Proton Magnetic Resonance Spectra of Adenine 5′-Nucleotides. Assignment of $H_{2'}$, $H_{3'}$, and $H_{4'}$ Resonance Bands and Their Structural Implications[1]

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Abstract: Proton magnetic resonance spectra (100 MHz) of adenosine 5′-monophosphate (5′-AMP) and adenosine 5′-triphosphate (5′-ATP) in which *all* the nonexchanging proton peaks are separate have been obtained. The $H_{2'}$, $H_{3'}$, and $H_{4'}$ bands were identified by comparison of the 5′-AMP spectrum with the spectra of 2′-AMP and 3′-AMP. The $H_{2'}$ assignment was verified by a double resonance experiment. The chemical shifts of these three protons and the $J_{1'-2'}$, $J_{2'-3'}$, and $J_{3'-4'}$ coupling constants in 5′-AMP and 5′-ATP have been calculated assuming no coupling between nonvicinal protons. Comparison of chemical shifts in various adenine nucleotides have furnished additional information concerning structure and stacking of 5′-AMP and 5′-ATP in D_2O solution, particularly with regard to the torsion angle about the glycosidic bond and the rotational freedom of the phosphate group.

Structural implications of the proton magnetic resonance (pmr) spectra of adenine nucleosides and nucleotides have been discussed earlier by several groups.[3-5] However, to date no one has presented a pmr spectrum of either adenosine or an adenine 5′-nucleotide in which the ribose $H_{2'}$, $H_{3'}$, and $H_{4'}$ resonance signals have been separate and assignable. Rather, all previous conclusions have been drawn from consideration of only the $H_{1'}$, $H_{5'}$, H_2, and H_8 resonances.

We present here pmr spectra of adenosine 5′-monophosphate (5′-AMP) and adenosine 5′-triphosphate (5′-ATP) in D_2O in which the $H_{2'}$, $H_{3'}$, and $H_{4'}$ signals are separate. These signals have been identified by comparison of the 5′-AMP spectrum with 2′-AMP and 3′-AMP spectra. The $H_{2'}$ assignment was verified by a spin-decoupling experiment. A study of the now complete 5′-AMP spectrum and its comparison with spectra of 2′-deoxy-5′-AMP (5′-dAMP) and 5′-ATP has added to existing structural knowledge of these compounds.

Experimental Section

All spectra were recorded with a JEOLCO 4H-100 (100 MHz) nmr spectrometer kept in a 24° constant-temperature room. The probe temperature was 27°. D_2O solutions of the nucleotides were prepared from aqueous solutions, which had been adjusted to the desired pH with concentrated sodium hydroxide, by lyophilizing the solutions overnight and then redissolving the powdery residues to the desired concentration with D_2O. The process of lyophilization of the solution and dissolution of the residue in D_2O was repeated three times. It was found that *three lyophilizations of D_2O solutions were required to minimize the HDO peak* in the spectra. The reported pD values were obtained by adding 0.4 to the pH meter readings.[6]

Chemical shifts were measured relative to 2% acetone as an internal standard. Although the frequency counter of the nmr spectrometer is accurate to 0.01 ppm, the character of the spectral peaks in the study limits the measurable chemical shifts to 0.005 ppm at best. The actual accuracy of the values reported are considered to be 0.01–0.02 ppm, so we will not interpret chemical shift changes of 0.02 ppm or less. For a subsequent paper pH adjustment was done using tetramethylammonium hydroxide (0.2–0.4 *M*) and the tetramethylammonium ion was used as the internal standard. The only change in the nucleotide spectra was an upfield shift of 0.96 ± 0.01 ppm for each signal in each spectrum. This value is equal to the chemical shift of $(CH_3)_4N^+$ relative to acetone as an internal standard in a D_2O solution containing only $(CH_3)_4NCl$ and 2% acetone. Hence, neither $(CH_3)_4N^+$ nor $(CH_3)_2O$ as internal standard affects the spectra in any way detectable by the nmr spectrometer.

The nucleotides used were either Sigma Chemical Co. Sigma grade or Calbiochem A grade and were used as obtained without further purification.

In the following discussion we use the convention that downfield chemical shift changes are positive values.

Results and Discussion

Assignment of $H_{2'}$, $H_{3'}$, and $H_{4'}$ Resonances in 5′-AMP Spectrum. In Figures 1B, 1C, and 1D are presented the 100-MHz pmr spectra of 5′-AMP at three concentrations, 0.08, 0.20, and 1.0 *M*, all at pD 10 ± 0.2. It is seen that increasing the AMP concentration produces a downfield shift of the HDO resonance which completely unmasks a triplet which we have assigned to $H_{2'}$. We have assigned the apparent triplet next upfield of this $H_{2'}$ peak to $H_{3'}$. The poorly resolved signal next upfield of $H_{3'}$ is then, obviously, $H_{4'}$, the poor resolution being due to the fact that $H_{4'}$ is coupled to four atoms, $H_{3'}$, $H_{5'}$, $H_{5''}$, and P.

Coupling constants, $J_{1'-2'}$ = 5.3 cps, $J_{2'-3'}$ = 4.8 cps, and $J_{3'-4'}$ = 3.8 cps, were calculated for 5′-AMP using the assumption that there is no coupling in ribose between nonvicinal protons. The doublet character of $H_{1'}$ validates this assumption. Parameters were first estimated by first-order analysis and then refined by calculations performed on the IBM 360-44 computer

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(3) (a) C. D. Jardetzky and O. Jardetzky, *J. Am. Chem. Soc.*, **82**, 222 (1960); (b) C. D. Jardetzky, *ibid.*, **82**, 229 (1960); (c) C. D. Jardetzky, *ibid.*, **83**, 2929 (1961); (d) C. D. Jardetzky, *ibid.*, **84**, 62 (1962); (e) O. Jardetzky, *Biopolymers Symp.*, **1**, 501 (1964).

(4) (a) A. D. Broom, M. P. Schweizer, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **89**, 3612 (1967); (b) M. P. Schweizer, A. D. Broom, P. O. P. Ts'o, and D. P. Hollis, *ibid.*, **90**, 1042 (1968).

(5) S. S. Danyluk and F. E. Hruska, *Biochemistry*, **7**, 1038 (1968).

(6) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).

Table I. Comparison of Chemical Shifts of Adenosine Nucleotides[a]

Nucleotide	pD	H_8	H_2	$H_{1'}$	$H_{2'}$	$H_{3'}$	$H_{4'}$	$H_{5'}(H_{5''})$
5′-AMP	6.4	6.19	5.79	3.80	2.49	2.29	2.17	1.92
5′-AMP	10	6.31	5.82	3.83	2.55	2.31	2.17	1.83
2′-AMP	10	6.10	5.86	3.90	2.80	2.38	2.10	1.68
3′-AMP	10	6.06	5.82	3.82	~2.63[b]	~2.58[b]	2.23	1.71
5′-dAMP	10	6.22	5.82	4.14	~0.48[c]	~2.50[d]	2.05	1.75
5′-ATP	10	6.23	5.82	3.84	2.56	2.42	2.21	2.08

[a] At 27° and 0.2 *M*. All values are tabulated in parts per million (ppm) *downfield* from 2% acetone as internal reference. [b] Approximate values estimated from 5′-AMP chemical shifts as explained in text. [c] Center of two-proton 2′,2″ band. [d] Partial overlap of HDO and $H_{3'}$ bands cause slight uncertainty in $H_{3'}$ location.

using the LAOCOON II program.[7] Spectra computed using various combinations of frequencies and coupling constants were matched with the experimental spectra. For a given set of experimental frequencies coupling constants accurate to 0.1 cps could be determined. However, the actual accuracy of the reported values is

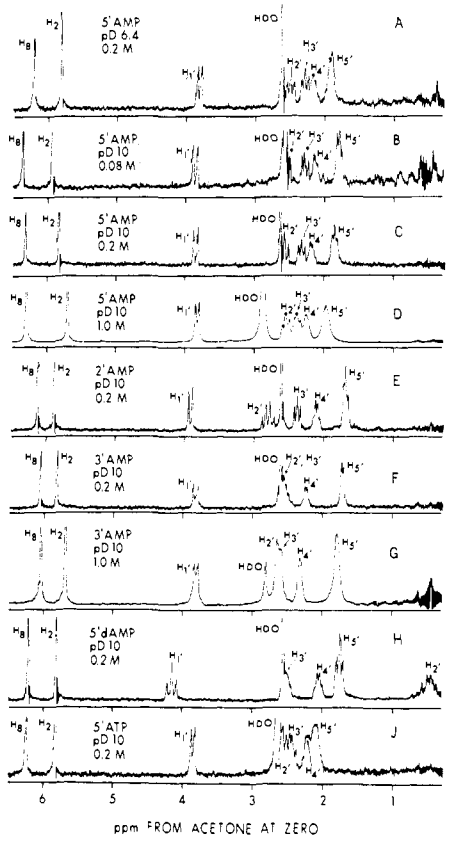

Figure 1. Pmr spectra (100 MHz) of various adenine nucleotides at 27°. Conditions are given on each curve. Chemical shifts are measured downfield from 2% acetone as internal standard.

only about 0.25 cps because of the natural widths of the peaks and the noise. In theory the $H_{2'}$ and $H_{3'}$ signals are quartets, but the closeness of the three relevant coupling constants makes them pseudo triplets. Because of the complicated coupling schemes of $H_{4'}$ and $H_{5'}$, involving geminal proton coupling as well as vicinal and also proton to phosphorus coupling, the resolution of these two peaks does not allow extraction of $J_{4'-5'}$, $J_{5'-5''}$, or $J_{5'-P}$.

(7) S. Castellano and A. A. Bothner-By, *J. Chem. Phys.*, **41**, 3863 (1964).

In our work, the $H_{2'}$ triplet was fully revealed at 0.2 *M* when the pD was about 6 (Figure 1A) but not until 0.4 *M* at pD 10. However, since the middle (highest) peak of the $H_{2'}$ signal is completely exposed at 0.2 *M* and pD 10, the chemical shift of $H_{2'}$ is measurable even in this case. The downfield movement of the HDO peak with increasing AMP concentration is due to at least two factors: (i) the fact that the rapid exchange of HDO and ribose hydroxyl protons becomes more spectrally significant as the concentration of ribose groups increases, since it is evident from Gatlin and Davis' spectrum[8] of adenosine in dimethyl sulfoxide that ribose hydroxyl protons resonate at lower field than water protons, and (ii) the polarization of the solvent HDO by the phosphate group.[9]

Assignment of the H_2, H_8, $H_{1'}$, and $H_{5'}$ spectral bands of 5′-AMP was first made by Jardetzky and Jardetzky,[3a] but the $H_{2'}$, $H_{3'}$, and $H_{4'}$ resonances were treated as an unresolved group with an averate frequency. The only attempt to identify these latter three resonances seems to have been the *purely arbitrary* assignment by Gatlin and Davis who incorrectly, we believe, placed $H_{3'}$ downfield from an unresolved two-proton band, which they labeled $H_{2'}$,$H_{4'}$.

Our assignment of $H_{2'}$, $H_{3'}$, and $H_{4'}$ of the 5′-AMP spectrum in order of increasing magnetic field is based primarily upon comparison of the 0.2 *M* 5′-AMP, 2′-AMP, and 3′-AMP spectra (Figures 1C, 1E, and 1F, respectively). In Table I are presented the chemical shifts for these three protons, as well as the other protons, at 0.2 *M* nucleotide concentration and pD 10 ± 0.2 relative to 2% acetone as internal reference.

Due to the electron-withdrawing ability of a phosphate group,[4b] moving it from the 5′ position of AMP to the 2′ position should produce a significant downfield shift in the $H_{2'}$ resonance and smaller, but almost equal, downfield shifts for its vicinal neighbors, $H_{1'}$ and $H_{3'}$. The $H_{4'}$ signal should move slightly upfield, however, since the small deshielding effect on $H_{4'}$ of a 5′-phosphate group is replaced in 2′-AMP by the even smaller deshielding effect of a 5′-OH radical. Indeed, this change in phosphate position does cause downfield shifts of 0.07, 0.25, and 0.07 ppm, respectively, for $H_{1'}$, $H_{2'}$, and $H_{3'}$ and an upfield shift, −0.07 ppm, for $H_{4'}$, the latter value as expected being much smaller than the −0.15-ppm change in $H_{5'}$.

By analogy with the $H_{1'}$ and $H_{2'}$ changes observed when phosphate is transferred from the 5′ position to the 2′ position, we should expect the $H_{2'}$ and $H_{3'}$ signals in the 3′-AMP spectrum (Figure 1F) to be, respectively, about 0.07 and 0.25 ppm downfield of

(8) L. Gatlin and J. C. Davis, Jr., *J. Am. Chem. Soc.*, **84**, 4464 (1962).
(9) J. N. Schoolery and B. J. Alder, *J. Chem. Phys.*, **23**, 805 (1955).

their locations in the 5′-AMP spectrum (Figure 1C). The most obvious difference seen in comparing these spectra is a downfield shift of $H_{3'}$ of at least 0.2 ppm. The expected $H_{2'}$ movement would put $H_{2'}$ under the HDO peak, which does cover most of the left half of an apparently two-proton band. Shift of the HDO peak downfield when the 3′-AMP concentration is increased to 1.0 *M* (Figure 1G) exposes a two-proton doublet whose peaks are 0.06 ppm apart. We cannot be certain as to which of the peaks in this doublet is $H_{2'}$ and which is $H_{3'}$. If the left peak is $H_{2'}$, it indicates downfield shifts of 0.10 and 0.20 ppm for $H_{2'}$ and $H_{3'}$, respectively, in going from 5′-AMP to 3′-AMP at 1.0 *M* concentration. If the left peak is $H_{3'}$, then the $H_{2'}$ and $H_{3'}$ movements are 0.04 and 0.26 ppm, respectively. Either choice gives shifts near the expected values of 0.07 and 0.25 ppm. The significant thing here is that in either case it is the 5′-AMP signal assigned to $H_{3'}$ which moves downfield the most when the phosphate transfers from the 5′ to the 3′ position.

The peak which is obviously $H_{4'}$ in the 5′-AMP spectrum moves upfield −0.06 ppm in the 3′-AMP spectrum. We would have predicted a smaller upfield shift than this since the deshielding action on $H_{4'}$ that results when the 3′-OH is replaced by 3′-phosphate is partially compensated by the shielding action on $H_{4'}$ resulting from replacing a 5′-phosphate group by 5′-OH. However, since chemical-shift effects are not strictly additive, especially for ring protons, we believe the most important observation in this case is the correct direction of the shift rather than its magnitude.

A spin-decoupling experiment[10] verified the $H_{2'}$ assignment. Irradiation of the $H_{1'}$ proton caused collapse of the $H_{2'}$ triplet to a doublet with peak separation ~5 cps, and irradiation of $H_{2'}$ collapsed $H_{1'}$ to a singlet. This assignment automatically verifies the $H_{3'}$ and $H_{4'}$ assignments because of the fine structure in $H_{3'}$ and the lack of resolvable fine structure in $H_{4'}$.

Correlation of Nucleotide Structure and Stacking with Proton Resonance Frequency Changes. For the sake of clarity in our later discussion, we will give a very brief résumé of the ideas of Broom, Schweizer, Ts'o, and Hollis,[4] whom we will henceforth refer to as the JH (*i.e.*, Johns Hopkins) group. These authors have obtained vapor-pressure osmometry and pmr data to show that nucleosides and nucleotides self-associate in vertical stacks to a degree dependent on concentration, pH, and the presence and position of a phosphate group. They have presented two geometric models[4a] for association in which the six-membered rings of stacked neighbors overlap considerably and the five-membered rings overlap to a lesser extent. They favor the model referred to as the "alternate stack" arrangement in which, relative to its stacked neighbor, the purine base is rotated 180° about an axis passing through the centers of the two purine rings. Thus, N_3 of one nucleotide lies almost opposite C_6 of the neighbor, and N_7 of one lies nearly opposite N_9 of the other. Constancy of line width with changing pD or concentration shows that these stacks break and re-form very rapidly irrespective of these variables.

According to the JH group the extent of shielding of H_2 measures the closeness and degree of six-membered ring stacking and, *if* the specific deshielding[11] of H_8 by phosphate remains constant, the H_8 shielding reflects five-membered ring stacking.[12] These authors also use the $H_{1'}$ frequency as an indicator of five-membered ring stacking. However, if one examines molecular models constructed from Corey–Pauling–Koltun (CPK) space-filling atomic models it becomes evident that the shielding of $H_{1'}$, and of all the other ribose protons, must depend also on the torsion angle,[14] ϕ_{CN}.

Two groups[4b,5] have concluded from pmr evidence that 5′-AMP is in the *anti* conformation with H_8 in juxtaposition with the phosphate group. This conformation allows the existence of a hydrogen bond between N_3 and 2′-OH, and in fact their evidence shows that there is a mutual dependence of the specific phosphate deshielding of H_8 and this hydrogen bond. The 5′-AMP molecular model indicates that at a ϕ_{CN} of *ca.* −25° the phosphate group can be placed in direct, and maximum, juxtaposition with H_8 and that the hydrogen bond between N_3 and 2′-OH should be near its maximum strength. At this ϕ_{CN} value a line through $H_{1'}$ and $H_{2'}$ is almost perpendicular to the plane of the purine rings, but $H_{2'}$ is about 0.7 Å further from the ring plane than $H_{1'}$ is. Both protons probably are situated slightly inside the shielding region of the purine rings of the given nucleotide ($H_{2'}$ more so than $H_{1'}$) and each would probably be slightly shielded by a neighboring nucleotide of a stack[12] ($H_{1'}$ more so than $H_{2'}$). Of course, for dimeric stacking only one of these protons on a given nucleotide is shielded by a stacked neighbor at a given instant, but on the average there would be shielding of both protons since the stacks break and re-form rapidly and since there is probably very little preference as to which side associates. The JH group stated that Donohue and Trueblood[14] had concluded that ϕ_{CN} is *ca.* −30° for the *anti* conformation, which agrees well with the −25° angle just cited as most favorable for both specific phosphate deshielding of H_8 and hydrogen bonding of N_3.

However, we would like to point out that what Donohue and Trueblood actually concluded was that the *anti* range is *centered* at ~−30° and, in fact, covers somewhat over 90°. Although the electrostatic attraction of the phosphate group for H_8 and the energy of the hydrogen bond to N_3 would be maximum when ϕ_{CN} is near −25 or −30°, the packing effects of stacking with its accompanying increase in interphosphate repulsion of stacked neighbors could change this angle. There is crystal structure evidence[15] for this view since ϕ_{CN} values ranging from −4 to −69° have been obtained for adenine nucleosides and nucleotides, albeit a ϕ_{CN} of −20° was found for crystalline 5′-AMP.[16] Now, it seems from the molecular model that if ϕ_{CN} of 5′-AMP is changed in a positive direction both $H_{1'}$ and $H_{2'}$ be-

(10) This spin decoupling experiment was very kindly performed for us by Dr. L. D. Colebrook of the University of Rochester Chemistry Department.

(11) According to the JH group[4b] the deshielding of H_8 by a 5′-phosphate is due to electrostatic attraction of the slightly labile H_8 by the negatively charged phosphate which polarizes the C_8–H_8 bond, thereby lowering the electron density at the proton. In contrast, a phosphate group would have an electrostatic shielding effect on a nonlabile proton in its vicinity.

(12) The justification for considering the heteronuclear rings of the purine base to have shielding and deshielding zones due to ring anisotropies seems to be ample.[13]

(13) (a) M. P. Schweizer, S. I. Chan, G. K. Helmkamp, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **86**, 696 (1964); (b) S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, *ibid.*, **86**, 4182 (1964).

(14) J. Donohue and K. N. Trueblood, *J. Mol. Biol.*, **2**, 363 (1960).

(15) A. E. V. Haschemeyer and A. Rich, *J. Mol. Biol.*, **27**, 369 (1967).

(16) J. Kraut and L. H. Jensen, *Acta Cryst.*, **16**, 79 (1963).

come more deshielded by the given nucleotide but more shielded by a stacked neighbor. If ϕ_{CN} changes in a negative direction both $H_{1'}$ and $H_{2'}$ become more deshielded by the given nucleotide. However, although $H_{1'}$ becomes more shielded by a stacked neighbor, $H_{2'}$ becomes more deshielded by a stacked neighbor. This difference results from the fact that the rotation of the $C_{1'}$–$C_{2'}$ bond about the N_9–$C_{1'}$ bond as ϕ_{CN} becomes more negative causes $H_{2'}$ to move further away from both purine rings. The effect of a given small torsion angle change is much greater on the $H_{2'}$ chemical shift than on the $H_{1'}$ shift because the length of arc traversed is much greater for $H_{2'}$ than for $H_{1'}$, since $H_{2'}$ is about 0.7 Å further from the purine plane than $H_{1'}$ is when ϕ_{CN} is $\sim -25°$. Thus, as the JH group contended, stacking should always cause increased shielding of $H_{1'}$, but the shielding effect on $H_{2'}$ produced by stacking would be at least partially reduced when the torsion angle becomes more negative.

The chemical shifts of $H_{3'}$, $H_{4'}$, and $H_{5'}$ also depend on both the stacking and the torsion angle but to a much smaller extent than $H_{2'}$ does, since $H_{2'}$ is much closer to the center of the five-membered ring than either $H_{3'}$, $H_{4'}$, or $H_{5'}$. We deduce from the CPK model that the latter three protons are all in the deshielding regions of the five-membered ring of the given nucleotide and of stacked neighbor nucleotides and should remain deshielded for small changes in the torsion angle. However, they should become more deshielded if ϕ_{CN} is made more negative than $-25°$ and less deshielded if ϕ_{CN} becomes more positive. We estimate from the molecular model that a ϕ_{CN} of $\sim -40°$ would give maximum deshielding for $H_{3'}$, $H_{4'}$, and $H_{5'}$.

Effects of Varying pD and Nucleotide Concentration. In Table II are presented the changes in the chemical shifts relative to the spectrum of 0.2 *M* 5′-AMP at pD 10 brought about by decreasing the pD to 6 and by raising the AMP concentration to 1.0 *M*.

Table II. Changes of Chemical Shifts in 0.2 *M* 5′-AMP Spectrum Produced by Varying the pD and Concentration

Proton	pD effect[a]	Concn effect[b]
H_8	−0.13	−0.04
H_2	−0.04	−0.14
$H_{1'}$	−0.03	−0.03
$H_{2'}$	−0.06	−0.01
$H_{3'}$	−0.02	+0.07
$H_{4'}$	0	+0.08
$H_{5'}$	+0.09	+0.13

[a] pD lowered from 10 to 6; AMP concentration, 0.2 *M*. [b] AMP concentration raised to 1.0 *M*; pD 10.

The pD and concentration effects on H_8, H_2, $H_{1'}$, and $H_{5'}$ have been discussed previously by others.[4b,5] They attributed the increased shielding of H_8 as pD decreases from 10 to 6 to the decrease in the net negative charge of phosphate as it protonates, resulting in a smaller specific deshielding action of the phosphate on H_8. The downfield shift of $H_{5'}$ as pD decreases was believed to be due to the greater electron-withdrawing power of phosphate after protonation. The small upfield shifts of H_2 and $H_{1'}$ were considered to reflect greater stacking of the purine rings because of decreased repulsion between phosphate groups of stacked neighbors after protonation of the phosphate. Following our discussion in the previous section, we believe that the increased shielding of $H_{2'}$ as the pD is lowered suggests that the torsion angle becomes a little less negative, since the $H_{2'}$ shielding is increased to a significantly larger extent, −0.06 ppm, than that of either H_2, −0.04 ppm, or $H_{1'}$, −0.02 ppm.

When the 5′-AMP concentration is raised from 0.2 to 1.0 *M*, at constant pD of 10, significantly large downfield shifts of $H_{3'}$, $H_{4'}$, and $H_{5'}$ develop despite the fact that H_8, H_2, and $H_{1'}$ become more shielded. $H_{2'}$ remains virtually unaffected. These observations, of course, are related to the large increase in stacking,[4] past the dimer stage,[17] as the AMP concentration is so greatly increased. As the stacks get past the dimer stage the large interphosphate repulsion tends to force the phosphate groups to maximize the distance between themselves by pulling away from H_8 as far as possible. That this occurs to some extent is indicated by the increased shielding, −0.04 ppm, of H_8. This value is, however, definitely smaller than the change of H_8 caused by pD lowering or by addition of NaCl (*vide infra*) because the charge neutralization of phosphate is even more effective than the enhanced interphosphate repulsion of larger stacks. The large concentration-induced deshielding effects on $H_{3'}$, $H_{4'}$, and $H_{5'}$ are, we believe, due to the paramagnetic effect of the purine ring anisotropy, which we explained above. The almost insignificant change in $H_{2'}$, despite the fact that H_2 and $H_{1'}$ are more shielded, suggests to us that the ϕ_{CN} torsion angle becomes about 10° more negative when the stacks get above the dimer stage.

The JH group[4b] has attributed the downfield shift of $H_{5'}$ with increasing 5′-AMP concentration at pD 5.9 to "the effect of concentration on the ionization of phosphate groups, as anticipated from the Debye–Hückel theory on electrolytes." We believe the evidence negates this explanation. First, the Debye–Hückel theory is not applicable at the high ionic strengths in which they obtained this concentration-induced chemical shift, *i.e.*, 0.05–0.85. If one must invoke activity coefficients here, it would seem that the only thing one can do is to assume that the ratio of activity coefficients, $(\gamma_{AMP^{2-}})/(\gamma_{HAMP^{-}})$, is approximated by $\gamma^3_{\pm Na_2HPO_4}/(\gamma^2_{\pm NaH_2PO_4} \cdot \gamma_{\pm NaCl})$. Using these mean activity coefficients for ionic strengths of 0.3, 0.6, and 0.9, we calculate this activity coefficient ratio to be 0.39, 0.31, and 0.26, respectively, at these three ionic strengths. Thus, if it were the cause of the concentration-induced $H_{5'}$ movement, the ionic strength effect of an increasing AMP concentration would probably increase the ionization of the phosphate with concomitant increased shielding of $H_{5'}$ similar to the effect of *increasing* pD. As a matter of fact, we find only an insignificant change, −0.01 ppm, in the $H_{5'}$ chemical shift when 2 *M* NaCl is added to 0.2 *M* 5′-AMP either at pD 6.4 or at pD 10.

Comparison of 5′-AMP and 5′-dAMP Spectra. Comparison of the chemical shifts in the 5′-AMP and 2′-deoxy-5′-AMP (5′-dAMP) spectra, Figures 1C and 1H, respectively, presented in Table I shows that replacement of the 2′-OH radical of 5′-AMP with a proton causes increased shielding of H_8, $H_{4'}$, and $H_{5'}$, the chemical shift changes being, respectively, −0.09,

(17) G. P. Rossetti and K. R. Van Holde, *Biochem. Biophys. Res. Commun.*, **26**, 717 (1967).

Table III. Chemical Shift Changes Produced by Adding 2 *M* NaCl to 0.2 *M* Nucleotide

Proton	5′-AMP	5′-dAMP	2′-AMP
H_8	−0.10	−0.09	−0.03
H_2	−0.10	−0.08	−0.07
$H_{1'}$	−0.02	−0.08	−0.04
$H_{2'}$	−0.02	−0.01	−0.03
$H_{3'}$	−0.04	∼−0.04	−0.02
$H_{4'}$	−0.01	0	−0.01
$H_{5'}$	−0.01	−0.02	−0.02

−0.12, and −0.08 ppm, and decreased shielding of $H_{1'}$, +0.31 ppm, and $H_{3'}$, +0.19 ppm. $H_{2'}$ was unchanged. Similar changes for H_8 and $H_{1'}$ were reported by the JH group[4b] for pD 5.9. They felt that in 5′-AMP there is greater shielding of H_2 than of H_8 or $H_{1'}$, because of closer stacking of the six-membered adenine rings than of the five-membered rings, and that removal of 2′-OH allows closer stacking of the five-membered rings, so that the stacking effect on this substitution at the 2′ position is felt more by H_8 and $H_{1'}$ than by H_2. $H_{1'}$ is affected even more, however, in a deshielding direction because the 2′-OH of 5′-AMP originally shielded the *cis*-$H_{1'}$ atom electrostatically.

While we do not disagree with this explanation, we feel that there is an additional factor—the relative freedom of rotation of the phosphate group. As mentioned earlier, the specific deshielding effect on H_8 by a 5′-phosphate group is due to the close juxtaposition of H_8 and a phosphate oxygen atom. Rotation about either the $C_{5'}$–O bond or about the $C_{4'}$–$C_{5'}$ bond is therefore somewhat restricted. In such a restricted arrangement stacking will cause the phosphate groups of neighboring nucleotides to lie right next to each other and considerable repulsion energy should develop. For the same H_8–O distance this repulsion energy would be greater in 5′-dAMP than in 5′-AMP because of the closer stacking of the five-membered rings in the former,[4b] but it can be completely relieved by movement of the phosphate group back away from the vicinity of H_8. This phosphate movement causes H_8 to be much more shielded in 5′-dAMP than in 5′-AMP.

This enhanced freedom of rotation in 5′-dAMP about the $C_{5'}$–O and $C_{4'}$–$C_{5'}$ bonds can bring phosphate oxygens near to the $H_{4'}$ and the $H_{5'}$ protons for a sufficient fraction of time to produce, on the average, a greater electrostatic shielding effect on these protons in 5′-dAMP relative to 5′-AMP.

The greater deshielding of $H_{3'}$ in 5′-dAMP is probably the net result of several factors: (i) the removal of the 2′-OH group which eliminates its electrostatic shielding effect on $H_{3'}$, (ii) the increased deshielding effect of the ring anisotropy because of the closer stacking in 5′-dAMP, and (iii) the increased shielding effect of the more freely rotating $CH_2PO_3^{2-}$ in 5′-dAMP.

Effects of NaCl Addition. Table III shows the spectral effects of adding 2 *M* NaCl to 0.2 *M* 5′-AMP, 5′-dAMP, and 2′-AMP. Comparing these results with the data in Table II we conclude that NaCl increased the closeness of dimeric stacking but had little effect on the degree of aggregation. That is, NaCl addition produced a relatively large shielding effect on H_2 in all three nucleotides, but, contrary to the deshielding effect of increasing 5′-AMP concentration, the $H_{3'}$, $H_{4'}$, and $H_{5'}$ were slightly more shielded in the presence of NaCl. It does not seem, therefore, that NaCl produces a solvent-influenced "salting-out" effect but rather a specific action on the nucleotides.

This belief is strengthened by the fact that the NaCl increased the shielding of H_8 much more in the two 5′ nucleotides than in 2′-AMP. This shows that the added Na^+ ion neutralizes the electrostatic charge of phosphate sufficiently to cause significant decrease in its specific deshielding of H_8. However, because of the noncovalent nature of any binding of Na^+ by phosphate, the NaCl does not increase the ability of phosphate to withdraw electronic charge from the adjacent $C_{5'}$ carbon atoms.

Spectrum of Adenosine 5′-Triphosphate (5′-ATP). The 5′-ATP spectrum (Figure 1J) seems to be sufficiently similar to the 5′-AMP spectrum (Figure 1C) to indicate that the proton resonances should be assigned in the same relative order in both spectra. Data in Table I show that $H_{3'}$, $H_{4'}$, and $H_{5'}$ resonances in 5′-ATP are at lower field than in 5′-AMP but that H_8 is more shielded in 5′-ATP.

It is important to note first that the protons which are least affected by lengthening the phosphate chain are $H_{1'}$, H_2, and $H_{2'}$, their changes being insignificant. Hence, there is probably little difference in either the adenine ring stacking or the torsion angle of 5′-AMP and 5′-ATP. Apparently, the triphosphate chains of neighboring members of a 5′-ATP stack can rotate and wiggle far enough away from each other to leave very little repulsion between them. For stacks larger than dimers, neighboring phosphate chains would probably execute a scissoring type of motion. Otherwise, a destacking effect in 5′-ATP with consequent deshielding of H_2, $H_{1'}$, and $H_{2'}$ would have been observed. Further evidence for this view is the fact that H_8 is 0.08 ppm more shielded in ATP than in AMP, which we attribute to the pulling away of the phosphate from the vicinity of H_8, thereby decreasing the specific deshielding of H_8.[18]

(18) Note Added in Proof. Since this paper was submitted Fujiwara and Uetsuki have published the same spectral assignments as ours (S. Fujiwara and M. Uetsuki in "Recent Developments of Magnetic Resonance in Biological System," S. Fujiwara and L. H. Piette, Ed., Hirokawa Publishing Co. Inc., Tokyo, Japan, 1968, pp 1–9). They report that spin decoupling identified the $H_{2'}$, resonance, but that spin–spin coupling constants were used to assign $H_{3'}$ and $H_{4'}$. It is not clear to us, however, how the latter assignments can be extracted from their reported $J_{1'-2'}$, $J_{2'-3'}$, and $J_{3'-4'}$, values: 5.0, 5.0, 5.0 for adenosine at 100°; 5.3, 5.0, 4.8 for adenosine in 1 *M* DCl; and 5.0, 5.0, 4.5 for AMP.