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Investigation of the Structure of Purines, Pyrimidines, Ribose Nucleosides and Nucleotides by Proton Magnetic Resonance. II[1]

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Several purine and pyrimidine proton resonances, as well as those of nucleosides and nucleotides, were observed in solutions of D_2O at different hydrogen ion concentrations. Tentative assignments for the heterocyclic ring proton peaks were made by comparing the shifts for these protons in the dissociated purines with those in the corresponding ribose and ribose-5'-mono- or tri-phosphate derivatives. The electron shielding effect of amino and oxy substituents of the purine ring, and a concentration dependence of the proton shifts which was found to be different for the various protons of a given purine were also observed. Titration of the dissociated oxy groups caused very little change in the chemical shifts of the heterocyclic ring protons, while titration of the amino groups had pronounced effects on the shifts of the base protons in both purine and pyrimidine nucleosides and nucleotides. Definite tautomeric structures for the cations of cytidine and adenosine and for the guanosine triphosphate ion were considered to be in agreement with the nuclear magnetic resonance results and with existing evidence from X-ray crystallography. On the basis of the coupling constant for the C_1' proton of ribose, it was suggested that the ribose ring configuration in the pyrimidine nucleosides may be different from that present in either the purine suggested or the purine nucleotides.

Introduction

Ultraviolet and infrared spectroscopy have greatly contributed to the understanding of the structure of the heterocyclic bases and, in particular, of the nucleic acid derivatives.[3-5] The dissociation constants for many compounds are known from titrimetric studies[4] and X-ray crystallography has established the fact that the ribose is in the N_9- or N_1-β-D-furanosyl form with the furanose ring almost perpendicular to that of the planar purine or pyrimidine base, respectively,[6,7] at least in the solid state. However, very little is known about the electron distribution in the ring structure of the bases and, thus, about the most important tautomeric and resonance forms in aqueous solution. On the other hand, the specificity with which these compounds are active in various enzymatic reactions and with which derivatives of similar structure may act, either as normal metabolites or as metabolic inhibitors, indicates that the reactivity or electron shielding at the various ring atoms may be directly linked with the role of the compound in a biological reaction. Correlations of calculated electron densities with the antitumor activity of purine and pyrimidine derivatives already have been made.[8,9]

Recently, important contributions to the understanding of structure and mechanism of interaction in solution have been made by the use of nuclear magnetic resonance spectroscopy.[10-12a] With the purpose of elucidating some of the problems mentioned, a study has been made of the proton resonance spectra of purines, pyrimidines and their ribose derivatives in solutions of D_2O at different hydrogen ion concentrations.[13]

Experimental

All spectra were obtained with the 40 megacycle high resolution n.m.r. spectrometer from Varian Associates in a field of about 9,400 gauss. The purines and pyrimidines were dissolved in sulfuric acid or sodium hydroxide of different normalities, and the final concentration was 30 g. in 100 ml. However, effects on the proton chemical shifts due to high concentration soon became apparent, and the solute concentration in all later studies was kept at 0.2 *M*. The nucleosides and nucleotides studied were dissolved in D_2O and the *p*H was adjusted to the desired value by addition of small amounts of anhydrous sodium peroxide crystals or concentrated hydrochloric acid. For exact *p*H measurements, 3 to 5 ml. samples in D_2O and 0.2 *M* in solute concentration were prepared and 0.2 to 0.3 ml. aliquots were removed after titrating and determining the *p*H with the Beckman Model G *p*H meter. The compounds used were obtained from the California Foundation for Biochemical Research, from the Sigma Chemical Company and from the Mann Research Laboratories.

The 0.2 to 0.5 ml. samples were pipetted into Pyrex glass tubes of 5 mm. outside diameter and were used for obtaining the spectra within a few hours after preparation or were stored at $-20°$ for a few days before the recording of the spectra. All spectra were obtained with the Sanborn Recorder in both the presence and absence of an external standard. (In many instances the reversibility of the spectrum of the substance in D_2O was assured by increasing or decreasing the *p*H with sodium peroxide or hydrochloric acid and then titrating back to the original *p*H.) The reference compound, toluene, was introduced in the sample by inserting a toluene containing capillary of about 1 mm. outside diameter. From the distance between the aromatic and the methyl peaks of toluene which was measured by the side band modulation method[14] and found to be equal to 196.6 ± 0.2 c.p.s. at a room temperature of 20°, the frequency shift for the other peaks was calculated. At least two spectra with the field increasing and two with the field decreasing were analyzed separately, and the average value varied within ±0.8 c.p.s. In some instances, additional checks on the frequency separation were obtained with the side band modulation method and the means from both de-

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(2) Biological Laboratories, Harvard University, Cambridge, Massachusetts.

(3) G. E. W. Wolstenholme and C. M. O'Connor, "Ciba Foundation Symposium on the Chemistry and Biology of Purines," Little, Brown & Company, Boston, Mass., 1957.

(4) E. Chargaff and J. N. Davidson, "Nucleic Acids," Vol. I, Academic Press, Inc., New York, N. Y., 1955.

(5) H. T. Miles, *Biochim. Biophys. Acta*, **27**, 46 (1958).

(6) S. Furberg, *Acta Chem. Scand.*, **4**, 751 (1950).

(7) L. Pauling and R. B. Corey, *Arch. Biochem. Biophys.*, **65**, 164 (1956).

(8) B. Pullman and A. Pullman, *Bull. soc. chim. France*, 973 (1958).

(9) A. Pullman and B. Pullman, *Compt. rend.*, **246**, 611 (1957).

(10) "Chemical Society Symposia," The Chemical Society, Burlington House, W 1, London, Special Publication No. 12, p. 211, 1958.

(11) O. V. St. Whitelock, *Ann. N. Y. Acad. Sci.*, **70**, 763 (1958).

(12) J. D. Roberts, "Nuclear Magnetic Resonance, Applications to Organic Chemistry," McGraw-Hill Book Co., New York, N. Y., 1959.

(12a) J. A. Pople, W. G. Schneider and H. J. Bernstein, "High-Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., New York, N. Y., 1959.

(13) C. D. Jardetzky and O. Jardetzky, *Federation Proc.*, **17**, 380 (1958).

(14) J. T. Arnold and M. G. Packard, *J. Chem. Phys.*, **19**, 1608 (1951).

terminations agreed within ±0.5 c.p.s. The chemical shifts reported refer to a zero chemical shift for the aromatic peak of toluene and are given in parts per million according to the formula

$$\frac{H_{\text{compound}} - H_{\text{reference}}}{H_{\text{reference}}} \times 10^6$$

where H refers to the magnetic field strength. Conversion to cycles per second is obtained by multiplying by forty.

In all instances the sweep rate was maintained at about 1.5 to 2.0 c.p.s. per mm. of paper. Since susceptibility measurements for these compounds are not available, the chemical shifts were not corrected for bulk diamagnetic susceptibility difference. However, these differences are believed to be very small, judging from the fact that the acetone methyl peak is observed at +4.24 ± 0.02 p.p.m., irrespective of the solute and the hydrogen ion concentration when acetone is used as an internal standard. Unfortunately, at *p*H about 14 acetone is not suitable as internal standard because of the splitting and broadening in the methyl peak due to enolization of the compound.

Results and Discussion

Purines and Pyrimidines.—The results for the chemical shifts of the non-exchanging protons of the purines and pyrimidines[15] studied in solutions of sulfuric acid and sodium hydroxide of varying normalities are seen in Tables I and II. It is noted from Table I that there are three groups of protons besides that of the solvent. The most shielded protons are those on the C_5 and C_6 atoms of the pyrimidines. Among these, the C_6 proton is the less shielded, judging from the spectrum of thymine. This is to be expected since C_6 is bonded to a carbon and a nitrogen atom, while C_5 is bonded to two carbon atoms. The peaks in uracil and cytosine are doublets due to the spin–spin splitting with a coupling constant of about 7 to 8 c.p.s. The purine stable protons, on the other hand, are seen at lower fields since both the C_2 and C_8 atoms are bonded to two nitrogen atoms. Within a given solvent the chemical shifts for the non-exchangeable protons of the solutes show little or no dependence on the hydrogen ion concentration.

TABLE I

PROTON CHEMICAL SHIFTS IN SULFURIC ACID[a]

H_2SO_4, *N*	33	27.5	22	11	5.5
Solvent	−5.50	−4.92	−3.80	−1.25	+0.62
Adenine-H_2, -H_8	−3.22	−3.32	−3.32	−3.12	−3.08
		−2.65	−2.60	−2.40	−2.30
Uracil-H_6	−1.92	−1.97	−1.75		
-H_5	−0.30	−0.40	−0.18		
Thymine-H_6	−1.80	−1.70	−1.47		
-methyl	+4.08	+4.11	+4.25		
Cytosine-H_6	−1.47				
-H_5	−0.30				

[a] Solute concentration is about 30 g. in 100 ml. Mean values vary within ±0.1 p.p.m. All chemical shifts in this and subsequent tables are given in parts per million from the aromatic peak of toluene which is used as external standard. Conversion to cycles per sec. is given by multiplying by 40.

TABLE II

PROTON CHEMICAL SHIFTS IN SODIUM HYDROXIDE[a]

NaOH, *N*	6	3	1.5
Solvent	+1.06	+1.36	+1.51
Adenine-H_2, -H_8	−1.31	−1.21	−1.31
Uracil-H_6	−1.21	−1.16	−1.00
-H_5	+0.71	+0.66	+0.61
Thymine-H_6	−1.09	−1.11	−0.91
-methyl	+4.49	+4.51	+4.63

[a] Solute concentration and variation of the means as in Table I.

The $-NH_2$ of adenine and cytosine as well as the NH protons have not been observed in either acidic or basic solution. These protons may be either exchanging very rapidly with the solvent, or their peaks may be covered up by the broad solvent resonance line. In the case of guanine in sulfuric acid, however, three broad lines appear which cannot, at the present time, be identified.

An attempt to identify the peaks due to the C_2 and C_8 protons of the purines was made by comparing the spectra of the free purines and their ribose derivatives in solutions of sodium peroxide in D_2O. As seen in Table III, the C_8 proton of either guanosine or xanthosine is shifted to lower field by 0.27 ± 0.01 p.p.m. as compared with guanine or xanthine. It is also noted that the C_2 and C_8 protons of inosine are shifted to lower field by 0.14 and 0.34 p.p.m. (respectively). Assuming that the change in charge distribution due to the imidazole dissociation in hypoxanthine is similar to that in guanine or xanthine, it is likely that the C_8 proton gives rise to the peak at −1.41 p.p.m. If the same argument is extended to the pair, adenine–adenosine, it may be concluded that the C_8 protons are responsible for the peaks at −1.48 and −1.78 p.p.m., respectively. This assignment of peaks makes the C_2 proton in adenosine more shielded than the corresponding one in inosine by 0.22 p.p.m.

Although it is not possible to assign with certainty the peaks due to the protons on the different purine carbon atoms, it is tempting to suggest that the most shielded proton belongs to the C_6 atom, assuming that the negative charge is equally distributed among the pyrimidine and imidazole nitrogens. Calculations of charge densities in the purine atoms indicate that the C_6 position is the most shielded one.[16] The assignment of peaks due to the various protons will be facilitated greatly by examining the spectra of the C^{13} derivatives of purines. In this case, the peak due to the proton attached on C^{13} will be a doublet due to the carbon spin of $^1/_2$.

In connection with the effect of substituents in mono- and disubstituted purines, interesting facts arise from an examination of the data in Table III.

a. The least shielded protons belong to the unsubstituted purine, while the most shielded ones are seen in the di-substituted purines.

b. The shielding is not proportional to the number of negative charges, due to the dissociated groups as seen by comparing the shifts in adenine and 2,6-diaminopurine, both of which have the imidazole proton dissociated, with the shifts in hypoxanthine which bears an additional negative charge due to the dissociation of the NHCO group.

c. There appear to be no differences in the shielding of the C_8 proton in the di-substituted compounds given an oxy or an amino group on the C_2 atom, as in xanthine and guanine or in 2,6-diaminopurine and isoguanine.

(15) The numbering system is that recommended in "Commission de Nomenclature de Chimie Organique," Reports of the 1955 IUPAC Meeting at Zurich.

(16) S. F. Mason, "Chemistry and Biology of Purines," G. E. W. Wolstenholme and C. M. O'Connor, 1957, p. 72.

TABLE III

CHEMICAL SHIFTS OF STABLE PROTONS IN PURINES, PYRIMIDINES AND THEIR DERIVATIVES[a]

Compound	Solvent	H_2	H_8	H_6	H_5	H_1'	H_2', H_3', H_4'	H_5'
Purine	+1.40	−2.31	−2.12	−1.83				
Adenine	+1.41	−1.62	−1.48					
2,6-Diaminopurine	+1.41		−1.30					
Hypoxanthine	+1.41	−1.61	−1.41					
Xanthine	+1.42		−1.17					
Guanine	+1.44		−1.14					
Isoguanine	+1.41		−1.27					
Cytosine	+1.37			−1.37	+0.54			
Uracil	+1.37			−1.23	+0.71			
Adenosine	+1.37	−1.53	−1.78			+0.62	+2.26	+2.57
Inosine	+1.38	−1.75	−1.75			+ .59	+2.26	+2.61
Xanthosine	+1.41		−1.43			+ .74	+2.29	+2.63
Guanosine	+1.37		−1.42			+ .71	+2.25	+2.60
Cytidine	+1.44			−1.37	+0.36	+ .54	+2.35	+2.56
Uridine	+1.37			−1.24	+0.56	+ .56	+2.34	+2.56

[a] The solvent is 0.75 M of Na_2O_2 in D_2O with pH about 14.0. Solute concentration in all cases in this and subsequent tables is 0.2 M. The variation of the means is within ±0.02 p.p.m. The primed hydrogen atoms refer to those of ribose.

d. Substitution of a dissociated oxy by an amino group in the sixth position results in unshielding the C_8 proton by 0.13 ± 0.03 p.p.m. as indicated from the shifts in guanine and 2,6-diaminopurine, as well as xanthine and isoguanine. The shielding power of the dissociated oxy as compared to the amino group is also evident by comparing cytosine and uracil where the difference in shielding in the C_5 and C_6 protons is 0.17 and 0.14 p.p.m., respectively.

Since both the amino and the dissociated oxy groups may participate in the resonance structures of the substituted purines and hence serve as a source of electrons it is not surprising that the C_2 and C_8 protons of the mono- and di-substituted purines are more shielded than the corresponding protons of unsubstituted purine, Tables III and IV.

TABLE IV

EFFECT OF CONCENTRATION ON THE SHIELDING OF SOME PURINE PROTONS[a]

Concn., M	0.2	2.0
Purine-H_2	−2.34	−1.82
-H_8	−2.14	−1.67
-H_6	−1.83	−1.55
Adenine-H_2	−1.70	−1.37
-H_8	−1.59	−1.37
Hypoxanthine-H_2	−1.69	−1.40
-H_8	−1.49	−1.30
2,6-Diaminopurine-H_8	−1.37	−1.23
Guanine-H_8	−1.26	−1.29[b]

[a] The solvent is 3.0 N NaOH. Variation ±0.02 p.p.m. [b] Concentration is about 1.0 M.

The large dependence of the chemical shifts on concentration is seen to decrease as the number of purine substituents is increased, as illustrated in Table IV. If similarly to hypoxanthine, the C_2 proton of adenine is considered to be the less-shielded one, it is interesting to note that the shift of the C_2 proton peak is more dependent on concentration than that of the C_8 proton peak. This is consistent also with the fact that the frequency of the C_8 proton resonance of the di-substituted purines is relatively little affected at high concentration.

In the case of purine, the frequency difference between the C_2 and C_8 proton peaks changes little with concentration as noted also for adenine and hypoxanthine (0.05 to 0.10 p.p.m.). However, the difference between the chemical shift of the C_6 and, either the C_2 or the C_8 proton peaks, is decreased by 0.24 and 0.19 p.p.m., respectively, as the concentration is increased ten times. A similar concentration dependence has been reported for the protons of pyridine.[17] In that case, however, the frequency difference between two proton peaks either remained unchanged or increased in going from a solution of pyridine at infinite dilution in CCl_4 to the pure liquid.

These results cannot be explained on the basis of hydrogen bonding at high concentration, since there are no available protons to bring about such an association, at least in the case of the purine and hypoxanthine ions. Also, the change in the chemical shift at 0.2 M as compared with 2.0 M solutions cannot be entirely accounted for by the difference in the concentration of sodium hydroxide, since adenine at a concentration of about 2.0 M is characterized by a single resonance peak which is fairly independent of the normality of the base (Table II). That the sodium ion effect is very small is evident also from the data of Tables III and IV. The proton peaks of the substituted purines in dilute solution are shifted to lower field by about 0.10 p.p.m., while those of purine are not affected as the sodium hydroxide concentration is changed from 1.5 to 3.0 M.

A possible explanation for the concentration dependence of the shifts would be that, for a preferred arrangement of molecules in solution the protons of a given molecule may experience, to a different extent, the diamagnetic shielding effect of the secondary magnetic field arising from neighboring anisotropic molecules.

Nucleosides.—The electron withdrawing effect of the ribose ring in the purine nucleosides has already been mentioned (Table IV).

At this point attention may be drawn to the ribose derivatives of the pyrimidines. It is noted

(17) W. G. Schneider, H. J. Bernstein and J. A. Pople, *Can. J. Chem.*, **35**, 1487 (1957).

TABLE V

PROTON CHEMICAL SHIFTS IN CYTIDINE (HEMISULFATE)[a]

pH	1.50	2.70	3.92	4.80	6.20	8.00	11.0	14.0
H_6	−1.71	−1.71	−1.62	−1.49	−1.40	−1.38	−1.38	−1.37
H_5	+0.17	+0.18	+0.24	+0.32	+0.41	+0.41	+0.41	+0.36
H_1'	+0.54	+0.54	+0.54	+0.57	+0.55	+0.56	+0.54	+0.54
Solvent	+1.59	+1.57	+1.58	+1.59	+1.59	+1.60	+1.60	+1.44
H_2', H_3', H_4'	+2.20	+2.20	+2.21	+2.20	+2.22	+2.25	+2.24	+2.35
$2H_5'$	+2.55	+2.55	+2.56	+2.54	+2.55	+2.58	+2.57	+2.50
Acetone[b]	+4.22			+4.23		+4.24		

[a] pK_{A_1} = 4.22 due to ionization of a cytosine hydrogen, pK_{A_2} = 12.3 due to ionization of a ribose hydrogen. [b] Used as internal standard.

that while the differences in shielding of the C_5 and C_6 protons in cytidine and uridine are 0.20 and 0.13 p.p.m., respectively, in agreement with those in the free pyrimidines, substitution of the $(CON)^-$ group by the (CONR) group, where R stands for ribose, does not cause a change in the shielding of the C_6 proton but brings about the unshielding of the C_5 proton by 0.18 and 0.15 p.p.m., respectively, in the cytosine and uracil derivatives. It is also interesting that the C_5 and C_6 protons of N_1-methylcytosine are more shielded by about 0.10 and 0.30 p.p.m., respectively, as compared with cytidine. However, titration of the amino group in both compounds causes these proton peaks to shift to lower field by about 0.20 and 0.33 p.p.m., respectively, as seen in Tables V and VI, indicating that both compounds must be characterized by the same tautomeric structure in acid solution.

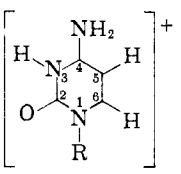
TABLE VI

PROTON CHEMICAL SHIFTS IN N_1-METHYLCYTOSINE[a]

pH	1.2	3.2	4.1	5.1	5.6	7.5
H_6	−1.37	−1.39	−1.33	−1.21	−1.09	−1.08
H_5	+0.31	+0.31	+0.33	+0.49	+0.49	+0.51
Solvent	+1.55	+1.57	+1.57	+1.57	+1.57	+1.54
Methyl	+3.01	+3.01	+3.03	+3.06	+3.11	+3.11

[a] Very generously supplied by E. B. Keller; pK_A is 4.55.

The fact that the C_6 proton is unshielded more than the C_5 proton upon titration of cytidine or N_1-methylcytosine suggests the possibility that the positive charge may be partially located on the N_1 atom. In this case the tautomeric structure should be such as to allow the distribution of the positive charge in the ring through the various resonance forms. The following structure is consistent with the above idea



Thus, the protonation of the amino group in these nucleosides brings about proton shifts to lower fields as in the amino acids[18] with the significant difference that, in the latter case, the protons nearest to the amino group are those which are shifted the most.

In contrast to the narrow lines for the protons on the purine or pyrimidine bases, one obtains a number of broad and not-well-resolved peaks due to the ribose protons with the exception of the C_1' proton peak. The C_1' proton is unique in that it is attached to a carbon atom which is bonded to an oxygen and a nitrogen atom and, consequently, its resonance peak is observed at a lower field than any other ribose proton. This peak is a doublet due to spin–spin coupling with the C_2' proton, and the magnitude of the coupling constant may reflect the orientation of these protons with respect to each other and to the plane of the ring.

As seen from Table XIV, the coupling constant for cytidine is 3.0 ± 0.2 c.p.s. Although it was not possible to measure accurately the coupling constant of the H_1' doublet in uridine, because of superposition of the doublet due to the C_5 proton of the base, the rest of the ribose spectrum is identical with that of cytidine as seen from Fig. 1a and 1e. The observed coupling constant for cytidine is in agreement with that described by Lemieux, *et al.*, for the *cis* protons of six-membered ring compounds[19] and with the value of 2.7 ± 0.2 c.p.s. for the coupling between axial–equatorial or equatorial–equatorial protons in dioxane.[20] On the other hand, the coupling constant between two axially oriented protons of neighboring carbon atoms was shown to vary between 5 and 8 c.p.s. in various acetylated aldopyranoses.[19] In the purine as opposed to the pyrimidine nucleosides the ribose ring may assume a conformation in which the C_1' and C_2' protons become axial as indicated from the magnitude of the coupling constant which is within the range of 5.8 to 6.6 c.p.s at *p*H 14. As the *p*H is lowered, however, the coupling constant also decreases to about 5 c.p.s. and a shift to lower field of the C_1' proton is observed for adenosine and inosine.

For the C_2', C_3' and C_4' protons of ribose, an average peak position is reported in some instances. Definite differences between the ribose spectra of pyrimidine *versus* purine nucleosides become apparent from an inspection of the figures. In the pyrimidine nucleosides the C_2', C_3' and C_4' proton peaks appear less well resolved and at higher field than those of the purine nucleosides.

In the case of the methylene protons on C_5', a doublet with a constant of about 2–4 c.p.s. may be seen in Figs. 1a, 2a and 2d or a rather broad line as in Figs. 1b, 2b, 2e and 2g. It is suggested that the broad line may result, in this case, from a slight nonequivalence between the C_5' protons with the two doublets (because of spin coupling with

(18) O. Jardetzky and C. D. Jardetzky, *J. Biol. Chem.*, **233**, 383 (1958).

(19) R. V. Lemieux, R. K. Kullnig, H. J. Bernstein and W. G. Schneider, THIS JOURNAL, **80**, 6098 (1958).

(20) Cohen, Sheppard and Turner, *Proc. Chem. Soc.*, 118 (1958).

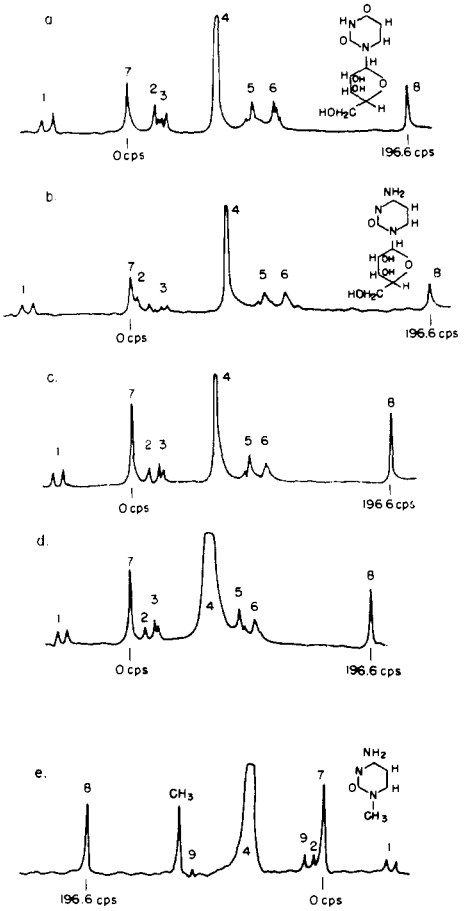


Fig. 1.—Pyrimidine nucleosides, a, uridine, *p*H 7.5. b, c and d, cytidine hemisulfate, *p*H 2.95, 6.0 and 10.1, respectively. e, N_1-methylcytosine, *p*H 3.2. Peak (1) H_6 doublet, (2) H_5 doublet, (3) H_1' doublet, (4) H_2O, (5) H_2', H_3', H_4', (6) $2H_5'$, (7), (8) aromatic and methyl protons of toluene, the external standard and (9) side band (due to the spinning of the sample). In this and subsequent figures the concencentration is 0.2 *M* in D_2O and the magnetic field is increasing from left to right except in case 1e.

H_4') due to the methylenic protons being very close to one another, but not exactly superimposed. This situation has been shown to exist in the 21-acetoxy-20-one steroids for the C_{21}-methylene resonance.[21] Further work is planned to resolve this point in the nucleosides and nucleotides.

The effect of protonation of the purine ribosides may be seen in Tables VII and VIII for adenosine and inosine. Only a limited *p*H range was studied because of the low solubility of the compounds. In the case of adenosine at high *p*H, the peaks due to the C_2 and C_8 protons were assigned on the basis of arguments discussed in the previous section. However, at low *p*H, where the difference in shift between these protons becomes much smaller, the assignment is purely intuitive. At any rate, there is a greater shift to lower field of the C_2 than of the C_8 proton. Furthermore, a shift to lower field is noted for the C_1' proton of ribose by about 0.30 p.p.m. between the extremes of the *p*H range

(21) J. N. Shoolery and M. T. Rogers, THIS JOURNAL, **80**, 5121 (1958).

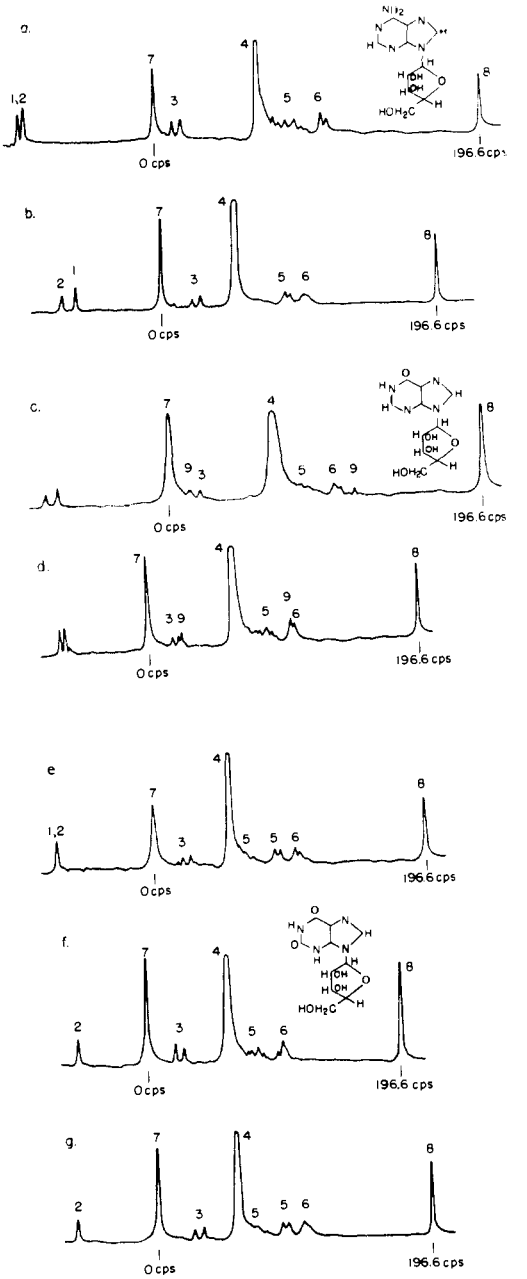


Fig. 2.—Purine nucleosides, a, b, adenosine, *p*H 1.82 and 14, respectively. c, d and e, inosine *p*H 2.2, 9.4 and 14, respectively. f, g, Xanthosine *p*H 10.7 and 14, respectively. Peak (1) H_2, (2) H_8. The rest of the peak identification and remarks as in Fig. 1.

studied. These results can be understood in terms of a definite tautomeric structure of adenosine in which the hydrogen is attached to N_1 and the positive charge is distributed among the nitrogen atoms of the adenine ring. Further evidence for such a structure is provided by the X-ray crystallographic data for adenine hydrochloride according to which the proton is located on N_1 rather than on N_{10} of the amino group.[22,23]

For inosine, on the other hand, one observes that protonation of the $(NCO)^-$ group with a

(22) W. Cochran, *Acta Cryst.*, **4**, 81 (1951).
(23) J. M. Broomhead, *ibid.*, **4**, 92 (1951).

TABLE VII

PROTON CHEMICAL SHIFTS IN ADENOSINE[a]

pH	1.82	3.42	12.5	14.0
H_8	−2.09	−1.96	−1.75	−1.78
H_2	−2.01	−1.83	−1.57	−1.53
H_1'	+0.32	+0.38	+0.60	+0.62
Solvent	+1.57		+1.54	+1.37
H_2', H_3', H_4'	+1.84	+1.85		
	+2.00	+1.99		
	+2.12	+2.22		
	+2.22	+2.21	+2.22	+2.26
$2H_5'$	+2.57	+2.56	+2.60	+2.57

[a] pK_{A_1} = 3.45 due to ionization of a hydrogen from the adenine ring. pK_{A_2} = 12.5 due to ionization of a ribose hydrogen.

TABLE VIII

PROTON CHEMICAL SHIFTS IN INOSINE

pH	2.2	7.4	8.3	9.4	14.0
H_2, H_8	−1.87	−1.85	−1.79	−1.59	−1.75
	−1.69	−1.72	−1.66	−1.48	−1.75
H_1'	+0.40	+0.40	+0.45	+0.50	+0.59
Solvent	+1.59	+1.59	+1.59	+1.55	+1.38
$2H_5'$	+2.56	+2.58	+2.61	+2.60	+2.61

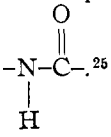
pK_A of 8.8[24] results in little change in the shielding of the purine protons as seen by comparing the data at pH between 2.2 to 8.3 and 14. Existing evidence from the infrared spectra of hypoxanthine points to the fact that the protonated group is probably in the form of  .[25]

TABLE IX

CHEMICAL SHIFTS OF SOME NON-EXCHANGEABLE PROTONS IN NUCLEOSIDES AND NUCLEOTIDES

Compound	H_2	H_8	H_6	H_1'	$2H_5'$
Adenosine	−1.55	−1.79		+0.61	+2.58
pH 12.5–14.0					
2′3′-AMP	−1.49	−1.79		+ .45	+2.53
pH 5.2–9.8					
5′-AMP	−1.51	−1.98		+ .43	+2.42
pH 6.0–11.0					
ATP	−1.56	−1.99		+ .38	+2.20
pH 6.5–10.0					
Inosine	−1.75	−1.75		+ .59	+2.61
pH 14.0					
5′-IMP	−1.63	−1.95		+ .39	+2.48
pH 11.6					
Guanosine		−1.42		+ .71	+2.60
pH 14.0					
2′3′-GMP		−1.50		+ .51	+2.57
pH 10.5					
GTP		−1.69		+ .52	+2.20
pH about 12					
Cytidine			−1.38	+ .55	+2.55
pH 6.2–14.0					
2′3′-CMP			−1.42		+2.56
pH 6.0–8.0					
5′-CMP			−1.63		+2.37
pH 6.5					

(24) The pK_A values were obtained from "Properties of Nucleic Acid Derivatives," California Corporation for Biochemical Research and those for guanosine triphosphate from the Pabst Circular ORIO.

(25) S. F. Mason, "Chemistry and Biology of Purines," G. E. W. Wolstenholme and C. M. O'Connor, 1957, p. 60.

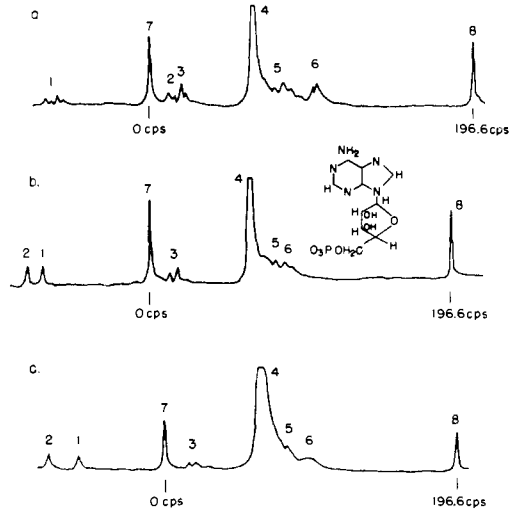


Fig. 3.—Mononucleotides, a, 2′, 3′-cytidilic acid, pH 5.35. b, and c, 5′-adenylic acid, pH 3.7 and 9.5, respectively. Peaks (1) and (2) in a are due to H_6 and H_5 respectively of the cytidine 2′- and 3′-phosphates. The rest of the peaks and remarks as in Figs. 1 and 2.

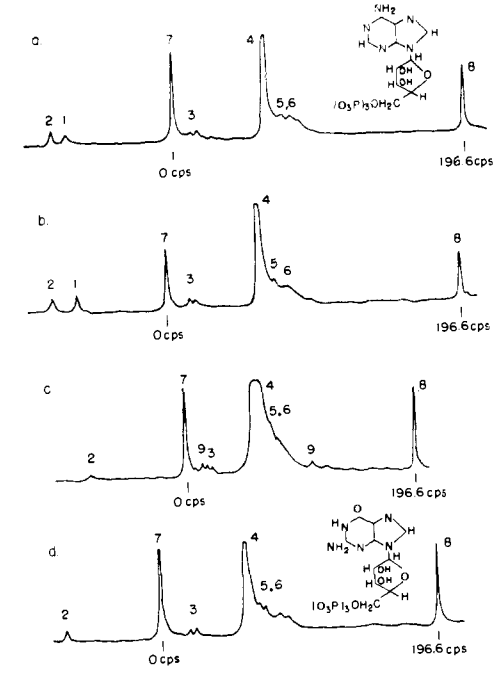


Fig. 4.—Nucleoside triphosphates, a, b, adenosine triphosphate pH 4.0 and 6.5, respectively. c, d, guanosine triphosphate pH 2.9 and 6.5, respectively. Peaks and remarks as in Fig. 2.

It is noted that at pH of about 2, where both adenosine and inosine exist in the protonated forms, the base protons of inosine are more shielded than those of adenosine. This is interesting in view of the fact that inosine has an additional ionization with a pK_A of 1.2 which refers to the ionization of one of the ring nitrogens. In the completely protonated form, therefore, the C_2 and C_8 protons of inosine must be seen at a lower field.

Nucleotides.—The presence of the phosphate group in the nucleotides may cause a further

TABLE X

PROTON CHEMICAL SHIFTS IN 5′-ADENYLIC ACID

pH	3.70	4.50	5.95	9.50	11.00
H_8	−2.01	−1.90	−1.94	−2.00	−2.01
H_2	−1.75	−1.56	−1.51	−1.49	−1.52
H_1'	+0.37	+0.44	+0.44	+0.42	+0.42
Solvent	+1.61	+1.56	+1.55	+1.56	+1.55
H_2', H_3', H_4'	+2.04	+2.06	+2.06	+1.92	+1.91
	+2.20	+2.21	+2.25	+2.05	+2.04
$2H_5'$	+2.33	+2.33	+2.38	+2.45	+2.42
Acetone			+4.25		+4.24

From the data on the titration of the adenine nucleotides, it is seen that the C_8 proton undergoes very little change in shielding, while the C_2 proton moves to lower field by about 0.40 p.p.m. In the case of 5′-adenylic acid (AMP), it was not possible to obtain a 0.2 M solution at pH less than 3.7 so that the titration data, although consistent with those of adenosine triphosphate (ATP), are not complete. The proton shifts due to titration of these nucleotides point to the fact that the proton is added to the pyrimidine portion of the ring since both the C_8

TABLE XI

PROTON CHEMICAL SHIFTS IN ADENOSINE TRIPHOSPHATE[a]

pH	2.00	3.00	4.00	4.75	5.90	6.50	7.35	9.95
H_8	−2.10	−2.09	−2.07	−2.00	−1.94	−2.00	−1.99	−1.98
H_2	−1.95	−1.93	−1.83	−1.70	−1.56	−1.58	−1.57	−1.54
H_1'	+0.33	+0.34	+0.35	+0.36	+0.36	+0.38	+0.37	+0.38
Solvent	+1.54	+1.55	+1.55	+1.57	+1.54	+1.56	+1.55	+1.56
H_2', H_3', H_4'	+1.87	+1.89	+1.85	+1.82	+1.78	+1.78	+1.76	+1.75
	+2.02	+2.00	+2.00	+2.02	+2.00	+2.04	+2.04	+2.06
$2H_5'$	+2.14	+2.12	+2.13	+2.17	+2.16	+2.19	+2.20	+2.21
Acetone							+4.24	

[a] $pK_{A_1} = 4.1$ refers to ionization of a hydrogen from the adenine ring. $pK_{A_2} = 6.5$ due to ionization of the phosphate groups.

TABLE XII

PROTON CHEMICAL SHIFTS IN GUANOSINE TRIPHOSPHATE[a]

pH	1.2	1.9	2.9	5.3	6.5	8.5	12[b]
H_8	−2.59	−2.58	−2.03	−1.64	−1.67	−1.65	−1.69
H_1'	+0.36	+0.41	+0.45	+0.54	+0.57	+0.55	+0.52
Solvent	+1.52	+1.54	+1.54	+1.55	+1.54	+1.50	
H_2', H_3', H_4'					+1.82	+1.83	
$2H_5'$					+2.15	+2.14	+2.20

[a] Disodium GTP·$3H_2O$. [b] pH 12 ± 1.0. $pK_{A_1} = 3.3$ due to ionization of a guanine hydrogen; $pK_{A_2} = 6.5$ due to ionization of the phosphate groups; $pK_{A_3} = 9.3$ due to —NHCO ionization.

unshielding of the C_8 proton in the purine, of the C_6 proton in the pyrimidine derivatives, and of various ribose protons as seen from Table IX. From the data on the various guanine derivatives, an estimate of 0.27 and 0.19 p.p.m. is obtained by which the C_8 proton is shifted to lower field in the triphosphate as compared with the nucleoside and the 2′3′-mononucleotides, respectively. In the adenine derivatives, an unshielding by 0.20 p.p.m. is observed for one proton which, from the data already considered, was tentatively identified as the C_8 proton. In this case it is noted that the C_2 proton is little, if at all, affected by the presence of the phosphate group. Similarly, the difference in shifts between the base protons of inosine and inosinic acid (IMP) at high pH may be used to distinguish between the C_2 and C_8 protons of inosinic acid. It is most interesting that the phosphate effect on the shift of the base protons is also dependent on its position on the ribose ring as seen from the quartet of lines obtained for the C_6 proton of 2′3′-cytidylic acid (CMP) in Fig. 3a.

In comparison with the nucleosides, the C_1' proton is shifted to lower field by about 0.20 p.p.m. in either the 2′,3′- or the 5′-phosphate and triphosphate derivatives, while the C_5' protons are not affected in the 2′,3′-compound and are shifted to lower field by about 0.15 and 0.40 p.p.m. in the 5′-mono- and tri-phosphate derivatives, respectively.

TABLE XIII

PROTON CHEMICAL SHIFTS IN 5′-INOSINIC ACID

pH	5.9	8.9	9.9	11.6
H_8	−1.92	−2.02	−1.96	−1.95
H_2	−1.66	−1.60	−1.62	−1.63
H_1'	+0.38	+0.39	+0.38	+0.39
Solvent	+1.56	+1.55	+1.57	+1.56
$2H_5'$		+2.46	+2.50	+2.48

TABLE XIV

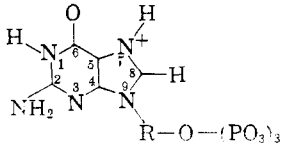
COUPLING CONSTANTS OF THE C_1' PROTON IN NUCLEOSIDES AND NUCLEOTIDES[a]

pH Range	1–2	3–4	5–6	7–8	9–10	11–12	14
Adenosine	5.2	5.0				5.0	5.8
Guanosine						6.4	6.5
Xanthosine						6.0	6.6
Inosine	5.0		5.0	5.3	5.4		6.2
Cytidine (hemisulfate)	3.0	2.8	3.1	3.0		2.9	3.0
Adenosine 5′-phosphate		4.6	4.6	4.5	5.0	4.5	
Adenosine triphosphate	4.2	4.0	4.6	3.9	4.5		
Guanosine triphosphate			4.1	4.3	4.5		

[a] The variation for each coupling constant is ±0.5 c.p.s.

and the C_1' protons are little affected by the protonation as seen in Tables X and XI. On the other hand, this does not appear to be the case with

guanosine triphosphate (GTP) (Table XII) where the titration of the group with pK_A equal to 3.30 causes a shift to lower field by about 0.90 and 0.20 p.p.m. of the C_8 and the C_1' protons, respectively. In this instance, the conclusion that the proton is probably attached to N_7 of the imidazole portion of the ring as shown below rather than to the $-NH_2$ group seems warranted. This conclusion



is further supported by the X-ray crystallographic data for the guanine cation which has four hydrogens on N_1, N_9, N_{10} and C_8 and two hydrogen bonds between N_3 and N_{10} and between O_6 and N_7.[23,24]

A comparison of the spectra in Figs. 4a and 4c, as compared with those in the Figs. 4b and 4d, respectively, reveals that the peaks due to the C_2 protons of ATP and to the C_8 proton of GTP are not only shifted to lower fields but are also broadened at *p*H's near the pK_A's of the titratable groups. This broadening may be accounted for by an exchange reaction of the type as depicted for ATP.

$$ATP^*—NH_2 + ATP—NH_3^+ \rightleftharpoons ATP—NH_2 + ATP^*—NH_3^+$$

Contrary to the marked effects due to protonation of the amino groups, essentially no effects in the chemical shifts were observed for the protonation of the $(CON)^-$ group as seen in the case of inosine (Table VIII), inosinic acid (Table XIII) and guanosine triphosphate (Table XII).

As may be seen from Table XIV the coupling constant for the C_1' proton in the purine nucleotides ranges between 4 and 5 c.p.s. Inferences concerning the conformation of the ribose ring from the magnitude of this coupling constant are discussed in a subsequent paper.[26]

Acknowledgment.—The authors are very grateful to Dr. John T. Edsall for a critical perusal of the manuscript and helpful suggestions.

(26) C. D. Jardetzky, THIS JOURNAL, **82**, 229 (1960).

CAMBRIDGE, MASS.

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Proton Magnetic Resonance Studies on Purines, Pyrimidines, Ribose Nucleosides and Nucleotides. III. Ribose Conformation[1]

BY CHRISTINE D. JARDETZKY[1a]

RECEIVED JULY 13, 1959

Specific conformations for D-ribose in nucleosides and nucleotides have been proposed on the basis of their proton magnetic resonance spectra. In the purine nucleosides C_2' is considered to be out of the plane defined either by C_1', O and C_4' or by C_1', O, C_3' and C_4' and is pointing on the same side as the C_4'-C_5' bond, while in the pyrimidine nucleosides, C_3' is out of the plane defined either by C_1', O and C_4' or by C_1', C_2', O and C_4'. Available information from X-ray diffraction and specific rotation studies further supports the existence of these structures.

Introduction

On the basis of proton magnetic resonance studies (p.m.r.), it was previously concluded that the five-membered ring of D-ribose in nucleosides assumes different puckered conformations depending on whether the base is a purine or a pyrimidine.[2]

Although the stereoconfiguration of five-membered rings has not been established, it has long been suspected that they are not planar structures. In the case of cyclopentane, Kilpatrick, Pitzer and Spitzer[3] have calculated a potential energy for the planar configuration which is higher by about four kcal./mole as compared with that corresponding to the two types of puckered structures which were considered possible. In the puckered conformations, either one atom is out of the plane of the other four, or two atoms are twisted with respect to one another and are out of the plane of the other three ring atoms. Thermodynamic and other measurements on cyclopentane disclose that the puckering of the ring is not of a definite type, but that the angle of maximum puckering rotates around the ring.[3] More recently information on the conformation of five-membered rings from X-ray diffraction studies has been summarized by Spencer.[4]

The purpose of this communication is to present specific three-dimensional structures for the furanose ring of D-ribose in nucleosides and nucleotides inferred from the p.m.r. measurements, and to point out that these structures are also in agreement with the available information from X-ray diffraction studies and with other physico-chemical properties.

Experimental

The spectra were taken with the Varian 40 mc. high resolution n.m.r. spectrometer at a field of about 9,400 gauss. The distance between the aromatic and methyl peaks of toluene, 196.6 c.p.s. was used to calculate the frequency difference between any two peaks in the spectrum. The results obtained in this manner agreed within 0.5 c.p.s. with those obtained by the side band modulation technique.[5]

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(2) (a) C. D. Jardetzky and O. Jardetzky, THIS JOURNAL, **81**, 222 (1959). (b) C. D. Jardetzky, presented at the Symposium on Physical Methods in Biochemistry, Federated Society Meetings, Atlantic City, April, 1959.

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(5) J. T. Arnold and M. G. Packard, *J. Chem. Phys.*, **19**, 1608 (1951).