phinatomethylcobaloxime, identical with authentic material by melting point, mixture melting point, and infrared comparison. No other product could be detected.

Methylation of Methylaniline Catalyzed by Vitamin B_{12r}. Details of the catalytic action of vitamin B_{12r} and the cobaloximes in the *de novo* synthesis of N-methyl groups have been described.[27] For the sake of completeness the procedure used in a specific example will be reported. To a solution of 0.5 g of cyanocobalamin in 50 ml of methanol under a stream of hydrogen there was added a trace of previously formed vitamin B_{12r} in methanol. The mixture was kept in the stream of hydrogen for 2 hr, reducing the volume of methanol to about one-half of the original. During this time the cyanocobalamin was reduced completely. To this solution there was added 0.5 g of N-methylaniline, and 0.5 g of 40% formaldehyde solution. This solution absorbed 18 ml (0.8 mmole) of hydrogen in 24 hr. The solution was diluted with 200 ml of water and extracted with benzene. Glpc analysis of the benzene layer indicated the presence of 0.5 mmole of N,N-dimethylaniline (10% yield, based on methylaniline).

Acknowledgment. We are indebted to Professor F. M. Huennekens, Scripps Clinic and Research Foundation, for a sample of 5-methyltetrahydrofolic acid.

Interaction and Association of Bases and Nucleosides in Aqueous Solutions. V. Studies of the Association of Purine Nucleosides by Vapor Pressure Osmometry and by Proton Magnetic Resonance[1a,b]

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Abstract: The physical properties of 14 purine nucleosides in aqueous solutions have been studied by vapor pressure osmometry and pmr. These compounds were shown to associate extensively in solution, not by hydrogen bonding, but through the formation of vertical stacks. The concentration dependence of the chemical shifts of various protons provided some detailed information about the nature and the orientation of the nucleosides in these partially overlapping stacks. Two plausible models were proposed. The tendencies of self-association among various nucleosides do not correlate with the dipole moment values of the corresponding bases but correlate reasonably well with the calculated polarizability. The spectral position of the chemical shifts of these nucleosides at infinite dilution provides much valuable information, for example, the indication of intramolecular hydrogen bonding of the 2'-OH group of the ribose to N-3 of the base in adenosine. Based upon consideration of π-charge density distribution, ring currents, and the effect of nitrogen magnetic anisotropy, the spectral positions of H-2 and H-8 for several nucleosides were calculated. The agreement between the theoretical calculations and experimental measurement is substantial.

Previous work from our laboratories has established that purine, 6-methylpurine, and pyrimidine nucleosides associate extensively in aqueous solution by a mechanism involving vertical stacking of bases.[2-5] The self-association of the purines was shown to be much greater than that of the pyrimidine nucleosides and the cross-interaction of purine with pyrimidine nucleosides was shown also to be substantial by measurements of solubilities and by pmr.[2,5] Recent investigation on the cooperative interaction of adenosine with polyuridylic acid demonstrates experimentally that the stacking energy involved in the interaction of the neighboring bases is the major force contributing to the stability of nucleic acid helices.[6]

In this communication, we wish to report the physical properties of 14 purine nucleosides in aqueous and neutral solutions as studied by vapor pressure osmometry and pmr. The major difficulty in this study is the low solubilities of these purine nucleosides, and this is why the properties of these compounds in solution have not been studied before.

Vapor pressure measurements and pmr results both show that, in general, the self-association of these purine nucleosides is even more extensive than that of purine. The effect of methylation clearly indicates that the mechanism of association is not by hydrogen bonding. For example, 1-methylinosine and N-6-dimethyladenosine, in spite of the fact the purine base hydrogen-bond donor sites of these two compounds have been completely removed by methylation, do associate substantially more than inosine or adenosine, respectively.

Studies by pmr on the concentration dependence of the chemical shifts indicate that the base protons (es-

(1) (a) Paper presented in part at the 150th National Meeting of American Chemical Society, Atlantic City, N. J., Sept 1965. (b) This work was supported in part by a Program Project Grant, National Institutes of Health (GM 10802-03), and by a grant from the National Science Foundation (GB-767).

(2) P. O. P. Ts'o, I. S. Melvin, and A. C. Olson, *J. Am. 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. M. Helmkamp, *ibid.*, **86**, 4182 (1964).

(5) M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, *ibid.*, **87**, 5241 (1965).

(6) W. M. Huang and P. O. P. Ts'o, *J. Mol. Biol.*, **16**, 523 (1966).

Table I. Molal Osmotic Coefficients (ϕ), Equilibrium Constants, and the Standard Free Energy Change of the Association for the Following Compounds in Water at 25° [a]

		Molal concn						
		0.025	0.05	0.10	0.15	0.20	K, m^{-1}	$\Delta F°$, $-RT \ln K$, cal
Inosine	(9)	0.994 ± 0.01	0.957 ± 0.02	0.888 ± 0.01	0.830 ± 0.02			
1-Methylinosine	(4)	0.962 ± 0.005	0.926 ± 0.005	0.860 ± 0.005	0.800 ± 0.01	0.750 ± 0.005	1.8–2.0	−360 to −410
Ribosylpurine	(5)	0.965 ± 0.01	0.930 ± 0.01	0.860 ± 0.01	0.810 ± 0.015	0.770 ± 0.01	1.9	−380
Purine[b]			0.917	0.849	0.794	0.749	2.1	−440
Adenosine	(4)	0.915 ± 0.01	0.836 ± 0.01	(0.740)[d] ± 0.005			4.5	−900
2′-O-Methyladenosine	(3)	0.908 ± 0.005	0.828 ± 0.01	0.723 ± 0.01	0.658 ± 0.01	0.611 ± 0.01	5.1	−970
2′-Deoxyadenosine	(4)	0.900 ± 0.01	0.800 ± 0.005	0.668 ± 0.005	0.598 ± 0.005		4.7–7.5	−(920–1195)
6-Methylpurine[c]			0.786	0.682	0.624	0.582	6.7	−1120
N-6-Methyladenosine	(4)	0.805 ± 0.002	0.685 ± 0.005	0.558 ± 0.005	0.480 ± 0.005		11.8–14.9	−(1460–1600)
N-6-Methyl-2′-deoxyadenosine	(2)	0.790 ± 0.005	0.680 ± 0.005	0.540 ± 0.005	0.468 ± 0.005		15.9	−1640
N-6-Dimethyladenosine	(2)	0.712 ± 0.005	0.608 ± 0.005	0.470 ± 0.005	0.408 ± 0.002	0.378 ± 0.002	22.2	−1840

[a] The number of determinations is indicated in the bracket following the name of each compound. All the experimental points were put on a graph and the line for the best fit was drawn. The spread of the data points is also given. [b] From ref 2. [c] From ref 3. [d] At solubility limit of 0.085 m.

pecially H-2), the methyl group on the bases, and also the anomeric proton H-1′ are shielded upfield progressively as concentration of the nucleosides in solution increases owing to the ring current effect as noticed previously in the case of purine.[3] These data provide some detailed information about the nature and the orientation of the nucleosides in stacks. In addition, some other important information was provided by the spectral position of the chemical shifts at infinite dilution. Among these is the indication of intramolecular hydrogen bonding of the 2′-OH group of the ribose to N-3 of the base in adenosine. Finally, the spectral positions of the chemical shifts of H-2 and H-8 for several nucleosides were calculated based upon consideration of π-charge density distributions, ring currents, and the effect of nitrogen magnetic anisotropy. Comparison between the theoretical calculations and experimental observations indicates that the agreement is substantial and encouraging.

Results

Molal osmotic coefficients, ϕ, for nine purine nucleosides at various concentrations are listed in Table I in order of decreasing ϕ. Previously determined values for purine and 6-methylpurine[2,3] are included for comparison. In the case of adenosine, solubility restriction prohibited measurements above 0.085 m. Guanosine and deoxyinosine are not sufficiently soluble (less than 0.05 m) for measurements. Among all these nucleosides, ribosylpurine (over 0.5 m) and 2′-O-methyladenosine[7] (up to 0.5 m) are more soluble than the others.

Figure 1 shows the curves of ϕ *vs.* m for these nucleoside solutions. Most of the ϕ values and the experimental deviation are also tabulated in Table I. The data show that adenine nucleosides associate to a greater extent than ribosylpurine, which in turn associates more extensively than inosine. For reasons not certain at present, experimental points for inosine tend to scatter more than those for other compounds especially at low concentrations such as 0.025–0.05 M (Table I). It appears that the inosine graph at this low region of concentration cannot be extrapolated smoothly to the origin in spite of considerable efforts (Figure 1). Comparison between the methylated and nonmethylated nucleosides indicates that methylation always increases association unless the methyl group is located on the sugar moiety. In this case, the effect of methylation is not significant.

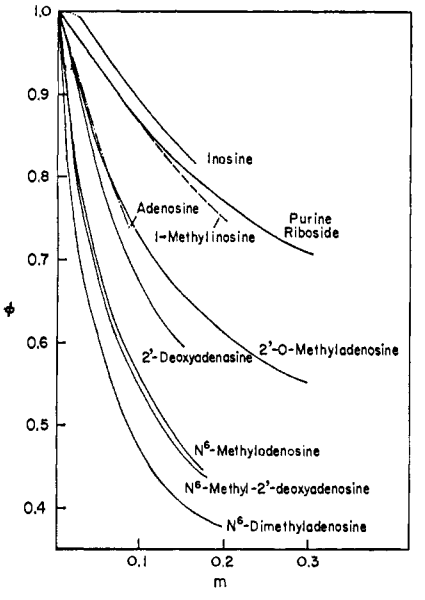

Figure 1. Osmotic coefficient, ϕ, *vs.* molal concentration, m, for nine purine nucleosides in H_2O.

The osmotic coefficients of eight nucleosides were further analyzed in terms of multiple equilibria with the application of eq 1 where K is the association constant

$$K = (1 - \phi)/m\phi^2 \qquad (1)$$

which is assumed to be the same for all the successive steps of association in the formation of dimer, trimer, etc.,

(7) A. D. Broom and R. K. Robins, *J. Am. Chem. Soc.*, **87**, 1145 (1965).

to N-mer.[2,8] The values for the equilibrium constant, K, as well as the corresponding change of standard free energy in the association are also given in Table I. The $(1 - \phi)$ *vs.* $m\phi^2$ plots of four nucleosides are shown in Figure 2 as selective examples. The line for 2′-deoxyadenosine and the line for N-6-methyl-2′-deoxyadenosine cannot be extrapolated through the origin. This indicates that the original assumption of the same K for the successive steps is not valid in these two cases and the values of the association constants for the early steps are likely to be lower than those for the more advanced steps.[3] Owing to the small number of the experimental points because of solubility limitation, it appears not justifiable to calculate the activity coefficient, γ, from these data and to apply more elaborate equations in relaxing the assumption about the K in eq 1.[3] Therefore, a range of K values for 2′-deoxyadenosine and N-6-methyl-2′-deoxyadenosine is given in Table I, representing the different slopes of the respective lines in Figure 2. Equation 1 also was not applied to inosine since the data from this compound cannot be extrapolated smoothly to the origin as shown in Figure 1.

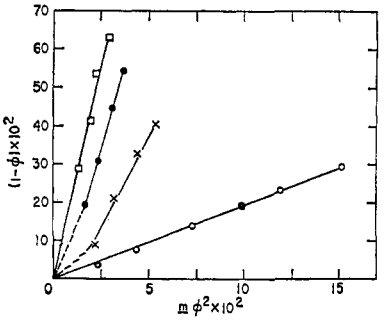

Figure 2. $(1 - \phi)$ *vs.* $m\phi^2$ for four purine nucleosides: ribosylpurine, O; 2′-deoxyadenosine, ×; N-6-methyladenosine, ●; and N-6-dimethyladenosine, □.

The concentration dependence of the chemical shifts for the base protons, the substituted methyl protons, and the anomeric proton H-1′ of 11 purine nucleosides is given in Table II.[9] All these protons are shifted to higher fields (the value for $\Delta\delta$ is positive) indicating an increase in shielding when the concentration is increased from 0 to 0.2 m. This observation, which mimics that of purine and 6-methylpurine,[4] indicates the association of these nucleosides by means of vertical stacking with partial overlap. This upfield shift is a manifestation of the ring-current diamagnetic anisotropy effects of the neighboring molecules in the stacks.

The order of the tendencies for association shown by the magnitude of these chemical shifts are in agreement with that determined by vapor pressure osmometry, *i.e.*, inosine < 1-methylinosine, ribosylpurine < 2′-O-methyladenosine, adenosine and deoxyadenosines < N-6-methylated adenosines.

(8) J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsburg, Ser. Chim.*, 29, 223 (1956).

(9) The concentration shifts of 2′-deoxyadenosine are given in both 0–0.1 and 0–0.2 m ranges so that the shifts for adenosine and 3′-deoxyadenosine over 0–0.1 m range can be compared with others in the table.

Table II. Concentration Dependence of Chemical Shifts for 11 Purine Nucleosides (0.0–0.2 m) in D_2O

Compound	Temp, °C	Δδ, cps H-2	H-8	H-6	H-1′	CH_3
Inosine	32	6.4	5.3		7.1	
1-Methylinosine[a]	33	8.9	6.4		6.8	5.3
Ribosylpurine	30	10.7	6.4	13.1	8.8	
Purine[b]	25–27	12.6	9.6	14.2		
2′-O-Methyladenosine	31	13.7	7.5		8.8	
6-Methylpurine[b]	25–27	19.4	13.3			17.0
2′-Deoxyadenosine	30	19.8	13.0		13.6	
N-6-Methyl-2′-deoxyadenosine	32	26.0	15.8		14.0	15.2
N-6-Dimethyladenosine	28	27.2	14.5		14.4	25.5
N-6-Methyladenosine	26	32.6	17.5		12.6	18.1
2′-Deoxyadenosine[c]	30	14.8	10.0		9.8	
Adenosine[c]	32	14.8	8.3		6.9	
3′-Deoxyadenosine[c]	25	15.8	9.0		9.6	

[a] Peak positions of H-8 and H-2 are reversed with respect to the other 6-substituted nucleosides studied. [b] See ref 4. [c] Differences measured over the concentration range 0–0.10 m because of solubility limitation.

Figures 3, 4, 5, and 6 are the typical plots of the chemical shifts of various protons *vs.* concentrations for these nucleosides. These plots indicate that the concentration shifts only occur at the base protons,

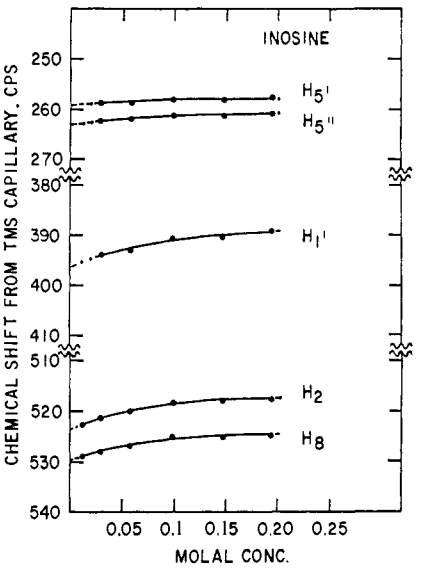

Figure 3. Concentration dependence of proton chemical shifts for inosine in D_2O.

particularly H-2, and at the anomeric proton H-1′ while the chemical shifts of the protons of the pentose are not concentration dependent, especially H-5′ and H-5″, which are farthest away from the base. This effect is identical with that observed for the chemical shifts of the pyrimidine nucleosides in solution with increasing concentration of purine.[5] Here the purine-induced shift was found to fall off progressively as the proton distance from the pyrimidine ring increases. Similarly, the chemical shifts of the methyl protons substituted at the 6-amino group are very concentration

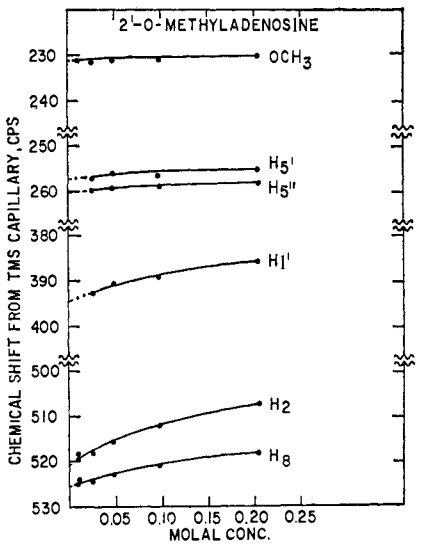

Figure 4. Concentration dependence of proton chemical shifts for 2'-O-methyladenosine in D_2O.

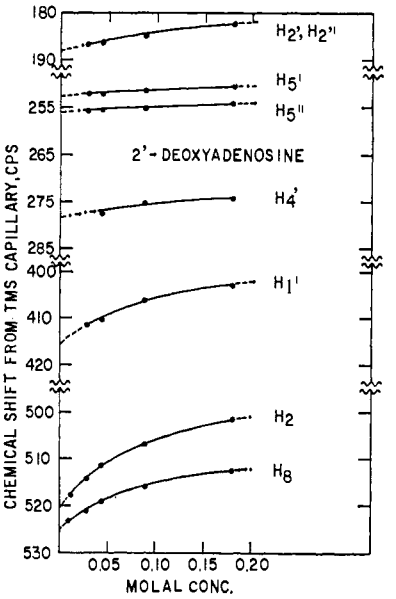

Figure 5. Concentration dependence of proton chemical shifts for 2'-deoxyadenosine in D_2O.

dependent (Figure 6), while the chemical shifts of the methyl protons substituted at the 2'-OH group of the pentose are not (Figure 4). All these data together indicated that the association of the purine nucleoside takes place mainly by the stacking of the base rings.

Table III. Proton Chemical Shifts for 14 Purine Nucleosides in D_2O at 28–33°

	Concn,	cps from TMS capillary				
	m	H-2	H-8	H-6	H-1'	CH_3
Ribosylpurine	0.0	565.5	549.7	577.6	402.0	
	0.1	558.2	545.3	568.6	395.9	
Ribosyl-6-chloro-	0.027	555.5	555.5		...	
purine	0.05	552.2	552.2		399.8	
1-Methylinosine	0.0	532.2	528.1		393.6	249.1
	0.1	526.4	524.0		389.0	245.5
6-Thioinosine	0.027	528.6	536.0		395.4	
	0.05	527.5	535.3		394.2	
Adenosine	0.0	524.0	528.5		392.0	
	0.1	509.0	520.0		385.0	
N-6-Methyladeno-	0.0	524.0	524.0		389.5	213.0
sine	0.1	498.5	510.1		379.4	198.7
Inosine	0.0	523.7	529.8		396.5	
	0.1	518.5	525.4		391.1	
N-6-Methyl-2'-	0.0	522.6	523.7		414.0	214.7
deoxyadenosine	0.1	500.6	510.2		403.2	201.8
2'-O-Methyladeno-	0.0	521.2	525.6		394.9	231.5
sine	0.1	511.7	520.7		388.7	230.7
2'-Deoxyadenosine	0.0	521.2	525.4		415.8	
	0.1	506.1	515.1		405.9	
3'-Deoxyadenosine	0.0	521.0	525.7		390.8	
	0.1	505.2	516.7		381.2	
N-6-Dimethyl-	0.0	517.7	521.2		390.8	233.2
adenosine	0.1	495.2	509.0		378.5	212.6
Ribosyl-2,6-di-	0.027		505.4		381.2	
aminopurine	0.05		504.1		381.0	
1-Methylguanosine	0.025		505.1		382.5	235.0

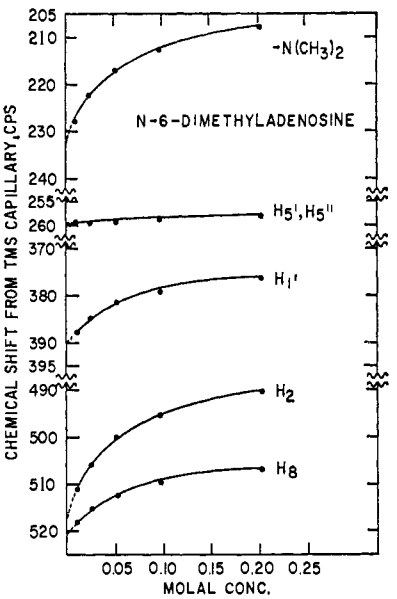

Figure 6. Concentration dependence of proton chemical shifts for N-6-dimethyladenosine in D_2O.

The chemical shifts of the base protons, the methyl protons, and the anomeric H-1' protons of 14 purine nucleosides are listed in Table III. These compounds are tabulated in order of decreasing chemical shift of the H-2 protons.

Table IV contains the measured coupling constants, $J_{1',2'(2'')}$, of four nucleosides. It is suggested from these data that 3'-deoxyadenosine (cordycepin) has a somewhat different furanose ring conformation than the

Table IV. The Coupling Constant, $J_{1',2'}$ (cps), for Several Adenine Nucleosides in D_2O (0.1 *M*)

2'-Deoxyadenosine	6.5[a]	2'-O-Methyladenosine	5.3
Adenosine	6.1	3'-Deoxyadenosine	2.2

[a] $J_{av} = (J_{1',2'} + J_{1',2''})/2$.

others. A detailed account of these results is presented in section C of the discussion of nmr results.

Discussion

Vapor Pressure Osmometry. It has come to our attention that some of the compounds which we have studied also were examined by Solie.[10] Our values of ϕ for ribosylpurine, uridine,[2] and thymidine (0.1 *m*, ϕ = 0.905 and 0.2 *m*, ϕ = 0.805) are in satisfactory agreement with his results. However, substantial deviation was found in the values for 2′-deoxyadenosine and inosine obtained by these two laboratories. For 2′-deoxyadenosine, at 0.05 *m*, ϕ = 0.72 ± 0.03 was reported by Solie and ϕ = 0.80 ± 0.005 is listed in Table I. For inosine at 0.05 *m*, ϕ = 0.878 ± 0.03 was reported by Solie and ϕ = 0.957 ± 0.02 is listed in Table I. Unfortunately no elemental analysis was reported in Solie's thesis and his calculations were apparently based on the assumption that the compounds were anhydrous. If the commercial 2′-deoxyadenosine used by Solie is indeed in monohydrate form as in our case, then his value of ϕ recalculated on the basis of 2′-deoxyadenosine monohydrate is in accord with ours, *i.e.*, 0.05 *m*, ϕ = 0.77 ± 0.03. It should be noted that our data on 2′-deoxyadenosine is very close to that for adenosine and 2′-O-methyladenosine, as may be expected. In the case of inosine, our compound is anhydrous. If the inosine used by Solie was the monohydrate (this form is also available commercially), then the recalculated value of Solie (0.936 ± 0.036) is again in agreement with ours. The real reason for the discrepancies is not certain at present.

One of the major objectives of this investigation is to study the effect of methylation on the association tendencies of these nucleosides. The results clearly indicate that the mechanism of association is not by hydrogen bonding. From the values of K and ϕ (Table I), the order of association tendencies with respect to the degree of methylation can be listed as follows: 2′-deoxyadenosine < N-6-methyl-2′-deoxyadenosine; adenosine < N-6-methyladenosine < N-6-dimethyladenosine; and inosine < 1-methylinosine. In every case examined, substitution of a hydrogen of the bases by a methyl group removes a hydrogen-bond donor, and the association tendency is enhanced significantly. 1-Methylinosine and N-6-dimethyladenosine, in spite of the fact the hydrogen-bond donor sites of those two compounds have been completely removed by methylation, do associate substantially more than inosine or adenosine, respectively. It is interesting to note that in order to obtain the promoting effect of methylation, the methylation has to take place at the base and not at the pentose. Thus, the association tendency of 2′-O-methyladenosine is about the same as that of adenosine and may be slightly less than that of 2′-deoxyadenosine. This observation confirms the conclusion from pmr studies in this as well as in a previous publication[5] that the sites of association of these nucleosides are at the bases. Thus, methylation at the pentose, distant from the sites of stacking, is not expected to exert an effect. It may even be inhibitory because of steric reasons. The probable steric hindrance of the sugar may account for the slight reduction in association tendency of ribosylpurine in comparison to purine.

It is interesting to note that substitution of a 6-amino group on the ribosylpurine to give adenosine substantially enhances the association of the nucleoside as studied by osmometry or by pmr. The substitution of a polar group is unlikely to reduce the solvation properties of the nucleoside; therefore, one cannot explain this enhancement on general hydrophobic terms. In Table V, the correlation among the osmotic coefficients (ϕ) of the nucleosides, the polarizabilities (α), and the dipole moments (μ) of the bases is listed. The values of α and μ are those calculated by Pullman's group.[11,12] The α values for adenine and cytosine from Pullman's paper are identical with those previously published by DeVoe and Tinoco.[13] The μ values from MO calculations for the methylated bases by DeVoe and Tinoco[13] are also similar to those by Pullman (9-methyladenine, 2.8 μ; 1,3-dimethyluracil, 3.7 μ; 3-methylthymine, 3.5 μ; 3-methylcytosine, 8.0 μ; and 9-methylpurine, 3.6 μ). These small differences do not affect the conclusion presented later in this paragraph. It is also gratifying to note that the experimental values of μ for the methyladenine, dimethyluracil, and methylpurine are in good agreement with the calculated values.[13] Comparison of the order of ϕ and the order of μ among the four purine nucleosides clearly indicates that these two quantities relate to each other in a reverse manner. Hypoxanthine has the greatest dipole moment value and the corresponding nucleoside, inosine, has the smallest tendency to associate; however, adenine has the smallest dipole moment value and the adenine nucleosides have the greatest tendency to associate. Comparison of the order of ϕ and the order of μ among the three pyrimidine nucleosides again shows no correlation between these two quantities. Thymine has the smallest dipole moment but thymidine has the greatest tendency to associate, while cytosine has the greatest dipole moment among the seven compounds in Table V but cytidine has the next to the

Table V. Correlation between the Osmotic Coefficients (ϕ) of the Nucleosides and the Polarizabilities (α^a) and Dipole Moments (μ^a) of the Bases

	ϕ (0.1 *m*, 25°)	α, A^3	μ, D.
Uridine[b]	0.943	10.2	(3.9)[c]
Cytidine[b]	0.935	11	7.2
Thymidine	0.905	12	3.6
Inosine	0.888	13.0	5.2
Ribosylpurine	0.860	12.5	4.2 (4.3)[c]
2′-O-Methyladenosine[d]	0.723	13.9	3.2 (3.0)[c]
2′-Deoxyadenosine[d]	0.668		

[a] From ref 11 and 12. [b] From ref 2. [c] Experimental value from ref 13. [d] Adenosine is not sufficiently soluble to give a 0.1 *m* solution.

(10) T. Solie, Ph.D. Dissertation, University of Oregon, 1965.

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

(12) B. Pullman in "Molecular Biophysics," B. Pullman and M. Weissbluth, Ed., Academic Press Inc., New York, N. Y., 1965, pp 154–157.

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

lowest tendency to associate. Therefore, it can be concluded with a considerable degree of certainty that as far as these nucleosides are concerned, permanent dipole moment attraction is not the most important driving force for their self-association. This conclusion is not surprising in view of the high dielectric constant of the solvent, water. It is also in accord with the model proposed in later sections for the mode of stacking of these purine nucleosides based on the pmr studies. In these models, the permanent dipole moment of the bases in stacks is likely to exert a negative influence rather than a positive promotion on association.

On the other hand, the correlation between the order of ϕ and the order of α, the polarizability is good (Table V). The only problem is that though hypoxanthine was calculated to have a larger polarizability than purine, the association tendency of inosine appears to be slightly less than that of ribosylpurine. This anomaly may be due to the negative influence of the large permanant dipole moment of hypoxanthine which may either hinder the stacking directly, or indirectly by more extensive hydration. Other than this exception, the rather good correlation here is in accord with the recent conclusion, based upon a correlation of polarizabilities and the effectiveness of a variety of denaturing agents, that London dispersion forces are responsible for the stability of the DNA helix.[14] In summary, the order of nucleoside osmotic coefficients, which is an index of association by stacking, is in good agreement with the reported polarizability values of the respective base and is not in agreement with the base dipole moment values.

It should be noted that the experimental osmotic coefficient data are from the nucleosides while the dipole moment calculations are done on the bases. The justification for the comparison between the data derived from the bases and those from the pentosyl bases is as follows. The inductive effect of the substituted pentosyl group on the μ of the bases should be rather small. From what is known about the orientation of μ in the bases,[13] the substitution of the electron-withdrawing pentosyl group should decrease slightly the absolute magnitude of μ of all the bases listed in Table V except in the case of cytosine. For cytosine, the pentosyl substitution is most likely going to increase the magnitude of μ to a small extent. Therefore, the effect of pentosyl substitution is not expected to change the order of the values of μ listed in Table V so as to give a positive correlation between ϕ and μ. Within the forseeable future the osmotic coefficient data of the bases in water as well as the experimental values of μ for the base component in the nucleoside are unlikely to be obtainable due to solubility and other problems.

Nuclear Magnetic Resonance. A. The Concentration Dependence of the Chemical Shifts ($\Delta\delta$). The close correlation between the magnitudes of $\Delta\delta$ (Table II) and the values of ϕ and K (Table I) for this series of nucleosides in solution not only establishes that stacking is the mode of association of these solutes but also verifies the usefulness of nmr as a tool for studies of association.[15] The differentials in the magnitude of $\Delta\delta$ for various protons of a given nucleoside may provide additional information about the average geometry of the stacks in solutions. We shall focus our attention on the H-2, H-8, H-1′, and CH_3 protons since they participate to a greater extent in the stacking interaction than others.

Data in Table II indicate that $\Delta\delta$ for H-2 is substantially larger than that for H-8. In addition, $\Delta\delta$ for the anomeric H-1′ is also slightly larger than that for H-8 in six nucleosides, while in the remaining five nucleosides, $\Delta\delta$ for H-1′ and H-8 protons are very close. There are three possible explanations for the enhancement of $\Delta\delta$ for the H-2 proton as compared to others: (a) steric influence of the pentose moiety at the N-9 position on H-8. This influence may be part of the reason, but it cannot be the major effect because of the following two arguments: (i) the larger $\Delta\delta$ for H-2 is also observed for purine and 6-methylpurine; (ii) this reasoning cannot explain why $\Delta\delta$ for H-1′ is greater or close to that for H-8. (b) From his study of purine, Jardetzky[16] suggested that the "electron currents" in the six-membered ring are greater and thus the pyrimidine ring exerts a larger shielding effect than the five-membered ring. This explanation is unlikely to be adequate for the purine nucleosides because of the following two reasons: (1) the greater value of $\Delta\delta$ for H-2 is observed even for inosine and 1-methylinosine (Table II), in which the ring current in the pyrimidine ring would be expected to be smaller than that of the imidazole ring due to the cyclic amide tautomeric structure[17] (see Figure 8a). (2) This reasoning again provides no explanation why $\Delta\delta$ for H-1′ is greater or close to that for H-8. (c) A preferred average orientation of the nucleoside bases in the stacks to account for the results observed. This is the explanation which we tentatively favor. This preferred orientation can be illustrated diagrammatically by two related models for the dimer (Figure 7). These models can be extended to trimer or stacks of higher order with the same geometric arrangement as the dimer by adding nucleosides on top or below. In these dimer models, one can see that the H-2 proton will be shielded strongly by the six-membered ring of the neighboring bases 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 these models, 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. These two models are related to each other by symmetry considerations. The base-stacking arrangement for model Figure 7a is face to back, and that for Figure 7b is face to face (or back to back), one of the nucleosides being rotated by 180° along an axis in the plane bisecting the C_4–C_5 bond. Thus, in model Figure 7a the two ribosyl substituents at N-9 will be on the *same side* of the dimer (straight stack). On the other hand, in model Figure 7b, the two ribosyl moieties at N-9 will be *opposite* to each other in the dimer resulting

(14) S. Hanlon, *Biochem. Biophys. Res. Commun.* 23, 861 (1966).

(15) The temperature control for the nmr is not as precise as that for vpo measurement; therefore the temperature for the measurement of each compound is given in Table II. The temperature range for purine and 6-methylpurine was cited from a previous publication.[4] Since the association process is exothermic,[2,4] the $\Delta\delta$ values, especially that of the sensitive H-2 proton of the nucleoside, become greater as temperature is lowered. This is probably the reason why the $\Delta\delta$ of N-6-methyladenosine (measured at 26°) is larger than that of the N-6-methyl-2′-deoxyadenosine (measured at 32°), not expected from the vpo data (Table I). The error in the measurement of $\Delta\delta$ is usually around ±5%.

(16) O. Jardetzky, *Biopolymers Symp.*, 1, 501 (1965).

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

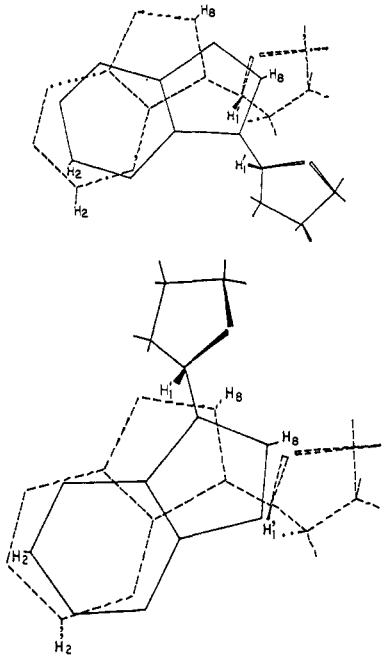

Figure 7. Models illustrating the proposed arrangements for two nucleosides in stacks (see text): (a) straight stack (face to back), the ribosyl substituents at N-9 are on the same side of the stack; (b) alternate stack (face to face or back to back), one of the nucleosides is rotated by 180° along an axis in the plane bisecting the C_4–C_5 bond. The ribosyl substituents are opposite to each other in the stack.

in an alternating arrangement (alternate stack). The alternate stack may be favored by two considerations: (i) the steric hindrance of the ribosyl group is reduced; (ii) this arrangement reduces the repulsion between the dipole moments of the adjacent bases expected in the straight stack. No critical evaluation of these two models can be pursued further at this time. These models are similar to the arrangement in 8-azaguanine monohydrate crystals[18] and to one of the stacking modes of purine or 6-methylpurine suggested by Pullman, *et al.*[19]

Two qualifying statements should now be mentioned in discussion of these models: (a) the standard free energy change for the association is in the order of the thermal energy kT, and no significant line broadening or separate shifts for stacked and free species were observed in the nmr studies; therefore, these stacks must break and re-form rapidly. What we observe and describe is an over-all average phenomenon. (b) The preferred orientation of the bases in the stack must vary to a certain extent depending upon the nature of the bases in the nucleosides. Substitution of an oxo group, amino group, methyl or dimethylamino group, etc., all should exert an influence on the geometric arrangement of the stacks. Nevertheless, these stacks will have a partially overlapped orientation so that the H-8 and H-1′ can be both shielded to a similar extent.

Methylation at the ring or at the exocyclic amino group enhances the association and the value of $\Delta\delta$ as expected from previous studies.[3] Furthermore, the methyl resonances of these compounds also show a marked concentration dependence, indicating that they are experiencing the effects of the anisotropy of neighboring bases as well. It is interesting to compare the values of $\Delta\delta$ for N-6-methyladenosine and N-6-dimethyladenosine (Table II). The $\Delta\delta$ for H-2 of the dimethyl nucleoside is smaller than the $\Delta\delta$ for H-2 of the monomethyl nucleoside, even though the association has been enhanced by methylation (Table I). We interpret this to mean that the effective distances from the H-2 proton to the neighboring base rings have been increased when the nucleosides are changed from the monomethyl to the dimethyl derivative. On the other hand, $\Delta\delta$ of the methyl group is substantially increased by methylation of the monomethylated compound. Apparently the dimethylamino group is, on the average, being influenced by neighboring purine rings in the stacks to a greater degree than the monomethylamino moiety. As a consequence of the dimethylamino steric requirements in the stacks, the distance of closest approach to neighboring rings is increased with respect to the region of H-2. Similar differences in the $\Delta\delta$ of H-8 are also found between the monomethyl and the dimethyl compounds (Table III), presumably for the same reason.

Previous investigations showed that purine interacts with pyrimidine nucleosides by a vertical stacking mechanism, resulting in increasing upfield shifts of pyrimidine ring protons with increasing purine concentration.[5] The same effect can be observed when a purine nucleoside is used in place of purine. For example, a mixture of 0.2 *m* 2′-O-methyladenosine and 0.1 *m* thymidine results in the following upfield shifts (in cps) of the thymidine protons: 8.2 (CH_3), 8.9 (H-6), 7.1 (H-1′), 4.3 (H-2′, H-2″). Similar effects were noted for N-6-methyladenosine and 2′-deoxyadenosine in thymidine solutions, while ribosylpurine is only 70% as effective as the adenosine derivatives.

B. The Spectral Position of the Chemical Shifts. Owing to the large concentration dependence, the intrinsic δ for protons of the purine nucleosides can be obtained only by extrapolation to infinite dilution. The results of ten nucleosides are given in Table III, the accuracy being within 1–3 cps. As for the other four nucleosides, solubility restrictions prohibited getting sufficient data for the extrapolation. In these cases, the values of δ at 0.025 and 0.05 *m* are listed. It is expected that the difference of the extrapolated value and the listed values should not be more than 3–5 cps.

Various kinds of interesting information were provided by comparative studies of the δ values in Table IV. We shall focus our attention first on the H-1′ proton. The chemical shifts for H-1′ of the adenine ribonucleosides occur at 22 ± 2 cps upfield from that of the adenine deoxynucleosides. This phenomenon has been observed previously in dimethyl sulfoxide solutions and was attributed to electrostatic shielding of H-1′ by the *cis* oxygen atom in the 2′ position.[20] This explanation is in accord with the following two observations. (a) The δ of H-1′ of 3′-deoxyadenosine has a similar value to that of adenosine and is 25 cps upfield from 2′-deoxyadenosine. (b) The δ for H-1′ of arabinosylcytosine, which has a 2′-OH group *trans*

(18) W. M. MacIntyre, *Science*, 147, 507 (1965).
(19) B. Pullman, P. Claverie, and J. Caillet, *Compt. Rend.*, 260, 5387 (1965).
(20) L. Gatlin and J. C. Davis, *J. Am. Chem. Soc.*, 84, 4464 (1962).

to H-1′, has a value 16 cps downfield from the δ of cytidine H-1′ in which a 2′-OH group is *cis* to the H-1′.[21]

It is interesting to note that the ring protons of adenine ribonucleosides are downfield several cycles per second from the corresponding adenine 2′-deoxy- or 2′-substituted nucleosides (Table III). This observation strongly suggests the existence of hydrogen bonding from the 2′-OH group of the pentose to the N-3 of the purine ring. Such a hydrogen bond has received support from studies of ultraviolet absorption spectroscopy[22] and of infrared spectroscopy[23,24] on nucleosides and nucleotides. Preliminary potentiometric titration experiments done in our laboratory (0.05 *M* nucleoside, 0.15 *N* NaCl, 25°) indicate that 2′-deoxyadenosine has a pK_a 0.1 pH unit higher than that of adenosine. We have found also that H-2 and H-8 of 2′-deoxyadenosine are shifted 10 cps downfield going from neutral solution to pH 3.6, whereas H-2 and H-8 of adenosine are shifted only 5 cps to lower field over the same pH range. Thus the base of 2′-deoxyadenosine is more readily protonated, indicating a higher pK_a. These results again are in accord with the concept of intramolecular hydrogen bonding. This intramolecular hydrogen bonding was suggested, in a recent paper from our laboratory,[25] to be responsible for the differences in conformation between the polyribonucleotides and the polydeoxyribonucleotides as studied by optical rotatory dispersion and other methods.

The δ's of the ring protons of 3′-deoxyadenosine are very similar to that of 2′-deoxyadenosine, indicating no intramolecular hydrogen bonding involving the 2′-OH group. However, as shown in a later section, the conformation of C-2′ in the ribose appears to be quite different from that in the 3′-deoxyribose. The C-2′ of ribose is much more in the *endo* position, *i.e.*, out of the plane toward C-4′–C-5′ bond, than the C-2′ of 3′-deoxyribose. This difference in conformation could account for why this hydrogen bond formation is not found in case of 3′-deoxyribose. The pK_a of the hydroxyl ionization of 3′-deoxyadenosine was reported to be higher than that of adenosine.[26] This is another indication that the properties of this 2′-OH group are different in these two compounds.

Comparison of the data on 1-methylinosine with inosine and other nucleosides reveals some interesting features. 1-Methylinosine is the only 6-substituted nucleoside to have δ of H-2 at a lower field than δ of H-8. H-2 of 1-methylinosine is shifted downfield by 8.5 cps as compared to H-2 of inosine, while the H-8 proton of 1-methylinosine is unchanged as compared to inosine (Table III). It has been shown previously that the H-8 of 7-methylguanosine[27] is shifted downfield by more than 1 ppm as compared to the H-8 of 1-methylguanosine[28] owing to the quaternization of the imidazole ring caused by methylation of the N-7 position.

(21) Dr. W. J. Wechter, The UpJohn Co., private communication.
(22) H. Witzel, *Ann. Chem.*, **635**, 182 (1960).
(23) J. Pitha, S. Chladek, and J. Smrt, *Collection Czech. Chem. Commun.*, **38**, 1622 (1963).
(24) A. M. Michelson, *Ann. Rev. Biochem.*, 30, 133 (1963).
(25) P. O. P. Ts'o, S. A. Rapaport, and F. J. Bollum, *Biochemistry*, **5**, 4153 (1966).
(26) R. M. Izatt, J. H. Rytting, L. D. Hansen, and J. J. Christensen, *J. Am. Chem. Soc.*, **88**, 2641 (1966).
(27) L. Townsend and R. K. Robins, *ibid.*, **85**, 242 (1963).
(28) A. D. Broom, Ph.D. Dissertation, Arizona State University, 1965.

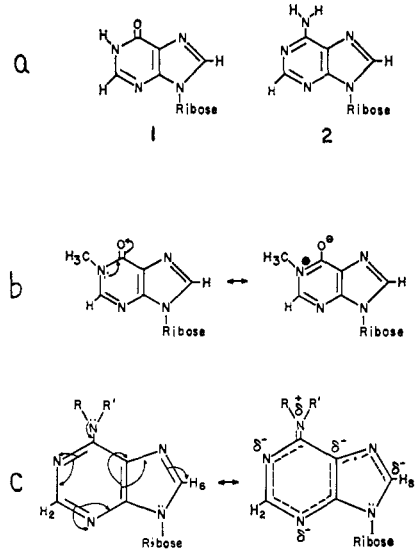

Figure 8. (a) Inosine in cyclic lactam form (1) and adenosine in lactim form (2). (b) Resonance forms for 1-methylinosine illustrating the methyl stabilization of partial positive charge at N_1. (c) For the series adenosine, N-6-methyladenosine, and N-6-dimethyladenosine, the figures indicate the stabilization of partial positive charge at N-6 when R and R′ are changed from H to methyl.

Therefore, the substantial and selective deshielding of H-2 in 1-methylinosine suggests that a more extensive degree of quaternization is occurring at N-1 in the 1-methyl derivative as compared with inosine itself. Such an effect can be explained as seen in Figure 8b; the electron-releasing inductive effect of the methyl group will tend to stabilize the positive charge on the N-1 nitrogen.[29]

Similar methyl-stabilized resonance forms can be adopted as an explanation for the difference in base proton chemical shifts of adenosine, N-6-methyladenosine, and N-6-dimethyladenosine (Table III). At infinite dilution, H-2 and H-8 are shifted progressively to higher fields as methyl groups are added to the N-6 amino position. H-2 and H-8 of adenosine are 527.0 and 530.4 cps, those of the monomethyl compound 524.0 and 524.0, and those of the dimethyl derivative 517.7 and 521.2. On the other hand, the methyl proton resonance shifts downfield from monomethyl to dimethyl compound, 213.0–233.2. These observations suggest that a progressively greater degree of resonance donation of electrons from the amino group to the ring takes place as R and R′ change from H to CH_3, due to methyl inductive stabilization of positive charge on N-6 as shown in Figure 8c.

A comprehensive survey of chemical shifts of N-methyl derivatives has been published by Ma and Warnhoff.[30] Comparison of the irdata on monomethyl and dimethyl N-substituted aromatic compounds, such as N-methylaniline *vs.* N,N-dimethylaniline, shows a similar deshielding of the methyl protons in the dimethylated compounds. In saturated systems, methyl protons are more shielded in N,N-dimethyl compounds as compared to monomethyl derivatives.

(29) We wish to thank Dr. Lloyd Stempel of our laboratory for suggesting this explanation.
(30) J. C. N. Ma and E. W. Warnhoff, *Can. J. Chem.*, 43, 1849 (1965).

The order of the intrinsic δ for H-8 and H-2 of the nucleosides listed in Table III is in agreement with the order reported by Jardetzky for H-8 of various anions of purines in 1 *M* NaOD.[16] The H-8 and H-2 position of ribosylpurine and ribosyl-6-chloropurine are located at lowest field, next are those of adenosine and inosine, and the δ's of the disubstituted ribosyl-2,6-diaminopurine and 1-methylguanosine are still further upfield. Up to the present time, no serious attempt has been made to account quantitatively for the effect of ring substituents upon base proton chemical shifts in the purine nucleosides. Coburn, *et al.*,[31] have reported a correlation of proton chemical shifts of 6-substituted purines with the reactivity parameters (Brown's electrophilic substituent constants) of the substituents. Diminution of ring currents by polar substituents has been proposed by Jardetzky[16] to explain the reduction of $\Delta\delta$ and intrinsic δ of anions of purine and substituted purines. This explanation may be invoked in the case of ribosylpurine *vs.* adenosine, but fails to account for the practically identical shifts observed for adenosine and inosine (Table III). Inosine, existing in the lactam form (Figure 8a), should have less ring current in the pyrimidine ring than does adenosine which exists in the lactim form[17] (Figure 8b). Thus according to this ring-current argument one would expect H-2 in inosine to be shifted upfield significantly, contrary to what has been observed.

A linear correlation between the observed chemical shifts and the calculated π-electron charge densities in the aromatic and heterocyclic systems can be established with a certain degree of satisfaction, as notably shown by the work of Schaefer and Schneider,[32] Veillard,[33] and more recently by the work of Lynch and Dou,[34] on protonated nitrogen heterocyclic compounds. These papers pointed out as well that the significant contribution of ring current and nitrogen atom magnetic anisotropy should also be properly considered for these systems. Following these lines of reasoning, we shall attempt here to provide a theoretical basis to account semiquantitatively for the observed chemical shifts of several purine nucleosides.

In the present situation, the method developed in one of our earlier papers[35] was employed, in which the calculated electron density at each atom of the base is taken into account, rather than only at that carbon atom to which the proton is bonded. These excess π-charge densities are handled in terms of a field effect upon the proton chemical shifts by the following equation[35]

$$\Delta\sigma = 12.5 \times 10^{-6}\,\epsilon_i \frac{\Delta\rho_i}{R_i^2}\cos\theta_i - 17.0 \times 10^{-6}\left(\frac{\epsilon_i \Delta\rho_i}{R_i^2}\right)^2 \qquad (2)$$

where $\Delta\rho_i$ is the excess π-charge density on the ith atom; R_i is the distance (A) from the ith atom to the proton in question; and θ_i is the angle of the field vector with respect to the C–H bond axis for the proton under consideration. This equation, eq 2, was developed from the familiar equation for field-effect calculations with the coefficients derived from the work of Schaefer and Schneider[32] and of Musher.[36] Equation 2 was shown to provide a good rationalization of experimentally determined proton resonance in purine, when combined with considerations of ring current and nitrogen atom anistropy.[35]

Though the π-charge densities of the bases in the nucleosides have not been calculated, such calculations for the bases themselves have been made by Veillard and Pullman[37,38] employing the self-consistent field method of Pariser and Parr.[39] Their π-charge density values for purine were shown to be applicable as a basis for further calculation since by the use of them one could account for the experimental chemical shifts.[35]

The comparison of experimental δ's of purine and ribosylpurine at infinite dilution in water indicates that H-6 and H-2 are deshielded by about 0.16 ppm and the H-8 by about 0.25 ppm owing to the N-9 ribosyl substitution. This observation is expected from the electron-withdrawing effect of this group. It is reasonable as a first approximation to consider that the effect of ribosyl substitution at N-9 on all the atoms in the base is the same for each of the several nucleosides in question. Thus, we assume that a comparison of the calculated π-charge densities of the bases can represent the comparison of the π-charge densities of the bases in the nucleosides.

The contribution to the screening constants due to ring currents was calculated with the following equation from Schaefer and Schneider[32]

$$\delta = -12.0(a^2/R^3) \qquad (3)$$

where δ is expressed in parts per million; a and R (A) are, respectively, the radius of the ring in question and the distance of the proton being considered from the center of the ring. Calculated ring-current values for purine bases have been reported by Giessner-Prettre and Pullman.[17] From four sets of values in their paper, the two which were results of most recent calculations were adopted for comparison. The validity of employing the five-membered ring figures is somewhat questionable since they were calculated for the bases and not for the nucleosides. Fortunately, the calculated ring-current differences for various imidazole rings among these bases is rather small.

The final problem is the effect of nitrogen atom magnetic anisotropy. For pyrimidine, this effect was evaluated in our earlier paper.[35] It was noticed that only slightly more than half of the shift difference between H_2 and H_6 (0.50 ppm)[40] could be accounted for by the difference in charge densities calculated by two different groups.[41,42] If the remaining 0.21 ppm (based on charge densities calculated from ref 41) or 0.24 ppm (based on charge densities calculated from ref 42) can be attributed to the difference in the effects of the nitro-

(31) W. C. Coburn, Jr., M. C. Thorpe, J. A. Montgomery, and K. Hewson, *J. Org. Chem.*, 30, 1114 (1965).
(32) T. Schaefer and W. G. Schneider, *Can. J. Chem.*, 41, 966 (1963).
(33) A. Veillard, *J. Chim. Phys.*, **59**, 1056 (1962).
(34) B. M. Lynch and H. J. M. Dou, *Tetrahedron Letters*, 2627 (1965).
(35) M. P. Schweizer, S. I. Chan, G. K. Helmkamp, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, 86, 696 (1964).
(36) J. I. Musher, *J. Chem. Phys.*, 37, 34 (1962).
(37) A. Veillard and B. Pullman, *Compt. Rend.*, **253**, 2277 (1961).
(38) A. Veillard and B. Pullman, *J. Theoret. Biol.*, 4, 37 (1963).
(39) R. Pariser and R. G. Parr, *J. Chem. Phys.*, 21, 466 (1953).
(40) S. Gronowitz and R. A. Hoffman, *Arkiv Kemi*, 16, 459 (1960).
(41) R. L. Miller, P. G. Lykos, and N. H. Schmeising, *J. Am. Chem. Soc.*, 84, 4623 (1962).
(42) A. Veillard and B. Pullman, *Compt. Rend.*, **253**, 2277 (1961).

gen magnetic anisotropy at the two protons, one can compute a magnetic anisotropy for the nitrogen atoms in the pyrimidine ring by the use of the following general equation based upon the dipole approximation[43]

$$\Delta\sigma = \epsilon_i \frac{\Delta\chi_i}{3R_i^3}(1 - 3\cos^2\gamma_i) \qquad (4)$$

where R_i is the distance between N_i and the proton in question, $\Delta\chi_i$ is the anisotropy of the susceptibility tensor, and γ_i is the angle between the axis of symmetry in N_i and the N_i–H line. $\Delta\chi$ was computed to be $+2.72 \times 10^{-6}$ and $\Delta\sigma$ for H-2 due to N_1 was computed to be -0.183 ppm. Considering the uncertainties and assumptions involved in both calculations, this value is in reasonable agreement with the -0.25 ppm calculated by Gil and Murrell[44] as the effect of the magnetic anisotropy of the N atom on the α protons in pyridine. As in the treatment by these authors, our reference for calculation was sp^2 carbon. But the actual comparison is the difference in magnetic anisotropy between pyridine-type nitrogen (N_1 in adenine) and cyclic amide nitrogen (N_1 in hypoxanthine or guanine). At present, we have no reliable evaluation of the magnetic anisotropy of the cyclic amide nitrogen so the value of -0.183 ppm may be either an over- or underestimate of the difference in the anisotropic effect exerted on the adjacent hydrogen by these two different types of nitrogen atoms. In Figure 9, the experimental δ for H-2 and H-8 of ribosylpurine, adenosine, inosine, and 1-methylguanosine are given *vs.* the theoretical values of δ calculated from the contributions of π-charge densities, the ring current, and nitrogen magnetic anisotropy. Guanosine is too insoluble for measurement; H-8 of 1-methylguanosine is used instead. The justification for this substitution is that δ of H-8 is the same for both inosine and 1-methylinosine (Table III). It should be mentioned also that the value of H-8 for 1-methylguanosine is not extrapolated to infinite dilution because of solubility limitations, and therefore likely to be about 0.05 ppm closer to the purine lines than the position presently plotted. The calculated spectral positions for purine are normalized to experimental positions for ribosylpurine. Therefore, we are concerned here with the relative chemical shifts between these three or four nucleosides employing purine (or ribosylpurine) as standard. The experimental observation that H-2 of ribosylpurine is 0.26 ppm downfield from H-8 is included in Figure 9, explaining why the two sets are offset. Two groups of calculated values are plotted, employing two sets of ring-current values reported[17] as discussed above. It appears that the b set (the fourth column from Giessner-Prettre and Pullman) gave better agreement for hypoxanthine. Within each set of calculations the dotted lines represent values obtained without correction for the difference in ring current of the five-membered ring and the solid lines are those with the correction for the ring current of the five-membered ring included. The best agreement between the theoretical and experimental values

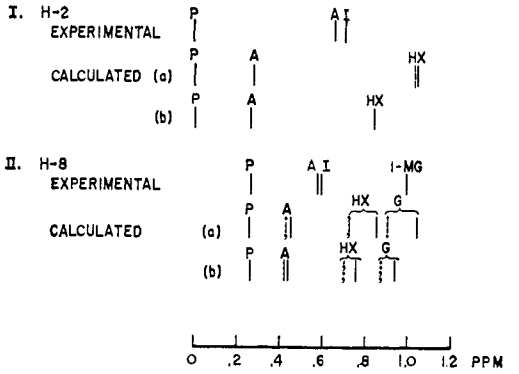

Figure 9. Experimental chemical shifts of four nucleosides compared with the calculated shifts of the respective bases from π-charged densities, π current, and nitrogen atom anisotropy. Values are normalized to ribosylpurine: P, purine or ribosylpurine; A, adenine or adenosine; I, inosine; HX, hypoxanthine; 1-MG, 1-methylguanosine; and G, guanine. The sets a and b include different π currents from ref 11. The dotted lines refer to calculations in which corrections for ring currents of the five-membered ring have been omitted.

of δ for hypoxanthine and guanine is within 0.05–0.2 ppm, for H-8 of adenine is about 0.15 ppm but comparison of adenine H-2 is poor, about 0.35 ppm. The disagreement in H-2 for hypoxanthine may be due to the overestimation of the N_1 anisotropic contribution as discussed above.

It appears, therefore, that a semiquantitative calculation of proton screening constants in purine nucleosides from a theoretical consideration of π-charge density, ring-current, and anisotropy effects can be achieved with some degree of success. Hence, the nmr data may provide an experimental guidance to values obtained from theoretical calculations on the electronic configurations of these types of compounds.

C. The Coupling Constants of Furanose Protons H-1′ and H-2′. The value of $J_{1',2'}$ is 6.1–6.4 cps for adenosine and 2.1–2.3 cps for 3′-deoxyadenosine, and is slightly concentration dependent. Jardetzky[45-47] has discussed the relationship of $J_{1',2'}$ to the conformation of the ribose ring for various nucleosides in D_2O.

From an analysis based on the Karplus[48] relationship between vicinal coupling constants and the dihedral angle ϕ, she concluded[47] that the ribose moiety of adenosine most likely exists in the $C_{2'}$-*endo* conformation, *i.e.*, C-2, tipped out of the plane of the furanoside ring in the direction of the C-4′–C-5′ bond, and the value of $\phi_{1',2'}$ is about 140–150° corresponding to $J_{1',2'}$ of about 6 cps. Although the nmr spectrum of 3′-deoxyadenosine has been reported previously,[49,50] the small value of $J_{1',2'}$ compared to adenosine was not mentioned. It should be noted that the substituents at C-2′ and C-1′ of 3′-deoxyadenosine are the same as adenosine and the difference between these two compounds is hydrogen *vs.* hydroxyl group at C-3′, respec-

(43) J. A. Pople, W. G. Schneider, and H. J. Bernstein, "High-Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York, N. Y., 1959, p 178.
(44) V. M. S. Gil and J. N. Murrell, *Trans. Faraday Soc.*, 60, 248 (1964).
(45) C. D. Jardetzky and O. J. Jardetzky, *J. Am. Chem. Soc.*, 82, 222 (1960).
(46) C. D. Jardetzky, *ibid.*, 82, 229 (1960).
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tively. Thus, in this case, some of the problems[51,52] in applying the Karplus relationship for analysis of $J_{1',2'}$, such as substituent perturbations, C-1'–C-2' bond lengths, etc., are minimized. Hence, we propose that the small $J_{1',2'}$ of 3'-deoxyadenosine arises from a smaller $\phi_{1',2'}$ than that of adenosine. If the Karplus plot is utilized, $\phi_{1',2'}$ will be about 120° for 2'-deoxyadenosine. A consequence of this smaller dihedral angle would be that C-2' is only slightly puckered out of the plane of the furanose ring in an *endo* fashion. Unfortunately, the other coupling constants involving protons at C-3' and C-4' were not obtainable due to the low solubility. From the stereochemical considerations, the hydrogen bond formation between the 2'-OH group and the N-3 will be much favored when the 2'-carbon is in the *endo* position than when it is not.[29] This finding, therefore, is in accord with the observation that the intramolecular hydrogen bonding was observed only for the adenosine and not for the 3'-deoxyadenosine.

In concluding, 14 purine nucleosides in aqueous solutions have been studied extensively by vapor pressure osmometry and pmr. Based on this study, several conclusions and calculations have been made on the properties of these compounds. In view of the importance of these conclusions and calculations, additional experimental observations and developments in theory are needed for further evaluation and improvement. Some of these programs are now in progress in our laboratory.

Experimental Section

When possible, commercially available compounds of the highest degree of purity were used without further purification. Elemental analyses on compounds studied by vapor pressure osmometry were obtained from Galbraith Laboratories, Knoxville, Tenn., and from Spang Microanalytical Laboratory, Ann Arbor, Mich. These analytical values were used to determine the extent of hydration. 2'-Deoxyadenosine monohydrate (found: N, 26.06), N-6-dimethyladenosine (found: N, 24.03), and inosine [found: C, 44.56; H, 4.74; N, 20.81 ± 0.3 (average of three determinations for N)] were obtained from Sigma Chemical Co. Adenosine (found: N, 26.12) was purchased from Calbiochem. 1-Methylinosine (found: C, 46.63; H, 5.05; N, 19.60) and ribosyl-2,6-diaminopurine were obtained from Cyclo Chemical Co. Ribosylpurine samples (specified as anhydrous by the suppliers) were obtained from both Sigma and Cyclo Chemical Co. Cordycepin (3'-deoxyadenosine) was kindly provided by Dr. Robert J. Suhadolnik of the Albert Einstein Medical Center, Philadelphia, Pa. 2'-O-Methyladenosine (found: N, 24.71), N-6-methyladenosine hemihydrate (found: N, 23.96), N-6-methyl-2'-deoxyadenosine, some 1-methylinosine (found: N, 20.08), and 1-methylguanosine were prepared according to published procedure.[7,53,54] In the case of N-6-methyl-2'-deoxyadenosine, although its identity was established by ultraviolet spectrum, nmr studies, melting point, and chromatography in three solvents,[55] no satisfactory elemental analyses could be obtained despite repeated efforts. It should be noted that no elemental analyses were reported either in the paper concerning the chemical synthesis[54] or in the paper concerning enzymatic synthesis.[55] At present, it is assumed to be anhydrous in the calculation of its osmotic coefficients.

All the nucleosides used were in neutral and deionized form. The solutions in H_2O or D_2O were prepared by directly dissolving the weighed nucleosides without additions of salt or any other chemicals.

Vapor pressure lowering measurements were made as previously described using a Mechrolab 301A vapor pressure osmometer.[2] Nuclear magnetic resonance spectra were obtained using Varian Associates A-60 or HA-100 spectrometers. Chemical shift values are reported in cps (60 Mc) from a TMS capillary; difference values are also in cps (60 Mc). Bulk susceptibility corrections were not made, but should be less than 1 cps.

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Effects of Site Symmetry and Sequential Metal Binding upon Protein Titration (Zinc Insulin)

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Abstract: A hydrogen ion titration curve for zinc insulin, in agreement with the experimental data, is computed on the basis of a model in which the metal ions are each bound to three imidazole nitrogens. The equations involved and the effects of the parameters in them are discussed in more general terms.

The hydrogen ion dissociation for metal-free and for zinc insulin are presented in two papers by Tanford and Epstein.[1,2] In the first paper, the metal-free case is quantitatively described. In the second, the titration behavior of insulin in the presence of zinc is reported to differ before and after exposure to acid or base, and

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