B. T. Keeler, and C. A. Birkhimer, *J. Am. Chem. Soc.*, **86**, 2478 (1964); D. P. Gross, G. W. Renner, R. C. Sheppard, and C. E. Stehr, *J. Chem. Soc.*, 2143 (1963).
(15) J. Karle and I. Karle, *Acta Crystallogr.*, **21**, 849 (1966).
(16) Initially pointed out by Y. Okaya, Y. Saito, and R. Pepinsky, *Phys. Rev.*, **98**, 1857 (1955).
(17) It is noteworthy that the first form of thiostrepton was tetragonal (ref 3) before it was isolated in the simple monoclinic form which permitted solving of the Patterson function.
(18) G. Germain, P. Main, and M. M. Woolfson, *Acta Crystallogr., Sect. A*, **27**, 368 (1971).
(19) C. Riche, *Acta Crystallogr., Sect. A*, **29**, 133 (1974); C. Riche, DEVIN program, Report C1, Institut de Chimie des Substances Naturelles, Gif, 1973.
(20) Diffusion factors were taken from "International Tables for X-Ray Crystallography", Vol. III, Kynoch Press, Birmingham, England, 1962.
(21) W. R. Busing, K. O. Martin, and H. A. Levy, Oak Ridge National Laboratory Report, ORNL-305, 1962. We used a modified version of the program OFRLS.
(22) D. E. Pilling, D. W. Cruickshank, A. Bujosa, F. M. Lovell, and M. R. Truter, "Computing Methods and the Phase Problem in X-Ray Crystal Analysis", Pergamon Press, Oxford, 1961, p 32.
(23) Anisotropic factors are of the form exp-($\beta_{11}h^2 + \beta_{22}k^2 + \beta_{33}l^2 + 2\beta_{12}hk + 2\beta_{13}hl + 2\beta_{23}kl$) and given in supplementary material.
(24) See paragraph at end of paper regarding supplementary material.
(25) See, e.g., M. J. Buerger in "Crystal Structure Analysis", 2nd ed, Wiley, New York, N.Y., 1967, p 542.
(26) E. T. Fossel, W. R. Veatch, Y. A. Ovchinnikiv, and E. R. Blout, *Biochemistry*, **13**, 5264 (1974); K. J. Rothschild and H. E. Stanley, *Science*, **185**, 616 (1974).
(27) K. Maeda, H. Kosaka, K. Yagishita, and H. Umezawa, *J. Antibiot., Ser. A*, **9**, 82 (1956).
(28) F. R. Ahmed, S. R. Hall, M. E. Pippy, and C. P. Huber, "NRC Crystallographic Programs for the IBM 360 System", 2nd ed. World List of Crystallographic Computer Programs, 2nd ed. Appendix, 1966, p 52.
(29) The closest equivalent feature we have compiled is the SO bridge in the macrocyclic poisonous compound α-amanitin, in which the sulfur atom is also included in a modified cystein residue.

The Protonation Site of Vitamin B_1 as Determined from Natural-Abundance ^{15}N Nuclear Magnetic Resonance Spectra[1]

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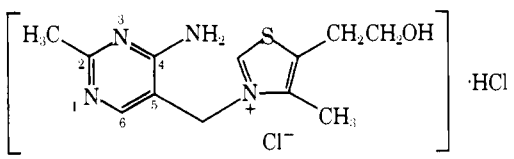
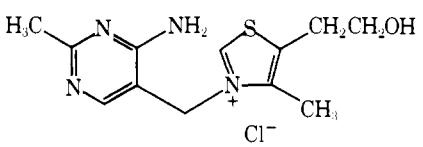
Abstract: Natural-abundance nitrogen-15 nuclear magnetic resonance spectroscopy has been used to determine the site of protonation in vitamin B_1. Selectively decoupling individual protons of the vitamin in water, D_2O, and ethylene glycol permitted assignment of the nitrogen resonances and thence the determination of the site of protonation, which was found to be N-1 of the pyrimidine ring.

Because of its important biological function, vitamin B_1 (thiamine hydrochloride, **1**) has been the subject of many



vitamin B_1 **1**

studies.[2] A problem that has never been solved is which nitrogen is protonated when thiamine (**2**) reacts with hydrogen



thiamine **2**

chloride. Common representations of the vitamin show protonation of the vitamin on N3 or the $-NH_2$ group.[3] These representations are used despite the spectroscopic studies of aminopyrimidines in acid solutions by Brown and co-workers,[4] from which it has been predicted that protonation of **2** would be most favorable on N1.[2] With the advent of practical natural-abundance nitrogen-15 spectroscopy, it should be possible to resolve the protonation question by examining the nitrogen chemical shift changes in going from thiamine to the conjugate acid.

Experimental Section

Proton spectra were taken using a Varian A-60 NMR spectrometer. Spectra in water and D_2O solutions used sodium 3-(trimethylsilyl)propanoate as reference, while those in ethylene glycol employed Me_4Si reference. No concentration dependence of the shifts was noted in dilute water solutions.

Nitrogen-15 spectra at 18.25 MHz were taken with a Bruker WH-180 NMR spectrometer using an external $H^{15}NO_3/H_2O$ capillary reference. Because of the poor solubility of thiamine in water, the protonation studies employed 0.9 M solutions in ethylene glycol with an internal Me_2SO-d_6 lock. Selective decoupling of vitamin B_1 was carried out in 2 M solutions in water with internal D_2O lock, or 2 M solutions in D_2O (solvent lock). The selective decoupling with thiamine employed a 1 M solution made up of 5.0 g of vitamin B_1, 13 mL of ethylene glycol, 1.0 mL of Me_2SO-d_6 (internal lock), and 0.75 mL of 18.3 M aqueous NaOH.

Results and Discussion

The ^{15}N NMR spectrum of thiamine hydrochloride in water solution taken at the natural-abundance level of ^{15}N without proton decoupling is shown in Figure 1. Four well-separated resonances were observed: singlets at 136, 166, and 208 ppm and a triplet at 268 ppm. The triplet (J = 91 Hz) can be assigned with certainty to the $-NH_2$ nitrogen (see Figure 1). If this nitrogen were protonated, its resonance should not be a triplet but a quartet. Furthermore, when the spectrum was run with broad-band proton decoupling, two of the nitrogen resonances had large nuclear Overhauser enhancements (NOE) and strong negative intensities as expected for *two* different nitrogens with attached protons. Finally, the nitrogen resonance which changed positions most prominently on addition of alkali was not the triplet at 268 ppm, but the broad singlet at 208 ppm (see Table I).

It should be noted that the addition of 1 equiv of base will result in deprotonation of **1** on the pyrimidine ring.[4] The pK_a of this titration is 4.85 (at 25 °C).[2] The second titration step of thiamine requires 2 equiv of base and occurs at pK_a =

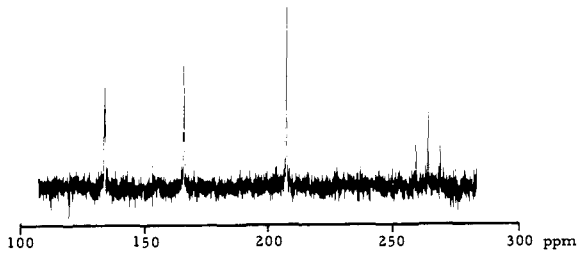


Figure 1. The coupled ^{15}N spectrum of vitamin B_1 (1).

Table I. Shifts of ^{15}N Resonances on Addition of Base to Thiamine Hydrochloride (1) in Ethylene Glycol

	Equiv of NaOH added			
	0	0.25	0.75	1.0
^{15}N shifts[a]	130.5	131.2	132.8	134.0
	164.7	163.9	162.8	161.8
	208.8	189.3	(159)[b]	(135)[b]
	266.8	272.2	280.8	288.0

[a] In parts per million upfield from nitric acid. [b] These peaks were broad and partially overlapped other resonances at this degree of neutralization of 1.

Table II. Half-Widths of ^{15}N Signals of Thiamine Hydrochloride

Solvent	Proton decoupling target	Proton frequency decoupled, ppm	Half-widths in Hz of ^{15}N resonances		
			136 ppm	166 ppm	208 ppm
H_2O	None		10.0	7.5	7.5
	Methyls	2.6	7.0	5.5	5.0
	Pyrimidine proton	8.1	8.0	7.0	7.0
	Thiazole proton	9.6	8.0	7.0	7.0
D_2O	None		7.0	7.5	8.5
	Methyls	2.6	3.5	6.5	5.0
	Pyrimidine proton	8.1	6.0	7.0	6.0

9.2-9.3.[2] Because the ambient pH of the aqueous solution was 2.1, we could be sure that the shift of the signal at 208 ppm was due to deprotonation of the pyrimidine ring.

The problem of assigning the other resonances is not a trivial one and was attacked by selective proton decoupling of 1 in D_2O and water. The results of these experiments are summarized in Table II. Assignment of the thiazole nitrogen to the resonance at 136 ppm was the most straightforward. A solution of 1 in D_2O showed the expected deuterium exchange with the thiazole C-H proton[5] through disappearance of the ^1H NMR absorption at 9.6 ppm. The ^{15}N spectrum of a corresponding D_2O solution showed a marked sharpening of the absorption at 136 ppm with the decoupler off compared to the absorption in an H_2O solution, also with the decoupler off. The effect of deuterium exchange on this nitrogen resonance was essentially the same as decoupling the proton resonance at 9.6 ppm in water solution. In addition, decoupling the pyrimidine ring proton attached to C6 in D_2O had little effect on peak width at half height (Figure 2). The assignment of the 136-ppm peak to the thiazole nitrogen is also consistent with the fact that this absorption showed only a 4-ppm shift during protonation.

The most difficult and important assignments are of N1 and N3 (one of which is protonated) to either the 166 or 208 ppm resonances. The only decoupling which significantly affected the nitrogen signal at 166 ppm, in either D_2O or water, was decoupling of the methyl group protons. Deuterium exchange

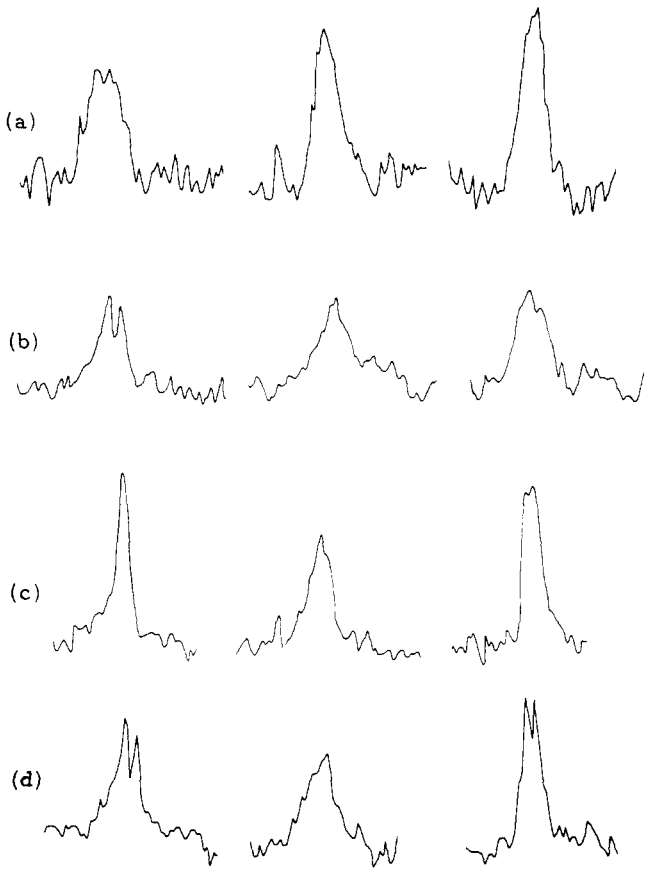


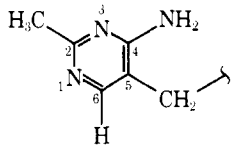
Figure 2. Selective proton decoupling experiments of 1 showing the line shapes of the ^{15}N absorptions at 136, 166, and 208 ppm, respectively, with (a) no decoupling, in water solution; (b) no decoupling, in D_2O solution; (c) both methyl groups decoupled, in D_2O solution; (d) pyrimidine proton at C6 decoupled, in D_2O solution.

did not affect this absorption, which suggests that this absorption is the one corresponding to N3 (Figure 2). The absorption at 208 ppm (the protonated nitrogen) is broadened slightly in D_2O, possibly because of quadrupole-induced relaxation when the nitrogen becomes deuterated. In D_2O, selective proton decoupling of the pyrimidine proton at C6 causes sharpening of the nitrogen signal at 208 ppm (Figure 2), which is consistent with the assignment of this resonance to the conjugate acid at N1.

Additional support for the assignment of the 208-ppm resonance to N1 can be derived from the pyrimidine-ring nitrogen shifts of thiamine (not the protonated form) with the shifts predicted using substituent effects which have been obtained for pyridines.[6] Neutralization of thiamine hydrochloride causes the 209-ppm resonance to shift to about 135 ppm and the 165-ppm resonance to shift to 162 ppm. These are the ^{15}N shifts to be correlated with the predicted values. Pyrimidine in dimethylformamide solution has a chemical shift of 82 ± 2 ppm,[7] and we augment this value with the known ^{15}N shift changed for substitution in pyridine as shown in Table III. Although small solvent effects may be observed, they will be small with respect to the protonation shifts. The observed shifts compare favorably with the predicted shifts.

In ^{15}N experiments involving pyridine and pyrimidine hydrochloride Lichter and Roberts[8] found that the N to α H couplings were much larger in pyridine than in its protonated form. These experiments suggested that neutralization of thiamine hydrochloride could result in a resolvable doublet for whichever resonance is that of N1. Incomplete neutralization of the vitamin in ethylene glycol did not give a clear doublet as was predicted; however, the resonance at 156 ppm (shifted from 209 ppm) was substantially broadened, while the reso-

Table III. Predicted Chemical Shifts for N1 and N3



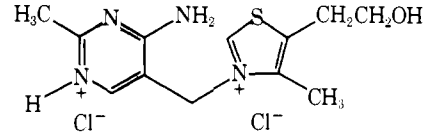
N1		N3	
Pyrimidine	82 ppm	Pyrimidine	82 ppm
2-CH_3	9	2-CH_3	9
4-NH_2	42	2-NH_2	65
3-CH_3	5	3-CH_3	5
Predicted N1 shift	138 ppm	Predicted N3 shift	161 ppm

Table IV. Half-Widths of ^{15}N Signals of **1** in Ethylene Glycol after Incomplete Neutralization

Proton decoupling target	Proton frequency decoupled, ppm	Half-widths in Hz of ^{15}N resonances		
		133 ppm	156 ppm	162 ppm
None		8.5	13.0	7.0
Pyrimidine proton	8.1	8.5	5.5	5.5
Thiazole proton	9.6	6.0	17-22	4.5

nance at 163 ppm (shifted from 165 ppm) was not. Selective proton decoupling of the pyrimidine C6 hydrogen sharpened the resonance at 156 ppm (see Table IV), which is in agreement with the assignments previously made using data from water and D_2O solutions of the hydrochloride.

The data in Table I show that the nitrogen signal which shifts the most on protonation of **1** is the signal at 209 ppm. With the assignment of this resonance to N1, thiamine hydrochloride, vitamin B_1, is properly represented as **3.**



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Why thiamine protonates on N1 is not so clear. Adenine, with a somewhat similar arrangement of aminopyrimidine nitrogens, apparently protonates at its ring nitrogen (now N1) next to the $-NH_2$ group.[9] An important effect is possibly the C–N inductive effect and, in this connection, it is probably significant that 2-aminopyridine is a much weaker base than 4-aminopyridine.[10]

References and Notes

(1) Supported in part by the National Science Foundation and by the Public Health Service, Research Grant 11072 from the division of General Medical Science.
(2) For a general review see (a) *Annu. Rev. Biochem.*, **38,** 213 (1969); or for a review of enzyme action see (b) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms", Vol. 2, W. A. Benjamin, New York, N.Y., 1966, Chapter 8.
(3) See for example "The Merck Index", 8th ed, Merck and Co., Rahway, N.J., 1968.
(4) D. J. Brown, E. Hoerger, and S. F. Mason, *J. Chem. Soc.*, 4035 (1955).
(5) (a) R. Breslow, *J. Am. Chem. Soc.*, **79,** 1762 (1957); (b) *ibid.*, **80,** 3719 (1958).
(6) M. Witanowski, L. Stafaniak, and H. Januszewski in "Nitrogen NMR", M. Witanowski and G. A. Webb, Ed., Plenum Press, New York, N.Y., 1973, pp 222–223.
(7) Reference 6, p 220.
(8) R. L. Lichter and J. D. Roberts, *J. Am. Chem. Soc.*, **93,** 5218 (1971).
(9) See V. Markowski, G. R. Sullivan, and J. D. Roberts, *J. Am. Chem. Soc.*, **99,** 714 (1977), for references and discussion.
(10) A. Albert, R. Goldacre, and J. Phillips, *J. Chem. Soc.*, 2240 (1948).

Magnetic Resonance Studies of Copper(II) Interaction with Nucleosides and Nucleotides

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Abstract: Complexes of copper(II) with the four common nucleosides have been studied by ESR and NMR at different pHs in water–Me_2SO solutions. For comparison some nucleotide complexes of copper were also studied. These studies provide information about the different types of complexes which can be formed and the effect of pH on the mode of interaction of the bases with copper. In the pH range between 8 and 12 copper ions are able to discriminate between 5′-ribonucleotides and 5′-deoxynucleotides. The information derived from these studies may be useful in understanding the role which copper ions play in certain enzymatic reactions and in metal polynucleotide interactions. These studies also suggest that copper(II) may be able to stabilize some very unusual polynucleotide structures.

Divalent metal ions have pronounced effects on the properties of polynucelotides. Magnesium, the divalent metal ion usually found in relatively high concentrations in vivo, stabilizes both DNA and RNA against thermal denaturation and in many cases is required for these molecules to function properly.[1,2] Mn^{2+} also stabilizes DNA and RNA and may substitute for Mg^{2+},[3] although with certain enzymes (e.g., reverse transcriptase, RNA and DNA polymerases) the replacement of magnesium by manganese induces translational errors.[4] In contrast to Mg^{2+} and Mn^{2+}, which stabilize secondary structures in DNA and RNA, copper ions destabilize DNA and RNA double helices,[5-7] and this is attributed to the ability of copper to bind to the nucleic acid bases.[5,7] Because of this special property we were interested in exploring the use of copper(II) as a probe of certain structural features in both RNA and DNA molecules. For example, tRNA molecules contain looped out regions[8] which might be expected to bind copper rather strongly, and this might be exploited in electron and nuclear magnetic resonance studies of these molecules. Certain special regions of the DNA (e.g., operator regions)