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Mononucleotides in Aqueous Solution: Proton Magnetic Resonance Studies of Amino Groups†

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ABSTRACT: Proton magnetic resonance spectroscopy was applied to mononucleotides to obtain information on hydrogen-bonding properties as well as the rates of proton exchange between 5′-ribonucleotide amino groups and solvent water. Symmetrical acid–base catalysis of exchange rates was observed for GMP, AMP, and CMP. The rates of proton exchange are relatively slow and comparable to those of amides, in contrast to the diffusion-controlled exchange of imino (ring nitrogenous) protons. Chemical shift measurements provide evidence of extensive conjugation of CMP and AMP amino groups into the aromatic rings, since sigmoidal titration curves with midpoints at base pK were observed. In favorable cases, titration at the pK for phosphate ionization could also be monitored. Amino groups of mononucleotides interact with externally added phosphate, as judged from line width and chemical shift changes. This is attributed to the dual role of phosphate, as a proton exchange catalyst and a hydrogen-bond acceptor. From concentration-dependence studies, it appears that the phosphate group of one CMP molecule interacts with the amino group of another. In contrast, little or no association by hydrogen bonding was observed for cytidine. Restricted rotation of amino group was evident not only in cytidine but also in adenosine at acid pH. This implicates N-1 as the site of protonation in AMP. Comparisons with nicotinamide derivatives revealed significant similarities between nucleotide amino and amide protons with respect to restricted rotation and proton exchange characteristics. However, due to extensive charge delocalization into nucleotide amino groups—not evident in NMN^+—the pK for amino protonation might be lower than that for amides. These studies may give a better understanding of the factors that underlie the hydrogen-bonding specificity of nucleotide interaction.

In our previous communication, it was shown that the amino groups of mononucleotides in aqueous solution can participate in hydrogen bonding (Raszka and Kaplan, 1972). Proton magnetic resonance (pmr) spectroscopy revealed that the amino protons give rise to broad spectral lines; this broadening can be related to the lability of amino protons in aqueous solutions. This report examines the pH dependence of amino proton line widths and chemical shifts. Comparisons with amide protons of nicotinamide mononucleotide are undertaken in support of the view that nucleotide amino groups possess a significant amount of amide character.

Pmr chemical shift measurements can yield information on intermolecular and intramolecular association in solution. In the case of nucleotides, chemical shifts of amino protons show sensitivity to the protonation of ionizable groups, particularly base protonation. This is important for pmr studies of nucleotide hydrogen bonding, which rely on relatively small chemical shift changes.

Analyses of pmr line widths of the amino proton resonances can give detailed data on the rates of proton exchange between the amino groups and the solvent. Nitrogenous protons are labile and exchange with water at finite rates. In the region of intermediate exchange rates, amino proton resonances are broad; the contribution of exchange broadening to the line width is additive with that of spin–spin relaxation T_2

$$\pi \Delta \nu_{1/2} = \frac{1}{T_2} + \frac{1}{\tau}$$

where $1/\tau$ is the rate of exchange (Johnson, 1965). A more detailed treatment of line-width dependence, its relationship to acid–base catalytic rate constants, and a discussion of possible mechanisms of nucleotide amino group proton exchange, has already been presented (McConnell and Seawell, 1972).

Figure 1 displays the 220-MHz pmr spectra of the three mononucleotides at pH 7.5 and 0°. In water, the amino proton resonances are broad and sensitive to temperature; at higher temperatures they broaden rapidly and become unobservable above 50–60°. A positive temperature coefficient is characteristic of proton exchange and reflects the activation energy of the exchange process.

The chemical shifts of amino protons also change drastically with temperature, due principally to breaking of hydrogen bonds with water. In favorable cases, temperature-dependence studies can be used to infer intramolecular hy-

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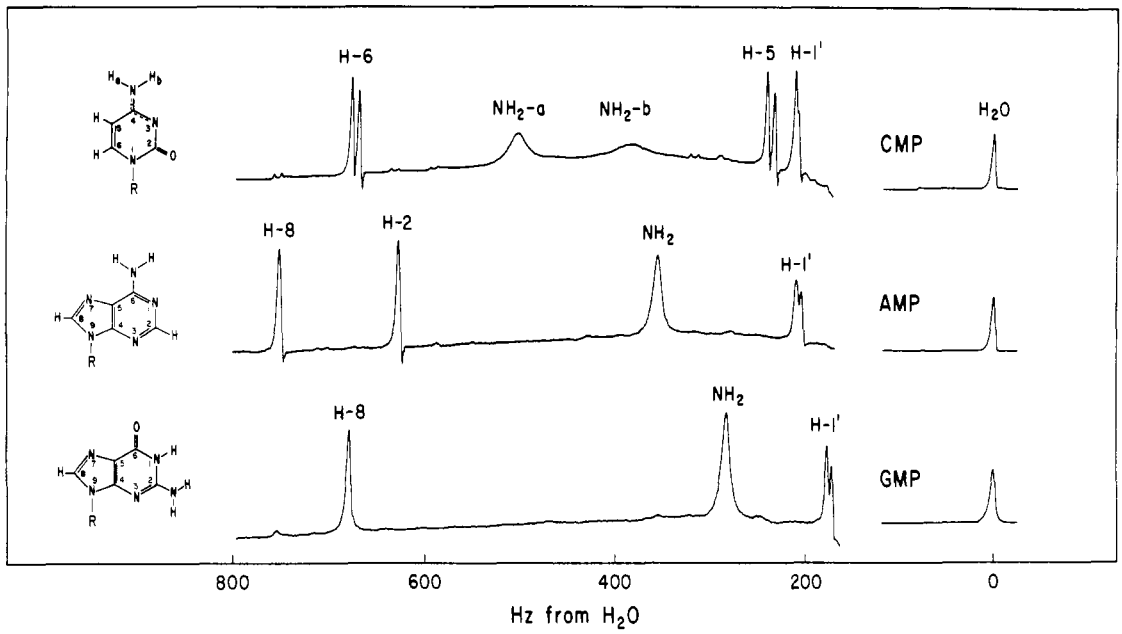

FIGURE 1: Pmr spectra at 220 MHz and 0° of GMP, AMP, and CMP. Each mononucleotide was 0.2 M and at pH 7.5. Bottom scale: Hz from solvent H_2O. The H_2O absorption was recorded at a greatly reduced vertical gain.

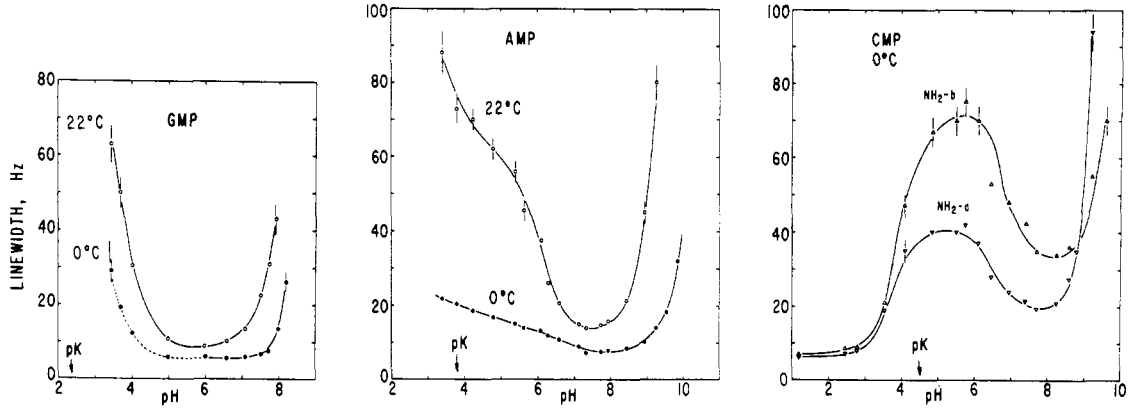

FIGURE 2: Observed line width of amino protons in GMP, AMP, and CMP as a function of pH. Line widths of GMP (0.025 M) and AMP (0.05–0.1 M) are shown at two temperatures, whereas CMP (0.05–0.1 M), whose line width was steeply temperature dependent, is shown only at 0°. Note that restricted rotation of the CMP amino group allows the line width of each amino proton to be monitored. GMP at 0° and below pH 6 forms a transparent gel, indicated in the figure with a broken line.

drogen bonding, as in the case of internal hydrogen bonding between adenine and flavine moieties of FAD (Raszka and Kaplan, 1974).

Materials and Methods

Ribonucleoside 5′-monophosphates were purchased from P-L Laboratories, Inc. in the form of sodium salts with the exception of AMP, acid form, which was neutralized in solution with 6 N NaOH and lyophilized. Mononucleotide stock solutions were prepared volumetrically in distilled water containing 0.001 M DSS[1] (sodium 2,2-dimethyl-2-silapentane-5-sulfonate, Merck), adjusted to desired pH, and pressure filtered through 0.45 μ Millipore filters (Millipore Corp. HATF 01300). Measurements of pH were repeated after spectroscopy.

Spectra were recorded on a Varian HR-220 nmr spectrometer operating in field-sweep mode. In some cases, enhancement of the signal-to-noise ratio was obtained by collecting repetitive scans in a Fabri-Tek time-averaging device. Lower temperatures were obtained with the aid of a Varian variable temperature controller and monitored with a standard methanol sample. Chemical shifts were measured by means of audio frequency side bands and chart calibrations from internal reference DSS.

Results and Discussion

Line Widths of Amino Proton Resonances. Figure 2 shows the experimental line widths of mononucleotide amino protons as a function of pH. The curves are reminiscent of "catalytic catenaries" that are characteristic of acid–base catalysis of proton exchange. Minimum line width of GMP amino protons occurred around pH 6. This pH coincides with that at which gel formation has been reported below 14° (Gellert *et al.*, 1962). According to that report, GMP gel formation (which does not occur at pH 7 or 2) is due to the combined action of hydrogen bonding and stacking of planar, hydrogen-bonded tetramers.

AMP in Figure 2 displays a minimum line width at pH 7.5. At this pH the rate of proton exchange with water is

[1] Abbreviation used is: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

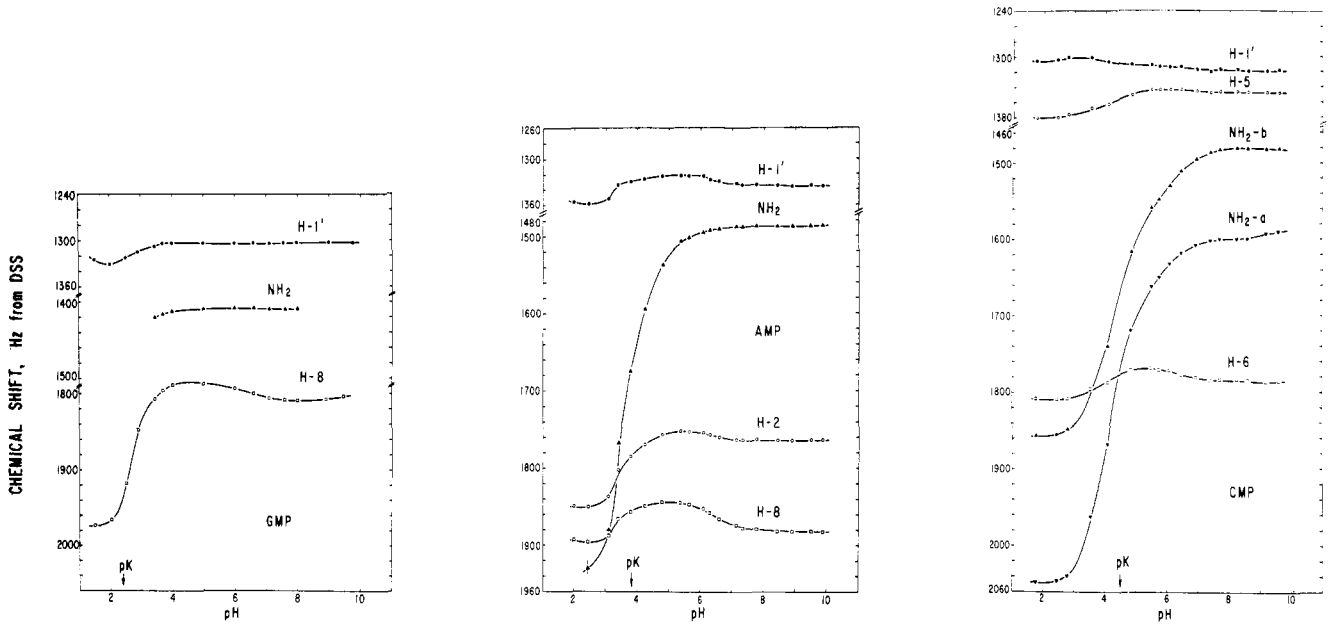

FIGURE 3: Proton chemical shift at 0° of GMP (0.25 M), AMP (0.05–0.1 M), and CMP (0.05–0.1 M) as a function of pH. All solutions were in water containing 1 mM DSS. Chemical shifts in Hz at 220-MHz pmr frequency, measured from DSS as internal reference.

minimal. The line width, and hence the rate, increased as the acidity or basicity of the solution was increased. In particular, the amino proton resonances were very broad in the vicinity of the pK for base protonation. At more acid pH, the amino proton resonances of GMP and AMP were not detectable.

In contrast, CMP amino protons exhibited rather narrow lines at low pH, indicating that base protonation prevents acid catalysis of proton exchange. This might be due to a large amount of positive charge carried by the amino nitrogen of the protonated base, preventing proton exchange catalysis by the hydronium ion, H_3O^+. At neutral pH, CMP amino group showed a high degree of restricted rotation at 0°, resulting in separate proton resonances for the two amino protons (Raszka and Kaplan, 1972); this may be interpreted as evidence of "partial" positive charge localized on the amino nitrogen even in the neutral molecule. The separate proton resonances exhibited different line widths, indicating that proton exchange occurs at unequal rates. Intermediate rates of amino group rotation also contribute to the line widths, but this contribution should be equal for both amino protons.

For the unprotonated molecule, the unequal pH for minimum line width of individual CMP amino protons as well as their unequal sensitivity to catalysis of proton exchange by hydronium–hydroxyl ions, indicates that one of the protons might be more electropositive in character. The NH_2-a proton was more sensitive to base catalysis, suggesting that it carries greater positive charge. Acid catalysis of CMP proton exchange is complicated by the effect of 5′-phosphate, since the line-width maximum at pH 5–6 (Figure 2) was not observed for cytidine.

Adenosine amino line-width dependence at neutral pH resembled that of GMP, whereas at acid pH it was more comparable to CMP. At pH values near zero, adenosine amino protons gave rise to a resolvable resonance (that will be discussed in connection with Figure 4); for AMP under these conditions only a very broad line could be discerned. Hence, the amino protons of AMP in Figure 2 are also under the influence of 5′-phosphate below pH 6. Earlier fits of catalytic rate constants from 100-MHz data (Raszka, 1971; McConnell *et al.*, 1972; McConnell and Seawell, 1972) did not reveal this complication for AMP or CMP, but the use of a 220-MHz spectrometer allows appropriately higher rates of proton exchange to be measured accurately. The earlier results were sufficient, however, to demonstrate that the calculated rate constants for monomers were several orders of magnitude lower than those surmised from experiments with DNA tritium exchange (see, for example, McConnell and von Hippel, 1970).

The observed rate constants differ from those for "normal" amino groups and reinforce the argument that a significant amount of amide character may be present to account not only for the planarity of nucleotide amino groups (Shoup *et al.*, 1972) but also for the relatively slow proton exchange rates. For amides, it is well established that proton exchange with water occurs at slower rates than maximal (diffusional) because amide pK's lie outside the range in which hydronium–hydroxyl catalysis is efficient (Eigen, 1964).

Chemical Shifts of Amino Protons. Pmr studies can provide important insight into the ionization of a molecule, because the resulting changes of charge distribution often drastically alter the diamagnetic environment of protons. Several studies have been carried out on nucleotide bases in deuterium oxide (Jardetzky *et al.*, 1963; Danyluk and Hruska, 1968), and sigmoidal titration curves for the aromatic protons and H-1′ have been related to the protonation of primary phosphate (pK ~ 1), secondary phosphate (pK ~ 6), and protonation of the base (pK = 2–5). These studies have been extended to nucleotide amino groups, as shown in Figure 3. Sigmoidal titration curves are evident for AMP and CMP, with midpoints that closely coincide with the titrimetric pK of the bases. A titration curve for the GMP amino group could not be obtained because of onset of rapid proton exchange near the pK. Gel formation of GMP at 0° limited the solubility, but produced only slight observable broadening of pmr lines.

Despite the sensitivity of amino proton chemical shifts to the titration of the base, the amino groups do not undergo

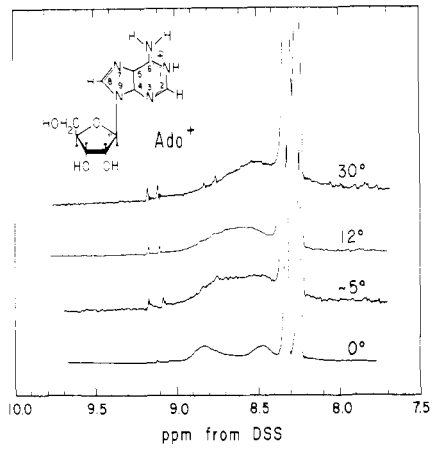

FIGURE 4: Restricted rotation of the amino group of 0.5 M adenosine in 2 N HCl, as a function of temperature. Pmr spectra at 220 MHz; bottom scale applies to lowest trace only.

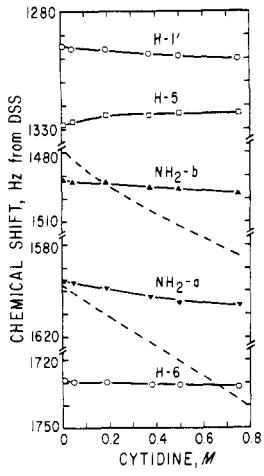

FIGURE 5: Chemical shift at 0° of selected protons of cytidine (—) and CMP (- - -) as a function of concentration, at pH 7.5. Chemical shift, in Hz at 220 MHz, measured from internal reference DSS.

direct protonation to yield $-NH_3^+$ within the usable range of pH (Shimanouchi *et al.*, 1964). The pmr titration behavior can be interpreted as evidence for the extensive conjugation of nucleotide amino groups into the rings, as would be expected from the initial observation of restricted amino group rotation of CMP at neutral pH (Raszka and Kaplan, 1972). Protonated cytidine derivatives are well known to exhibit restricted rotation (Becker *et al.*, 1965), and it becomes necessary to ask whether adenosine derivatives also show effects attributable to the presence of a partial double bond. Figure 4 displays pmr spectra of adenosine in 2 N HCl. The amino group of the protonated nucleoside—not detectable in AMP^+ presumably because of faster proton exchange rates—gave rise to two separate absorptions in the pmr spectrum, due to the individual amino protons. At temperatures around 20°, the rate of amino group rotation exceeded the time resolution of the spectrometer, and the separate proton absorptions coalesced into a single pmr line. At higher temperatures, the combined amino absorption would be expected to become narrow, but as a result of proton exchange with the solvent, it broadened rapidly and was virtually undetectable above about 40° (line width greater than 200 Hz).

No direct evidence of restricted rotation exists for AMP or adenosine at neutral pH. However, N^6,N^6-dimethyladenosine at pH 7.5 evidenced restricted rotation of the dimethylamino group, with a coalescence temperature about 0° (unpublished observations), similar to that reported for 2′,3′-*O*-isopropylidene-N^6,N^6-dimethyladenosine in chloroform (Martin and Reese, 1967). The monomethyl derivative of adenosine is currently under investigation.[2]

It is likely that the amino group of unprotonated adenosine or AMP is conjugated into the ring to some extent, although less so than in the case of cytidine or CMP. Upon protonation of the base, some positive charge is apparently localized on the respective amino nitrogens. This might serve to depress the expected p*K* for protonation of the amino group to $-NH_3^+$.

The similarities in restricted rotation and titration behavior of the amino groups of AMP and CMP might be taken to suggest that protonation occurs in both nucleotides at the endocyclic nitrogen closest to the amino group. Protonation of CMP at N-3 is well established (Becker *et al.*, 1965) but protonation of N-1 is more ambiguous for AMP. Jardetzky and Jardetzky (1960) first reported greater sensitivity of H-2 than H-8 to titration of AMP, also evident in Figure 3, but recent studies employing ^{13}C nmr favor protonation on the imidazole ring of adenosine 5′-triphosphate (Dorman and Roberts, 1970). Protonation at N-7 of GMP was inferred on the basis of a large shift of H-8 with pH (Jardetzky and Jardetzky, 1960; Danyluk and Hruska, 1968), as can be seen also in Figure 3.

The similarities in titration behavior of amino groups of AMP and CMP can be compared with their differences in proton exchange properties, Figure 2. The amino protons of CMP exhibited slow proton exchange at acid pH, a consequence of inability of H_3O^+ to catalyze proton exchange of an amino group that carries significant positive charge. This effect was less pronounced for AMP; in the case of GMP, however, proton exchange was relatively rapid near the base p*K*, and did not decrease at acid pH. Hence, delocalization of positive charge into the amino groups decreases with the p*K* as follows: $CMP^+ > AMP^+ > GMP^+$. This might imply that the positive charge is delocalized throughout the base of AMP^+ rather than constrained to N-1. However, the restricted rotation illustrated in Figure 4 provides evidence that a significant amount of protonation occurs at the N-1 position, in agreement with the conclusions derived from a synthetic study by Jones and Robins (1963).

Phosphate–Amino Group Interaction. Results of several pmr experiments implicate a direct interaction between phosphate and the amino groups of mononucleotides. These observations fall into three groups: (1) titration behavior of 5′-, 3′-, and 2′-nucleotides, as well as nucleosides; (2) concentration dependence of nucleotides and nucleosides; and (3) with externally added phosphate, the effect on chemical shifts and line widths of amino protons. Changes in the chemical shifts of amino protons can reflect, among others, hydrogen-bonding interaction. Phosphate may catalyze proton exchange or it may exhibit a salt effect, producing broadened amino proton lines.[3]

[2] The methyl pmr absorption of N^6-methyladenosine is a doublet, and the amino proton a spin-coupled quartet, around pH 6.5. The multiplets collapse upon addition of either acid or base, confirming acid–base catalysis of proton exchange. Experiments in progress indicate feasibility of quantitating the collapse of methyl doublet, as in the case of *N*-methylamides (Berger *et al.*, 1959), in terms of kinetics of proton exchange.

[3] Proton exchange is only indirectly related to hydrogen bonding between nitrogenous protons and water—a hydrogen-bonded intermediate is envisioned as an obligatory step in the mechanism of diffusional proton exchange (Eigen, 1964).

TABLE I: Phosphate–Amino Group Interaction in Aqueous Solution.

	GMP			AMP				CMP				
	H-8	NH_2	H-1′	H-8	H-2	NH_2	H-1′	H-6	NH_2-a	NH_2-b	H-5	H-1′
Sodium phosphate added												
Interaction shift[a]	+6	−10	+3	+2	+4	−2	+3	+1	−9	−10	−2	−1
Line width increase[b]		4				1			14	30		
Ribose 5-phosphate added												
Interaction shift[a]	+9	−6	+5	+1	+3	−1	0	+3	−12	−13	0	−1
Line width increase[b]		5				1			2	4		

[a] Upfield (+) or downfield (−) pmr shift in Hz at 220 MHz of 0.2 M 5′-ribonucleotides upon addition of 0.2 M sodium phosphate or ribose 5-phosphate, at pH 7.5 and 0°. Chemical shifts were measured from 1 mM DSS as internal reference. [b] Increase in line width of amino protons, in Hz, upon addition of 0.2 M sodium phosphate or ribose 5-phosphate, at pH 7.5 and 0°.

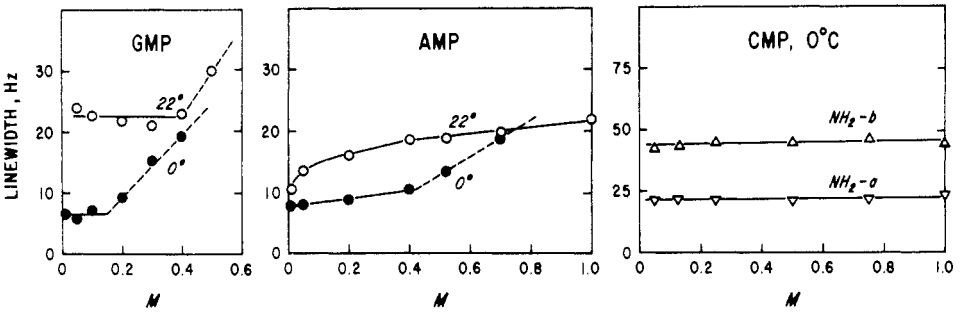

FIGURE 6: Line-width dependence of mononucleotide amino protons as a function of concentration, pH 7.5.

It is perhaps easiest to demonstrate phosphate–amino interaction with externally added sodium phosphate or ribose 5-phosphate. Alternatively, the concentration dependence of a nucleotide can be compared with that of a nucleoside. Only cytidine is sufficiently soluble; coincidentally, only CMP amino protons showed steep concentration dependence of chemical shifts, which we have earlier interpreted in terms of CMP–CMP hydrogen bonding (Raszka and Kaplan, 1972).

Figure 5 displays the chemical shift of representative protons of cytidine and CMP as a function of concentration. The slope of amino protons is widely different, and leads to a novel conclusion: that a significant contribution to CMP self-association involves the hydrogen-bonding interaction between a 5′-phosphate group of one CMP and the amino group of another molecule. Of course, we might expect that phosphate repulsion between CMP molecules might impose a geometry to favor interbase hydrogen bonding, and for cytidine Figure 5 indicates little or no C · C base pairing. However, we can confirm the importance of phosphate–amino group interaction independently, with externally added phosphate.

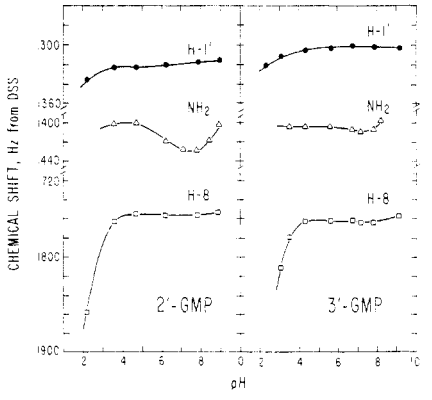

FIGURE 7: Chemical shift at 220 MHz and 0° of selected protons of 50 mM 2′-GMP and 3′-GMP as a function of pH.

Table I lists the interaction shifts (differential chemical shifts) observed upon addition of 0.2 M phosphate (pH 7.5) to solutions of the three nucleotides. Downfield shifts were especially large—and equal—for the separate amino proton resonances of CMP (or cytidine). Line-width measurements indicated that the amino protons exchanged at faster rates in the presence of phosphate.[4] As we have seen earlier, the amino groups of CMP and cytidine carry significant positive charge. It follows that they may show particular sensitivity to interaction with negatively charged phosphate.

The amino protons of mononucleotides showed similar interaction shifts if 0.2 M ribose 5-phosphate—but not D-ribose—was added at pH 7.5. As shown in Table I, only slight increase in the line width of amino protons could be observed upon addition of the sodium salt of ribose 5′-phosphate, in contrast to sodium phosphate. Alternative experiments confirm that ribose-bound phosphate at pH 7.5 is a poor catalyst of proton exchange, since the amino protons of CMP or AMP exhibited little or no line-width increase with concentration, as shown in Figure 6. The line-width increase noted for GMP, particularly at 0°, can be attributed to the formation of a soluble complex (Raszka and Kaplan, 1972).

The effects of externally added phosphate provide ample

[4] Since large amounts of externally added agents were required to produce an increase in amino proton line widths, it is likely that phosphate, as well as imidazole (Raszka, 1971) and aliphatic amines (unpublished observations) are not true catalysts of proton exchange in this case, but instead exhibit a salt effect (Bell, 1973).

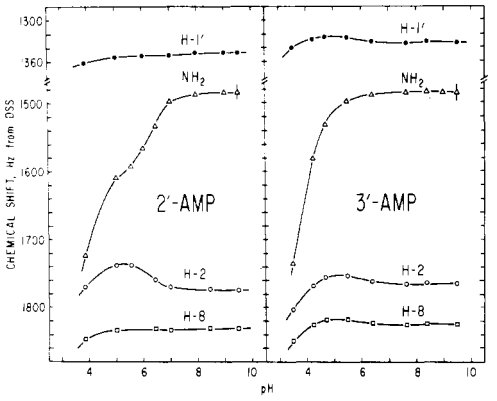

FIGURE 8: Chemical shift at 220 MHz and 0° of selected protons of 0.1 M 2'-AMP and 3'-AMP as a function of pH.

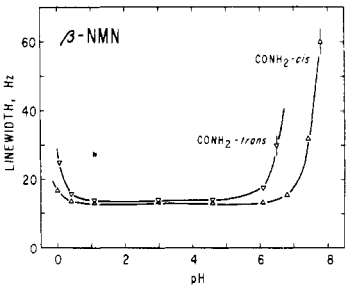

FIGURE 9: Observed line width at 21° of amide protons in 0.1 M NMN^+ as a function of pH. Restricted rotation of the amide group gives rise to separate signals for the individual cis and trans (to keto) protons.

evidence for intermolecular phosphate–amino group interaction. In favorable cases, we can find evidence for intramolecular interaction by focusing on the effect of nucleotide phosphate titration on the chemical shift of amino protons. Figure 7 displays the chemical shift of various protons in 2'- and 3'-GMP as a function of pH. In contrast, the amino protons of 5'-GMP showed no chemical shift variation at pH near the pK for secondary phosphate ionization (Figure 3).

Parallel results for 2'- and 3'-AMP are shown in Figure 8. The amino groups of 2' nucleotides were most sensitive to phosphate titration near pH 6, although close proximity is possible only in 2'- and 3'-GMP. Electrostatic field effects should be distance-dependent for mononucleotides, as has been discussed by Schweizer *et al.* (1968) and Danyluk and Hruska (1968). It is impossible at present to preclude intermolecular interactions on one hand and internal through-bonds polarization on the other. A special case of intramolecular hydrogen bond between the phosphate and an amide proton of NMN^+ was presented in a recent publication (Raszka, 1974).

Nicotinamide Mononucleotide. Useful insight into the nature of ribonucleotide amino groups[5] can be obtained from a comparison with derivatives of nicotinamide. Aliphatic amides have been the object of numerous studies, and pmr spectroscopy was found particularly useful in investigations of amide restricted rotation, proton exchange, and hydrogen bonding in aqueous solution. Unlike aliphatic amides, nicotinamide nucleotides offer the opportunity to study two unique model amides—NMN^+ with protonated and NMNH with unprotonated heterocyclic base.

A salient similarity is apparent from the pmr spectra of NMN^+ and CMP^+: both nucleotides show a high degree of restricted rotation about the exocyclic C–N bond. Specifically, separate pmr absorptions were observed for the two nitrogenous protons. On the other hand, both NMNH and CMP exhibited intermediate rates of rotation, with a coalescence temperature about 10° for NMNH (Raszka, 1974) and about 30° for CMP at neutral pH (Raszka and Kaplan, 1972). Above the coalescence temperature, the rotation rate is fast enough, on the pmr time scale, to average out any differences in diamagnetic environment of the individual protons. Consequently, a single pmr absorption is observed, with intensity equal to two protons, as in the case of the amino groups of AMP and GMP in Figure 1.

The parallel with nucleotide amino groups extends to nicotinamide proton line widths; as shown in Figure 9, acid-base catalysis of proton exchange is evident for the individual amide protons of NMN^+. The two protons exhibited unequal sensitivity to catalysis—also observed in the model compound 1-methylnicotinamide—as did the separate amino protons of CMP (Figure 2). However in NMN^+ (or DPN^+) the amide protons became unobservably broad around neutral pH, whereas in NMNH (or DPNH) the line widths remained narrow up to pH 9–10. This could be an indirect effect due to positive charge, because catalysis by a hydroxyl ion may be facilitated if the carboxamide is attached to a positively charged pyridinium ring.

However, there is no evidence that the positive charge is delocalized into the amide group of NMN^+. Chemical shift measurements indicated that the amide protons were not deshielded significantly in the positively charged 1-methylnicotinamide compared with uncharged nicotinamide. Further, at very low pH, Figure 9 shows that catalysis by hydronium ion was observed for NMN^+, whereas no linewidth increase was seen in 2 N HCl for the amino protons of CMP^+ (unpublished data). The extent of charge delocalization will influence the pK for NH_2 protonation in nucleotides. We might expect that this pK will be significantly lower than for analogous N-protonation of amides [pK of −7.8 was computed by Molday and Kallen (1972) for *N*-methylacetamide].

The amide-like properties of nucleotide amino groups were illustrated by restricted rotation and proton exchange behavior. The amino groups show sensitivity to ionization of the heterocyclic base or ribose-bound phosphate; they also interact with externally added phosphate. These factors may have bearing on the specificity of hydrogen bonding that involves nucleotides and polynucleotides in aqueous solution.

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[5] Similar observations were reported in a recent pmr study of thiamine and thiamine pyrophosphate (Suchy *et al.,* 1972). In thiamine the amino group is ortho to an endocyclic ring nitrogen, comprising an aromatic amidine structure, as in the case of ribonucleotides.

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The Enzymatic Synthesis of *N*-(Purin-6-ylcarbamoyl)threonine, an Anticodon-Adjacent Base in Transfer Ribonucleic Acid†

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ABSTRACT: Almost all *Escherichia coli* tRNAs which have codons with a 5′-adenosine have an unusual nucleoside adjacent to the 3′ end of the anticodon the systematic name of which is *N*-[*N*-(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine (t^6A). An enzyme has been isolated from *E. coli* which can synthesize this nucleoside in tRNA when provided with a tRNA deficient in t^6A and with L-threonine, bicarbonate, ATP, and Mg^{2+}. The t^6A-deficient tRNA was isolated from a threonine-starved culture of an *E. coli* strain which is a threonine auxotroph and is also a relaxed control mutant (capable of synthesizing RNA in the absence of protein synthesis). The t^6A-deficient tRNA was treated with periodate to destroy the 3′-ribose so that the enzyme assay could depend upon the incorporation of labeled L-threonine into the tRNA to form t^6A without interference by the threonine incorporating activity of the *E. coli* threonyl-tRNA synthetase. Using this assay, an enzyme was purified from *E. coli* extracts. It catalyzed the bicarbonate- and ATP-dependent incorporation of L-threonine into t^6A-deficient tRNA. The product of the reaction was established as t^6A by comparison with an authentic sample. The same purified enzyme could incorporate glycine in place of threonine into t^6A-deficient tRNA. Others had previously found a small amount of the glycine analog of t^6A in tRNA; the present findings indicate that a single enzyme incorporates threonine and glycine into the same tRNA species. Since glycine competes with threonine, the tRNA synthesized in threonine-deficient cells would have more than the normal content of the glycine analog of t^6A. This could explain why the t^6A-deficient tRNA used here as substrate was found to accept much less threonine than was predicted from the amount of tRNA that was synthesized during the threonine starvation.

Transfer RNAs whose codons have a 5′-adenosine usually bear in the position adjacent to the 3′-uridine of the anticodon an unusual nucleoside containing a threonine residue (Takemura *et al.,* 1969; Ishikura *et al.,* 1969; Kimura-Harada *et al.,* 1972a; Powers and Peterkofsky, 1972b). The structure of this nucleoside was determined by Chheda *et al.* (1969) and Schweizer *et al.* (1969) to be *N*-[*N*-(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine (t^6A).[1]

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[1] Abbreviations used are: t^6A, *N*-[*N*-(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine or the simpler and more descriptive name suggested by Dr. W. E. Cohn, Office of Biochemical Nomenclature, N^6-(*N*-threonylcarbonyl)adenosine; t^6Ade, *N*-(purin-6-ylcarbamoyl)-threonine or N^6-(*N*-threonylcarbonyl)adenine; SD enzyme, total cell soluble proteins freed of low molecular weight material and of nucleic acids by passage through Sephadex G-25 and DEAE-cellulose columns; rel^-, a mutation in *E. coli* which allows RNA synthesis to occur in the absence of protein synthesis.