

Adenosine 2′-Methyl Hydrogen Phosphate. Methylation of 2′-AMP, sodium salt, at the phosphate only, was accomplished with diazomethane in neutral aqueous 1,2-dimethoxyethane solution (DME).[16] Phosphomethylation, rather than reaction at the base, had been indicated in the work of Haines, *et al.*,[17] and Brimacombe, *et al.*,[18] with 5′-AMP. A typical set of reaction conditions was: 254 mg (0.73 mmol) of 2′-AMP (free acid) was dissolved in 25 ml of water with slight heating. The pH was adjusted to 5.5 with NaOH. A 22-mol excess of diazomethane in DME (0.9 mmol/ml, concentration determined with excess benzoic acid, back-titrating with standard sodium hydroxide) was added. The reaction was instantaneous (disappearance of diazomethane color). Tlc analysis of the reaction mixture indicated starting material as well as a faster migrating component. Separation of the mixture was accomplished on a 2.8 × 20 cm DEAE (bicarbonate) column, previously equilibrated with 0.01 *M* ammonium bicarbonate. A linear 0.01–0.5 *M* gradient of ammonium bicarbonate (pH 8.5) at a flow rate of 1 ml/min was used as eluent on a Gilson fraction collector, collecting 13-ml fractions. Combined fraction 1 (tubes 1–50) was shown by tlc to contain the component migrating faster than 2′-AMP, presumably the methylated product. Paper electrophoresis (0.05 *M* Tris–HCl, pH 7.5, 36 V/cm) indicated a main band with mobility relative to 2′-AMP of 0.69, plus traces of nonionic contaminants. Combined fraction 2 (tubes 51–110) was mainly 2′-AMP as indicated by tlc and electrophoresis, though a small amount of the methyl ester was present. 2′-Methyl-AMP (NH_4^+) was separated from the nonionic species by rechromatographing fraction 1 above on another DEAE column. After repeated evaporation to dryness of the main fraction to remove ammonium bicarbonate, followed by lyophilization, 170 mg (62%) of adenosine 2′-methyl-ammonium phosphate (NH_4^+) was obtained. The uv spectrum (Cary Model 15) in water, λ_{max} 259 mμ, λ_{min} 227 mμ, indicates no ring methylation. Jones and Robins[19] reported λ_{max} 265 mμ for N^6-methyladenosine and λ_{max} 257 mμ for 1-methyladenosine in water. The nmr spectrum (0.1 *M* in D_2O) provides additional evidence for phosphomethylation. At δ 3.59 ppm from external TMS the methyl resonance is split into a doublet due to coupling with P^{31} of phosphate, $J_{P^{31}-O-CH}$ = 11.0 cps.[20] This is identical with the $J_{P^{31}-O-CH}$ for methyls in 5′-methyl-AMP. By comparison of the chemical shifts of H-1′ in 2′-AMP, 2′-methyl-AMP, and 3′-AMP, it is concluded that the phosphomonomethyl group had not migrated from the 2′ to the 3′ position on the ribose ring. The electron-withdrawing phosphoryl or methylphosphoryl group would be expected to deshield H-1′ in the 2′ isomer more than in the 3′ isomer.[21] Thus $\delta_{H-1'}$ is 6.64 ppm in 2′-AMP, 6.52 in 3′-AMP, and 6.62 in 2′-methyl AMP (all solutions 0.1 *M*, pH (apparent) 5). Tlc R_f's for 2′-methyl AMP are solvent A, 0.55; solvent B, 0.61; and solvent C, 0.63, compared to 0.22, 0.29, and 0.46 for 2′-AMP.

Adenosine 3′-Methyl Hydrogen Phosphate. When 3′-AMP was treated in a similar manner as above for 2′-AMP, it was shown by nmr that 15% of the product was adenosine 2′-methyl hydrogen phosphate. Thus migration of the phosphate group occurred.

Separation of 3′(2′)-CMP Mixture. A mixture of 3′(2′)-CMP was separated on a Dowex 1 (formate) column essentially according to the method of Cohn[22] and Cathou and Hammes.[23] A 1.6 × 16 cm column of Dowex 1 (formate) was used to separate 100 mg of 3′(2′)-CMP (sodium salt) in 4 ml of H_2O. Elution was accomplished with 0.01 *M* formic acid. Fractions (15 ml) were collected at 0.9 ml/min. Tubes 60–100 comprised fraction 1 and tubes 110–170 fraction 2. Fraction 1 was 2′-CMP which has a higher pK_a (4.30) thus is more readily protonated by HCO_2H and released from the column than 3′-CMP (pK_a = 4.16). Also in the nmr spectrum of the solid isolated from fraction 1, H-1′ was found to absorb at a lower field than H-1′ of solid from fraction 2, thus confirming the assignment; tlc R_f's: 2′-CMP (0.78), 3′-CMP (0.73) in saturated ammonium sulfate, 1 *M* sodium acetate, 2-propanol (80:18:2).

Results and Discussion

Self-Association of Mononucleotides. Data in Table I summarize the effect of concentration on the proton chemical shifts of various mononucleotides. More detailed features of this concentration effect for 5′-AMP, 5′-AMP-methyl ester, and 2′-AMP are shown in Figures 1, 2, and 3, respectively. These results together with the data in Figure 4 are to be discussed in the following sections.

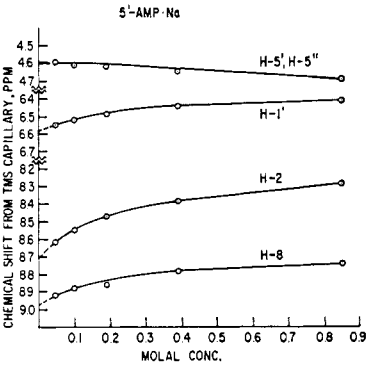

Figure 1. Chemical shift *vs.* molal concentration for 5′-AMP (Na) protons; 30°, D_2O, pD 5.9.

A. The Concentration Dependence. As shown in Figures 1 and 2 and Table I, the concentration dependence of various protons of these 5′-adenine nucleotides is very similar to that reported for the adenine nucleosides.[7] The δ value of the sugar protons located far away from the adenine base, such as the H-5′ and H-5″, are not sensitive to concentration changes. A small downfield shift observed for the H-5′ and H-5″ with increasing concentration may be due to the effect of concentration on the ionization of phosphate group, as anticipated from the Debye–Hückel theory on electrolytes. For the base protons, the magnitude of the high-field shift (Δδ) in response to the increase of concentration is largest for the H-2. The Δδ values for H-8 are about the same as those for the anomeric proton, H-1′. This comparison is dependent on individual nucleotides and will be discussed further below. These

(13) From Dr. Ross Hall, Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada.
(14) From Dr. William J. Wechter, Department of Chemistry, The Upjohn Co., Kalamazoo, Mich.
(15) M. Smith, J. G. Moffat, and H. G. Khorana, *J. Amer. Chem. Soc.*, **80**, 6204 (1958).
(16) A. D. Broom and R. K. Robins, *ibid.*, **87**, 1145 (1965).
(17) J. A. Haines, D. B. Reese, and Lord Todd, *J. Chem. Soc.*, 1406 (1964).
(18) R. L. C. Brimacombe, B. E. Griffin, J. A. Haines, W. J. Haslom, and C. B. Reese, *Biochemistry*, **4**, 45 (1965).
(19) J. W. Jones and R. K. Robins, *J. Amer. Chem. Soc.*, **85**, 93 (1963).
(20) N. Muller, P. C. Lauterbur, and J. Goldenson, *ibid.*, **78**, 3557 (1956).
(21) H. P. M. Fromageot, B. F. Griffin, C. B. Reese, J. E. Sulston, and D. R. Trentham, *Tetrahedron*, **22**, 705 (1966).
(22) W. E. Cohn, *J. Amer. Chem. Soc.*, **72**, 811 (1950).
(23) R. E. Cathou and G. G. Hammes, *ibid.*, **86**, 3240 (1964).

Table I. Effect of Concentration upon the Chemical Shifts of Various Protons of the Mononucleotides[a,b]

Mononucleotides	Concn, *m*		δ, ppm from TMS capillary H-8	H-2	H-1′	H-5′,H-5″	H-2′,H-2″,CH₃
5′-AMP·Na	0.0		8.957	8.705	6.578	4.602	
	0.3		8.805	8.412	6.463	4.658	
		Δδ	0.152	0.293	0.115	−0.056	
5′-AMP·Na₂	0.0		9.055	8.717	6.587	4.483	
	0.3		8.897	8.392	6.472	4.580	
		Δδ	0.158	0.325	0.115	−0.097	
5′-MeAMP·Na	0.0		8.883	8.717	6.605	4.575	3.990 (CH₃)
	0.3		8.730	8.408	6.447	4.585	4.020
		Δδ	0.153	0.309	0.158	−0.010	−0.030
5′-dAMP·Na	0.0		8.861	8.640	6.922		
	0.3		8.720	8.375	6.730		
		Δδ	0.141	0.265	0.192		
5′-dAMP·Na₂	0.0		8.960	8.672	6.945		
	0.3		8.803	8.395	6.753		
		Δδ	0.157	0.277	0.192		
3′-AMP·Na	0.0		8.805	8.697	6.530		
	0.3		8.613	8.347	6.407		
		Δδ	0.192	0.350	0.123		
2′-AMP·Na	0.0		8.773	8.653	6.610		
	0.3		8.697	8.328	6.555		
		Δδ	0.076	0.325	0.055		
2′-AMP·Na₂	0.0		8.810	8.700	6.597	4.323	
	0.3		8.730	8.462	6.562	4.365	
		Δδ	0.080	0.238	0.035	−0.042	
5′-dGMP·Na₂	0.049		8.568		6.712	4.373	3.133
	0.90		8.520		6.598	4.480	3.073
		Δδ	0.048		0.104	−0.110	0.060
			H-6	CH_3	H-1′	H-5′,H-5″	H-2′,H-2″
5′-TMP·Na₂	0.052		8.272	2.392	6.812	4.518	2.838
	0.98		8.280	2.438	6.830	4.580	2.912
		Δδ	−0.008	−0.046	−0.018	−0.062	−0.074

[a] Temperature 28–31°. [b] Δδ values are all positive unless otherwise noted, indicating upfield shifts with increase in concentration. Values of chemical shifts at 0.0 *m* were obtained from extrapolations.

results not only suggest the association of 5′-AMP in water by vertical stacking but also suggest that the geometry for the mode of association of the 5′-AMP is probably very similar to that proposed for the association of adenine nucleosides (see ref 7, Figures 7a and 7b). In this model the six-membered ring is partially overlapping the neighboring six-membered ring so that the H-2 proton will be strongly shielded by the neighboring base most of the time. The H-8 proton and the H-1′ proton will be shielded mainly by the five-membered ring. In addition, according to the model, H-8 and H-1′ will spend less time on the average in the proximity of the five-membered ring than will H-2 in the proximity of the six-membered ring.

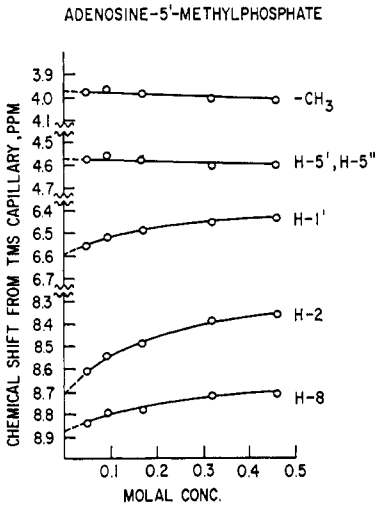

Figure 2. Chemical shift *vs.* molal concentration for protons of adenosine-5′-methyl phosphate (5′-MeAMP(Na)); 31°, D_2O, pD 5.9.

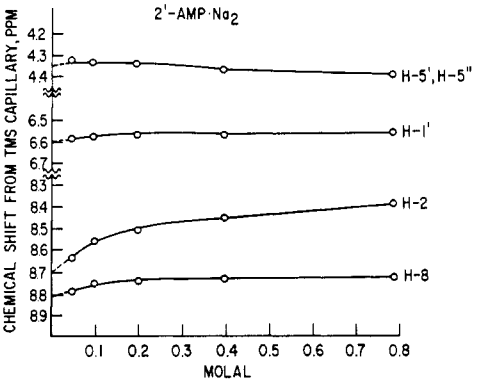

Figure 3. Chemical shift *vs.* molal concentration for 2′-AMP $(Na)_2$ protons; 27°, D_2O, pD 7.4.

It is interesting to compare the Δδ of the H-8, H-2, and H-1′ of the monosodium 5′-AMP (pD 5.8), the monomethyl-5′-AMP, and disodium 5′-AMP (pD 7.4) in Table I and from Figures 1 and 2. Diionization and the methylation of 5′-phosphate have very little effect on the Δδ of these protons. This observation suggests to us that the model in Figure 7b (the alternate stack) in ref 7 is preferred over the model in Figure 7a (the straight stack). The geometrical arrangement of the stacking

bases is the same in these two models, except that the ribosyl phosphate groups of the neighboring nucleotides will be on the *same* side for the "straight stack" and these groups will be on the *opposite* side in the "alternate stack" model. The alternating arrangement in separating the ribosyl phosphate substituents will result in the reduction of electrostatic and steric repulsion. This may be the reason why the increase in the charge of the phosphate group by further ionization has very little effect on the $\Delta\delta$ values. The chemical shifts of the methyl protons of the adenosine 5′-methyl phosphate (Figure 2) are not sensitive to concentration. This is another indication that the phosphate groups of the nucleotide stacks are far away from any diamagnetic moieties such as the purine rings and the hydroxyl groups.

Comparison of the $\Delta\delta$ values of the 5′-dAMP at a range of 0.0–0.2 *m* with that of the 2′-deoxyadenosine[7] does indicate a reduction in the extent of interaction, by about 40% at H-8 and about 30% at H-2 and H-1′. The greater reduction in the $\Delta\delta$ of H-8 may be related to the specific effect of the 5′-phosphate on the H-8 protons which will be discussed later.

The maximum value of $\Delta\delta$ for the H-2 proton is around 0.40 ppm and for the H-8 proton is around 0.20 ppm for 5′-AMP (Figure 1). The difference of the δ value of the H-8 proton of the poly A (8.287 ppm, measured at neutral pH) to the intrinsic δ of H-8 of 3′-AMP is 0.52 ppm, and to the intrinsic δ of the H-8 of 5′-AMP is 0.67 ppm (Table I). The reason for the larger difference in the δ value of the H-8 between poly A and 5′-AMP is because the H-8 proton is specifically deshielded by the 5′-phosphate in the 5′-AMP. This will be fully described in later paragraphs. The difference of the δ value of the H-2 proton of the poly A (8.167 ppm) to the intrinsic δ of the H-2 proton in 3′- or 5′-AMP is 0.53–0.55 ppm (the polymerization shift). On the other hand, the difference of the δ value of the H-8 and H-2 protons of the 3′-AMP in ApA (adenosine dinucleoside monophosphate) as compared to the monomeric 3′-AMP is 0.2 ppm (the dimerization shift). The difference of the δ value of the H-8 and H-2 protons of the 5′-AMP in ApA as compared to monomeric 5′-AMP is 0.32–0.35 ppm (the dimerization shift). Therefore, one may conclude that the maximal value of the $\Delta\delta$ for the H-2 proton in the stacks of 5′-AMP in solution (0.0 to 0.85 *m*) surpasses the dimerization shifts in the ApA and is about 80% of the polymerization shift of the H-2 in the poly A. On the other hand, the values for dimerization or for polymerization shift of the H-2 proton and H-8 proton in ApA or poly A are much closer to each other than the values of $\Delta\delta$ for the H-2 and H-8 in the 5′-AMP stacks or even the adenine nucleoside stacks.[7] This difference probably reflects the restraint of the phosphodiester linkage in bringing the five-membered ring into the diamagnetic environment.

B. The Position of the Phosphate Group. Comparison of the $\Delta\delta$ of the protons of the 2′-AMP and 3′-AMP with the $\Delta\delta$ of the protons of the 5′-AMP reveals some very interesting effects of the position of the charged phosphate group. When the charged phosphate group is moved from the 5′ position to the 2′ position which is much closer to the five-membered ring and the H-1′, the $\Delta\delta$ values of the H-8 and the H-1′ were reduced by about 50% (Table I and Figure 3). The $\Delta\delta$ value for the more distant H-2 proton located at the six-membered ring remains unaffected by this 2′-phosphate group until the phosphate is doubly ionized. Then there is a reduction of $\Delta\delta$ of about 30% for the H-2, 50% for the H-8, and 70% for the H-1′ as compared to the $\Delta\delta$ value of the 5′-AMP (Table I). Thus, placement of the phosphate near the site of the interaction results in a different stacking orientation for 2′-AMP·Na, chiefly involving overlap of the six-membered rings.

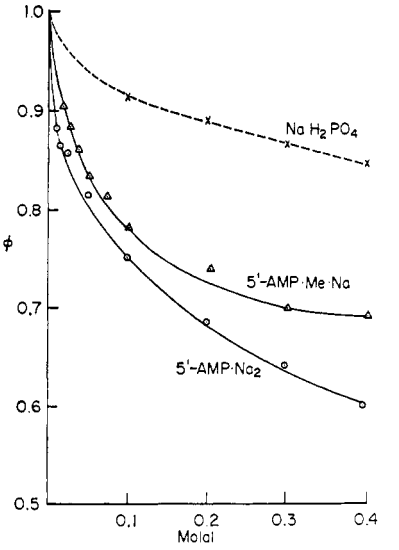

Figure 4. The osmotic coefficient, ϕ, *vs.* molal concentration (*m*) for NaH_2PO_4 (···×···×···), 5′-AMP monomethyl ester sodium salt (—△—△—), and 5′-AMP disodium salt (–⊙–⊙–). The data on NaH_2PO_4 are from ref 28.

When the phosphate is moved from the 5′ position to the 3′ position, the $\Delta\delta$ values appear to be higher especially for the H-8 proton. Examination of the molecular model indicates that now the 3′-phosphate group is distant from the base rings regardless of the rotation around the glycosyl bond (N_7–$C_{1'}$). Thus, the specific interaction of the 5′-phosphate group with the H-8 proton, which occurs when the nucleotide is in an "*anti*" conformation, is abolished. This point will be discussed in greater detail in a later section. This is undoubtedly the reason for the enhancement of the $\Delta\delta$ value of the H-8 proton of the 3′-AMP as compared to the H-8 proton of the 5′-AMP. The H-8 proton of the 3′-AMP can now be overlapped closer by the neighboring molecules in the stack.

C. Difference in Ribose 5′-AMP and Deoxyribose 5′-AMP. Deoxyribose 5′-AMP also stacks to a similar extent as ribose 5′-AMP. The $\Delta\delta$ for the H-1′ of deoxyribose AMP, however, is even larger by about 0.06 ppm (Table I) than the $\Delta\delta$ for the H-1′ of the ribose AMP. Evidently, the replacement of the 2′-OH group by the hydrogen atom allows the neighboring base rings to approach the H-1′ region more readily. Similar behavior was noted for the deoxyribose *vs.* ribose adenine nucleosides.[7]

D. Association of Mononucleotides Consisting of "Nonaromatic" Bases. The $\Delta\delta$ value for the 5′-dGMP·Na_2 is very low even at a large concentration range (0.05–0.90 *m*, Table I). At high concentration, the nucleotide solution is viscous and other physical

evidence indicates that the molecules are stacked.[24-26] Similarly, the $\Delta\delta$ value of the 5′-TMP·Na_2 is also very small even over a large concentration range (Table I). This low value of $\Delta\delta$ has been observed previously for all the pyrimidine nucleosides even though these molecules are shown to associate in water by vapor pressure osmometry.[2,5,7] The observation on 5′-TMP is expected since the value of $\Delta\delta$ is dependent upon the magnitude of the ring current anisotropies as well as the tendency to form stacks. The thymine in the TMP is not "aromatic" in character; theoretical calculation shows that indeed the ring current (or π current) of thymine, uracil, and cytosine is much less than that of the adenine.[27]

The situation for the 5′-GMP is more complicated. Its six-membered ring has very little ring current and is "nonaromatic," but its five-membered ring (the imidazole) has approximately the same calculated ring current as that of the adenine.[27] Furthermore, association of GMP in aqueous solution is caused not only by the stacking of the bases but also by hydrogen bonding of the bases (including N-7) to yield multiple strands.[25,26] It is possible that the noticeable $\Delta\delta$ value for the H-1′ (0.10 ppm) for the 5′-dGMP·Na_2 at this large concentration range (Table I) originates from the ring current effect of the five-membered ring in the stacks. This $\Delta\delta$ value for the H-1′ is substantially larger than the $\Delta\delta$ value for the H-8 proton (0.05 ppm). The only explanation we can propose at the present is the possible occurrence of intermolecular hydrogen bonding at N-7 which will deshield the C-8 protons and offset the high-field shift caused by stacking of the five-membered ring.

E. Other Evidence for the Association of AMP. Besides the pmr data presented here and that reported by Jardetzky,[6] there is other evidence for the association of AMP. In Figure 4, the osmotic coefficients (ϕ) of the adenosine 5′-monomethyl phosphate, 5′-disodium AMP, and monosodium dihydrogen phosphate[28] in various molal concentrations are presented. The values for the nucleotides were obtained with the assumption that these compounds are fully ionized. Clearly, the ϕ value of the 5′-AMP methyl ester is considerably lower than that of the NaH_2PO_4. This is another strong evidence for the association of the 5′-AMP methyl ester. Unfortunately, these data cannot be treated quantitatively without making an assumption about the ionization of the sodium phosphate part of the 5′-AMP with respect to the change of concentration. Recently, by the method of sedimentation equilibrium, Rossetti and Van Holde[29] have found an increase of weight-average "apparent molecular weight" of 5′-AMP (monoanion) for concentrations of 0.05 *M* (40% increase) and higher (up to about 110% increase). It is therefore virtually certain that the AMP does stack in aqueous solution as does adenosine. This observation again provides an explanation for the observed concentration-dependent interaction of AMP with poly U.[30]

F. Association of AMP in the Presence of Poly-L-lysine and Mg^{2+}. Attempts were made to increase the association of AMP by providing Mg^{2+} or poly-L-lysine in solution. It was found in our laboratory that 1.6 equiv of $Mg(ClO_4)_2$ could be added to 5′-AMP·Na (0.1 *M*) in D_2O, pD 5.4, without precipitation. The formation constant of the Mg^{2+} complex with AMP has been reported to be about 100.[31] When the nmr spectra were taken of these samples (0.1 *M*), some small changes were noted when compared with a Mg^{2+}-free control. Specifically, H-2 was shifted upfield 0.033 ppm, H-8 shifted downfield 0.066 ppm, and the respective line widths increased from 1.5 to 1.8 and from 1.5 to 3.1 cps. Though the effect of Mg^{2+} is small, the downfield shift and the line broadening at H-8 are of interest. This suggests the possible interaction of Mg^{2+} with the N-7 of the adenine. Further work on this aspect is needed. Cu(II) and Zn(II) have been reported to interact with the N-7 of the 5′-dAMP and the N-7 of the purine, respectively.[32,33] In these studies, the H-8 line is broadened in the presence of these metal ions and the Zn also causes a downfield shift of the H-8 of the purine.

When poly-L-lysine (free base) and 5′-dAMP acid were mixed in D_2O at a lysine/PO_4 ratio of 2 to give pH 5.4, a precipitate, unsuitable for pmr experiments, was formed which indicates the formation of a complex. Precipitation can be avoided by careful addition of only small amounts of free 5′-dAMP acid or by only partial removal of the bromide ion from the poly-L-lysine hydrobromide by the Dowex I (OH^-) resin. Spectra obtained under these conditions did indicate formation of poly-L-lysine–dAMP complex. The adenine H-2 and H-8 protons were broadened and shifted upfield by about 0.08 ppm which is an increase of $\Delta\delta$ to 50% at the concentration of 0.08 *M*. Solubility difficulties prevented further quantitative experiments.

The Influence of the Phosphate Group in Mononucleotide Spectra. Jardetzky and Jardetzky[9] first studied the pmr spectra of mononucleotides at different pH and they noted the specific influences of the phosphate on the H-8 of the purine nucleotides and H-6 of the pyrimidine nucleotides. These investigators at that time were mainly interested in the problems of the assignment of the proton chemical shifts and the site(s) of protonation of the bases. No discussion was offered about the nature of this specific phosphate influence or any implications regarding the conformation of mononucleotides in aqueous solution.

We are interested in this problem for three main reasons. Firstly, the specific phosphate effect has a direct bearing on the analysis of the dinucleoside monophosphate spectra to be presented in a forthcoming publication. Secondly, accurate knowledge about the conformation of the nucleotide in solution, especially about the rotation around the glyclosyl bond, which defines the geometrical relationship between the base and the pentose, is a prerequisite for the understanding of the conformation of dinucleotides or even poly-

(24) R. K. Ralph, W. J. Connors, and H. G. Khorana, *J. Amer. Chem. Soc.*, **84**, 2265 (1962).
(25) M. Gellert, M. N. Lipsett, and D. R. Davies, *Proc. Natl. Acad. Sci. U. S.*, **48**, 2013 (1962).
(26) H. T. Miles and J. Frazier, *Biochim. Biophys. Acta*, **79**, 216 (1964).
(27) C. Giessner-Prettre and B. Pullman, *Compt. Rend.*, **261**, 2521 (1965).
(28) R. A. Robinson and R. H. Stokes, "Electrolyte Solutions," Butterworth & Co., (Publishers) Ltd., London, 1959, p 484.
(29) G. P. Rossetti and K. R. Van Holde, *Biochem. Biophys. Res. Commun.*, **26**, 717 (1967).
(30) W. M. Huang and P. O. P. Ts'o, Abstracts of the Biophysical Society, 10th Annual Meeting, Boston, Mass., 1966, p 16.
(31) E. Walaas, *Acta Chem. Scand.*, **12**, 528 (1958).
(32) G. Eichhorn, P. Clark, and E. Becker, *Biochemistry*, **5**, 245 (1966).
(33) S. N. Wang and N. C. Li, *J. Amer. Chem. Soc.*, **88**, 4592 (1966).

nucleotides in terms of definite coordinates. Thirdly, the specific phosphate effect reveals some subtle but important intramolecular interaction in the nucleotides.

For convenience in discussing the conformation of the sugar relative to the base, Donohue and Trueblood[34] first defined the sugar–base torsion angle, ϕ_{cn}, as "the angle formed by the trace of the plane of the base with the projection of the C–O bond of the furanose ring when viewed along the C–N bond. This angle will be taken as zero when the furanose-ring oxygen atom is antiplanar to C_2 of the pyrimidine or purine ring, and positive angles will be taken as those measured in a clockwise direction when viewing from C to N." They further concluded that there are two ranges of ϕ_{cn} for the nucleosides: *ca.* $-30°$ for the *anti* conformation, and *ca.* $+150°$ for the *syn* conformation. All the X-ray data and stereochemical analysis based on X-ray data[35–37] indicated that most of the purine and pyrimidine nucleosides and nucleotides exist in the solid state with the *anti* conformation including 5′-AMP[38] and 3′-AMP,[39] except the case of deoxyguanosine (*syn* conformation) in a mixed crystal with 5-bromodeoxycytidine.[40] The *anti* conformation of the dianion 5′-AMP is illustrated in Figure 5. The situation about the conformation of nucleosides or nucleotides in solution is far less clear. However, the X-ray studies also showed that there is a substantial rotational barrier between these two conformations, and a rapid conversion between them is unlikely. In a series of papers Ulbricht and his associates attempted to correlate the sign of the Cotton effect as studied by ORD of various cyclonucleosides, which have fixed *syn* or *anti* conformations, to those of the naturally occurring compounds in the assignment of the conformation for the latter.[41–44] For instance, they reported that the sign of the Cotton effect (negative) of C-8,5′-cycloadenosine which has a fixed *anti* conformation is the same as the adenosine,[43,45] while the Cotton effect is positive for the 2′,3′-isopropylidene-N-3,5′-cycloadenosine which has fixed *syn* conformation. Recently, Klee and Mudd[46] stated that the 2′,3′-isopropylidene-N-3,5′-cycloadenosine is very unstable in solution, and they expressed doubts about using this compound as a model. These authors found that the substitution of the 5′-OH of adenosine by SCH_3 to give 5′-methylthioadenosine causes the sign of the Cotton effect to change from negative to positive. They suggested that the adenosine may exist in a *syn* conformation. Furthermore, Hampton and Nichol,[47] as well as Miles, *et al.*,[48] had also presented ORD data on various N-3,5′-

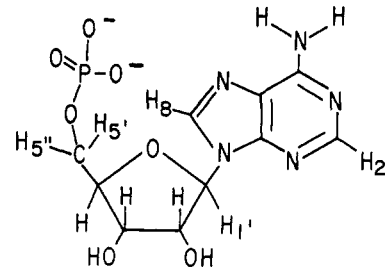

Figure 5. The 5′-AMP disodium salt depicted in an *anti* conformation with the H-8 proton in juxtaposition with the phosphate group.

purine cyclonucleosides. They reported that while the N-3,5′-cycloxanthosine[47] (or its 2′,3′-O-isopropylidine derivative)[48] has a positive Cotton effect, the 2′,3′-O-isopropylidene-N-3,5′-cycloguanosine and the 2′,3′-O-isopropylidene-N-3,5′-cycloadenosine still have a negative Cotton effect. The ORD pattern of the same cycloadenosine compound obtained by these authors is in direct disagreement with that obtained by Emerson, *et al.*[43] Lin, *et al.*, reported that they have observed an effect of pH at the range of 4.9 to 8.9 (pK_a of the secondary phosphate) on the ORD curve of the 5′-AMP at the region of 220–210 mμ.[49] They interpret this result to mean an intramolecular hydrogen bonding of the 5′-phosphate hydrogen to the N-3 of the purine. This intramolecular hydrogen bonding requires that the 5′-AMP has the *syn* conformation. However, in spite of repeated effort, we were not able to confirm the observation reported by Lin, *et al.*[50] We can only observe the effect of pH on the ORD pattern at the range of the pK_a of the adenine. In pmr studies, Cushley, *et al.*, have compared the chemical shifts of the acetyl group of various uracil nucleosides (both pyranose and furanose) and that of the corresponding dihydrouracil nucleoside in DMSO.[51] They concluded that there is an anisotropic effect of the 5,6 double bond on these acetyl groups. Therefore, they proposed that there is a restriction of the rotation around the glycosyl bond of these uracil nucleosides and that these compounds have an *anti* conformation in DMSO.[52]

A. Influence of the Phosphate Group as Shown by the Ionization of the Secondary Phosphate. Effect of Change in pD from 5.9 to 7.4. The effect of pD on the chemical shifts of 5′-AMP and 5′-AMP monomethyl ester at 0.09 *M* is shown in Figure 6. At the region of pD 4.5 to 6.0, the effect of the pD is on the deprotonization of the base. The loss of the proton from the adenine moiety causes the upfield shift of the base protons both by the removal of the intrinsic deshielding effect of the positive charge and by the increase in the intermolecular association of the bases. This interpretation is confirmed by the identical results obtained from both the 5′-AMP and 5′-AMP monomethyl ester.

(34) J. Donohue and K. N. Trueblood, *J. Mol. Biol.*, 2, 363 (1960).
(35) J. Kraut, *Ann. Rev. Biochem.*, 34, 247 (1965).
(36) V. Sasisekharan, A. V. Lakshminarayanan, and G. N. Ramachandran in "Conformation of Biopolymers," Vol. 2, G. N. Ramachandran, Ed., Academic Press Inc., New York, N. Y., 1967, p 641.
(37) A. E. V. Haschemeyer and A. Rich, *J. Mol. Biol.*, 27, 369 (1967).
(38) J. Kraut and L. H. Jensen, *Acta Cryst.*, 16, 79 (1963).
(39) M. Sundaralingam, *ibid.*, 21, 495 (1966).
(40) A. E. V. Haschemeyer and H. M. Sobell, *ibid.*, 19, 125 (1965).
(41) T. L. V. Ulbricht, T. R. Emerson, and R. J. Swan, *Biochem. Biophys. Res. Commun.*, 19, 643 (1965).
(42) T. L. V. Ulbricht, T. R. Emerson, and R. J. Swan, *Tetrahedron Letters*, 1561 (1966).
(43) T. R. Emerson, R. J. Swan, and T. L. V. Ulbricht, *Biochem. Biophys. Res. Commun.*, 22, 505 (1966).
(44) T. R. Emerson, T. J. Swan, and T. L. V. Ulbricht, *Biochemistry*, 6, 843 (1967).
(45) J. T. Yang, T. Samejima, and P. K. Sarkar, *Biopolymers*, 4, 623 (1966).
(46) W. A. Klee and S. H. Mudd, *Biochemistry*, 6, 988 (1967).
(47) A. Hampton and A. W. Nichol, *J. Org. Chem.*, 32, 1688 (1967).
(48) D. W. Miles, R. K. Robins, and H. Eyring, *Proc. Natl. Acad. Sci. U. S.*, 57, 1138, (1967).
(49) C. Y. Lin, D. W. Urry, and H. Eyring, *Biochem. Biophys. Res. Commun.*, 17, 642 (1964).
(50) We wish to thank Professor J. T. Yang, University of California, San Francisco, Calif., for his effort in doing independent experiments to check our results.
(51) R. J. Cushley, K. A. Watanabe, and J. J. Fox, *J. Amer. Chem. Soc.*, 89, 394 (1967).
(52) In ref 51, Cushley, *et al.*, used the description of "5,6 double bond of the aglycon is *endo* to the five-membered sugar ring" which is equivalent to the *anti* conformation.

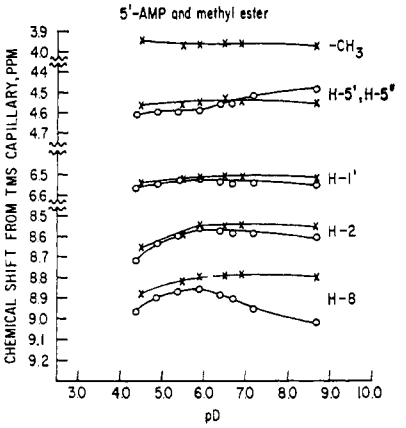

Figure 6. Chemical shift *vs.* pD for protons of 5′-AMP (—⊙—, 35°, 0.08 *M*) and 5′-AMP monomethyl ester (— × —, 30°, 0.08 *M*).

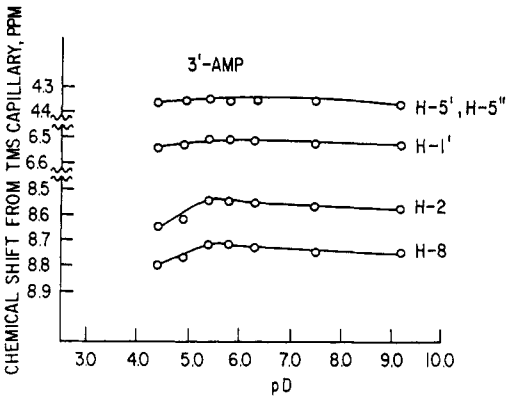

Figure 7. Chemical shift *vs.* pD for protons of 3′-AMP (0.09 *M*, 30°).

At the region of pD 6.0 to 8.7, the secondary phosphate is being ionized and thus increases its electron density. This is illustrated by the increase in shielding of the H-5′,H-5″ protons of the 5′-AMP at higher pD, while the H-5′ proton of the methyl ester is unchanged as expected. At this region, the H-8 proton of the 5′-AMP is specifically and substantially deshielded (~0.1 ppm), while the H-8 and H-2 protons of the corresponding methyl ester are unaffected. There is a slight downfield shift (~0.02 ppm) of the H-2 proton of the 5′-AMP. This shift is most likely due to the small effect of the ionization of the secondary phosphate on the stacking of the 5′-AMP and to the fact that the H-2 proton is most sensitive to the degree of stacking as described above. This interpretation also explains the even smaller deshielding of the H-1′ proton (~0.01 ppm) of the 5′-AMP upon increasing pD. Therefore, since the deshielding effect due to the increase of pD from 6 to 8 occurs only in the 5′-AMP and not in its monomethyl ester, and since this deshielding effect occurs only in H-8 and not in H-2, this effect must be a direct result of the ionization of the secondary phosphate on the H-8 proton. This specific effect of the phosphate group on the H-8 suggests strongly that the 5′-AMP is in an *anti* conformation as depicted in Figure 5. In this conformation, the H-8 proton is in juxtaposition to the 5′-phosphate group.

In Figure 7, the effect of pD on the chemical shifts of the 3′-AMP is shown. Now, when the phosphate

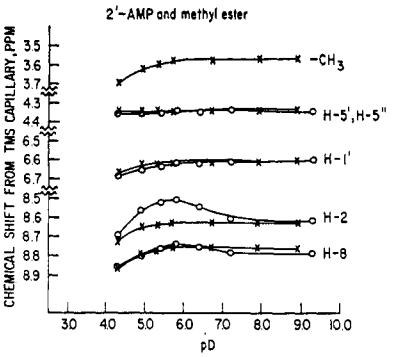

Figure 8. Chemical shift *vs.* pD for protons of 2′-AMP (—⊙—, 0.09 *M*, 30°) and adenosine 2′-methyl phosphate (— × —, 0.09 *M*, 25°).

group is moved from the 5′ position to the 3′ position, the specific effect of ionization of the secondary phosphate essentially disappears. The small deshielding effect upon increase in pD from 6 to 9 observed for the H-2, H-8, and H-1′ protons (0.01–0.02 ppm) is probably due to a slight decrease in stacking because of the increase in the charge of the phosphate group as explained above for the similar effect on the H-2 proton of the 5′-AMP. The shielding effect upon increase in pD from 4.5 to 6 due to the deprotonation of the adenine ring is also observed here.

In Figure 8, the effect of pD on the chemical shifts of the 2′-AMP and of the 2′-AMP monomethyl ester is shown. The curves of the H-1′, H-5′, H-5″, and H-8 protons are nearly superimposible but not those for H-2. The 2′-AMP is more shielded at the monoanion level compared to the methyl ester, but upon the ionization of the secondary phosphate, the H-2 of the 2′-AMP is equivalent to that of the methyl ester. These results can be partially explained by reference to Table I. The data reported in Table I show that the dianion of the 2′-AMP associates to a lesser degree than the monoanion of the 2′-AMP, when monitored by the $\Delta\delta$ of H-2. This is one reason for the deshielding of H-2 observed upon dianion formation at higher pD. Other possible intramolecular effects of the 2′-phosphate will be further discussed in later paragraphs. A small effect of deshielding on the H-8 observed is presumably also due to the decrease in association at higher pD. It is interesting to note from Figure 8 that the stacking of the monomethyl ester of the 2′-AMP is less than that of the monoanion of the 2′-AMP and is equivalent to the dianion, as far as the chemical shift of the H-2 is concerned. Evidently, the steric effect of the methyl group exerts an influence specifically on the stacking of the H-2 region of the molecules while it has no effect on 5′ isomers (Table I). In agreement with this interpretation, note also that the methyl protons in the 2′-AMP methyl ester shift upfield in response to the deprotonization of the adenine bases. This is an indication that the methyl group is close enough to the base to be influenced by the diamagnetic effect of the ring current in the stacks.

Table II shows the effect of the secondary phosphate ionization (from pD 5.9, 10% dianion to pD 7.4, 90% dianion) on the chemical shift of various mononucleotides. For the purine nucleotides, the change of pD from 5.9 to 7.4 has virtually no effect on the H-2 protons save that for the 2′-AMP as has been discussed

Table II. The Effect of the Secondary Phosphate Ionization (from pD 5.9 to 7.4) on the Chemical Shift of Various Mononucleotides ($\Delta\delta$ = δpD 5.9 − δpD 7.4)[a]

A. Purine nucleotides	Concn, *M*	δ[a] H-8 pD 5.9	δ[a] H-8 pD 7.4	H-8 $\Delta\delta$	δ[a] H-2 pD 5.9	δ[a] H-2 pD 7.4	H-2 $\Delta\delta$
5′-AMP	(0.08)	8.853	8.950	0.097	8.567	8.588	0.021
5′-AMP	(0.25)	8.792	8.895	0.103	8.425	8.442	0.071
5′-MeAMP	(0.08)	8.795	8.795	0	8.550	8.553	0.003
3′-AMP	(0.09)	8.717	8.737	0.020	8.547	8.562	0.015
2′-AMP	(0.09)	8.733	8.780	0.047	8.508	8.608	0.100
2′-MeAMP	(0.09)	8.758	8.758	0	8.628	8.625	+0.003
5′-IMP	(0.09)	8.888	9.000	0.112	8.645	8.662	0.017
5′-GMP[b]	(0.02)	8.500	8.613	0.113			
5′-MeGMP	(0.09)	8.462	8.465	0.003			
3′-GMP[c]	(0.02)	8.393	8.423	0.030			
5′-dAMP	(0.08)	8.788	8.883	0.095	8.533	8.562	0.029
Tubercidin 5′-phosphate	(0.1)	8.000	8.008	0.008	8.510	8.513	0.003
B. Pyrimidine nucleotides		**δ[a] H-6**			**δ[a] H-5**		
5′-CMP	(0.02)[d]	8.424	8.504	0.080	6.588[e]	6.600	0.012
5′-MeCMP	(0.09)	8.383	8.383	0		*f*	
3′-CMP	(0.03)	8.303	8.287	+0.016		*g*	
2′-CMP	(0.03)	8.241	8.233	+0.008		*g*	
5′-UMP[h]	(0.02)	8.403	8.533	0.130	6.370	6.398	0.028
5′-MeUMP	(0.1)	8.352	8.357	0.005	6.372	6.378	0.006
5′-dUMP	(0.09)	8.443	8.527	0.084	6.400	6.430	0.030
5′-TMP	(0.095)	8.190	8.240	0.050	2.361[i]	2.371[i]	0.010
5,6-Dihydro-5′-UMP	(0.1)	4.097	4.115	0.018	3.220	3.235	0.015
5′-dCMP	(0.09)	8.411	8.472	0.061	6.551	6.554	0.003
C. Nucleosides							
Uridine	(0.1)	8.370[j]	8.368[k]	+0.002	6.398[j]	6.373[k]	+0.005
		H-8			H-2		
Inosine	(0.05)	8.762[l]	8.755[m]	+0.007	8.653[l]	8.642[m]	+0.011

[a] Measured from TMS capillary; $\Delta\delta$ values negative unless indicated. [b] In mixture with 0.02 *M* 3′-AMP. [c] In mixture with 0.02 *M* 5′-AMP. [d] In mixture with 0.02 *M* 2′(3′)-CMP. H-5 not discernible. [e] In 0.09 *M* concentration measured with 60-Mc instrument. The absolute δ value is not as accurate as that quoted in Table III, but $\Delta\delta$ is accurate. [f] H-5 was not unambiguously determined. [g] Difficult to measure shifts due to broad resonances. [h] In mixture with 0.02 *M* 3′(2′)-UMP. [i] Methyl protons. [j] 0.2 *M* NaH_2PO_4. [k] 0.2 *M* Na_2HPO_4. [l] Sodium acetate buffer. [m] Adjusted by NaOD checked before and after run.

above. As controls, this change of pD has no effect on the H-2 or H-8 protons of all the monomethyl esters of 5′-AMP, 2′-AMP, and 5′-GMP or on the nucleoside, inosine. Little or no effect on the H-2 can also be observed for the dianion formation of the 3′-phosphate of the AMP and the CMP. The small effect on the H-8 of 2′-AMP is probably due to destacking as discussed above. The ionization of the secondary phosphate has a large effect (0.1 ppm) on the H-8 protons of the 5′-AMP, 5′-deoxy-AMP, 5′-IMP, and 5′-GMP. This ionization has no effect on the H-8 or H-7 protons of tubercidin 5′-phosphate which will be discussed later in relation to the mechanism.

As for the pyrimidine nucleotides, this change of the pD from 5.9 to 7.4 has virtually no effect on the H-5 protons. As controls, this increase of pD has no effect on the H-5 or H-6 protons of the methyl esters of 5′-CMP and 5′-UMP, or on the protons of the nucleoside, uridine, which was dissolved in 0.2 *M* phosphate buffer. This ionization of the secondary phosphate has a specific and substantial effect (~0.08 ppm) on the H-6 proton of the 5′-CMP, 5′-dUMP, 5′-TMP and 5′-CMP. The ionization has no effect on the H-6 of 3′-CMP or 2′-CMP, or on the H-6 proton(s) of the 5,6-dihydro-5′-UMP, as will be discussed later. It should be recalled also that the chemical shifts of the base protons in pyrimidine nucleosides are not influenced by the degree of their self-association.[5] We may conclude, therefore, by analogy to the 5′-purine nucleotides, that the 5′-phosphate group of the pyrimidine nucleotide also has a direct and specific effect on the H-6 proton and not on the H-5 proton. Again, in order to produce this intramolecular deshielding, the 5′-pyrimidine phosphate must be in the *anti* conformation. In this conformation, the H-6 proton is in juxtaposition to the 5′-phosphate group.

B. Influence of the Phosphate Group as Shown by the Comparison of the Nucleoside and the Monoanion of the Corresponding Nucleotides at pD 5.9. Table III shows the influence of the phosphate group by the comparison of the nucleoside with the corresponding nucleotide in monoanion form. The chemical shifts of all the adenine derivatives are obtained by extrapolation to infinite dilution. The chemical shifts of the other compounds at low concentration are not sensitive to concentration.[5,7] Therefore, the influence of the intermolecular stacking is essentially eliminated from the data in Table III.

First of all, if the monoanionic phosphate group is substituted at the 3′ position, no difference in chemical shift can be found for the base protons (H-8 and H-2 of purine, H-5 and H-6 of pyrimidine) and for the H-1′ and H-5′ and H-5″ protons between the nucleoside and the corresponding 3′-nucleotide as exemplified by the adenosine *vs.* 3′-AMP and the cytidine *vs.* 3′-CMP. If the phosphate group is substituted at the 5′ position, the H-8 protons of the purine nucleotides, such as 5′-AMP, 5′-dAMP, 5′-GMP, and 5′-IMP, are deshielded specifically by about 0.12 ppm, while the H-2 protons are unaffected. In an analogous manner and

Table III. Influence of the Phosphate Group as Shown by the Comparison of the Chemical Shifts of the Nucleosides and Nucleotides (pD 5.9); Chemical Shifts Measured from TMS Capillary

		H-8	H-2	H-6	H-5	H-1′	H-5′,H-5″
5′-AMP[a]		8.957	8.705			6.578	4.602
Adenosine[a]		8.820	8.730			6.533	4.353
	Δδ	0.137	−0.025			0.045	0.249
Adenosine 5′-methyl hydrogen phosphate[a]		8.883	8.717			6.605	4.572
Adenosine[a]		8.820	8.730			6.533	4.353
	Δδ	0.063	−0.013			0.072	0.219
5′-dAMP[a]		8.861	8.640			6.922	
2′-Deoxyadenosine[a]		8.756	8.686			6.930	
	Δδ	0.105	−0.046			−0.008	
3′-AMP[a]		8.805	8.697			6.530	
Adenosine[a]		8.820	8.730			6.533	
	Δδ	−0.015	−0.033			0.003	
2′-AMP[a]		8.773	8.653			6.610	
Adenosine[a]		8.820	8.730			6.533	
	Δδ	−0.047	−0.077			−0.077	
5′-GMP[b]		8.544				6.381	
1-Methylguanosine		8.418				6.375	
	Δδ	0.126				0.006	
5′-IMP[c]		8.888	8.662			6.592	4.603
Inosine[c]		8.762	8.653			6.542	4.367
	Δδ	0.126	0.009			0.050	0.236
5′-CMP[c]				8.424	6.546	6.402	4.587
Cytidine[c]				8.292	6.513	6.367	4.333
	Δδ			0.132	0.033	0.035	0.254
Cytidine 5′-methyl hydrogen phosphate[c]				8.383	*d*	6.463	4.625
Cytidine[c]				8.292	6.513	6.367	4.333
	Δδ			0.091		0.096	0.292
5′-UMP[b]				8.403	6.370	*d*	*d*
Uridine[c]				8.275	6.327	6.343	4.295
	Δδ			0.128	0.043		
Uridine 5′-methyl hydrogen phosphate				8.352	6.372	6.406	4.566
Uridine[c]				8.275	6.327	6.343	4.295
	Δδ			0.077	0.045	0.063	0.271
3′-CMP[c]				8.303	6.533	6.372	4.325
Cytidine[c]				8.292	6.513	6.367	4.333
	Δδ			0.011	0.023	0.005	−0.008
5′-TMP[c]				8.190	2.350[e]	6.774	4.486
Thymidine[c]				8.087	2.318[e]	6.715	4.283
	Δδ			0.103	0.032	0.059	0.203

[a] Extrapolated infinite dilution shifts. [b] 0.02 *M*, in mixture with 0.02 *M* 3′(2′)-UMP. 1-Methylguanosine was used because of solubility problem of guanosine. Since the chemical shift of the H-8 proton is the same for inosine and 1-methylinosine, this substitution of guanosine was considered justifiable.[7] [c] 0.09 *M*. [d] These resonances not discernible. [e] Methyl protons.

with a similar magnitude, the H-6 protons of the pyrimidine nucleotides such as 5′-CMP, 5′-UMP, and 5′-TMP are deshielded to a much greater extent than the H-5 protons. Owing to the electron-withdrawing effect of the phosphoryl group, the H-5′ and H-5″ protons which are neighboring to the phosphoester are deshielded. The electron density of the phosphoryl group is expected to be the highest in the dianion form, next as the monomethyl ester, and lowest in the monoanion form. Indeed, this is what was indicated by the chemical shifts of the H-5′,H-5″ protons listed in Table I, especially for the AMP series. The H-5′,H-5″ protons are at the highest field when there is no substitution at the 5′-OH group and at the lowest field when there is substitution by the singly ionized phosphoryl group. The values of the chemical shift of the H-5′, H-5″ protons are in between these two limits when the 5′-OH group is substituted with dianionic phosphate or phosphoryl monoester. On the other hand, the deshielding effect on the H-8 (for purine) or H-6 (for pyrimidine) of the 5′-phosphate is strongest when it is dianionic, as anticipated (Table II), weaker when it is monoanionic (Tables II and III), and weakest when it is in the form of monomethyl ester (Table III). The only reason we can offer to explain why the deshielding effect is stronger when the phosphoryl group is in the monoanionic form than when it is in the monomethyl ester form is that the methyl group on the phosphate may provide a steric hindrance in reducing the deshielding effect. The 5′-substituted phosphate, especially when it is a monoester, also appears to have a small deshielding effect on the H-1′ proton (Table III). This effect is not observable for 5′-GMP and 5′-dAMP as well as for the 3′-substituted nucleotides such as 3′-AMP and 3′-CMP. The explanation of the small effect is not certain at present and will be further discussed in later paragraphs.

Substitution of the phosphate group at the 2′ position, as in the case of 2′-AMP, has caused the shielding of H-8 and especially the H-2 protons (0.05–0.08 ppm) and expectedly a deshielding of the H-1′. The effect of the 2′-phosphoryl group on the base protons can be examined from two points of view: (A) the direct effect of the phosphoryl group, and (B) the indirect effect of the removal of the 2′-OH group which is likely to form a hydrogen bond with the N-3 of the base.

In our previous publication,[7] this intramolecular hy-

drogen bonding was proposed to explain the deshielding of the base protons of the adenosine in comparison to those of 2′-deoxyadenosine and of 2′-O-methyladenosine, as well as the slight lowering (by 0.1∼0.15 pH) of the pK_a of adenosine in comparison to deoxyadenosine. So, if the chemical shifts of the base protons of the 2′-AMP are to be compared with 2′-deoxyadenosine (or 2′-O-methyladenosine cited in ref 7) as shown in Tables IV or V, these values differ only by ±0.02–0.03 ppm, which is within experimental error since all the values have to be obtained from extrapolation to infinite dilution. Thus, most of the shielding effect of the 2′-phosphoryl group is an indirect one and this can be accounted for by the removal of the intramolecular hydrogen bonding of the 2′-OH group. Upon examination of the molecular model, however, one is impressed with the close proximity of the charged 2′-phosphate to the bases. It appears that some interaction is likely to take place. Data in Table IV indicated that when the 2′-phosphoryl group is further ionized from the monoanionic state to the dianionic state, there is a small deshielding effect on the base protons. Preliminary data from our laboratory[53] indicated that the negative Cotton effect of the ORD curve of the 2′-AMP in dianionic form ($[M]_{277m\mu} = -2900°$) is about the same as that from the monoanionic form ($[M]_{277m\mu} = -3000°$). The monoanionic 2′-AMP was reported by Klee and Mudd[46] to have an ORD pattern practically identical with that of adenosine. Therefore, we can tentatively conclude that the direct effect of the phosphate group in 2′-AMP is a small and subtle one and unlikely to cause a major change in conformation. In a mixture of 2′-AMP and 3′-AMP, the base protons of the two isomers are readily separable in the pmr spectrum, with the protons of 2′-AMP at higher field (Table I). Similarly as shown in Table II, the δ H-6 of 2′-CMP is about 0.05–0.06 ppm to higher field than the δ H-6 of 3′-CMP. This difference is readily observed in a 2′(3′)-CMP mixture in our studies by a 100-Mc instrument, and this was briefly reported earlier by the Jardetzkys[9] though they did not separate and assign the δ position to the individual nucleotides. Such a separation of the base protons from the isomers, however, was not found in 2′(3′)-UMP and 2′(3′)-GMP mixtures. By spectroscopic titration, Fox, *et al.*,[54] has shown that 2′-CMP has a pK_a for the protonation of the base higher by 0.15–0.2 pH than the 3′-CMP unit, and they interpreted these data in terms of the removal of the intramolecular hydrogen bonding of the 2′-OH group to the 2-keto group of the base. The 2′(3′)-nucleotide mixtures of cytosine, adenine, and guanine are separable from each other by their chromotographical behavior in ion-exchange resin columns, which indicates that the pK_a of the bases are indeed slightly different from one another in these pairs of isomers.[55] All we can conclude at the present is that the differences in these isomers are subtle and small, likely to be involved in the problem of the intramolecular hydrogen bonding of the 2′-OH group, and unlikely to result from a major conformational change such as from "*anti*" to "*syn*."

Table IV. Chemical Shifts of the Adenine Nucleosides and Nucleotides at Infinite Dilution

	H-8	H-2	H-1′
Adenosine	8.820	8.730	6.533
2′-Deoxyadenosine	8.756	8.686	6.930
5′-AMP·Na	8.957	8.705	6.578
5′-dAMP·Na	8.861	8.640	6.922
5′-MeAMP·Na	8.883	8.717	6.605
5′-AMP·Na_2	9.055	8.717	6.587
5′-dAMP·Na_2	8.960	8.672	6.945
3′-AMP·Na	8.805	8.697	6.530
2′-AMP·Na	8.773	8.653	6.610
2′-AMP·Na_2	8.810	8.700	6.597

Table V. Chemical Shifts of H-1′ and H-2′,H-2″ in Deoxynucleotides

	Concn, *M*	pD	Shifts,[a] ppm H-1′	Shifts,[a] ppm H-2′,H-2″
5′-TMP	0.095	5.95	6.774	2.800
	0.095	7.6	6.770	2.814
5′-dUMP	0.092	5.95	6.797	2.847
	0.092	7.40	6.808	2.857
5′-dCMP	0.087	5.95	6.754	2.795
	0.087	7.50	6.769	2.794
5′-dAMP	0.084	5.9	6.824	3.113
	0.084	7.4	6.851	3.125
5′-dGMP	0.11	7.7	6.702	3.122

[a] From TMS.

Intramolecular Hydrogen Bonding in Nucleotides. As mentioned in the previous section, a proposal has been made by us and by others (see review in ref 56 and 7) about the intramolecular hydrogen bonding of the 2′-OH group of the ribose to the N-3 of purine or to the 2-keto of the pyrimidines. Most of the experimental data pertaining to this question came from the studies on the nucleosides. Here we wish to report and to discuss the pmr data on the nucleotides pertinent to this question. Before going any further, it should be noted that in order for this intramolecular hydrogen bonding to take place, the conformation of both purine and pyrimidine nucleotides has to be in the "*anti*" form and not in the "*syn*" form. The data presented so far strongly suggest that the conformation of the nucleotide is indeed in the "*anti*" form. As a corollary to this conclusion, there is *unlikely* to be an intramolecular hydrogen bonding between the 5′-OH and the 2-keto of the pyrimidine nucleoside or N-3 purine nucleoside, since it requires the conformation of the nucleosides to be in the form of "*syn*."

A. The Difference between the Chemical Shifts of the H-2 Protons of the Ribose and Deoxyribose Adenine Nucleotides. Table IV listed the δ values of the H-8, H-2, and H-1′ protons of the deoxyribose and ribose adenine nucleosides and nucleotides at infinite dilution. The chemical shifts for H-1′ of the adenine ribosides and 5′-nucleotides occur at about 0.35–0.4 ppm upfield from that of the corresponding deoxyribose compounds. As reported and discussed in our previous paper,[7] this phenomenon was attributed to the magnetic shielding of H-1′ by the *cis*-oxygen atom in the 2′ position and it is very useful for identification purposes for 2′-deoxy, ribose, or arabinose nucleosides. It should be noted here (Table IV) that the δ value of the H-1′ proton is

(53) R. S. Umans and H. Holmes, unpublished data.

(54) J. J. Fox, L. F. Cavalieri, and N. Chang, *J. Amer. Chem. Soc.*, **75**, 4315 (1953).

(55) W. E. Cohn and E. Volkin, *Nature*, **167**, 483 (1951).

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

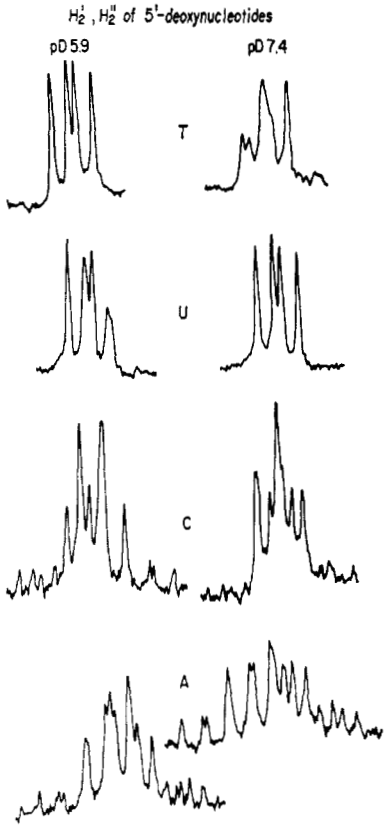

Figure 9. The effect of the 5′-secondary phosphate ionization on the H-2′,H-2′′ pattern of deoxynucleotides; pD 5.9 (10% dianion) to pD 7.4 (90% dianion); T = 5′-TMP, U = 5′-dUMP, C = 5′-dCMP, A = 5′-dAMP.

more deshielded in the 2′-AMP as compared to the 3′-AMP, 5′-AMP, or the adenosine due to the presence of neighboring phosphoryl groups. This phenomenon, therefore, can be used for identification purposes in pmr studies to distinguish the 2′-phosphoryl nucleotides from the 3′-phosphoryl nucleotides. This phenomenon had also been studied previously by Fromageot, *et al.*[57]

The H-2 protons of adenine nucleotides are not greatly influenced by the phosphate group and will be discussed here. As shown in Table IV, and in a previous publication,[7] the H-2 proton of 2′-deoxyadenosine is about 0.05 ppm more shielded than the H-2 proton of adenosine. This difference of the chemical shift of H-2 protons can be found in the comparison between the 5′-AMP and the 5′-dAMP regardless of the state of ionization of the phosphate. Hence, these data suggest that there is also an intramolecular hydrogen bonding of 5′-AMP similar to that in adenosine. The chemical shift of the H-2 proton of 3′-AMP is very close to that of the 5′-AMP and the chemical shift of the H-2 proton of the 2′-AMP, which has no free 2′-OH group, is very close to the 2′-deoxy-AMP. These data also suggest that an intramolecular hydrogen bonding may exist in 3′-AMP as well. This interpretation is far less certain, however, as compared to that concerning the 5′-AMP. The main reason is that data on 3′-dAMP are not easily obtainable, and therefore no direct comparison was made. Furthermore, as mentioned earlier, it is not certain whether the 2′-phosphoryl group is exerting an effect on the H-2 proton in the case of the 2′-AMP.

(57) H. P. M. Fromageot, B. E. Griffin, C. B. Reese, J. E. Sulston and D. R. Trentham, *Tetrahedron*, 22, 705 (1966).

B. The Influence of the 5′-Phosphoryl Group on the H-6 Proton (Pyrimidine Nucleotide) and on the H-8 Proton (Purine Nucleotide) as Related to the Intramolecular Hydrogen Bonding Involving the 2′-OH Group. The procedure of the above section A is not applicable to pyrimidine nucleosides or nucleotides for the testing of the intramolecular hydrogen bonding involving the 2′-OH group. Such a hydrogen bonding to the exocyclic oxygen is unlikely to affect the protons of the double bond in the ring especially since the ring is not extensively conjugated. Indeed, the δ value of the H-5 proton is very similar between the corresponding ribose *vs.* deoxyribose nucleotide such as 5′-CMP (Table III) *vs.* 5′-dCMP (Table II). On the other hand, the deshielding influence of the 5′-phosphoryl group on the H-6 proton is dependent on the *anti* conformation which in turn is likely to be stabilized by a hydrogen bond from 2′-OH group to the 2-keto group. Thus, it might be expected that the loss of these stabilizing forces would reduce the proportion of the molecules in a proper *anti* conformation and consequently the phosphate influence would be less.

Data in section B of Table II show the deshielding effect on the H-6 protons of the ionization of the secondary phosphate (from pD 5.9 to 7.4) of various pyrimidine nucleotides. The deshielding due to this secondary ionization is indeed less for the deoxynucleotides, only to the extent of about 60–70% of the corresponding ribonucleotides, such as 5′-dUMP (0.084 ppm) *vs.* 5′-UMP (0.13 ppm) and 5′-dCMP (0.062 ppm) *vs.* 5′-CMP (0.08 ppm). Thus, the deshielding effect of the 5′-phosphate group on the H-6 protons is dependent to a certain extent on the presence of the 2′-OH group in the ribose. We may conclude, therefore, though there is no direct pmr evidence in the case of the pyrimidine nucleotides (or nucleosides) to support the intramolecular hydrogen bonding involving the 2′-OH group and the 2-keto group, there is some indirect evidence from the dependence of the phosphate effect on the presence of a 2′-OH group to infer the existence of such bonding. In the case of the adenine nucleotide, such a dependence appears to be very small. The secondary ionization effect on the H-8 of 5′-AMP is about 0.10 ppm, practically identical with that of the 5′-dAMP which is about 0.095 ppm. The details of the stereochemical and energy considerations may be different in this case. The *anti* conformation may be more favored in the purine nucleotides than in the pyrimidine nucleotides and thus not affected by the presence or absence of the intramolecular hydrogen bond.

Effect of the 5′-Phosphate Ionization on the Spectral Pattern of the Deoxyribose Protons. Figure 9 shows the spectral change of the multiple patterns of the H-2′,H-2′′ resonances upon the ionization of the secondary phosphate from pD 5.9 to 7.4. Whereas for 5′-TMP the H-2′,H-2′′ pattern goes from the usual quartet seen for the A_2MX system to something more complicated, the reverse is observed for 5′-dUMP. Much larger effects are seen for 5′-dCMP and 5′-dAMP. Hence, these effects are base specific. This suggests that the phosphate ionization may alter slightly the

spatial arrangement of the base and pentose ring to bring about a change in magnetic environment of the H-2′ and H-2″. The possibility also exists that small conformational changes are brought about in the deoxyribose rings and thus change the spin–spin couplings between H-3′ and H-2′,H-2″. The change in coupling with H-1′ is likely to be small, since the H-1′ pattern is not affected by the ionization of the secondary phosphate.

The chemical shifts of the H-2′,H-2″ protons in purine 2′-deoxynucleotides (0.1 *M*, pD 7.4) are 0.3 ppm to lower field than those of pyrimidine 2′-deoxynucleotides (Table V). This is unlikely to be caused by a difference in the inductive effect of the purine *vs.* pyrimidine ring on the 2′-carbon since the H-1′ of dAMP is only about 0.05 ppm lower field than the H-1′ of pyrimidine nucleotides, and the H-1′ of dGMP is even at higher field. The most plausible explanation is that these protons in the purine derivatives are experiencing deshielding by the ring current anisotropy by being nearly in the plane of the purine ring with the nucleotides in the *anti* conformation. This anisotropy causes the H-2′ and H-2″ to be magnetically nonequivalent to a great extent and thus provides the reason why the pattern of the H-2′ and H-2″ of the dAMP is so much more complicated than the patterns from TMP, dUMP, and dCMP. The pattern from dCMP is more complicated than TMP and UMP, because cytosine has a slightly larger ring current than thymine and uracil.[27] Since the chemical shifts of H-2′ and H-2″ in 5′-dGMP are similar to the 5′-dAMP, these data further suggest that the H-2′ and H-2″ are much closer to the five-membered ring than to the six-membered ring. We came to this conclusion because the calculated magnitude of the ring current of the five-membered ring is very similar to both guanine and adenine, but the ring current of the six-membered ring of the guanosine is much less than that for the adenine.[27] This conclusion is also self-evident if we examine a model of the nucleotide in an *anti* conformation.[38]

Mechanism of the Deshielding Effect of the 5′-Phosphoryl Group on the Base Protons. The mechanism of the deshielding effect of the 5′-phosphate on the H-8 proton of the purine nucleotides or on the H-6 proton of the pyrimidine nucleotides deserves further inquiry. One possible mechanism was suggested during the early phase of the investigation. It was considered that the hydrogen of the 5′-phosphate monoanion may form a hydrogen bond with the base, such as with N-7 of the purine, which may lead to deshielding of the base protons. This proposal was rejected on two grounds.

1. This hypothesis requires that the N-7 of the purines or the π electrons of the 5,6 double bond of the pyrimidines serve as a hydrogen bond acceptor site. Though N-7 of the purine is a good hydrogen bond acceptor, the stereochemical situation is not favorable for hydrogen bonding with the 5′-phosphate group.[38] As for the pyrimidine, although the π electrons have been mentioned in this sense in the case of intramolecular hydrogen bonding in methyl mandelates in chloroform[58] and the stereochemical position is favorable, this type of hydrogen bonding in aqueous media should be very unlikely due to effective competition by the solvent.

(58) N. Mori, Y. Tanaka, and Y. Tsuzuki, *Bull. Chem. Soc. Jap.*, **39**, 1490 (1966).

2. This hypothesis completely fails to account for the observation that further ionization of the secondary phosphate brings about a larger deshielding effect on the base protons. No hydrogen is left on the dianion of the phosphoryl group, yet the dianion definitely has a larger deshielding influence than that of the monoanion (Table II). This hypothesis also cannot explain the fact that the monomethyl ester of the 5′-phosphate group, which also has no hydrogen left, still exerts a considerable deshielding effect though less than that by the monoanion.

One possible mechanism is the paramagnetic effect of the anisotropy of the phosphoryl group which may be spatially oriented in a proper manner toward the sensitive protons (H-8 or H-6) in order to produce the deshielding effect. It should be noted, however, the phosphoryl group presents a complex case with regard to its magnetic anisotropy, because not only does the P=O bond resonate between the three oxygen atoms (Figure 5) but also the P–O ester bond can rotate freely. This consideration leads us to expect that the anisotropic effect of the phosphoryl group on the H-8 or the H-6 proton may be minimized on a time-average basis. Another possible mechanism is the direct electrostatic field effect of the negatively charged phosphate. This mechanism hypothesizes that the electrostatic field of the negative charge tends to attract the positive proton and thus polarizes the C–H bond to a greater extent resulting in lower electron density at the proton, and hence a deshielding effect. In other words, the former mechanism emphasizes the influence of the *magnetic* field effect of the phosphate while the latter emphasizes the *electrostatic* field effect of the phosphate on the proton. One way to distinguish these two proposals is to compare the deshielding effect of the phosphate group on the sensitive protons which have different degrees of "acidity." It is also important for the success of this experiment that the spatial relationship of the phosphate group to the sensitive protons of these various analogs remain essentially constant.

Following this line of reasoning, one can examine the data on 5′-TMP and 5′-dUMP in Table II. The 5′-TMP, bearing an electron-releasing methyl group at the C-5 position, gives a chemical shift of the H-6 proton about 0.25 ppm upfield in comparison to the H-6 of the 5′-dUMP, as expected. The less "acidic" H-6 of the 5′-TMP is shifted only 0.05 ppm downfield over the pD 5.9–7.4 range compared with the 0.084 ppm for the more "acidic" H-6 of the 5′-dUMP. If the 5,6 double bond of the 5′-UMP is saturated as in 5,6-dihydrouridine 5′-phosphate, the effect of pD change is practically eliminated (0.018 ppm, Table II). One cannot be sure, however, in the case of the 5′-dihydro-UMP that the spatial relationship of the 5′-phosphate group to the H-6 is the same as the other unsaturated nucleotides. It would be instructive to demonstrate the reverse situation, namely, that the electron-withdrawing substituents at the C-5 position of the 5′-UMP would increase the deshielding effect of the charge of the 5′-phosphate on the H-6 proton. Unfortunately, this cannot be done since these substituents substantially lowered the pK_a for ionization of the hydrogen at N-3. The ionization of the hydrogen at N-3 would cause the base protons to shift to higher field, which would counteract the deshielding effect brought about by the phos-

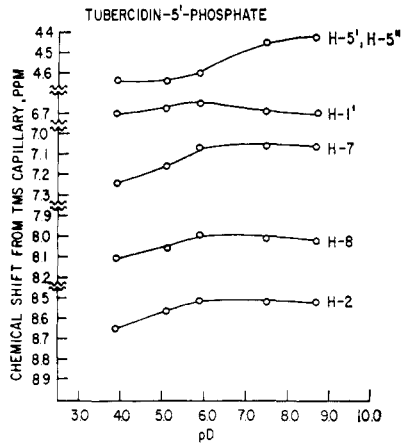

Figure 10. Chemical shift *vs.* pD for protons of tubercidin 5′-phosphate; 0.1 *M*, 32°.

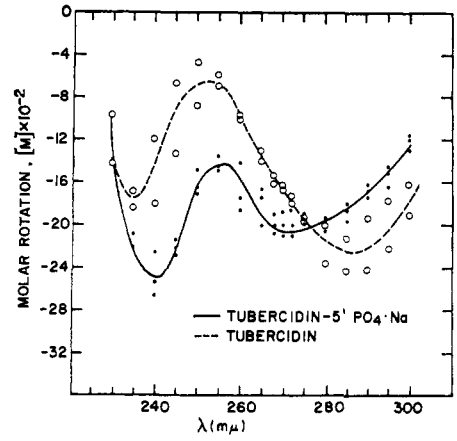

Figure 11. Optical rotatory dispersion of tubercidin (- - - -) and tubercidin 5′-phosphate sodium salt (——) in sodium phosphate buffer, 0.05 *M*, pH 6.1, room temperature.

phate ionization. Thus, whereas the N_3–H ionization has a spectrophotometric pK_a of 9.5 in 5′-UMP,[59] in 5-bromouridine 5′-phosphate (5′-BrUMP) it is 8.25[60] and in 5-fluorouridine 5′-phosphate (5′-FUMP) it is 7.75.[61] At pD 7.4, about 5.5% anion is formed in the 5-bromo derivative, while at this pD, 15% anion is formed in the 5-fluoro derivative. So while the 5-bromo substitution does lower the electron density at C-6, as indicated by δ H-6, 0.3 ppm to lower field in 5′-BrUMP than in 5′-UMP, the pD 5.9–7.4 dependence is only 0.049 compared to 0.130 ppm (Table II). The 5-fluoro compound displays the opposite dependency, as a result of the N_3–H ionization, shifting upfield 0.075 ppm. It is of interest to note that at pD 5.9, H-6 of 5′-FUMP resonates only 0.13 ppm to lower field than H-6 of 5′-UMP, even though fluorine is more strongly electron withdrawing than bromine. Possible p-orbital resonance donation of electrons from fluorine to the ring counteracts the inductive effect. Had the appropriate N_1-methyl-substituted 5-halouridine 5′-monophosphates been available, the problem of N_3–H ionization could have been circumvented.

As for the 5′-purine nucleotides, the H-8 proton is well known for being "acidic," judging from its rate of exchange with deuterium.[10,62] In regard to the change of the "acidity" of the H-8 proton, we have studied the pD shift of the tubercidin 5′-phosphate [4-amino-7-β-D-ribofuranosyl 5′-phosphate-7H-pyrrolo[2,3-*d*]pyrimidine] which is a 7-deazaadenosine 5′-phosphate (Table II and Figure 10).[63] The "acidity" of the H-8 (purine numbering system) of this molecule is decreased as compared to the "acidity" of the H-8 of the adenine due to the replacement of the electronegative N-7 with C–H in the five-membered ring of the adenine. This is evidenced by the fact that the H-8 resonance of the tubercidin 5′-phosphate is moved upfield to 8.00 ppm (Table II) as compared to H-8 of 5′-AMP (8.85 ppm). As clearly shown in Figure 10 and Table II, neither the pyrrole ring protons H-8 and H-7 nor H-2 in the pyrimidine ring are deshielded upon dianion formation of the phosphate. The effect of deprotonation on the ring protons and the effect of the secondary phosphate ionization on the H-5′,H-5″ are shown in Figure 10, as anticipated. We expected that the general conformation of the tubercidin 5′-phosphate is similar to that of the 5′-AMP since the five-membered rings of the base remain aromatic (therefore, planar) in both molecules. In examining this question further, we have studied the ORD patterns of tubercidin and tubercidin 5′-phosphate (Figure 11). The data show that both compounds have negative Cotton effects similar to adenosine and 5′-AMP but with much smaller amplitude.[45,46] Also, the rotations of these two compounds stay in the negative region from 230 mμ to longer wavelengths, evidently under the influence of other strong transition(s) at wavelengths lower than 230 mμ. The inflection point of these ORD patterns (around 270 mμ) is at the uv absorption maximum (λ_{max} 270 mμ (ϵ 11,800)).[64] It is interesting to note the effect of 5′-phosphate on the ORD pattern of tubercidin is larger than the effect of 5′-phosphate on the adenosine.[45] The same sign (negative) of the Cotton effect in the ORD patterns of 5′-AMP and tubercidin 5′-phosphate suggests that these compounds are both in *anti* conformation. However, since these ORD patterns are not identical, no detailed comparison between the conformations of these two compounds can be made at present. Therefore, it is most likely that the loss of the deshielding effect caused by the secondary ionization of the phosphate on the H-8 proton of the tubercidin 5′-phosphate is due to the decrease in the "acidity" of the H-8 proton and not to a major change in conformation.

The foregoing experiments on analogs of 5′-pyrimidine and 5′-purine nucleotides do provide some reliable evidences that this deshielding effect of the phosphate is reduced when the "acidity" of the sensitive protons is decreased. Unfortunately, no experimental data are yet available to prove or to disprove the reverse situation. Nevertheless, we view these data as in favor of the "electrostatic mechanism" over the "paramagnetic

(59) Pabst Laboratory Circular, OR-10, 1956.
(60) A. M. Michelson, J. Dondou, and M. Grunberg-Manago, *Biochim. Biophys. Acta*, **55**, 529 (1962).
(61) M. Szer and D. Shugar, *Acta Biochim. Pol.*, **10** (2), 219 (1963).
(62) M. P. Schweizer, S. I. Chan, G. K. Helmkamp, and P. O. P. Ts'o, *J. Amer. Chem. Soc.*, **86**, 696 (1964).
(63) Although the nomenclature for this compound in *Chemical Abstracts* is 4-amino-7-β-D-ribofuranosyl-5′-phosphate-7H-pyrrolo-[2,3-*d*]pyrimidine, the numbering system for the purine is retained to facilitate the comparison with the 5′-AMP. Thus, the 2, 7, and 8 positions in purine are equivalent to the 2, 5, and 6 positions in tubercidin, respectively.
(64) J. E. Pike, L. Slechta, and P. F. Wiley, *J. Heterocyclic Chem.*, **1**, 159 (1966).

mechanism," since the latter mechanism should not be concerned with the "acidity" of the sensitive protons.

There have been other observations on the deshielding effect of a phosphoryl group on the proton chemical shifts through space. In a recent paper, Lira and Huffman[65] have reported a deshielding influence of a phosphonate group upon H-8 in N^9-bis(β-chloroethyl)-β-adenin-9-yl ethyl phosphonate. These authors suggested that the deshielding arose as a result either of the P=O anisotropy or hydrogen bonding of H-8 to the phosphoryl oxygen. More direct evidence for the phosphate deshielding effect is forthcoming from the data on arabinosylcytosine 2′,5′-cyclic phosphate in which the phosphate anion is held in a position very close to H-3′.[66] It is found that the δ H-3′ of this cyclic phosphate is about 0.5 ppm to lower field than in arabinosylcytosine 5′-phosphate. In view of the mechanism discussed above, it would be of interest to examine the rate of hydrogen–deuterium exchange of this H-3′ proton.

It should be strongly emphasized here that, although we favor the proposal of the electrostatic field effect of the 5′-phosphate on the sensitive protons in order to cause the deshielding phenomenon, we do not mean that the phosphate group is strongly influenced by these protons such as by formation of a hydrogen bond to the phosphoryl oxygen atom. We view that our data and our proposal are in essential agreement with the conclusion of Phillips, *et al.*[67] They have studied the pK_a of the secondary phosphate group of various 5′-nucleoside mono-, di-, and triphosphates by potentiometric titration. They found that the pK_a of the secondary phosphate is not very sensitive to the nature of the bases attached to the nucleotides and concluded that there is no "ring–chain interaction" in nonmetal complexed mononucleotides. As a corollary, we do not consider that this phosphate–proton interaction provides much of the stabilization energy for the *anti* conformation. To the best of our knowledge from the X-ray data[35,37] and ORD data,[45] the nucleosides and the corresponding 3′-nucleotides and 5′-nucleotides all have the same *anti* conformation.

Regardless of the nature of the mechanism for this specific deshielding effect of the 5′-phosphate on the H-8 (purine) or H-6 protons (pyrimidine), the results of this pmr study strongly indicate that the 5′-nucleotides are in an *anti* conformation. This specific shielding effect of the 5′-phosphate has also been found in the dinucleoside monophosphates.[68] This effect can be used for purposes of identification to distinguish the resonance signal arising from the 3′-linked nucleoside (more shielded) from that arising from the 5′-linked nucleoside. This observation also infers that the 5′-linked nucleosides in the dinucleoside monophosphate are in an *anti* conformation.[69]

(65) P. Lira and C. W. Huffman, *J. Org. Chem.*, **31**, 2188 (1966).
(66) Dr. W. Wechter, Upjohn Co., private communication.
(67) R. Phillips, S. J. P. Eisenberg, P. George, and R. J. Rutman, *J. Biol. Chem.*, **240**, 4393 (1965).
(68) M. P. Schweizer, P. O. P. Ts'o, and D. P. Hollis, unpublished data.
(69) NOTE ADDED IN PROOF. We wish to thank Drs. S. S. Danyluk and F. E. Hruska of Argonne National Laboratory for sending their preprint to one of us (P. O. P. T.) on Jan 5, 1968. In this paper, which will appear in the March issue of *Biochemistry*, they have examined the pD dependence of the chemical shifts of the base protons and H-1′ proton of the nucleotides of adenosine, guanosine, xanthosine, and thymidine and have reached a conclusion similar to ours, *i.e.*, the 5′-nucleotides are in an *anti* conformation.

Communications to the Editor

The Kinetics of the Reduction of *t*-Butyl Chloride and *t*-Butyl Bromide by Organotin Hydrides[1]

Sir:

Kuivila and coworkers have shown[2] that the reduction of an organic halide, RX, with an organotin hydride, R'_3SnH, proceeds by a free-radical chain reaction. A reasonable reaction scheme is given by eq 1–6. Although the relative reactivities of a number of

Initiation

$$\longrightarrow R\cdot \qquad (1)$$

Propagation

$$R\cdot + R'_3SnH \longrightarrow RH + R'_3Sn\cdot \qquad (2)$$

$$R'_3Sn\cdot + RX \longrightarrow R'_3SnX + R\cdot \qquad (3)$$

Termination

$$\left.\begin{array}{ll} R\cdot + R\cdot \longrightarrow & \\ R\cdot + R'_3Sn\cdot \longrightarrow & \text{inactive products} \\ R'_3Sn\cdot + R'_3Sn\cdot \longrightarrow & \end{array}\right\} \qquad (4)\ (5)\ (6)$$

halides with some tin hydrides have been measured,[2] the kinetics of the reaction have not previously been reported.

We have examined the reaction of *t*-butyl chloride and bromide with tri-*n*-butyltin hydride and triphenyltin hydride in cyclohexane at 25°. The reaction was initiated photochemically at wavelengths above 3600 Å with the light from a filtered 200–250-V, 250-W ME/D B.T.H. ultraviolet lamp. α,α'-Azobis(cyclohexylnitrile) (ACHN) was used as the photoinitiator. The reaction was monitored by following the rate of heat evolution in the reaction. The apparatus has been described previously.[3] The rates of reaction were estimated from the heat of the over-all process

$$R'_3SnH + RX \longrightarrow R'_3SnX + RH + \Delta H$$

(1) Issued as N.R.C. No. 9987.
(2) H. G. Kuivila, L. W. Menapace, and C. R. Warner, *J. Am. Chem. Soc.*, **84**, 3584 (1962); L. W. Menapace and H. G. Kuivila, *ibid.*, **86**, 3047 (1964).
(3) D. J. Carlsson and K. U. Ingold, *ibid.*, **89**, 4885, 4891 (1967).