Carbon 13 Magnetic Resonance Studies of DL-2-(α-Hydroxyethyl)thiamin and Related Compounds

RELATION OF KINETIC ACIDITY TO ELECTRONIC FACTORS IN THIAMIN CATALYSIS*

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Carbon 13 NMR spectra have been obtained for aqueous solutions of DL-2-(α-hydroxyethyl)thiamin, DL-2-(α-hydroxybenzyl)thiamin, DL-2-(α-hydroxybenzyl)oxythiamin, and related N-3 methyl and N-3 benzyl analogs. The unusually large downfield shift of the 13C resonance of C-2 of hydroxyethylthiamin suggests that this carbon bears a partial positive charge. This result stands in contrast to results of x-ray crystallographic studies of hydroxyethylthiamin, which place a partial negative charge on C-2 (Pletcher, J., and Sax, M. (1974) J. Am. Chem. Soc. 96, 155-165). A partial positive charge on C-2 helps to explain the facility of carbanion formation at the α carbon both enzymatically and in model systems. The rates of proton-deuteron exchange of (C-α)-H with solvent deuterium, and of release of aldehyde to regenerate thiamin have been measured for hydroxyethylthiamin and analogs. The differences in kinetic acidity of (C-α)-H and of rates of aldehyde release are rationalized in terms of differing electron-withdrawing abilities of the substituents attached to N-3, and appear not to be related to intramolecular basic catalysis of these processes by the C-4' amino group.

Since Breslow (1, 2) proposed the mechanism of thiamin action which is broadly accepted today, numerous attempts have been made to correlate the catalytic efficiency of thiamin in each step of the mechanism with a description of the electronic structure of this catalyst, but these attempts have been only modestly successful. Molecular orbital calculations of the thiamin rings by Pullman and Pullman (3, 4) placed a partial negative charge on C-2 of the thiazolium ring of thiamin and suggested that the amount of electron excess on C-2 plays an important role in determining the ease of deprotonation of thiazolium salts. When the relative order of kinetic acidity of the C-2 protons of thiazolium and oxazolium salts were found to be opposite to that predicted (5), revised molecular orbital calculations were undertaken by Collin and Pullman (6), and a partial positive charge was placed on C-2. Neither the original nor revised molecular orbital calculations of π charge densities showed any correlation with 13C chemical shifts (7), as opposed to the rough correlation between these parameters found for purine and pyrimidine nucleosides (8). However, more recent CNDO/2 and extended Hückel molecular orbital calculations (9) of net atomic charges give values which correlate more successfully with 13C chemical shifts.

Since Breslow (1, 2) proposed the mechanism of thiamin action which is broadly accepted today, numerous attempts have been made to correlate the catalytic efficiency of thiamin in each step of the mechanism with a description of the electronic structure of this catalyst, but these attempts have been only modestly successful. Molecular orbital calculations of the thiazolium ring of thiamin and related compounds have been undertaken by Pullman and Pullman (3, 4) and by Collin and Pullman (6), and a partial positive charge was placed on C-2 of the thiazolium ring of thiamin. The differences in kinetic acidity of (C-α)-H and of rates of aldehyde release are rationalized in terms of differing electron-withdrawing abilities of the substituents attached to N-3, and appear not to be related to intramolecular basic catalysis of these processes by the C-4' amino group.

From x-ray crystallographic studies (10, 11) of thiamin and HET, contributing resonance forms and charge distribution of the thiazolium ring have been deduced. The resonance forms and their relative contributions are similar for these two compounds except that C-2 is more positive in thiamin than in HET; indeed, analysis of the proposed resonance forms places a partial negative charge on C-2 of HET. In an attempt to clarify the electronic structure of thiamin and HET, especially with regard to the charge at C-2, we have continued our investigation of the 13C NMR spectra of these and related compounds.

Carbon 13 magnetic resonance can be valuable for investigating charge distribution in aromatic systems if used cautiously. Maciel (12) states that discussions "should not include an implied assumption that 13C shielding varies linearly with electron density unless a reliable theoretical treatment has demonstrated that such dependence is at least approximately valid for the type of system of interest." Although there is no detailed study of the thiazole ring, other five-membered aromatic heterocyclic compounds have been investigated. An assumption of an exact linear dependence of 13C shielding with electron density is plainly unwarranted in some cases, but this dependence does appear to be approximately valid for the

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The abbreviations used are: HET, DL-2-(α-hydroxyethyl)thiamin; HBT, DL-2-(α-hydroxybenzyl)thiamin; HBT-PP, HBT pyrophosphate; HBOT, DL-2-(α-hydroxybenzyl)oxythiamin; DSS, sodium 2,2-dimethyl-2-silapentane sulfonate; TMS, tetramethylsilane.
general class of heteroaromatic five-membered ring systems. More details on this subject are presented under "Discussion."

In the present study, complete 13C spectral data are presented for HET and related compounds. The kinetic acidity of the C-α proton and aldehyde release reactions of these compounds are compared with an electronic description of thiamin deduced from the NMR results.

EXPERIMENTAL PROCEDURE

The experimental procedures, including preparation of materials, NMR instrumentation and methods, and a description of kinetic experiments, is discussed in the miniprint following this paper. The compounds studied are shown in Fig. 1P.

RESULTS

The complete 13C NMR spectrum of 0.6 M dl-2-(α-hydroxyethyl)thiamin (Ib, Fig. 1P) in D₂O is shown in Fig. 2P. The assignment is based on the splitting of the resonance lines under conditions in which proton noise decoupling is absent (Table I) and on the reported assignment of thiamin (7). The detailed rationale for the assignment of HET and its analogs and a tabular presentation of chemical shifts are presented in the miniprint.

The resonance of C-2 of 4b undergoes a downfield shift of 21.4 ppm (Table I) relative to the same carbon of 4a. This shift is similar to that observed for the quaternary carbon of α-phenylethanol (C-1', 6, Fig. 1P) of 17.4 ppm downfield relative to benzene (Table I). Carbons 4 and 5 of 4b were assigned by the similarity in chemical shift of these resonances of 4b and 4a (Table I). The C-α and α-CH₃ carbon resonances were assigned based on the similarity in chemical shift of these resonances to those of the corresponding resonances of α-phenylethanol, 6, (Table I). When the 13C NMR spectrum of HET, Ib, is compared to thiamin, Ia (Table I), an even larger downfield shift, of 24.0 ppm, is observed for the C-2 resonance. The assignment of C-4 and C-5 of HET is assisted by the small downfield shift of C-4 and the small upfield shift of C-5 expected for these resonances upon substitution at C-2 (compare 4b with 4a). There is no ambiguity in assigning C-4, C-5, and C-6' since C-6' is a doublet and the former as singlets under conditions of proton coupling (Table I). The assignments of C-2', C-4' and C-6' resonances of Ib are further verified by their pH dependence (Fig. 1S, panel A) similar to that found in thiamin (7).

The 13C NMR spectra of HBT, Ic, and HBOT, 2c (Fig. 1P and Table I) were also obtained and are compared with HET in Table I. The spectrum of Ic is very similar to that of Ib except for the loss of the α-CH₃ resonance and the addition of these resonances assigned to the C-2', (C-3', C-4'), and C-1' carbons. The most upfield of this group is assigned to C-2' and the most downfield resonance to C-1', based on the reported assignment of α-phenethylol, benzyl alcohol, and toluene (13, 14). The C-3' and C-4' resonances were not resolved.

Replacement of the phenyl ring of α-phenylethanol by a thiazolium ring to give 4b, or of the α-methyl group of α-phenethylol by the same thiazolium ring to give 4c, has only a slight effect on the (C-α)-H coupling constant (Table I). This result suggests that there is the same amount of s-character in the (C-α)-H bond (15) in these model compounds and in the parent compound, HET. The 13C-1H coupling constants for the phenyl carbon of 1c, 2c, 3c, 4c, and 5c are similar to those of benzene (16). These coupling constants are significantly smaller than the (C-6')-H coupling.

There is no confusion between the resonances of the thiazolium carbon atoms, C-4 and C-5, and the phenyl resonances, C-2', and (C-3', C-4'), since these latter resonances appeared as doublets and the former as singlets under conditions of proton coupling (Table I). Another doublet resonance, C-6', is not confused with the phenyl carbon resonances because this signal was shifted downfield upon deprotonation of N-1', as in thiamin and HET (Fig. 1S, panel B). A completely unambiguous assignment of C-5 and C-1' cannot be made because these resonances differ by only 1.7 ppm and both appear as singlets with proton coupling. However, the near identity of the chemical shifts of C-5 of Ic with that of Ib, (Table I) which has no C-1' resonance, strongly supports the original assignment. Further arguments bearing on this point are presented in the supplement. The assignment of C-2' and C-4' resonances was achieved by comparison with the spectra of model compounds, 4c and 5c, which have no pyrimidine ring (Table I). The assignments for 2c are similar to those for 1c and are discussed in the miniprint.

13C-1H coupling constants for HET and related compounds, shown in Table IP, are similar to those of thiamin (7). Replacement of the phenyl ring of α-phenethylol, 6 by a thiazolium ring to give 4b, or of the α-methyl group of α-phenethylol by the same thiazolium ring to give 4c, has only a slight effect on the (C-α)-H coupling constant (Table IP). This result suggests that there is the same amount of s-character in the (C-α)-H bond (15) in these model compounds and in the parent compound, HET. The 13C-1H coupling constants for the phenyl carbon of 1c, 2c, 3c, 4c, and 5c are similar to those of benzene (16). These coupling constants are significantly smaller than the (C-6')-H coupling.

**Fig. 1P.** Structures of HET and its analogs. All structures represent the molecules as they exist at pH 1. The counterion may be Br⁻, Cl⁻, or I⁻, variously. 1a, thiamin; 1b, HET; 1c, HBT; 2a, oxythiamin; 2b, 2-(α-hydroxyethyl)oxythiamin; 2c, HBOT; 3a, 3-benzyl-4-methyl-5-(2-hydroxyethyl)thiazolium bromide; 3b, 2-(α-hydroxyethyl)-3-benzyl-4-methyl-5-(2-hydroxyethyl)thiazolium bromide; 4a, 3,4-dimethyl-5-(2-hydroxyethyl)thiazolium iodide; 4b, 2-(α-hydroxyethyl)-3,4-dimethyl-5-(2-hydroxyethyl)thiazolium iodide; 4c, 2-(α-hydroxybenzyl)-3,4-dimethyl-5-(2-hydroxyethyl)thiazolium iodide; 5a, 4-methyl-5-(2-hydroxyethyl)thiazolium chloride; 5b, 2-(α-hydroxyethyl)-4-methyl-5-(2-hydroxyethyl)thiazolium chloride; 6c, 2-(α-hydroxybenzyl)-4-methyl-5-(2-hydroxyethyl)thiazolium chloride.
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FIG. 2P. Natural abundance $^{13}$C NMR spectrum of HET, Ib, 0.5 M, observed pH 1.1 in D$_2$O. The spectrum was obtained under conditions of proton noise decoupling. The unmarked signal at TMS, 67.4 is due to added dioxane, the internal standard. The spectrum represents the accumulation of 800 transients of 1.3 s recycle time (total time, 17.3 min).

Table II

Coupling constants, $J_{CH}$, of HET and related compounds

All solutions were approximately 200 mg/ml in D$_2$O except 3c, in 2/1 D$_2$O/methanol; 6, in 1/1 D$_2$O/methanol; and 7, in 1/2 D$_2$O/methanol; pH 6.1 except 5c, pH 0.5.

| Compound | $J_{CH}$ | C-6-H | C-3'-H | C-4'-H | C-2'-H | (C)-H | CH$_2$OH | 5'-CH$_2$-3 | 5'-CH$_2$ | $\alpha$-CH | N-CH$_2$ | 2'-CH$_2$ | 4'-CH$_2$ |
|----------|----------|--------|--------|--------|--------|--------|----------|------------|------------|------------|----------|----------|----------|
| HET, Ib  | 179      | 151    | 146    | 146    | 131    | 128    | 128       | 131         | 128         | 131        |
| HBT, lc  | 176      | 164    | 159    | 149    | 146    | 146    | 131       | 128         | 146         | 131        |
| HBOT, 2c | 181      | 161    | 159    | 149    | 146    | 146    | 131       | 128         | 146         | 131        |
| 4b       |          | 151    | 144    | 131    | 128    | 146    | 131       |             |             |             |
| 4c       |          | 164    | 159    | 151    | 144    | 146    | 131       | 128         | 146         | 131        |
| 3c       |          | 164$^a$| 161$^a$| 144    | 146    | 131    | 131       |             |             |             |
| 5c       | 164      | 166    | 161    | 149    | 144    | 131    | 131       |             |             |             |
| 6        | 159      | 161    | 159    | 144    |        |        | 131       |             |             |             |
| 7        | 159      | 157    | 159    | 144    |        |        |           |             |             |             |

$^*$ The (C-3'-, C-4'') and (C-3'', C-4'') resonances and the C-2' and C-2'' resonances are unresolved.

Table II

Rate of exchange of the C-$\alpha$ proton and rate of aldehyde release of HET and related compounds

The kinetic experiments were performed under identical conditions of substrate concentration (0.25 M, except 3c, 0.18 M), buffer concentration (0.60 M Tris), observed pH, 8.30 ± 0.02 in D$_2$O, and temperature, 43°.

| Compound | Exchange $t_{1/2}$ | Aldehyde release $t_{1/2}$ |
|----------|--------------------|-----------------------------|
| HBT, 1c  | 35 ± 6$^a$        | 2.8 ± 0.5                   |
| HBOT, 2c | 43 ± 6             | 7.5 ± 1.5                   |
| 3c       | 78 ± 10            | 5.0 ± 1.0                   |
| 4c       | 102 ± 10           | >27$^b$                     |
| HET, 1b  | >40 hrs$^c$        |                             |

$^a$ At pH 8.3, 50°; HBT, $t_{1/2}$ = 8.5 min.; HBOT, $t_{1/2}$ = 14 min. $^b$ At pH 8.1, 30°; HBT, $t_{1/2}$ = 73 min; HBOT, $t_{1/2}$ = 120 min (28). The effect of buffer concentration, ionic strength, and metal ions on exchange of (C-$\alpha$)-H and on aldehyde release are discussed in the miniprint. $^c$ No detectable aldehyde release observed after 2-hour incubation at 43°. A greater than 27-hour half-life is estimated assuming that less than 5% aldehyde release had occurred at that time.

$^d$ At pH 8.1, 50°; HET, $t_{1/2}$ = 5 hours (17, 27). $^e$ No detectable exchange observed after 3 hours of incubation at 43°. A greater than 40-hour half-life is estimated assuming that less than 5% exchange had occurred at that time.

Discussion

The $^{13}$C NMR spectrum of HET has been assigned unambiguously. The chemical shift of C-2, 179.3 ppm downfield from TMS, to our knowledge is the largest downfield shift yet.

A mixture of 0.25 M HBT chloride hydrochloride and 0.6 to 1.0 M Tris base was brought to a pH of 8.30 with NaOH. At this pH, the following concentration of ions are present: 0.25 M HBT$^-$ Cl$^-$; 0.08 M Tris$^+$ H$^+$ Cl$^-$; 0.17 M Na$^+$ Cl$^-$; the remaining amount of Tris is present as the free base. An ionic strength of 0.50 is calculated for this solution. Since HBT-PP exists as a dianion at like pH, the ionic strength of 1.00 is calculated for a 0.25 M solution of HBT-PP. A 0.17 M solution of symmetrical dimethylpyrophosphate is calculated to have an ionic strength of 0.31 at pH 8.30.
reported for a carbon atom of an aromatic carbocyclic or heterocyclic ring (18, 19). This chemical shift is well outside the range of heterocyclic ring carbon shielding usually found, i.e. δc, 122 to 168 ppm (19), and falls into the range δc, 165 to 180 ppm, usually associated with the carbonyl carbon atoms of esters and amides. The unusual downfield shift suggests that C-2 of HET bears a partial positive charge, since carbon shielding in five-membered ring aromatic heterocyclic compounds has been shown to depend primarily on σ electron densities (19). Bloor and Breen (20) found reasonable correlation between total CNDO/2 charge densities and 13C shifts of nitrogen and oxygen heterocyclics. Page et al. (21) and Takahashi et al. (22) found a correlation between 13C and 1H shifts of attached protons in substituted pyrrole, furan, and thiophene compounds, suggesting that the 13C chemical shifts are dominated by the σ electron distribution. In two recent studies of substituted isothiazoles (23, 24) poor correlation was found between charge densities calculated by CNDO methods and actual 13C chemical shifts. On the other hand, Jordan’s (9) CNDO/2 and extended Hückel molecular orbital calculations of thiamin gave net atomic charges which correlate roughly with 13C chemical shifts which we observed (7), but both theoretical methods place a larger partial positive charge on C-4 rather than on C-2 of the thiazolium ring of thiamin. This charge distribution does not agree with x-ray crystallographic data (10), nor does it predict the correct order of carbon shielding of the thiazole ring of thiamin (7). It is difficult to judge whether the lack of correlation of σ charge densities and 13C chemical shifts in some cases lies in the inadequacies of molecular orbital calculations or in our insufficient understanding of the factors which affect 13C chemical shifts. Even when uncertainties concerning the exact linearity of 13C chemical shifts and σ charge densities are taken into account, the conclusion is nevertheless reached that the NMR method should at least predict the sign of the charge on a particular carbon atom, if not the exact magnitude. A partial positive charge on C-2 of HET should stabilize the adjacent α carbonanion, and thus helps to explain the ease of formation of the α carbamion both enzymatically (95, 96) and in model studies (17, 97, 98). This result stands in contrast to results of x-ray crystallographic studies of HET which place a partial negative charge on C-2 (11). The discrepancy probably reflects the more indirect approach used in the x-ray crystallographic study. 13C NMR spectroscopy is a direct approach to the charge on C-2, whereas the x-ray crystallographic technique obtains the σ charge distribution in the thiazolium ring indirectly from bond lengths.

Schellenberger (29) has proposed that interaction of thiamin-PP with its apoenzymes causes enhanced basicity of the pyrimidine amino group. He points out that from steric considerations, the amino group is favorably situated for participation in proton removal from substrate adducts at C-2. Other studies have shown that the amino group is neither protonated nor deprotonated between pH 0 and pH 9 and, consequently, is an extremely weak base. The results of Table III, also support the assumption that 14C chemical shifts of C-2 of 2-substituted derivatives of thiamin are a measure of charge density at this carbon, and further that the charge density at C-2 is an important factor in determining kinetic acidity of (Cat)-H.

The effect of inductive differences between substituents at N-3 on the rate of aldehyde release is more difficult to evaluate.

\[ \text{pK}_a \text{ of } C-4'\text{-OH of } 2e \text{ is approximately } 8.2 \text{ (30).} \]

As a result, this group is partially ionized at the pH of the α-CH exchange study, i.e. pH 8.3.

\[ 4^\text{a}, \text{inductive and } \sigma^* \text{ (polar) are defined by the Taft equation (39).} \]

\[ \text{These compounds are analogs of } 3a \text{ and } 4a \text{ which lack the substituent at position 5.} \]
since this reaction involves several steps (Scheme 1);

![Scheme 1]

If step A is slow and carbon carbon bond cleavage, step B, is fast, then the inductive effect of the substituent at N-3 may accelerate the rate somewhat, since a partial negative charge develops at (Cα)-O in the transition state. On the other hand, if step B is slower than A, the rate of aldehyde release may also be accelerated somewhat by an electron-withdrawing substituent at N-3, since in this step the negative charge is maintained but moved closer to the substituent. The substituent at N-3 may also affect the pK of the α-OH group. In this case, even if step B is slower than A and unresponsive to differing inductive effects at N-3, the amount of 9 present would be increased by an electron-withdrawing substituent at N-3, and the overall rate would be accelerated. The results in Table II suggest that an inductive effect is indeed operative, since the ratio of rates of aldehyde release for 1c, 3c and 4c, with an aminopyrimidyl substituent, a benzyl, and a methyl substituent at N-3 are faster than the normal 2'-methylpyrimidine ring.

The results of the present study contribute to a developing understanding of the chemistry of thiamin and further suggest that the electronic structure of thiamin is in accord with the natural suitability of thiamin to enzymatic carbanion-forming processes.

Note Added in Proof—Recently Jordan (36) has calculated charge densities from theoretical considerations and assigns a positive charge to C-2, in agreement with our experimental observations.

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Table I
Effects of methyl thiole on the rate of [3-carbon exchange and glutamate, threonine, and alanine, (a) unmodified, and (b) modified with methyl thiole (a) unmodified, and (b) modified with methyl thiole.

| Condition | Rate of 3-carbon exchange | Glutamate | Threonine | Alanine |
|-----------|--------------------------|-----------|-----------|--------|
| a         | 1.00                     | 1.00      | 1.00      | 1.00   |
| b         | 0.50                     | 0.50      | 0.50      | 0.50   |
| c         | 0.25                     | 0.25      | 0.25      | 0.25   |
| d         | 0.12                     | 0.12      | 0.12      | 0.12   |

(a) unmodified (b) modified with methyl thiole

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Figure 1: Effects of methyl thiole on the rate of 3-carbon exchange and glutamate, threonine, and alanine (a) unmodified, and (b) modified with methyl thiole. The conditions were as follows: (a) unmodified and (b) modified with methyl thiole.

Figure 2: Effects of methyl thiole on the rate of 3-carbon exchange and glutamate, threonine, and alanine (a) unmodified, and (b) modified with methyl thiole. The conditions were as follows: (a) unmodified and (b) modified with methyl thiole.

Figure 3: Effects of methyl thiole on the rate of 3-carbon exchange and glutamate, threonine, and alanine (a) unmodified, and (b) modified with methyl thiole. The conditions were as follows: (a) unmodified and (b) modified with methyl thiole.

Figure 4: Effects of methyl thiole on the rate of 3-carbon exchange and glutamate, threonine, and alanine (a) unmodified, and (b) modified with methyl thiole. The conditions were as follows: (a) unmodified and (b) modified with methyl thiole.
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