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# Proton Magnetic Resonance Study of 8-(6-Aminohexyl)-aminoadenosine 5′-Monophosphate

FREDERICK E. EVANS, CHI-YU LEE,[1] HANNELORE KAPMEYER,[2] AND NATHAN O. KAPLAN

*Department of Chemistry, University of California, San Diego, La Jolla, California 92093*

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The nucleotide 8-(6-aminohexyl)-amino adenosine 5′-monophosphate (8-AHA-AMP) has been investigated by 220-MHz proton magnetic resonance spectroscopy. The conformation and ionization state of the nucleotide have been determined. The *anti*-conformation about the glycosyl bond is the preferred form. The interaction between the hexyldiamino chain and the ribose moiety in this conformation gives rise to unusual ribosyl conformation results. The distribution of conformations about the glycosyl bond has little influence on the effectiveness of this nucleotide analog in the purification of dehydrogenases by affinity chromatography. The chemical shift dependence on pH has been carried out on 8-methylaminoadenosine 5′-monophosphate. The 8-aminoadenine ring is protonated at N1 (p$K_a$ 5.0) and at N7 (p$K_a$ 1.5) in acidic solutions. The protonation at N7 is apparently stabilized by a delocalization of charge onto the 8-amino group. The neutrality of the 8-aminoadenine ring at physiological pH is consistent with the efficient binding of the nucleotide by dehydrogenases. An improved method for the preparation of the 8-AHA-AMP is described.

## INTRODUCTION

Elucidation of the mechanism of coenzyme binding by dehydrogenases may lead to better understanding of how enzymes function. The analog 8-(6-aminohexyl)-aminoadenosine 5′-monophosphate (8-AHA-AMP)[3] is as good a competitive inhibitor of dehydrogenases as is AMP (*1*, *2*) in spite of the presence of a bulky group on the adenine ring. Such additions generally cause a nucleotide to adopt *syn* conformation about the glycosyl bond. The classical example of this is 8-Br-AMP (*3*). The parent nucleotide AMP is preferentially in the *anti* conformation.

In a preliminary communication (*4*) we have reported that 8-AHA-AMP retains the *anti* conformation. We present a proton magnetic resonance study on 8-AHA-AMP. The results are discussed in relationship to the binding by dehydrogenases and their purification of enzymes by affinity chromatography. A modified experimental procedure for the synthesis of 8-AHA-AMP is also presented.

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[1] Present address: Environmental Mutagenesis Branch, NIEHS, P.O. Box 12233, Research Triangle Park, North Carolina 27709.

[2] Recipient of a fellowship from the Deutsche Forschungsgemeinschaft.

[3] Abbreviations used: 8-AHA-AMP, 8-(6-aminohexyl)-aminoadenosine 5′-monophosphate; 8-NHCH$_3$-AMP, 8-methylaminoadenosine 5′-monophosphate; AMP, adenosine 5′-monophosphate; NMR, nuclear magnetic resonance; 6-AHA-AMP, 6-(6-aminohexyl)-monophosphate; 8-N(CH$_3$)$_2$-AMP, 8-dimethylaminoadenosine 5′-monophosphate; 8-NH$_2$-AMP, 8-aminoadenosine 5′-monophosphate; 8-Br-AMP, 8-bromoadenosine 5′-monophosphate.

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## EXPERIMENTAL PROCEDURE

The synthesis of 8-Br-AMP is carried out in aqueous solution by reaction of bromine with AMP (*1, 2, 5*). 8-Br-AMP is subsequently converted to 8-AHA-AMP (*1, 2, 6*). The following is a revised procedure for this synthesis.

*Synthesis of 8-AHA-AMP.* AMP (5 g, 13.5 mmol) is dissolved in 1 *M* sodium acetate solution (350 ml, pH 3.9) (*1*). Liquid bromine (2.5 ml, 45 mmol) is slowly added dropwise to the rapidly stirring solution. The bromine globules solubilize immediately. After 30 min the reaction is stopped by the addition of carbon tetrachloride and the unreacted bromine is extracted into the organic phase. The aqueous solution containing 8-Br-AMP is further extracted with ether until no further color enters the organic phase. The aqueous phase has a reddish-brown color. The solution is adjusted to pH 9, diluted 40-fold with water, and applied to a column (4 × 60 cm) of DEAE-Sephadex A-25 (formate form) resin. After washing with water, the column is eluted with a linear gradient of 0–0.12 *N* formic acid (formed from 3 liters of each solution). The column is then washed with constant 0.12 *N* formic acid until the 8-Br-AMP with an absorbance maximum at 263 m, is eluted. A yellow peak following the 8-Br-AMP fraction is discarded. The 8-Br-AMP is pooled and lyophilized as a white powder in a yield of 78% (4.4 g).

*Anal.* Calcd for $C_{10}H_{13}N_5O_7PBr \cdot H_2O$ (447.2): C, 27.04; H, 3.41; N, 15.77; Br 17.99. Found: C, 27.10; H, 3.74; N, 15.84; Br, 18.68.

To a 150-ml solution of 1.6 *N* diaminohexane (58 g, 500 mmol) in water, pH 10, is added 8-Br-AMP (4.4 g, 9.8 mmol). The mixture is heated at 60°C in a sealed tube.[4] After 14 hr no further shift of the absorbance maximum from 263 to 280 nm is observed. The mixture is cooled and poured into 750 ml of cold ethanol and left at −10°C overnight. The precipitate is centrifuged and washed with 50 ml of ethanol. For further purification the precipitate is dissolved in 1000 ml of water and the pH is adjusted to 10 with NaOH. This solution is applied to a column (4 × 10 cm) of DEAE-Sephadex A-25 (bicarbonate form) resin. After washing with water, the column is eluted with a linear gradient of 0–0.03 *N* ammonium bicarbonate (each 500 ml), and then by constant 0.03 *M* $NH_4HCO_3$ until 8-AHA-AMP is released. The fractions with an absorbance maximum at 280 nm are pooled and lyophilized. The amount of 8-AHA-AMP obtained was 4.3 g, for a yield of 86%. The overall yield from AMP was 66%.

*Anal.* Calcd for $C_{16}H_{29}N_7O_7P \cdot 3H_2O$ (515.4): C, 37.28; H, 6.66; N, 19.08. Found: C, 33.88; H, 6.61; N, 19.82; Br, 0.

The 8-$NHCH_3$-AMP was synthesized by an analogous procedure using methylamine instead of diaminohexane. The nucleotide 6-AHA-AMP was synthesized as described elsewhere (*7*).

*NMR methods.* $^1H$ NMR spectra were recorded on a Varian HR220 spectrometer and a Nicolet Technology Corp. TT-100 Fourier transform system. Samples were lyophilized from commercial 99.8% $D_2O$ and then dissolved in "100%" $D_2O$. Tetramethylammonium chloride was used as the internal standard $TMA_{int} = DSS_{int} +$ 3.176 ppm. The p*D* values were obtained from pH meter readings by addition of 0.4 units. Peak assignments, chemical shifts, and coupling constants were fit by computer simulation using the Nicolet ITRCL program and the HR220 plotting system. Spin-

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[4] The reaction may alternatively be run at room temperature for 24 hr and at pH 12.

lattice relaxation times ($T_1$) were calculated using the inversion recovery method of Vold *et al.* (*8*). The relaxation rates ($1/T_1$) due to $Mn^{2+}$ ions were calculated by subtracting the relaxation rates measured in the absence of $Mn^{2+}$ ions from those measured in the presence of $Mn^{2+}$ ions.

## RESULTS AND DISCUSSION

### *Conformation of 8-(6-Aminohexyl)-amino-AMP*

Association of a paramagnetic ion at the phosphate group of a nucleotide makes it possible to render the relative distance of various protons from the phosphate group by the measurements of the relaxation rates. From this information predictions can be made on the conformation around the glycosyl bond (*3*, *9*). For example in 8-$SCH_3$-AMP the methylmercapto protons relax less by $Mn^{2+}$ than by adenine H2, indicating that the adenine H2 proton is nearer to the phosphate group, and also that this distance requirement is only fulfilled for the *syn* conformation (*3*).

The effect of added paramagnetic ions on the proton relaxation rates in 8-AHA-AMP solutions has been measured. The ribose 5′ protons were relaxed to a greater degree than any other ribosyl or adenyl proton. This observation is consistent with a strong $Mn^{2+}$ binding site at the phosphate group as is the case in AMP (*3*, *9*, *10*).

The multiplicity of conformational probes on the hexyl chain should make the nucleotide most suitable for paramagnetic studies. It was found that $Mn^{2+}$ relaxes all of the hexylmethylene protons and that the magnitude of the relaxation is greater than that of adenine H2 (Fig. 1). This result is opposite to that reported for 8-$SCH_3$-AMP (*3*).

Further information on the conformation about the glycosyl bond may be obtained from selected chemical shifts and coupling constants. The NMR parameters (Table 1) are obtained by computer simulation of the 220-MHz $^1H$ NMR spectrum (Fig. 2).

The chemical shift of the ribose 2′ proton (*3*, *11–16*) as well as of the 2′ carbon (*17*) can be used as an indicator of the *syn* conformation. For example, in the *syn* nucleotide 8-Br-AMP, the H2′ chemical shift is 0.50 ppm downfield from that in AMP (*3*), which is preferentially in the *anti* form (*3*, *9*, *10*). Molecular orbital computations on the different contributions to the variation in chemical shifts of the protons of the ribose ring have been reported as a function of the angle about the glycosyl bond (*15*). The H2′ chemical shift in 8-AHA-AMP is similar to that in AMP (Table 1).

The conformation of the C4′–C5′ bond is also sensitive to glycosyl conformations. Both magnetic resonance investigations and theoretical calculations have shown that *syn* adenyl nucleotides have low (≈20%) *gauche–gauche* populations about the C4′–C5′ due to repulsion between the adenine base and the phosphate group (*3*). As will be discussed later the *gauche–gauche* population is very *high* in 8-AHA-AMP. These data suggest that very little *syn* conformation is present. Each of the above studies points to the same conclusion that 8-AHA-AMP is preferentially in the *anti* form. The 8-amino and 8-alkylamino nucleotides are exceptions among 8-substituted nucleotides which normally are in the *syn* form (*13*). The preferred conformation about the glycosyl bond of 8-AHA-AMP is the same as that reported for 8-$NH_2$-AMP and 8-$NHCH_3$-AMP (*13*). The nucleosides 8-$NH_2$-adenosine and 8-$NHCH_3$-adenosine have been reported from another laboratory to be in a flexible *syn–anti* equilibrium (*16*). No preferred con-

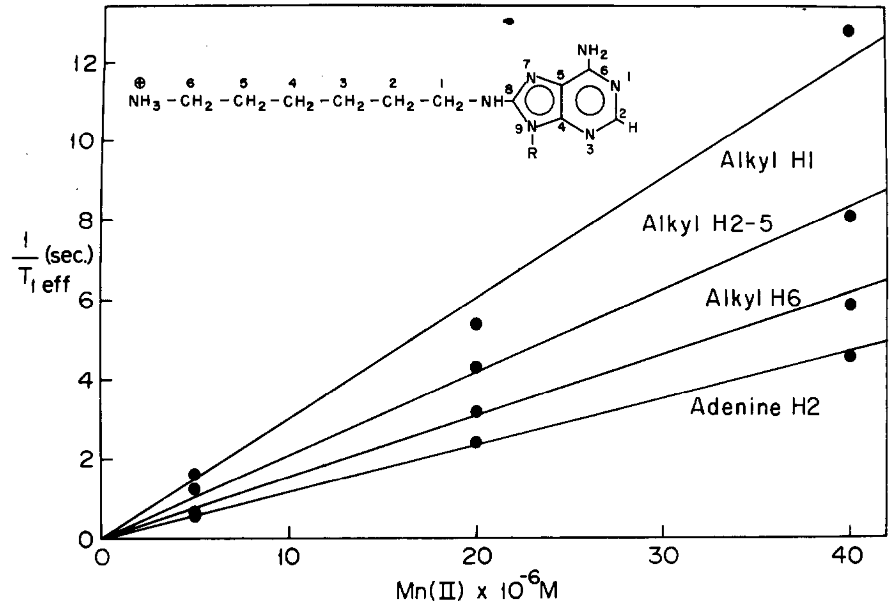


FIG. 1. The effect of $Mn^{2+}$ ion concentration on the spin-lattice relaxation rate for the alkylaminomethylene and adenine H2 protons of 8-AHA-AMP at 0.007 *M*, p*D* 8.5 and 22°C.

formation was suggested for these nucleosides. In 8-$N(CH_3)_2$-AMP (*13*), as well as in its nucleoside (*16*), the *syn* form predominates. Evidence was presented showing that 8-$N(CH_3)_2$-AMP is not locked into the *syn* form, but rather is in equilibrium with a small but biologically significant *anti* conformer population (*13*).

TABLE 1

PROTON NMR PARAMETERS FOR 8-AHA-AMP, 6-AHA-AMP, AND AMP[a]

| | Coupling constants of ribose moiety (Hz)[b] | | | | Chemical shift (ppm) | | |
|---|---|---|---|---|---|---|---|
| | 8-AHA-AMP | 6-AHA-AMP | AMP | | 8-AHA-AMP | 6-AHA-AMP | AMP |
| 1′2′ | 8.0 | 5.9 | 6.0 | 1′ | 2.849 | 2.897 | 2.950 |
| 2′3′ | 5.8 | 5.2 | 5.1 | 2′ | 1.612 | 1.607 | 1.621 |
| 3′4′ | 2.1 | 3.5 | 3.5 | 3′ | 1.298 | 1.315 | 1.327 |
| 4′5′ | 2.7 | 3.2 | 3.2 | 4′ | 1.150 | 1.169 | 1.179 |
| 4′5″ | 2.4 | 3.3 | 3.3 | 5′ | 0.911 | 0.825 | 0.836 |
| 5′5″ | −11.9 | −12.0 | −11.8 | 5″ | 0.793 | 0.805 | 0.806 |
| 4′P | 1.6 | 1.6 | 1.7 | Alkyl 1 | 0.30 | 0.21 | |
| 5′P | 7.2 | 4.5 | 4.6 | Alkyl 2.5 | −1.50 | −1.57 | |
| 5″P | 3.1 | 4.5 | 4.6 | Alkyl 3.4 | −1.77 | −1.85 | |
| | | | | Alkyl 6 | −0.20 | −0.28 | |
| | | | | 2 Adenine 2 | 4.820 | 4.934 | 5.081 |
| | | | | 8 Adenine 8 | | 5.351 | 5.442 |

[a] The concentration of nucleotides was 0.02 *M*, p*D* 8.5, 22°C.

[b] The accuracy of the computer-simulated coupling constants is estimated to be ±0.15 Hz.

Comparison of the individual relaxation rates of the alkyl chain methylene protons yields qualitative information on the orientation of the alkyl chain relative to the phosphate group. $Mn^{2+}$ relaxes the set of methylene protons nearest the adenine ring most efficiently (Fig. 1). The relaxation of the other methylene protons progressively decreases with the terminal methylene proton (Fig. 1) being relaxed the least. It can be concluded that the time-averaged distance from the phosphate group progressively decreases toward the free end of the hexyl chain. This is consistent with the recent report that ionization of the terminal amino group does not effect the conformation of this nucleotide (*18*). In the *syn* conformation, the amino group would be spatially far from the phosphate group and it was proposed that this nucleotide, like other 8-substituted nucleotides, is predominantly in the *syn* form (*18*). Our data are in disagreement with this conclusion. Space-filling models oriented in the *anti* conformation indicate that a very close approach between the positively charged terminal amino group and the phosphate moiety is possible, but such interaction is not significant.

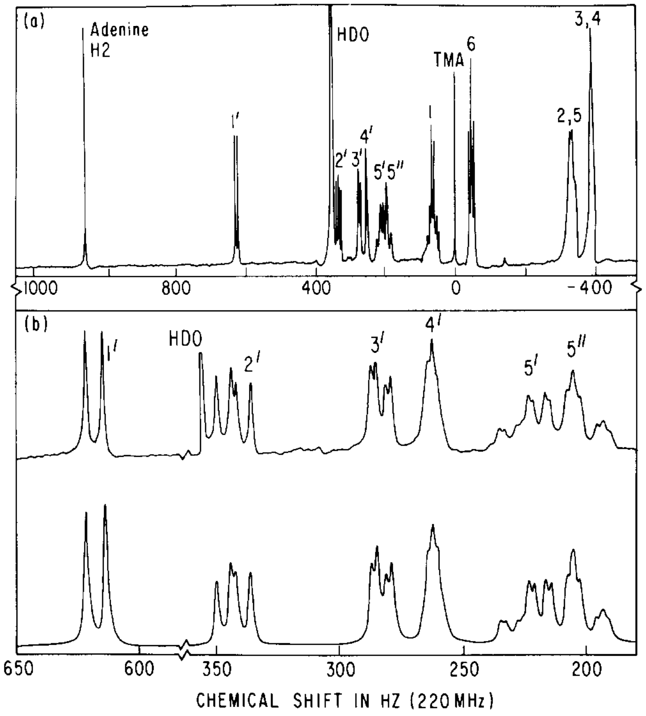


FIG. 2. (a) The 220-MHz $^1H$ NMR spectrum of 0.02 *M* 8-AHA-AMP is obtained in $D_2O$ at p*D* 8.5 and 22°C. The resonances between +100 and −400 H2 labeled from 1 through 6 are assigned to the six sets of alkyl methylene protons (Fig. 1). The resonances between +150 and +650 H2 are the ribosyl protons. (b) An expansion of the NMR spectrum of the ribose region and its computer simulation.

The conformation of the exocyclic C4′–C5′ and C5′–O5′ bonds and of the ribose ring may be treated in terms of a rapid equilibrium between various conformational forms (*3*). Populations of the conformations may be estimated by a Karplus-type analysis of the coupling constants (Table 1). According to procedures described elsewhere (*3*), we estimate that there is 80% *gauche–gauche* form about C4′–C5′, 85% *gauche′–gauche′* form about C5′–O5′, and 80% C2′ *endo* form for the ribose ring. Though the *gauche–gauche* and C2′ *endo* forms are the preferred conformations in naturally occurring ribonucleotides (*19, 20*), they are both 20% higher than normal. The large difference between the values of $J_{H5'-P}$ and $J_{H5''-P}$ (Table 1) indicates a conformation asymmetry about C5′–O5′. This has not previously been detected in other 5′-*β*-nucleotides.

It is of interest to conjecture the causes of the conformation adopted by 8-AHA-AMP. This is aided by comparing the conformation of 8-AHA-AMP with that of 6-AHA-AMP. The alkylamino chain of 6-AHA-AMP is structurally required to be far removed from the ribose moiety regardless of the conformation about the glycosyl bond of 6-AHA-AMP. Hence any differences in conformation should be attributable to interactions between the 8-alkylamino chain and the ribose. The ribosyl coupling constants of 6-AHA-AMP (Table 1) are identical to those reported for AMP (Table 1), which shows identity in their ribose conformations. It is not surprising that conformational adjustments of the ribose moiety occur in 8-AHA-AMP because of the placement of the large hexyldiamino group in proximity to the ribose moiety.

The unusually high C2′ *endo* population of 8-AHA-AMP may be the result of steric interactions. Space-filling models show that there is more room to accommodate a substituent at the 8 position in a C2′ *endo* form than in a C3′ *endo* form of the ribose ring when the orientation about the glycosyl bond is *anti.*

It remains to discuss the exceptionally high *gauche–gauche* population as well as the unique occurrence of *anti* conformation in an 8-substituted nucleotide. A possible factor causing the high *gauche–gauche* orientation about C4′–C5′ is an interaction between the exocyclic $CH_2OPO_3^{2-}$ moiety and the amino group at the 8 position of the adenine ring (N8). These two groups are only in proximity during simultaneous *gauche–gauche* and *anti* conformations. Examination of space-filling models suggests that a hydrogen bond between 5′-oxygen and N8 is structually possible during the *gauche–gauche, anti* conformation.

A closely related interaction would be possible if there existed a formal positive charge on N8. The charge could lead to a strong electrostatic interaction with the nearby phosphate group, which also could explain the presence of both the *anti* and *gauche–gauche* conformations. Measurement of the various ionizations of 8-AHA-AMP, to be discussed below, shows that N8 does not carry a charge under physiological conditions.

### *Ionization Study*

Chemical shift dependence on p*D* measurements on 8-AHA-AMP is complicated by interference from titration of the terminal amino group as well as by nonequivalence of the methylene proton chemical shifts. The nucleotide 8-$NHCH_3$-AMP does not have these limitations. It should be a good substitute for ionization studies in that the proton chemical shifts and coupling constants (*13*) are similar to those of 8-AHA-AMP (Table 1). The ionization profiles for adenine H2 and the methylamino protons of 8-$NHCH_3$-AMP have been measured (Fig. 3). Just as adenine H2 and H8 of AMP are sensitive to ionizations of the adenine ring (*21, 22*), one expects the same for H2 and the methylamino protons in the 8-methylaminoadenine ring. As indicated above, the question of the charge of N8 is of primary interest.

The adenine H2 and H8 chemical shifts of AMP are insensitive to p*D* changes above p*D* 8, except near p*D* 14 at which point the sugar hydroxyl titration (*23*) is detected (Fig. 3). The adenine H2 and methylamino protons of 8-NHCH$_3$-AMP chemical shifts display a similar insensitivity in the range p*D* 8–13. In contrast, we have observed that the methylene protons adjacent to the terminal amino group (alkyl H6) in 8-AHA-AMP undergo a 120-Hz shift in this p*D* range resulting from the titration of the *terminal* amino group (p$K_a \approx 10$). Clearly the 8-amino group in 8-NHCH$_3$-AMP does not titrate in the basic p*D* range as is typical of an aliphatic amine.

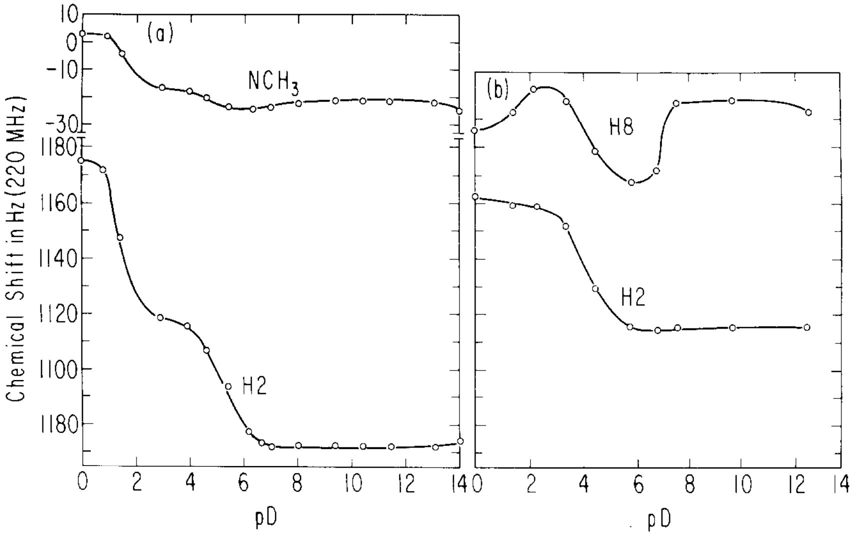


FIG. 3. (a) The chemical shift dependence on p*D* of the methylamino protons and adenine H2 of 0.007 *M* 8-NHCH$_3$-AMP. (b) The chemical shift dependence on p*D* of the adenine H2 and H8 protons of 0.007 *M* AMP.

The chemical shift of the H8 proton of AMP exhibits three different titrations in the p*D* range 0–8 (Fig. 3). These titrations correspond to the primary and secondary phosphate ionizations and the protonation at N1 (*21*, *22*). The phosphate protonations effect the H8 chemical shift by a through-space *shielding* mechanism which may be interpreted to show the presence of the *anti* conformation at high (*21*, *22*) and at low p*D* ranges. The adenine H2 of AMP is apparently too far from the phosphate group to detect the phosphate ionizations (*21*, *22*). Protonation at N1, which has a p$K_a$ of 3.8, causes the 34-Hz deshielding of H8 and the 45-Hz *deshielding* of H2 in AMP (*21*, *22*) (Fig. 3).

The p*D* profiles for 8-NHCH$_3$-AMP (Fig. 3) reveal two separate titrations. The methylamino protons, unlike H8 of AMP, are unaffected by phosphate ionization. For example, in the p*D* range 6–8, the phosphate is being titrated as evidenced by the substantial perturbation of the ribosyl H5′ chemical shifts, yet the methylamino protons as well as H2 are hardly effected (Fig. 3). In the forthcoming discussion, we shall analyze the magnitude and direction of the chemical shift changes in 8-NHCH$_3$-AMP in an attempt to identify the site of the apparent ionizations at p$K_a$ 5.0 and 1.5 (Fig. 3).

A titration with p$K_a$ 5.0 for 8-NHCH$_3$-AMP results in a 45-Hz deshielding of adenine H2 and an 8-Hz deshielding of the methylamino protons upon protonation (Fig. 3). The magnitude of the deshielding of H2 in 8-AHA-AMP equals that caused by N1 protonation in AMP which suggests that N1 is the site of ionization. The small magnitude of the deshielding of the methylamino protons is also consistent with this interpretation because these protons are far removed from N1.

The protonation of 8-NHCH$_3$-AMP which has an apparent p$K_a$ of 1.5 results in a 60-Hz deshielding of H2 and a 20-Hz deshielding of the methylamino protons (Fig. 3).

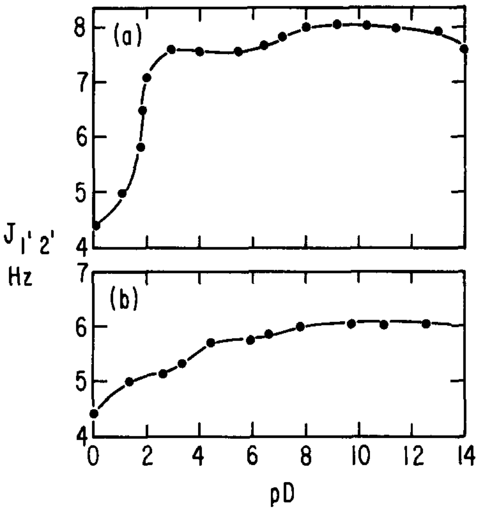


FIG. 4. The $J_{H1'-H2'}$ dependence on p$D$ for 0.007 $M$ 8-NHCH$_3$-AMP (a) and 0.007 $M$ AMP (b).

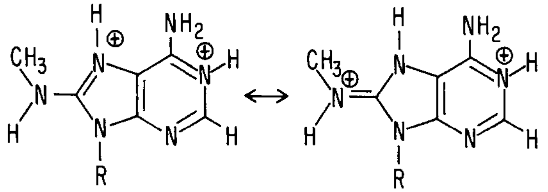
An identical result has been observed in 8-AHA-AMP. Little if any of the *deshielding* is associated with phosphate protonation, as discussed earlier. In fact, in the case of AMP, phosphate protonation causes *shielding* of H8. The deshielding in 8-AHA-AMP is caused by an ionization that is not present in AMP, and this implicates the 8-amino group.

The p$D$ dependence of the ribosyl coupling constant $J_{H1'-H2'}$ has also been measured (Fig. 4), and the results support the suggestion that the p$K_a$ 1.5 titration is associated with the 8-amino group. In AMP, both phosphate protonations as well as the N1 protonation cause small decreases in the coupling constant $J_{H1'-H2'}$(Fig. 4). Likewise in 8-NHCH$_3$-AMP the secondary phosphate protonation and the protonation assigned to N1 cause small decreases in $J_{H1'-H2'}$. However, the p$K_a$ 1.5 titration of 8-NHCH$_3$-AMP causes a substantial decrease in $J_{H1'-H2'}$ (Fig. 4). A first-order estimate of the ribosyl coupling constants at pH 0 indicates a 3.4-Hz decrease of $J_{H1'-H2'}$ and a 2.6-Hz increase in $J_{H3'-H4'}$ compared to the high pH values (Table 1). The greater change in $J_{H1'-H2'}$ could be accounted for by electronegativity changes in the base. The data indicate a large conformational change of the ribose ring. Based on the change in $J_{H3'-H4'}$, it is estimated that the C3′ *endo* population increases by 25–30%. There is a near equal distribution of C3′ *endo* and C2′ *endo* forms in strong acid solution.

The large change is absent in AMP (Fig. 4). The effect on the ribose conformation in 8-$NHCH_3$-AMP may be rationalized on the basis of the proximity of the adenine 8-amino group to the ribose moiety and the subsequent sensitivity to charge associated with the amino group.

Assignment of the site of protonation to the adenine 8-amino group is not consistent with the behavior of the methylamino protons which are deshielded by only 20 Hz (Fig. 2). As was discussed earlier, protonation of an aliphalic amino causes a 120-Hz deshielding of adjacent methylene protons. In addition the 60-Hz deshielding of adenine H2 in 8-$NHCH_3$-AMP (Fig. 3) is more than expected for protonation directly on the distance 8-amino group.

We propose that N7 of the 8-aminoadenine ring is the site of protonation for the $pK_a$ 1.5 ionization. The positive charge could then be stabilized by charge delocalization to the 8-amino group. This helps explain the relatively small deshielding (20 Hz) experienced by the methylamino protons, since the positive charge is delocalized. It also helps to rationalize the sensitivity of the adenine H2 chemical shift to the protonation, since the protonation occurs on the adenine ring.



The $pK_a$ values for 8-$NHCH_3$-AMP compared to those of AMP imply that electronic charge from the 8-amino group is delocalized throughout the purine ring. This is deduced from two observations. (i) The 8-amino group is not titrated in the normal aliphatic amine range of pH 10/11. This is somewhat analagous to the well-known case of aniline, which has a $pK_a$ of 4.6 due to the electron delocalization. (ii) The $pK_a$ of the N1 protonation is 1.2 pH units higher in 8-$NHCH_3$-AMP than in AMP. This is consistent with an increase in electron density in the purine ring as a result of the presence of the 8-amino group. In turn, one may argue that the protonation at N1 greatly effects the $pK_a$ associated with the 8-amino group because of the positive charge delocalization throughout the ring system. Since the purine ring system already carries a positive charge due to N1 protonation, the $pK_a$ of the N7 titration is lowered.

The ionization study reveals that at physiological pH the adenyl base of 8-AHA-AMP is neutral just like that in AMP. In strongly acidic solution the adenyl base of 8-AHA-AMP carries two positive charges. One protonation site is at N1 and the other is probably at N7.

### *Dehydrogenase Binding*

Enzyme binding and its relevance to the purification of dehydrogenases using 8-AHA-AMP may be discussed in terms of the above studies. The charge of the adenine ring should effect the binding affinity since the binding of the adenyl base takes place in a hydrophobic pocket of dehydrogenases (*22–24*). The 8-aminoadenyl part of the nucleotide is neutral in the pH range used in enzyme purifications making it suitable for dehydrogenase binding. A positively charged adenyl base would be expected to interfere with the hydrophobic interaction.

The positive charge at the terminal amino group of the alkylamino chain could decrease binding affinity, but because the charge is removed by eight bonds from the hydrophobic adenyl base, it probably is not a sizable factor. Examination of enzyme kinetic data between 8-AHA-AMP and AMP (*2*) support this view. Shortening of the alkylamino chain brings the charge closer to the adenyl ring. One anticipates that substantial shortening of the alkyl chain would decrease the binding affinity. This proposal can be tested by enzyme kinetic studies on alkyl and alkylamino nucleotides. For enzyme purifications by affinity chromatography, the nucleotide is connected to Sepharose through the terminal amino group by an N-substituted isourea linkage (*27*). A positive charge remains on the terminal nitrogen (*27*).

It is expected that the *anti* conformation preferred by 8-AHA-AMP will also be conserved for 8-AHA-AMP bound to a solid support, since conformation of mononucleotides is conserved in destacked polymers (*19*). The *anti* conformation of adenyl nucleotides has been observed in all cases for various nucleotide–dehydrogenase complexes in the solid state (*25*, *26*). This would seem to make 8-AHA-AMP suitable for binding by dehydrogenases. Recently it has been observed that in aqueous solution the distribution of conformations about the glycosyl bond does not affect the competition of the nucleotide for lactate dehydrogenase (*13*). Thus for 8-AHA-AMP, it is only necessary that the *anti* conformation be accessible in order for the nucleotide to bind efficiently with dehydrogenases.

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