The Effect of pH upon the Nuclear Magnetic Resonance Spectra of Nucleosides and Nucleotides*

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ABSTRACT: The pD dependence of the proton chemical shifts of a number of purine and pyrimidine bases, their nucleosides, and nucleotides has been studied in D_2O at 32°. It is concluded from the nuclear magnetic resonance results that a weak ring–chain interaction of an electrostatic type is operative in both the 5′-purine and pyrimidine nucleotides over the pD range of 1–8.2. No interaction of this type is indicated for the isomeric 2′- and 3′-nucleotides. The present results are consistent with a preferred *anti* conformation for 5′-nucleotides in solution and also indicate that the presence of a possible hydrogen bond between the 2′-OH group and the N-3 position in purine nucleotides has little effect upon the ring–chain interaction.

The conformations of nucleotides in aqueous solutions are of considerable interest because of the possibility of pH-dependent base ring–phosphate interactions and the resultant effect of such interaction upon the metal ion complexing ability of the nucleotide (Cohn and Hughes, 1962; Hammes and Miller, 1967). Simple stereochemical considerations indicate that a ring–chain interaction would be particularly favored in purine 5′-nucleotides in the *anti* conformation at low pH values. Under these conditions a strong electrostatic interaction can occur between the negatively charged phosphate group and the positively charged purine ring. Similar interaction might also be expected for 5′-pyrimidine nucleotides although in this case the over-all effects would not be as pronounced because the pyrimidine ring is uncharged at low pH.

A number of recent optical studies (Cushley *et al.*, 1967; Emerson *et al.*, 1967; Miles *et al.*, 1967; Ulbricht *et al.*, 1964, 1965) have provided strong evidence that naturally occurring β-nucleosides and nucleotides exist predominantly in the *anti* conformation in solution at neutral pH. It has also been suggested (Philips *et al.*, 1965) from ionization studies that a ring–chain interaction occurs for 5′-nucleotides in the pH range 2–5 but is absent at pH >5.5. In view of the importance of the conformation in determining the metal ion binding properties of the nucleotides additional information on ring–chain interactions would be desirable. In this communication we report a study of the pH dependence of the proton chemical shifts of ring protons for a variety of purine and pyrimidine nucleosides and nucleotides. Jardetzky (Bullock and Jardetzky, 1964; Jardetzky and Jardetzky, 1960) has previously reported the spectra for a number of nucleotides at various pH values but the measurements did not cover a sufficiently wide range to yield information about ring–chain interactions. The present results indicate that secondary phosphate ionization gives rise to a deshielding of the H-8 protons in 5′-purine nucleotides. The deshielding suggests an electric field interaction between the phosphate group and the H-8 protons in the pH range 5.5–8.2.

Experimental Section

Materials. Adenine, adenosine 5′-monophosphate, and guanosine 5′-monophosphate were obtained from Calbiochem; adenosine 2′-monophosphate and adenosine 3′-monophosphate were obtained as an approximately equimolar mixture from C. F. Boehringer & Sons Ltd., Germany. The remaining nucleosides and nucleotides were purchased from Sigma Chemical Co. All chemicals were of the highest purity commercially available and were used without any further purification.

Buffer solutions were made up in D_2O (99.8% isotopic purity; obtained from the U. S. Atomic Energy Commission) following the usual procedures and the pD's were checked with a Beckman pH meter. The ionic strength of the solutions was adjusted to 0.10 M by adding NaCl. The nucleoside and nucleotide concentrations were kept at approximately 0.01 M in order to minimize any stacking effects upon the shifts (Broom *et al.*, 1967; Jardetzky, 1964; Schweizer *et al.*, 1965).

Nuclear Magnetic Resonance Measurements. The proton spectra were measured with a Varian DA-60 spectrometer locked on an external tetramethylsilane reference. The signal to noise ratio of the spectra was increased with the aid of a Varian C-1024 time-averaging computer. All spectra were recorded at ambient temperature, ~32 ± 1°. Line positions were measured by interpolation relative to external TMS[1] and are accurate to 0.4 cycle/sec.

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[1] Abbreviation used: TMS, tetramethylsilane.

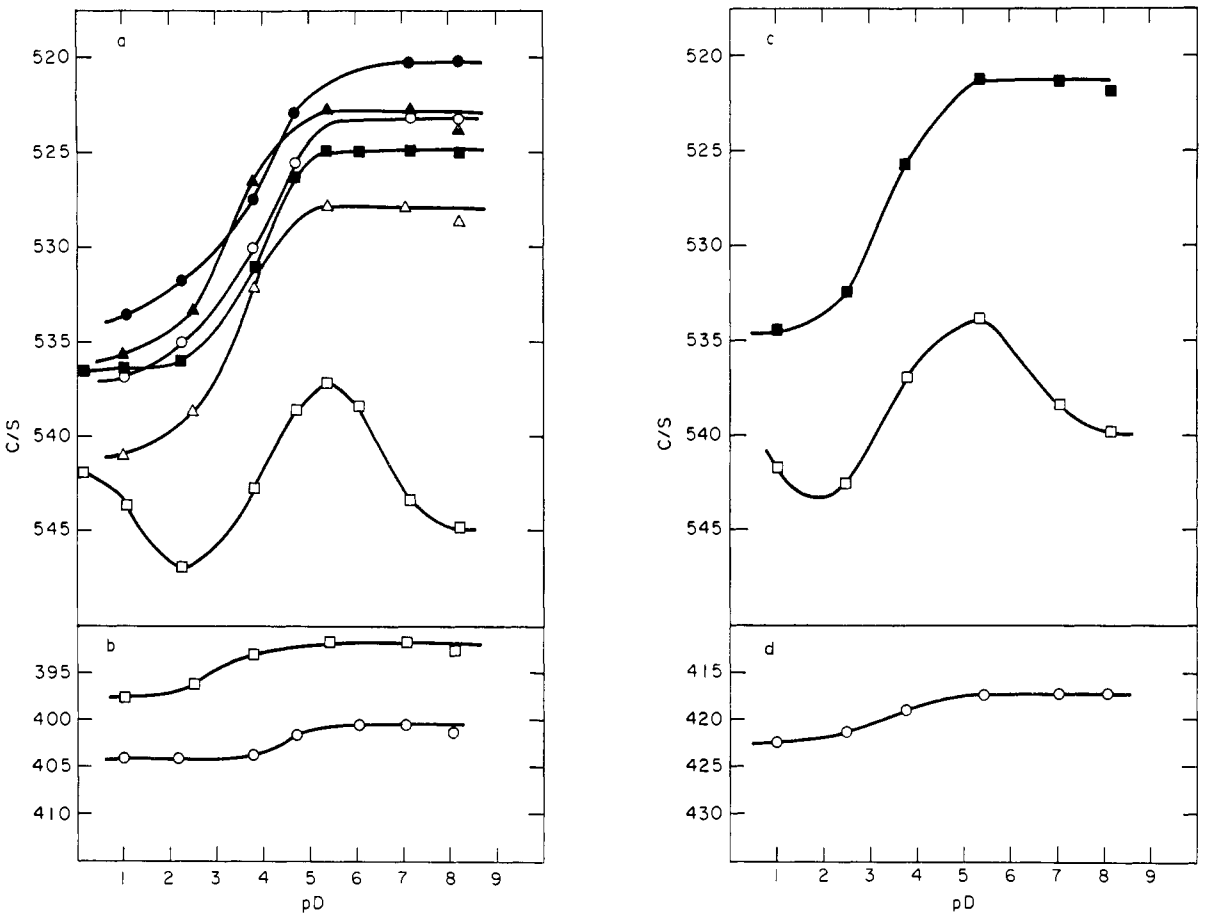

FIGURE 1: Proton chemical shifts (cycles per second relative to external TMS) *vs.* pD of the solution. (a) Adenine H-2 (●) and H-8 (○), adenosine H-2 (▲) and H-8 (△), and adenosine 5′-monophosphate H-2 (■) and H-8 (□). (b) H-1′ of adenosine (□) and of adenosine 5′-monophosphate (○). (c) 2′-Deoxyadenosine 5′-monophosphate H-2 (■), H-8 (□), and (d) H-1′ (○).

TABLE I: p*K* Values from Nuclear Magnetic Resonance Shifts.

Compound	Base pK^a	Secondary Phosphate pK^a	Primary Phosphate pK^a
Adenine	3.8 (4.2)		
Adenosine	3.2 (3.5)		
Adenosine 5′-monophosphate	3.9 (3.8)	6.7 (6.0–6.7)[b]	<1.2 (0.9)
Adenosine 2′- and 3′-monophosphate (mixture)	3.6 (3.7)	(6.0)	(0.9)
Adenosine 2′,3′-cyclic monophosphate	3.3		
Adenosine 3′,5′-cyclic monophosphate	3.6		
2′-Deoxyadenosine 5′-monophosphate	3.3 (4.4)[c]	6.7 (6.4)[c]	<1.3 (1.0)[c]
Guanosine	<2.2 (1.6)		
Guanosine 5′-monophosphate	<2.1 (2.4)	6.2 (6.1)	(0.7)[c]
2′-Deoxyguanosine 5′-monophosphate	<2.5 (2.9)[c]	6.3 (6.4)[c]	(0.8)[c]
Xanthosine	<1.7		
Xanthosine 5′-monophosphate	<1.6 (0.8)[d]	>6.7	
Thymidine	(9.8)[c]		
Thymidine 5′-monophosphate		>6.8 (6.5)[c]	<1.6 (1.6)[c]

[a] Previously reported titration values in parentheses were obtained from Steiner and Beers (1961), except *b* from Philips (1966) and *c* from Schwarz BioResearch Chart (1967). [d] Value for xanthine (Broom *et al.*, 1967).

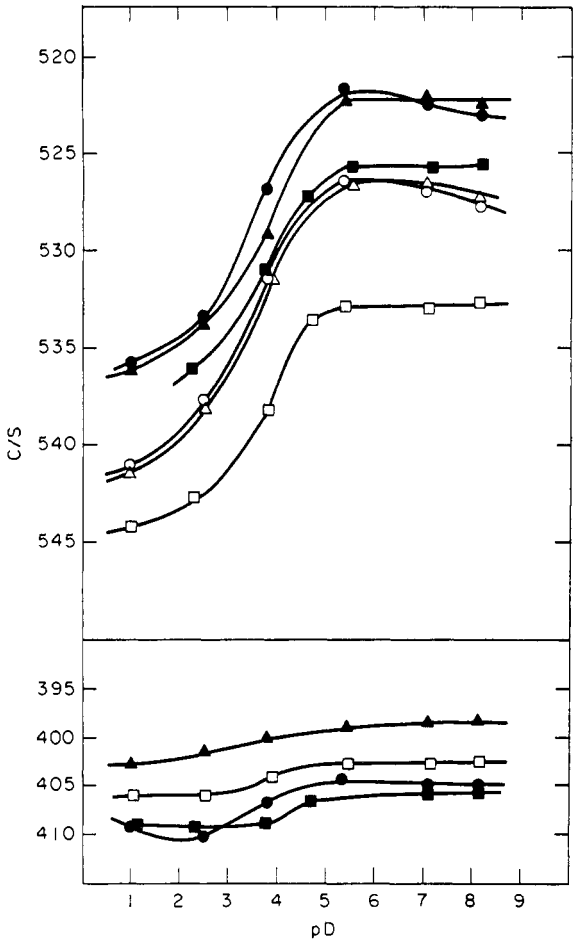

FIGURE 2: Proton chemical shifts (cycles per second relative to external TMS) *vs.* pD of the solution. (a) Adenosine 2′,3′-cyclic monophosphate H-2 (●) and H-8 (○), adenosine 3′,5′-cyclic monophosphate H-2 (▲) and H-8 (△), and adenosine 2′- and 3′-monophosphate (mixture) H-2 (■) and H-8 (□). (b) H-1′ (●), (▲), (■), and (□) of compounds in a, respectively.

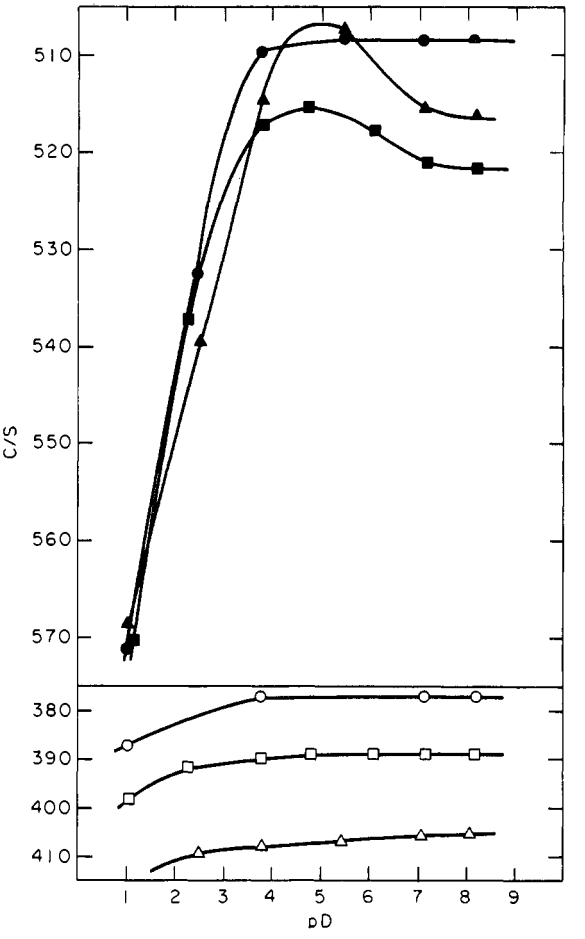

FIGURE 3: Proton chemical shifts (cycles per second relative to external TMS) *vs.* pD of the solution. Guanosine H-8 (●) and H-1′ (○), guanosine 5′-monophosphate H-8 (■) and H-1′ (□), and 2′-deoxyguanosine 5′-monophosphate H-8 (▲) and H-1′ (△).

Results

Adenine Derivatives. Figure 1a shows the pD dependence of the shifts for the base ring protons of adenine, adenosine, and adenosine 5′-monophosphate while Figure 1b shows the pD shift dependence for the H-1′ protons of adenosine and adenosine 5′-monophosphate. The pD dependence for the ring and H-1′ protons of 2′-deoxyadenosine 5′-monophosphate is summarized in Figure 1c,d, respectively. In Figure 2 is shown the pD shift dependence for adenosine 2′,3′-cyclic monophosphate and adenosine 3′,5′-cyclic monophosphate, and for the 2′- and 3′-monophosphate isomers. The ring proton signals were assigned on the basis of deuterium-exchange rates and are in agreement with the results of other workers (Bullock and Jardetzky, 1964). All of the adenine derivatives show significant deshielding effects of up to 15 cycles/sec with decreasing pD below pD ~5 in accord with the results of earlier studies (Jardetzky and Jardetzky 1960). The sigmoid shapes of the curves are characteristic of pH-dependent ionization processes and in this case presumably result from protonation of the adenine ring. This is confirmed by the generally good agreement between pK values derived from titration measurements and the present chemical shift curves (Table I). Above pD 5 the shifts for H-2 and H-8 are constant with increasing pD for all of the derivatives except the 5′-nucleotides. In both of the 5′-nucleotides the H-2 proton shifts are constant up to pD ~8 whereas the H-8 protons shift to low field by about 8 cycles/sec. The shift changes for H-8 fall in the pD range for the secondary phosphate ionization and the nuclear magnetic resonance pK values are in accord with titration values (Table I).

The ribose H-1′ protons of the nucleosides and nucleotides studied also reflect protonation of the adenine ring at pD's <5. The deshielding effect, however, is appreciably smaller (~5 cycles/sec). No significant deshielding was observed for this proton in the pD region corresponding to primary or secondary phosphate ionization.

Guanine Derivatives. The pD dependences of the ring and H-1′ proton shifts for guanosine, guanosine 5′-monophosphate, and 2′-deoxyguanosine 5′-monophosphate are shown in Figure 3. A striking shift to low field is noted for the H-8 proton of each compound below pD ~4.5. The over-all shift changes are very similar to those reported for guanosine 5′-triphosphate

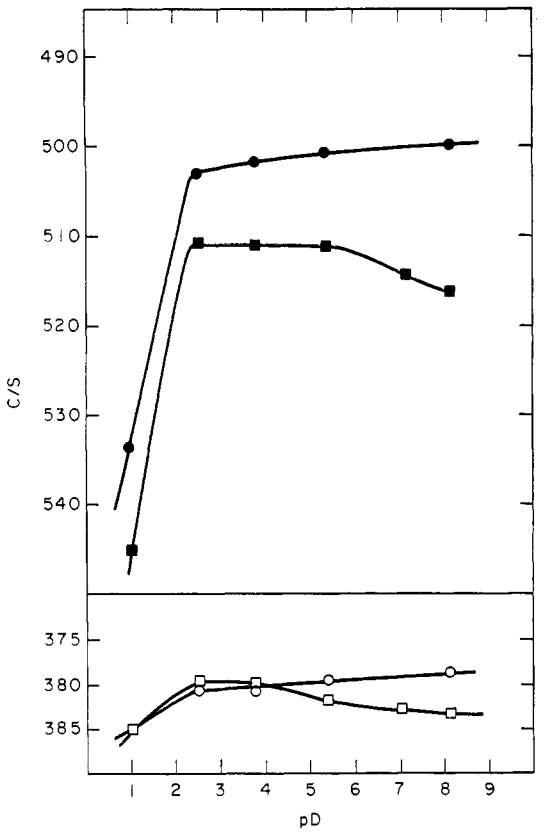

FIGURE 4: Proton chemical shifts (cycles per second relative to external TMS) *vs.* pD of the solution. Xanthosine H-8 (●) and H-1′ (○) and xanthosine 5′-monophosphate H-8 (■) and H-1′ (□).

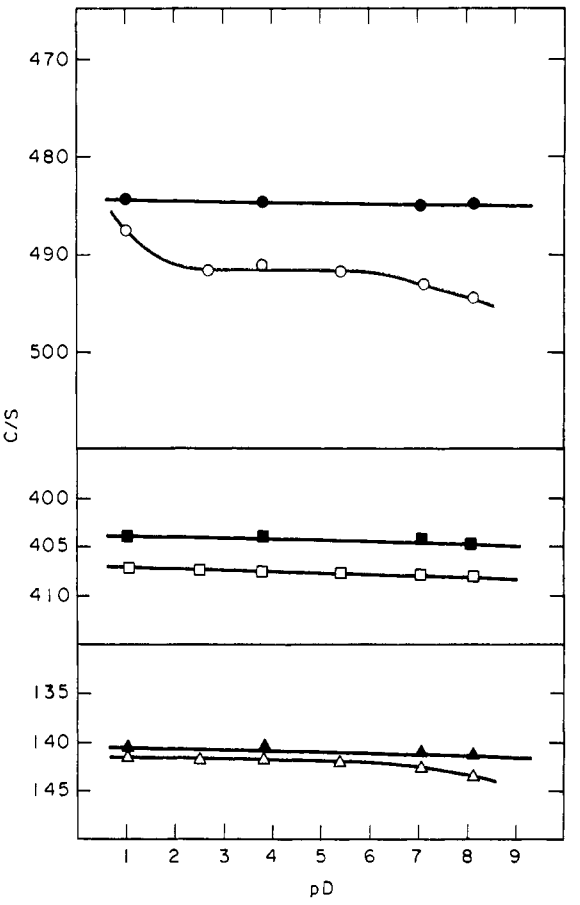

FIGURE 5: Proton chemical shifts (cycles per second relative to external TMS) *vs.* pD. Thymidine H-6 (●), H-1′ (■), and methyl protons (▲), and thymidine 5′-monophosphate H-6 (○), H-1′ (□), and methyl protons (△).

(−0.95 ppm: Jardetzky and Jardetzky, 1960) and guanosine (−0.93 ppm:[2] Bullock and Jardetzky, 1964), and are presumably due to protonation of the base ring. The p*K* values derived from the nuclear magnetic resonance shifts are in good agreement with values obtained titrimetrically (Table I). Above pD 4.5 the H-8 shift of guanosine is constant with increasing pD while for the 5′-nucleotides the H-8 proton shifts by ~7.5 cycles/sec to low field in the pD range 5–8. This shift change for the 5′-nucleotides can be associated with the secondary phosphate ionization. There is good agreement between p*K* values determined in this work and those previously reported (Table I).

The H-1′ shifts for guanosine and both 5′-nucleotides reflect protonation of the base ring but the effect is considerably smaller than for the H-8 proton. No noticeable effect of phosphate ionization on the H-1′ shifts is evident.

Xanthosine and Xanthosine 5′-Monophosphate. In Figure 4 are shown the pD chemical shift curves for xanthosine and xanthosine 5′-monophosphate. In both cases the H-8 proton shows a marked shift to low field with decreasing pD below pD ~2.5. Above this pD the H-8 proton shift for xanthosine varies only slightly with increasing pD while the same proton of the 5′-nucleotide shows a deshielding effect in the range pD 5.5–8.2. The shift changes for H-8 occur in pD ranges corresponding to protonation of the base ring and secondary ionization of the phosphate groups, respectively (Table I).

The H-1′ proton also shows a shift of −5 cycles/sec to low field with decreasing pD below pD ~2, and is presumably due to protonation of the ring. For the 5′-nucleotide a pD increase at pD >4 results in a shift of ~6 cycles/sec to low field which can be associated with the secondary phosphate ionization.

Thymidine and Thymidine 5′-Monophosphate. No shift change with pD was observed for the H-6, H-1′, and methyl protons of thymidine over the entire range studied (Figure 5). For thymidine 5′-monophosphate, however, the H-6 proton shows a shift to low field with increasing pD in the pD ranges 1–2.5 and 5.5–8.2 (Figure 5). A small deshielding is also indicated for the methyl protons at pD ~6–8. The shift changes for this nucleotide occur in ranges roughly corresponding to the primary and secondary phosphate ionization (Table I).

Discussion

Purine Derivatives. pD <5. The shift to low field of the ring proton signals for the free bases, nucleosides,

[2] The shift change for guanosine (Bullock and Jardetzky, 1964) refers to solutions under conditions different from those of the present work, *i.e.*, 0.05 M solution in D_2O at 80° and 0.1 M D_2SO_4.

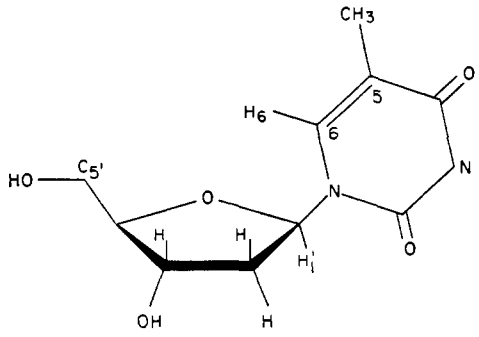

FIGURE 6: *anti* conformation of thymidine.

and nucleotides with decreasing pD below pD ~5 can be attributed to protonation of the base ring. This is confirmed by the good agreement observed between nuclear magnetic resonance pK values and those obtained by other methods (Table I). For the adenine derivatives the magnitude of the deshielding for the H-8 proton, about −12 to −14 cycles/sec, is indicative of protonation in the six-membered ring, most probably at the N-1 position (Cochrane, 1951; Kraut and Jensen, 1963). Other studies have shown that protonation of nitrogen heterocycles may lead to a deshielding of up to −30 cycles/sec in neighboring rings (Blears and Danyluk, 1967). The deshielding arises primarily from a redistribution of π-electron densities in the heterocyclic rings (Gil and Murrell, 1964). It is likely that a similar redistribution of charge deficiency occurs in the adenine ring. Little difference is noted in the magnitude of the over-all deshielding in various adenosine derivatives, *e.g.*, −13 cycles/sec for the H-8 proton of both adenine and adenosine. Substituents in the N-9 position accordingly have a negligible inductive effect upon proton shifts in the cations.

For the guanosine and xanthosine derivatives, on the other hand, the large deshieldings of the H-8 protons (−0.95 ppm) are in accord with protonation of the imidazole ring at the N-7 position (Broomhead, 1951; Staab and Mannschreck, 1962). The magnitude of this deshielding is appreciably larger than the values observed for a proton in the majority of mono- and bicyclic nitrogen heterocycles (−0.25 to −0.70 ppm) but is similar to that observed for imidazole (−0.91 ppm) (Staab and Mannschreck, 1962). It seems likely that the large deshieldings are the result of a localization of the π-electron deficiency mainly in the imidazole ring. In this connection it can be noted that the number of resonance structures possible in which the positive charge is distributed in both the pyrimidine and imidazole rings of the cation is much more restricted for guanine as compared with adenine derivatives. The small deshielding effect upon the H-1′ shifts is presumably due to the larger inductive effect of the positively charged base moiety relative to the uncharged species and also to the electric fields associated with the positive charge.

pD >5. The absence of any shift change with increasing pD in the pD range 5–8.2 is not unexpected for the free bases and nucleosides since these compounds do not possess ionizable groups in this region. For nucleotides, on the other hand, this pD range covers the region in which secondary phosphate ionization occurs in all of the nucleotides studied.

If favorable steric conditions are initially present, a change in ionization state could lead to shift changes of the base ring and ribose protons. The present results indicate that this is the case for all of the 5′-nucleotides, but not for the 2′, 3′, cyclic-2′, 3′, and cyclic-3′,5′ derivatives. The deshielding changes observed for the naturally occurring β-5′ nucleotides and the lack of any effect for the other isomers can be rationalized on the basis of a preferred *anti* conformation for the base and furanosyl groups. It can be shown with Courtauld's models that in this conformation rotation of the phosphate group about the C_5'–O bond in the 5′-nucleotides can bring the phosphate group into close proximity to the H-8 proton. In the β-2′ and -3′ isomers (and the cyclic isomers) a similar approach is sterically hindered by the furanosyl ring.[3] Furthermore, in this conformation the distance between the phosphate group and H-2 is too great for any effect upon the shift of this proton. Ionization of the phosphate group can then give rise to a deshielding of the H-8 proton either by an increase in the electric field effect due to the PO_4^{2-} group or by a hydrogen-bonding interaction between H-8 and the oxygen atoms. Of the two types of interactions the former would appear to be more favored in aqueous solution.[4] A simple calculation of the deshielding expected from electric field effects (Buckingham, 1960) indicates a shift change of −20 cycles/sec for H-8 and less than −2 cycles/sec for H-2[5] in the 5′ isomer. For the 2′- and 3′-nucleotides the corresponding deshieldings are estimated to be less than 1 cycle/sec in both cases. The contribution from field effects accordingly lies in the range of observed deshieldings for the 5′-nucleotide. A comparable assessment of shift changes from a potential hydrogen-bonding interaction is not possible.

From the present nuclear magnetic resonance results it can be concluded that a ring–chain interaction is operative in the pH range 5.0–8.2. This is in contrast with the conclusions reached from extensive optical (Cushley *et al.*, 1967) and ionization studies (Philips *et al.*, 1965) which indicate an electrostatic ring–chain interaction below pH ~5.0 where the base ring has a positive charge and no interaction above this pH where the ring is uncharged. The apparent anomaly arises because the chemical shift is more sensitive to weak local environmental changes than are the other measurements.

It is of interest to note that the shift changes in the pD range 5.0–8.2 for adenosine 5′-monophosphate and 2′-deoxyadenosine 5′-monophosphate (and also for the

[3] The distance of approach of the 5′-phosphate group to H-8 is approximately the same in the *anti* conformation as the approach of this group to H_2 in the *syn* conformation.

[4] Recent work completed in this laboratory (C. L. Bell, F. E. Hruska, and S. S. Danyluk, unpublished data) indicates the possibility of a hydrogen-bonding interaction between H-8 protons of purines and suitable proton acceptors in both aqueous and nonaqueous media.

[5] Calculated assuming a *trans* conformation of the 5′-nucleotide and using bond distances and angles reported in the literature (Kraut and Jensen, 1963).

corresponding guanosine derivatives) are quite similar, *i.e.*, 7.5 and 6.0 cycles/sec, indicating that any conformational differences due to the presence of a possible hydrogen bond between 2′-OH and N_3 (Ts'o *et al.*, 1966) are not reflected in the effect of the PO_4^{2-} group upon the ring shift.

Pyrimidine Derivatives. The absence of a shift change for thymidine over the entire pD range is in accord with the fact that the base ring does not undergo any change in ionization state in this region. For the 5′-nucleotide, on the other hand, the deshieldings observed at pH <2 and pH 5–8.2 can be attributed to the effects of primary and secondary phosphate ionization respectively. As in the case of purine nucleosides, optical studies (Emerson *et al.*, 1967; Ulbricht *et al.*, 1964; 1965) have established that the *anti* conformation is favored for β-pyrimidine nucleosides. For thymidine the preferred conformation is one in which the thymine ring is approximately perpendicular to the furanosyl ring with the C-5–C-6 double bond located above the sugar ring (Figure 6). A similar conformation is likely for thymidine 5′-monophosphate, and on this basis it is reasonable to expect that a change in ionization state of the phosphate group will result in chemical shift changes of both the methyl protons and H-6 as observed.

Added in Proof

Professor P. O. P. Ts'o has kindly forwarded to us a preprint of a paper (Schweizer *et al.*, 1968) on stacking and pH effects upon the nuclear magnetic resonance spectra of nucleotides. The conclusions arrived at indendently by Professor Ts'o and his coworkers are in substantial agreement with those reported by us.

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