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Interaction and Association of Bases and Nucleosides in Aqueous Solutions. III. A Nuclear Magnetic Resonance Study of the Self-Association of Purine and 6-Methylpurine[1b]

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The association of bases and nucleosides in aqueous solutions has recently been established by osmotic studies. To further elucidate the nature of this association, the proton magnetic resonance spectra of purine and 6-methylpurine have now been studied in detail as a function of concentration. A pronounced concentration effect has been observed. This concentration dependence of the proton magnetic resonance spectra was found to be both temperature and solvent dependent. These data, together with additional results on purine hydrochloride, substantiate the interpretations of the osmotic studies and further strongly suggest that the mode of association is that of vertical stacking of rings in a partial overlapping fashion. Model calculations show that the n.m.r. results are in quantitative agreement with the osmotic results and indicate that the average distance between rings in the complex is 3 to 4 Å.

Introduction

In an effort to understand the factors that contribute to the stabilization of nucleic acids as well as the basic mechanism of the recognition process involved in the enzymatic replication of nucleic acids *in vitro*, Ts'o and co-workers[3,4] have recently studied the interaction of bases and nucleosides in aqueous solutions. The motivation for this research was the logical deduction that the specificity of helix formation by strands of nucleic acids must reside in the interaction of the nitrogenous bases on the polynucleotide chains.

In the work of Ts'o, *et al*, these investigators examined the thermodynamic properties and solution behavior of purine, 6-methylpurine, uridine, 5-bromouridine, and cytidine at various concentrations in water. In particular, they determined molal osmotic coefficients and activity coefficients of these molecules over the concentration range 0.1 to 1.0 *m*. From these studies, they concluded that a high degree of association of these bases and nucleosides occurs in aqueous solution. Thus purine was found to associate in successive steps with an equilibrium constant of ~2 for each step. In the case of 6-methylpurine, the association constant was 6.7. For uridine and cytidine, the corresponding equilibrium constants for the association process were somewhat smaller, 0.6 and 0.9, respectively. However, the self-association of uridine was significantly enhanced by bromine substitution at the 5-position. In 5-bromouridine the association does not appear to proceed beyond the tetramer stage; here the association constant for the first step was determined to be approximately 1 and for the remaining three steps it was approximately 3.

In addition to these results on self-association, Ts'o, *et al.*, have also investigated cross interactions between bases and nucleosides. Their results on the solubility enhancement of adenine and thymine in aqueous solutions of purine, pyrimidine, uridine, and cytidine again provided evidence for complex formation.

The work of Ts'o, *et al.*, left little doubt that the association of bases and nucleosides is an energetically favorable process in aqueous solution. However, while the general extent of the association was established for a number of bases and nucleosides, the nature of the complex was only inferred. A number of mechanisms are possible, such as vertical stacking of rings due to π-electron interactions, horizontal interactions due to hydrogen bonding, etc. From a comparison of the association tendencies of bases and nucleosides with the association tendencies of systems where the association mechanism is without question that of hydrogen bonding, Ts'o, *et al*, concluded that hydrogen bonding is most likely not responsible for the stability of the purine and pyrimidine nucleoside polymers. This conclusion, which is based upon somewhat indirect evidence, is however not inconsistent with the results of recent experimental[5-8] and theoretical[9] studies on nucleic acids which suggest that hydrophobic interaction of bases contributes significantly to the stability of the DNA helix.

In view of the lack of more conclusive experimental evidence on the details of the interactions between bases and nucleosides, we have embarked on a nuclear magnetic resonance study of these solutions. It is well known that nuclear magnetic shielding is a very sensitive probe of inter- and intramolecular interactions. In this case, vertical stacking interactions are easily distinguished from hydrogen bonding interactions as these interactions will manifest themselves differently in the n.m.r. spectra. It is, therefore, hoped that the concentration dependence of the n.m.r. spectra of aqueous solutions of bases and nucleosides will shed some light on the association mechanism. In this paper, we wish to report the results of such studies on aqueous solutions of purine and 6-methylpurine.

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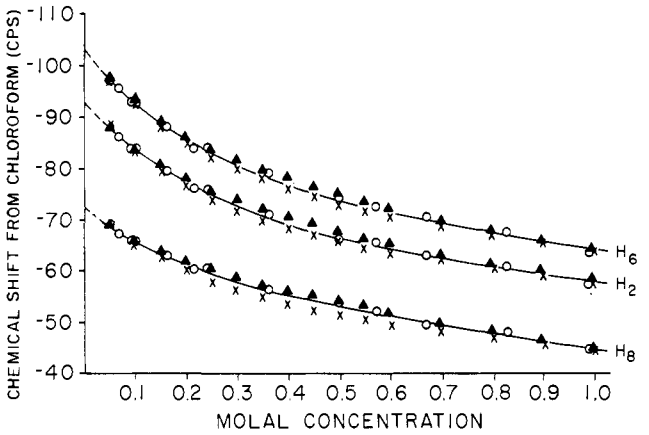


Fig. 1.—Concentration dependence of the proton chemical shifts for purine in aqueous solution at 25° (corrected for bulk susceptibility); shifts measured from external chloroform reference: —O—, experimental values; —X—, calculated values from over-all average model; --▲—, calculated values from statistical partial-overlapping model.

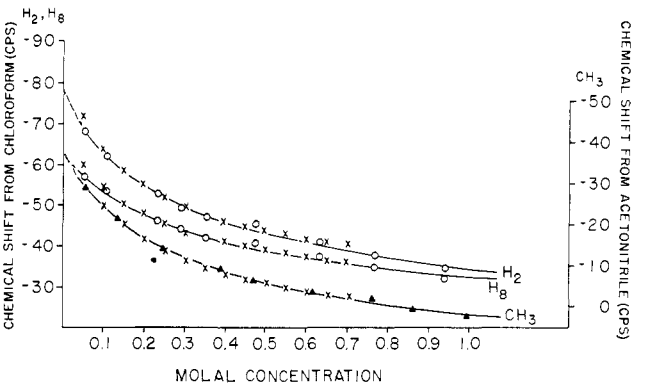


Fig. 2.—Concentration dependence of the proton chemical shifts for 6-methylpurine in aqueous solution at 25° (corrected for bulk susceptibility): —O—, experimental values for H-2, H-8 protons, shifts measured from external chloroform reference (left-hand coordinates); —▲—, experimental values for methyl protons, shifts measured from external acetonitrile reference (right-hand coordinates); —X—, calculated values from over-all average model.

An attempt has also been made in this work to correlate the n.m.r. spectral data with the osmotic results in a quantitative manner. Such a correlation is facilitated by the results presented in the preceding papers of this series, where analyses of the osmotic data have led to a knowledge of the population distribution of the solute molecules among the various associated species in solution.[3,4]

Experimental

Materials.—Purine was obtained from California Corporation for Biochemical Research, Los Angeles (A-grade), and Cyclo Chemical Corporation, Los Angeles, Calif. The purine was purified by dissolving in hot ethanol and recrystallizing after charcoal absorption and hot filtration (m.p. 217–218°). 6-Methylpurine from Cyclo Chemical Corporation was resublimed *in vacuo* before use.[4]

Synthesis of Purine Hydrochloride.—Purine hydrochloride was prepared by treating 100 mg. of purine in 50 ml. of dioxane with anhydrous HCl gas for several hours. The precipitate was isolated by filtration and dried. Sublimation of the hydrochloride did not affect the melting point, which was 206–208°.

Purine hydrochloride molal solutions were prepared by dissolving the sublimed material in 0.1 *M* HCl.

Instrumentation.—All n.m.r. spectra were recorded with a Varian V-4300 spectrometer operating at 56.4 Mc. Probe operating temperature was normally 25 ± 1°. High temperature studies were made with the aid of a V-4340 Variable Temperature n.m.r. probe accessory purchased from Varian Associates, Palo Alto, Calif. The spectrometer utilized V-4365 field homogeneity coils with V-K3506 flux stabilizer for maximum field uniformity. Peak positions were measured using a Hewlett-Packard wide-range audio oscillator and a HW 521 C electronic counter monitored the audio frequency output.

Chemical shifts were measured using the standard audio sideband technique. Chloroform was used as an external reference, except in the case of the methyl protons of 6-methylpurine where acetonitrile was used instead. Bulk susceptibility corrections have been made where necessary. The accuracy of the measured shifts are well within ±0.5 c.p.s., being somewhat limited by line widths and reduced signal intensities at low solute concentrations. Even though certain Pascal constants[10] had to be assumed in the bulk susceptibility corrections, it is estimated that the chemical shifts reported here are good to ±1 c.p.s.

Results

Nuclear Magnetic Resonance Spectra.—The n.m.r. spectrum of purine in aqueous solution consists of three resonance peaks. The N-9 proton was not observed because of exchange. Two independent experimental assignments as well as a theoretical rationalization of the n.m.r. spectrum of purine have now appeared in the literature.[11,12] These studies assigned the highest field peak to H-8, the center field peak to H-2, and the lowest field peak to H-6. The correctness of the n.m.r. assignment will have important bearing on the interpretation of the large concentration effect to be described below.

There are also three resonance peaks in the n.m.r. spectrum of 6-methylpurine. The high-field resonance can be assigned to the methyl protons with no ambiguity. The assignment of the two remaining peaks was made on the basis of deuterium exchange studies. When 6-methylpurine was boiled in D_2O for 75 min., deuterium was introduced at C-8 and the higher field ring proton peak was reduced in intensity by 70%. We have therefore assigned the center-field peak to H-8 and the low field peak to H-2. This assignment of the proton n.m.r. spectrum of 6-methylpurine is in agreement with that recently proposed by Reddy and co-workers.[13]

Concentration Effect.—The n.m.r. spectra of purine and 6-methylpurine have been studied over the concentration range of 0.05 to 1.0 *m*. A pronounced concentration effect has been observed. The chemical shifts of the three protons in purine are plotted *vs.* the concentration in Fig. 1. Similar chemical shifts *vs.* concentration curves for 6-methylpurine are presented in Fig. 2. The experimental data as displayed have been corrected for bulk susceptibility in the standard manner.

The pronounced concentration effect depicted in Fig. 1 for purine has been previously noted by Jardetzky and Jardetzky[14,15] in alkaline medium at two concentrations, namely, 0.2 and 2.0 *m*.

The proton resonances in purine and 6-methylpurine are all shifted to higher fields as the solute con-

(10) J. A. Pople, W. G. Schneider, and H. J. Bernstein, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Company, Inc., New York, N. Y., 1959, p. 18.

(11) M. P. Schweizer, S. I. Chan, G. K. Helmkamp, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **86**, 696 (1964).

(12) S. Matsuura and T. Goto, *Tetrahedron Letters*, **No. 22**, 1499 (1963).

(13) G. S. Reddy, L. Mandell, and J. H. Goldstein, *J. Chem. Soc.*, 1414 (1963).

(14) C. D. Jardetzky and O. Jardetzky, *J. Am. Chem. Soc.*, **82**, 222 (1960).

(15) Private communication from Dr. O. Jardetzky in the course of preparing this manuscript indicated that he also has made the concentration studies of purine in D_2O recently and has observed similar results.

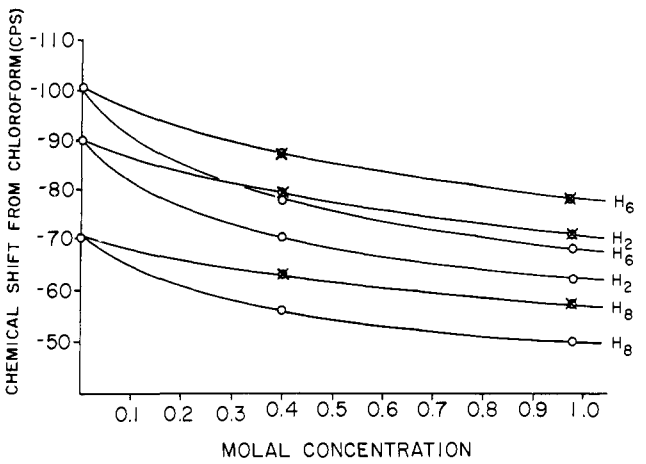


Fig. 3.—Temperature effect upon the concentration dependence of purine proton chemical shifts (data not corrected for bulk susceptibility); shifts measured from external chloroform reference: —O—, purine in water at 25°; —X—, purine in water at 50°.

centration is increased. Shifts to high fields with concentration are well known for aromatic systems and are generally attributed to the magnetic anisotropy associated with the ring currents in neighboring molecules. Because of the mobile π-electrons, a large diamagnetic current is induced in the plane of the ring by an external magnetic field when the field is perpendicular to the plane of the molecule. This ring current gives rise to a small secondary magnetic field which reinforces the primary field at the peripheral protons in the plane of the ring. In the region directly above and below the molecular plane, the two fields are, however, opposed. As the concentration of a solution of aromatic molecules is increased, the average distance between molecules decreases and the protons of a given molecule will feel the secondary magnetic fields produced by the ring current of neighboring molecules. Since it is much more probable to find a molecule somewhere above or below the molecular plane of another aromatic molecule due to the disk-shaped nature of aromatic molecules, this magnetic anisotropy of the ring current effect will lead to a high-field shift with concentration or a low-field shift upon dilution. Shifts of this nature have been observed in solutions of benzene in hexane[16] and solutions of toluene, mesitylene, chlorobenzene, and benzonitrile with aliphatic solvents.[17] Even larger concentration shifts have been noted for naphthalene[17] and azulene[18] in dioxane, the larger shifts apparently because of the larger ring current diamagnetism in these compounds.

Dilution shifts for aromatic molecules are generally of the order of 1 p.p.m. in going from the pure liquid to infinite dilution. For smaller concentration changes, the shifts are correspondingly smaller. Thus the dilution shift of azulene in dioxane is 0.1 p.p.m. for the five-membered ring protons and 0.25 p.p.m. for the seven-membered ring protons in going from a 20 mole % solution to a 3 mole % solution. In contrast, the concentration effect observed for purine from infinite dilution to 1.0 *m* (about 2 mole per cent) is 0.60–0.70 p.p.m. for the protons on the pyrimidine ring and 0.48 p.p.m. for the proton on the imidazole ring. The corresponding shifts in 6-methylpurine are 0.76 and 0.53 p.p.m., respectively. The shifts in purine and 6-methylpurine are much larger and, accordingly, cannot be rationalized in terms of a simple dilution effect. Molecular association is therefore suggested.

It is noteworthy that the protons on the pyrimidine ring of purine and 6-methylpurine exhibit essentially parallel concentration dependences. This interesting behavior was found for 6-methylpurine even though the "6-protons" are really methyl protons and are not ring protons. For the 8-proton on the imidazole ring, this parallel dependence exists at the high concentration end, but deviates at low concentrations.

Temperature Effect.—To further elucidate the nature of the pronounced concentration effect in purine and 6-methylpurine, the proton resonance shifts of purine have also been studied in aqueous solution at 50°. Two concentrations were considered, namely, 0.4 and 0.98 *m*. At infinite dilution, the same extrapolated chemical shift value obtained at 25° was assumed for the higher temperature.

A comparison of the concentration effect at 25 and 50° is given in Fig. 3. One can see that the concentration shifts at 50° are roughly 50–60% those at 25°. This result, of course, is consistent with the picture of molecular association and further suggests that the process is exothermic.

Solvent Effect.—Similar concentration studies of the n.m.r. spectra of purine have been conducted in other solvents. In Table I, experimental results are shown for dimethyl sulfoxide and dimethylformamide. The drastic effect of solvent on the concentration shifts is striking indeed. In contrast to the pronounced shifts observed in aqueous solution, the proton resonances are essentially independent of concentration in these solvent systems.

The n.m.r. spectrum of purine has also been investigated in a 50–50 mixture of dimethyl sulfoxide in water. Here the concentration shifts are comparable to those observed in water (see Table I).

TABLE I

EFFECT OF SOLVENT ON THE NUCLEAR MAGNETIC RESONANCE SPECTRUM OF PURINE

Solvent	Concentration, *m*	Chemical shifts from $CHCl_3$, c.p.s.[a] H-6	H-2	H-8
Water	0.20	−86.3	−77.6	−61.2
	1.00	−68.2	−62.5	−50.0
DMS	0.206	−94.7	−82.5	−65.9
	0.966	−92.9	−80.8	−64.9
DMF	0.204	−78.1	−66.5	−53.1
	0.952	−78.3	−66.5	−54.2
50% DMS in water	0.094	−100.5	−89.9	−71.2
	0.381	−95.8	−85.4	−68.2
	0.915	−85.5	−76.1	−61.8

[a] Data presented in this table are not corrected for bulk susceptibility.

Purine Hydrochloride.—The n.m.r. spectrum of the purine hydrochloride has been studied as a function of concentration in 0.1 *M* HCl. One might expect the protonated species to exhibit a much smaller tendency to associate due to the extra positive charge on the ring. This concentration appears to be confirmed by the evidence provided in Table II, where the purine hydro-

(16) J. R. Zimmerman and M. R. Foster, *J. Phys. Chem.*, **61**, 282 (1957).
(17) A. A. Bothner-By and R. E. Glick, *J. Chem. Phys.*, **26**, 1651 (1957).
(18) W. G. Schneider, H. J. Bernstein, and J. A. Pople, *J. Am. Chem. Soc.*, **80**, 3497 (1958).

TABLE II

CONCENTRATION SHIFTS OF PURINE HYDROCHLORIDE IN 0.1 *M* HCl SOLUTION

Concentration, *m*	Chemical shifts from $CHCl_3$, c.p.s.[a] H-6	H-2	H-8
0.11	−121.5	−111.0	−95.8
0.27	−121.3	−110.5	−95.7
0.47	−121.6	−111.0	−95.8
1.01	−120.4	−109.5	−94.7

[a] Data not corrected for bulk susceptibility.

chloride shifts are listed as a function of the concentration.

The proton resonances of the purine hydrochloride are all shifted considerably downfield. The shifts are strongly dependent upon the acid concentration, and for a given purine concentration the shifts are larger the stronger the acid concentration. Of the three resonances, H-6 is affected most and H-8 the least, at 1.0 *m* concentration, but there are essentially no differences at 0.2 *m* concentration. The much larger absolute shifts (45–50 c.p.s.) compared with the smaller relative shifts (5–7 c.p.s.) suggests that N-1, N-3, and N-7 are roughly equally susceptible to protonation. Finally, the resonances for the purine hydrochloride are considerably broader than those for purine in neutral media. This is undoubtedly due to proton exchange.

Discussion

We have presented in the previous section strong evidence for the association of purine and 6-methylpurine in aqueous solution. The pronounced concentration effect on the proton resonance shifts can only be interpreted in terms of molecular association, as the observed concentration shifts are an order of magnitude larger than those normally associated with ring-current magnetic anisotropic effects arising from the closer packing of molecules with concentration. This conclusion is strongly supported by three additional observations: the decrease in concentration shifts at higher temperatures; the concentration independence of the shifts for purine hydrochloride in contrast to those for purine; and the pronounced effects of solvents on the concentration shifts. The interpretations for the first and second observations are straightforward and require no further discussion. The striking effect of solvent on the concentration shifts is very interesting particularly in view of recent work on nucleic acids in organic solvents.[19-22]

The pronounced effect of solvent on the concentration shifts can be understood in terms of the following three competitive processes: (i) solute–solute interaction; (ii) solute–solvent interaction; (iii) solvent–solvent interaction. In aqueous solution, solvent–solvent interactions are expected to predominate over solute–solvent interaction. Because of the strong water–water hydrogen bonds, a solvent molecule cannot readily interact with the solute. Consequently, purine molecules in solution are encouraged to interact with each other. In dimethyl sulfoxide and dimethyl formamide, solvent–solvent interactions are considerably reduced due to the absence of hydrogen bonding donor sites in the solvent molecules. These solvents, however, are hydrogen bond acceptors and can, therefore, interact with purine. As a result, solute molecules cannot associate as readily. The results with dimethyl sulfoxide–water mixtures as solvent are not inconsistent with this interpretation of the solvent effect. Here one would expect a strong interaction between dimethyl sulfoxide and water molecules. Solute–solute interaction can, therefore, take place as observed. Dimethyl sulfoxide and dimethyl formamide have both been found to be effective solvents for the denaturation of DNA.[19] These observations are probably not irrelevant and can be understood on the basis of the above considerations.

It is clear that the chemical shift variation of the proton resonances of purine and 6-methylpurine with concentration in aqueous solution cannot be interpreted in terms of a solute–solute interaction *via* horizontal hydrogen bonding. Such a mechanism of association would produce shifts in the opposite direction than those observed. This point has been raised.[14,23] Shifts to higher fields with concentration can only be interpreted in terms of molecular association *via* vertical stacking of rings or π-complexing of the type proposed for chloroform in benzene.[24,25]

In the case of the π-complex, the C–H bond under consideration is directed at right angles to the plane of an aromatic ring, with the proton adjacent to the ring. Because of this interaction, the proton is drawn closer to the ring and, accordingly, its proton resonance is displaced more to higher fields by the ring current anisotropy than it would have been in the absence of such preferred interaction. Similar interactions have been proposed by Schaefer and Schneider[26] to interpret the ring proton chemical shifts of *para*-substituted toluenes in benzene.

With π-complexing, it seems reasonable to expect concentration shifts for different protons to be dependent upon their relative acidities. In the case of 6-methylpurine, we would therefore have expected very different concentration shifts for the methyl protons and the ring protons. Instead, as seen in Table III,

TABLE III

A SUMMARY OF THE CONCENTRATION SHIFTS FOR PURINE AND 6-METHYLPURINE

Purine	H-6 (or CH_3), p.p.m.	H-2, p.p.m.	H-8, p.p.m.
$\delta(0.2\ m) - \delta(0)$	0.31	0.27	0.19
$\delta(1.0\ m) - \delta(0.2\ m)$	0.38	0.35	0.29
$\delta(1.0\ m) - \delta(0)$	0.69	0.62	0.48
6-Methylpurine			
$\delta(0.2\ m) - \delta(0)$	0.37	0.41	0.25
$\delta(1.0\ m) - \delta(0.2\ m)$	0.34	0.37	0.28
$\delta(1.0\ m) - \delta(0)$	0.71	0.78	0.53

the methyl protons and H-2 exhibit practically parallel concentration shifts. This same parallel dependence is also observed for H-2 and H-6 in purine. For both molecules, the shifts for H-8 are somewhat less than the corresponding shifts for the protons on the pyrimidine ring. Such an interesting dependence of the concen-

(19) G. K. Helmkamp and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **83**, 138 (1961).
(20) P. O. P. Ts'o, G. K. Helmkamp, and C. Sander, *Biophys. Biochem. Acta*, **55**, 584 (1962).
(21) G. K. Helmkamp and P. O. P. Ts'o, *ibid.*, **55**, 601 (1962).
(22) P. O. P. Ts'o, G. K. Helmkamp, C. Sander, and F. W. Studier, *ibid.*, **76**, 54 (1963).
(23) L. Gatlin and J. C. Davis, Jr., *J. Am. Chem. Soc.*, **84**, 4464 (1962).
(24) C. M. Huggins, G. C. Pimentel, and J. N. Schoolery, *J. Chem. Phys.*, **23**, 1244 (1955).
(25) G. Korinek and W. G. Schneider, *Can. J. Chem.*, **35**, 1157 (1957).
(26) T. Schaefer and W. G. Schneider, *J. Chem. Phys.*, **32**, 1218 (1960).

tration shifts upon the ring to which the proton is attached seemingly is more conceivable when the mode of association is that of vertical stacking. On this basis, we have therefore excluded π-complexing as the probable mode of association. Note that an incorrect assignment of the proton resonance spectrum would have led to entirely different conclusions.

We now turn our attention to the vertical stacking interaction. Without a doubt the complex here arises from electrostatic interaction between the π-electron clouds. Since there is considerable charge separation in heterocyclic aromatic molecules, an estimate of the enthalpy of interaction between two rings may be made on the basis of a point charge approximation and the π-electron densities predicted by MO calculations.[27,28] In this way, interaction energies of the proper order of magnitude, *i.e.*, a few hundred calories (per mole) can be readily accounted for with a reasonable geometry for the dimer when the rings are somewhat displaced and their planes are 3–4 Å. apart.

In order to place our interpretation of the concentration shifts on a mole quantitative basis, we shall now attempt to correlate the present n.m.r. data with the osmotic results. In the preceding papers of this series[3,4] the osmotic coefficients and activity coefficients of purine and 6-methylpurine were interpreted in terms of a model of multiple equilibria and on this basis populations of the various associated species in solution were computed. The numerical results, as well as a full description of the model, the assumption involved, and the method of analysis are fully presented there and will not be discussed again.

Within the framework of this model, for a given base concentration, m, the observed chemical shift of a proton can be related to the concentration of the various associated species in solution as follows:

$$\delta_{\text{obsd}} = \sum_n \bar{\delta}_n F_n \qquad (1a)$$

Here $\bar{\delta}_n$ is the average chemical shift of this proton for those solute molecules in the nth associated state: $n = 1$ for monomers, $n = 2$ for dimers, etc. F_1, F_2, F_3 . . . denote respectively the mole fraction of base existing in solution as monomers, dimers, trimers, etc.

Since

$$F_n = \left[n \left(\prod_{i=1}^{n-1} K_i \right) (m_1)^n \right] / m$$

where m_1 is the monomer concentration, $K_1 = 1$, and the remaining K's are the association constants for the successive association steps, eq. 1a may be rewritten as

$$\delta_{\text{obsd}} = \sum_{n=1} \left[\bar{\delta}_n n \left(\prod_{i=1}^{n-1} K_i \right) (m_1)^n \right] / m \qquad (1b)$$

Using eq. 1b, we can extract $\bar{\delta}_1$ from the experimental data, as

$$\lim_{m_1 \to 0} \delta_{\text{obsd}}/F_1 = \lim_{m_1 \to 0} \delta_{\text{obsd}} \frac{m}{m_1} = \bar{\delta}_1 \qquad (2)$$

This procedure of determining $\bar{\delta}_1$ is in principle equivalent to the usual method of extrapolating $\bar{\delta}_{\text{obsd}}$ to infinite dilution ($m \to 0$, $m_1 \to 0$, $F_1 \to 1$). However, in practice where the limit is taken by a graphical extrapolation, our present procedure should yield a more reliable extrapolation and hence a more reliable chemical shift value for the "monomer" proton. Once $\bar{\delta}_1$ is determined $\bar{\delta}_2$ may be determined in an analogous manner. From eq. 1b

$$\lim_{m_1 \to 0} (\delta_{\text{obsd}} - \bar{\delta}_1 F_1)/F_2 = \bar{\delta}_2 \qquad (3)$$

Considering the nature of the interaction giving rise to the n.m.r. concentration shifts, we feel it is now physically reasonable to make the following assumption

$$\bar{\delta}_3 \cong \bar{\delta}_4 \cong \bar{\delta}_5 \ldots = \bar{\delta}_\infty \qquad (4)$$

With this approximation, eq. 1 can be rewritten in closed form instead of a series and is therefore in a considerably more tractable form insofar as further treatment of the data is concerned. In fact, $\bar{\delta}_3$ is then immediately determined by solving

$$\bar{\delta}_{\text{obsd}} = \bar{\delta}_1 F_1 + \bar{\delta}_2 F_2 + \bar{\delta}_3 (1 - F_1 - F_2) \qquad (5)$$

In order to examine the validity of this approximation, we can obtain $\bar{\delta}_\infty$ by extrapolating $\bar{\delta}_{\text{obsd}}$ to infinite concentration; *i.e.*

$$\lim_{F_1 \to 0} \delta_{\text{obsd}} = \bar{\delta}_\infty \qquad (6)$$

The values of $\bar{\delta}_\infty$ so obtained must be equal to the value of $\bar{\delta}_3$ determined from eq. 5 in order for the assumption under consideration to be valid.

In the above manner, $\bar{\delta}_1$, $\bar{\delta}_2$, $\bar{\delta}_3$, and $\bar{\delta}_\infty$ have been determined for each of the three ring protons in purine. Linear extrapolation was used. The numerical results are summarized in Table IV. It is gratifying to note the remarkable agreement between $\bar{\delta}_3$ and $\bar{\delta}_\infty$, thus justifying the assumption invoked. The extent to which the experimental concentration shifts are fitted by eq. 5 is indicated by a comparison of the calculated points with the experimental points in Fig. 1. The calculated chemical shift variation with concentration is based upon the population distribution of the various associated species obtained from analyses of the osmotic results[4] and the values of $\bar{\delta}_1$, $\bar{\delta}_2$, and $\bar{\delta}_3$ tabulated in Table IV. The excellent fit depicted indicates that the n.m.r. and the osmotic results are at least not inconsistent.

TABLE IV

AVERAGE CHEMICAL SHIFT VALUES[a] FOR PURINE PROTONS IN VARIOUS ASSOCIATED STATES[b]

Proton	$\bar{\delta}_1$	$\bar{\delta}_2$	$\bar{\delta}_3$	$\bar{\delta}_\infty$
H-6	−103.8	−68.5	−43.2	−44.8
H-2	−95.5	−55.0	−41.6	−41.7
H-8	−74.0	−46.0	−30.7	−29.8

[a] In c.p.s. from chloroform. [b] All the values are good to ±0.5 c.p.s.

The identical procedure was adopted for the analysis of the 6-methylpurine concentration shifts. Here, however, it was found, upon application of eq. 2, 3, and 6, that linear extrapolations were possible only with respect to the $\bar{\delta}_1$ and $\bar{\delta}_\infty$ determinations, but not with respect to that for $\bar{\delta}_2$. Consequently, $\bar{\delta}_3$ was assumed to be equal to $\bar{\delta}_\infty$ and $\bar{\delta}_2$ was obtained using eq. 5 at the concentration of 6-methylpurine where the per cent

(27) R. L. Miller and P. G. Lykos, *Tetrahedron Letters*, No. **11**, 493 (1962); R. L. Miller, P. G. Lykos, and H. N. Schmeising, *J. Am. Chem. Soc.*, **84**, 4623 (1962).

(28) A. Veillard and B. Pullman, *Comp. rend.*, **253**, 2277 (1961).

dimer in solution is a maximum. These results are listed in Table V. Again, as illustrated in Fig. 2, the concentration shifts calculated from these values of $\bar{\delta}_1$, $\bar{\delta}_2$, and $\bar{\delta}_3$ and the populations of the various polymeric species are in satisfactory agreement with experiment.

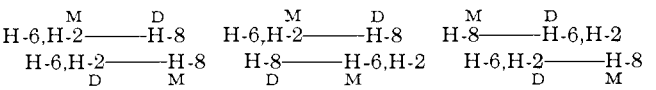
TABLE V

AVERAGE CHEMICAL SHIFT VALUES[a] FOR 6-METHYLPURINE PROTONS IN VARIOUS ASSOCIATED STATES[b]

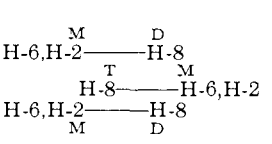
Proton	$\bar{\delta}_1$	$\bar{\delta}_2$	$\bar{\delta}_\infty$
H-2	-85 ± 2	-61 ± 1	-24 ± 0.5
H-8	-68 ± 3	-56 ± 1	-23.2 ± 0.5
Methyl	-46 ± 3	-24 ± 1	$+14.0 \pm 1$

[a] In c.p.s. [b] The aromatic protons are referred to chloroform and the methyl protons to acetonitrile.

As defined above, $\bar{\delta}_2$ and $\bar{\delta}_3$ denote respectively the chemical shift values of a proton averaged over the several magnetic environments possible in a dimer and a trimer state. By themselves, these numbers are not particularly informative. Upon association a ring proton can find itself in several quite different magnetic environments even in the same associated state. Thus, in the formation of the dimer unit, the two H-8 protons may find themselves in what we shall call monomeric (M) and dimeric (D) magnetic sites. This point can be illustrated schematically for purine as follows



The data observed requires the construction of the above models in such a manner that the rings are on the average stacking with their ring axes somewhat displaced. Clearly, H-8 will experience a different magnetic perturbation depending upon whether it finds itself in a M or D site in the dimer. Since the dimer structure is rapidly breaking up and reforming, only the average magnetic environment, however, will contribute to the observed resonance shifts. For trimer species, the ring proton can also be found in a trimeric (T) magnetic environment. Here, the proton will experience ring current effects from two rings, one below and one above as shown



For higher polymeric species, tetrameric and pentameric magnetic environments are also possible, but these should be quite similar magnetically to the trimeric magnetic sites due to the rapid drop off of the ring-current effect with distance.

It is clear that a satisfactory quantitative account of the concentration shifts can only come after some careful considerations concerning the details of the stacking interaction. Specifically, we need to know the relative probabilities of finding a proton in magnetically equivalent or similar environments in the various associated species. This aspect is complicated by preferred orientations which the rings might assume in the association process. *A priori* knowledge on the preferred orientations of rings is pretty much out of the question. However, under the present circumstances, where the free energies of association are of the same order of magnitude as kT, any preferred orientations of the rings will be reduced in importance and one may perhaps assume a somewhat random process and resort to the use of statistics. We have attempted an analysis along these lines, and in Table VI we list the various relative probabilities of finding a proton in monomeric, dimeric, and trimeric magnetic environments for the various associated species through the pentamer. These weighting factors have been obtained by statistical counting. All so-called tetrameric magnetic environments have been included as trimer sites. We have also ignored polymeric species higher than the pentamer since the results of the osmotic studies indicate that their concentrations are negligible.

TABLE VI

RELATIVE PROBABILITIES OF FINDING A PROTON IN MONOMERIC (M), DIMERIC (D), AND TRIMERIC (T) MAGNETIC ENVIRONMENTS FOR THE VARIOUS ASSOCIATED SPECIES THROUGH THE PENTAMER

	Monomer	Dimer	Trimer	Tetramer	Pentamer
M	1	$^1/_2$	$^5/_{12}$	$^3/_8$	$^7/_{20}$
D	0	$^1/_2$	$^1/_2$	$^1/_2$	$^1/_2$
T	0	0	$^1/_{12}$	$^1/_8$	$^3/_{20}$

Using this approach, the chemical shift of a proton as a function of base concentration can be re-expressed as follows

$$\delta_{\text{obs}} \sum_{n=1} = \delta_n f_n \tag{7}$$

where δ_1, δ_2, δ_3 . . . . . now denote respectively the chemical shift the proton would have if it were in monomeric, dimeric, trimeric, ... magnetic environments, and $f_1, f_2, f_3, \ldots$ give the relative probabilities of finding it in these environments. Because of our assumption that

$$\delta_3 = \delta_4 = \delta_\infty \tag{8}$$

eq. 2 readily reduces to the following simple form

$$(\delta_{\text{obs}} - \delta_3) = (\delta_1 - \delta_3)f_1 + (\delta_2 - \delta_3)f_2 \tag{9}$$

In accordance with our model here

$$f_1 = \frac{m_1}{m} + \frac{m_2}{m} + {}^5/_4 m_3/m + {}^3/_2 m_4/m + {}^7/_4 m_5/m + \ldots .$$

and

$$f_2 = m_2/m + {}^3/_2 m_2/m + 2m_4/m + {}^5/_2 m_5/m + \ldots . \tag{10}$$

Equations analogous to 2, 3, and 5 can be written down for the determination of δ_1, δ_2, and δ_3. The results for purine are reported in Table VII. Chemical shift *vs.* concentration curves for this model are given in Fig. 1. The agreement with experiment is seen to be satisfactory for purine.

For 6-methylpurine, it was found that δ_1 and δ_2 cannot be obtained by linear extrapolation. Apparently the stacking of 6-methylpurine does not proceed in a statistically random fashion because of stronger association tendencies of this base compared to purine and possibly because of steric influence of the methyl groups.

Finally, the distance between planes in the purine complex may be inferred from the values of $\delta_2 - \delta_1$ given

TABLE VII

CHEMICAL SHIFT VALUES[a] FOR PURINE PROTONS IN MONOMER, DIMER, AND TRIMER MAGNETIC ENVIRONMENTS[b]

Proton	δ_1	δ_2	δ_3
H-6	−104.0	−28.5	−5.6
H-2	−94.0	−25.8	−5.6
H-8	−72.7	−28.3	−11.9

[a] In c.p.s. from chloroform. [b] All the values are good to ±0.5 c.p.s.

in Table VII. Using the standard formula,[29] a distance of 3–4 Å. is obtained.

In conclusion, the n.m.r. data obtained for purine and 6-methylpurine in aqueous solution clearly support a model of vertical stacking with average partial ring overlap for the mode of association of these solutes in preference to one of horizontal hydrogen bonding. Furthermore, a numerical correlation between the n.m.r. data and the osmotic data has been successful in the sense that they reinforce and support the interpretations of each other.

It is noteworthy to point out that guanosine 5′-phosphate in water was shown to stack in a vertical, partially overlapping manner when forming a helix.[30] Desoxycholate in water has also been shown to form helical polymers by Blow and Rich.[31] It is tempting to suggest that such a situation may exist for the solutes mentioned here when their chain length attains a certain value.

(29) J. A. Pople, W. G. Schneider, and H. J. Berstein, "High-Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Company, Inc., New York, N. Y., 1959, p. 177–182.

(30) M. Gellert, M. N. Lipsett, and D. R. Davies, *Proc. Natl. Acad. Sci. U. S.*, **48**, 2013 (1962).

(31) D. B. Blow and A. Rich, *J. Am. Chem. Soc.*, **82**, 3572 (1959).

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH OF THE UNIVERSITY OF WISCONSIN, MADISON, WISCONSIN]

Studies on Polynucleotides. XXXIV.[1] The Specific Synthesis of $C^{3'}$–$C^{5'}$-Linked Ribooligonucleotides.[2] New Protected Derivatives of Ribonucleosides and Ribonucleoside 3′-Phosphates. Further Syntheses of Diribonucleoside Phosphates[3]

BY R. LOHRMANN AND H. G. KHORANA

RECEIVED MAY 25, 1964

Methods for the preparation of $O^{5'}$-monomethoxytrityladenosine, $O^{5'}$-monomethoxytritylcytidine, and $O^{5'}$-monomethoxytritylguanosine are described. Using these derivatives and the previously known $O^{5'}$-trityluridine, the following acylated derivatives of ribonucleosides bearing free 5′-hydroxyl groups were prepared: $N,N',O^{2'},O^{3'}$-tetrabenzoyladenosine, $N,O^{2'},O^{3'}$-tribenzoylcytidine, $O^{2'},O^{3'}$-diacetylguanosine, $N,O^{2'},O^{3'}$-triacetylguanosine, $O^{2'},O^{3'}$-dibenzoyluridine, and $N,O^{2'},O^{3'}$-tribenzoyluridine. Methods for the large-scale preparation of cytidine 3′-phosphate and guanosine 3′-phosphate are described. Condensations of the $N,O^{2'},O^{5'}$-triacetyl derivatives of these ribonucleotides with the protected ribonucleosides gave generally satisfactory yields of the following compounds: cytidylyl-(3′→5′)-adenosine, cytidylyl-(3′→5′)-cytidine, cytidylyl-(3′→5′)-guanosine, cytidylyl-(3′→5′)-uridine, guanylyl-(3′→5′)-adenosine, guanylyl-(3′→5′)-cytidine, guanylyl-(3′→5′)-guanosine, and guanylyl-(3′→5′)-uridine.

The most satisfactory method so far developed for the synthesis of the $C^{3'}$–$C^{5'}$ interribonucleotidic linkage is that which involves, as the key step, the protection of 2′-hydroxyl groups in ribonucleoside 3′-phosphates by an acyl (acetyl, benzoyl) group.[2c,e] The use of the protected ribonucleoside 3′-phosphates, in turn, requires suitably protected ribonucleosides bearing free 5′-hydroxyl groups. Our previous studies in the field of ribooligonucleotides using this approach have been limited to uridine 3′- and adenosine 3′-phosphates and to the use of a few derivatives of the ribonucleosides.[2] In initiating a comprehensive attack on the stepwise synthesis of ribopolynucleotides containing all of the four commonly occurring ribonucleoside units in predetermined sequences, we have devoted attention to (a) the preparation of useful amounts of the protected derivatives of ribonucleosides and nucleotides and (b) a generalized study of the synthesis of the interribonucleotidic linkage using these derivatives. This first phase of the study is reported in the present paper. The work parallels the systematic study recently reported of the corresponding problems in the deoxyribonucleotide series.[4] A brief report of this work has previously been made.[5] Recently, Chladek and Smrt[6] have also reported on the synthesis of several of the dinucleoside phosphates reported in this paper using the tetrahydropyranyl group[2a,b] to protect the 2′-hydroxyl group in ribonucleoside 3′-phosphates.

Protected Ribonucleosides.—The classical approach to the synthesis of protected ribonucleosides bearing free 5′-hydroxyl groups has involved the direct tritylation of the nucleosides, acetylation of the trityl derivatives, and subsequent detritylation under acidic conditions. In this way, several ribonucleosides bearing acetyl groups on the 2′- and 3′-hydroxyl groups have been available. With the realization of the need for the additional protection of the amino groups in the heterocyclic rings, methods for the preparation of $N,N',O^{2'},O^{3'}$-tetrabenzoyladenosine and $N,O^{2'},O^{3'}$-tribenzoylcytidine[2a,b] recently were described. In the present work, the selective formation of the $O^{5'}$-

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(2) Previous papers which deal directly with this topic: (a) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 430 (1962); (b) D. H. Rammler and H. G. Khorana, *ibid.*, **84**, 3112 (1962); (c) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963); (d) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3852 (1963); (e) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3857 (1963); (f) C. Coutsogeorgopoulos and H. G. Khorana, *ibid.*, **86**, 2926 (1964).

(3) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, the National Science Foundation, and the Life Insurance Medical Research Fund, Rosemont, Pa.

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