Determination of Rates of Proton Exchange of Thiamine Hydrochloride by ^{1}H NMR Spectroscopy

A Bioorganic Experiment for the Undergraduate Laboratory

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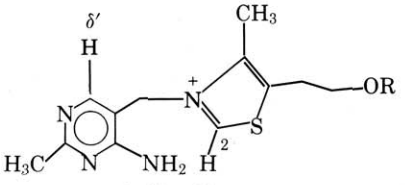
The reactivity of carbon acids is of central importance in synthetic and mechanistic organic chemistry, and there are many undergraduate laboratory experiments that illustrate the synthetic utility of carbanions. A standard example in several undergraduate textbooks is the synthesis of benzoin a reaction catalyzed by thiamine hydrochloride (*1*, *2*). However, students often have a difficult time grasping the concepts of kinetic and thermodynamic acidity of carbon acids, and there are few experiments that address the reactivity of carbon acids from a kinetic perspective (*3*).

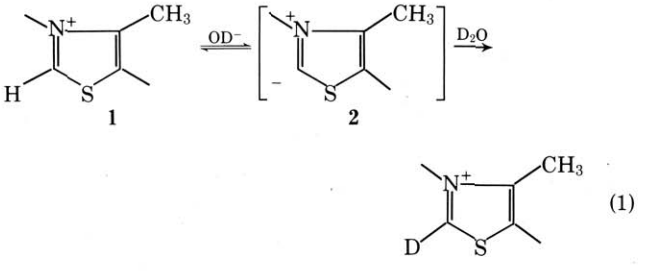
We present here a series of kinetic experiments involving the base-catalyzed exchange of the C(2)-H proton of thiamine hydrochloride monitored by ^{1}H NMR spectroscopy in D_2O. The experiments give students experience in the analysis of simple first-order kinetics and in the determination of an equilibrium constant from a ratio of rate constants. When carried out in conjunction with the thiamine catalyzed synthesis of benzoin, these experiments also provide mechanistic insight into the aldol-type reactions of thiazolium ylides.

Vitamin B_1 **(1a)** in the form of the cofactor thiamine pyrophosphate **(1b)** is required by enzymes that catalyze aldol-type addition reactions between thiamine and a variety of biological carbonyl compounds (*4*, *5*).



1a R = H
b R = $P_2O_6H_3$

The structural basis for thiamine catalysis was elucidated by Breslow in one of the first applications of ^{1}H NMR spectroscopy to organic reaction mechanisms (*6*). The first step involves the base-catalyzed removal of the C(2)-proton from the thiazolium ring to form the thiazolium ylide (**2**; eq 1).



(1)

The unique properties of thiazolium carbanions arise from the dipole stabilized ylide that is both a potent carbon nucleophile and a reasonably stable leaving group ($pK \sim 18$ in water). The ability of cofactors such as thiamine pyrophosphate to enlarge the biochemical transformations available to the amino acid side chains of polypeptides by generating nucleophilic and electrophilic centers serves as an important introduction to the biochemical relevance of mechanisms in organic chemistry.

Experimental Section

All chemicals were of analytical or reagent grade and were used without further purification. Thiamine hydrochloride was purchased from Sigma. Deuterium oxide (99.9 atom % D) was from Isotec. Spectra were recorded on a Varian *EM-360* continuous wave 60-MHz spectrometer. The temperature inside the probe was determined at the beginning of the laboratory period using the chemical shift differences of the methylene and hydroxyl protons of ethylene glycol containing 0.03% (v/v) HCl (*7*). Students should have prior lecture exposure to NMR instrumentation and lab experience in NMR operation.

In a typical experiment, an initial spectrum (0–10 ppm) was recorded in D_2O by dissolving 200 mg of thiamine hydrochloride in 1.0 mL of 10 mM DCl in a 1.5-mL microcentrifuge tube. The sample was immediately vortexed on a vortex mixer, placed in a 5-mm NMR tube, and then inserted into the spectrometer probe. The exchange reaction was initiated in the same manner by dissolving thiamine hydrochloride in acetate ion/acetic acid buffers. The buffer solutions were adjusted to an ionic strength of 2 M with NaCl, and total buffer concentrations ranged from 0.05 to 1.0 M. The spectrum was scanned from 10 ppm to 8 ppm and the integrated areas of the C(2) proton ($\delta \approx 10$ ppm) and the C(6′) proton ($\delta \approx 8$ ppm) of the pyrimidine ring (as a nonexchanging internal standard) were determined. The first-order rate constants k_{obsd} were obtained from the slopes of semilogarithmic plots of $A_2/A_{6'}$ against time, where A is the integrated area of the C-(2)–H or C(6′)–H protons, respectively. These plots were linear for $>3t_{1/2}$ with 10–15 time points.

After the reaction was over, the sample was carefully transferred back to the microcentrifuge tube and the pH was determined with an Orion Model 701A pH meter and a Radiometer GK2321C combination microelectrode thermostated at 30 °C. The value of pD was obtained by adding 0.40 to the observed pH of the solution (*8*).

Results

The exchange reaction shown in eq 1 is a two-step process with the thiamine ylide **2** as a steady-state intermediate. The rate of C(2)–H exchange is first-order in thiamine and deuteroxide ion concentration. At low buffer concentrations the rate law is given by

$$-\mathrm{d}[\mathbf{1a}]/\mathrm{d}t = k_{obsd}[OD^-][\mathbf{1a}] \qquad (2)$$

where k_{obsd} is obtained from the slope of the plot of ln ($A_2/A_{6'}$) against time.[2]

Because the reaction is buffered and the pD does not change during the course of the reaction, the second-order rate constant for deuteroxide ion catalyzed exchange can be obtained from

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[2] The concentration of thiamine-H at any time need not be known. As long as any physical measurement of the concentration is a linear function of the real concentration, the physical measurement can be used directly for the concentration value (*9*).

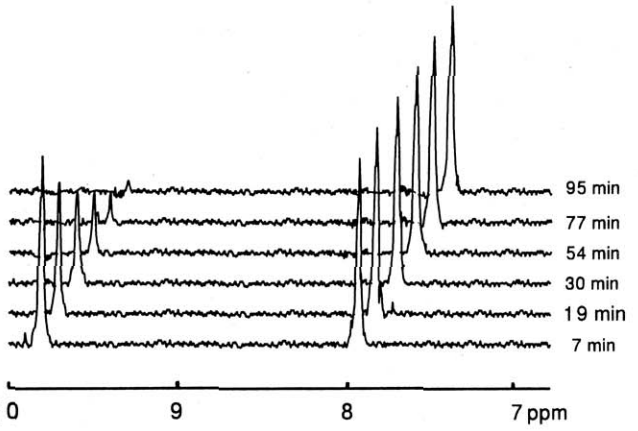


Figure 1. Time-dependent 60-MHz ^{1}H NMR spectra for C(2)–H exchange of thiamine in D_2O at 34 °C.

$$k_{OD} = k_{obsd}/[OD^-] \quad (3)$$

where concentration of deuteroxide ion at any measured pD is given by[3]

$$[OD^-] = 10^{(pD - pK_w)} \quad (4)$$

Figure 1 shows typical time-dependent ^{1}H NMR spectra for the base-catalyzed exchange of thiamine deuterochloride in 0.1 M acetate buffer at pD 4.61 and at 34 °C. The intensity of the C(2)–H signal decreases steadily relative to the nonexchangeable C(6′)–H signal. Figure 2 shows a semilogarithmic plot of the relative integrated areas of the two peaks against time. From these data a value of $k_{OD} = 5.5 \times 10^6$ $M^{-1}s^{-1}$ is obtained. Considering the temperature difference, the results show reasonable agreement with the literature value of $k_{OD} = 8.3 \times 10^6$ $M^{-1}s^{-1}$ measured at 30 °C (*4*).

Many undergraduate experiments in kinetics require an accurate infinity value or a complex derivation of the concentration of the changing species in terms of the observed experimental parameter (volume of titrant, absorbance, etc.). In this experiment an accurate infinity value is not required to analyze the data. Students can follow the reaction for two to three half-lives and obtain excellent experimental results. Furthermore, because the semilogarithmic plot of $A_2/A_{6'}$ against time intercepts the y-axis of Figure 2 at a ratio of 1.0, students can more directly see the relationship between the observable parameter (relative integrated area) and concentration.

This experiment lends itself to some interesting variations especially for students at a more advanced level. The concept of acid–base catalysis can be illustrated by determining the effect of increases in the buffer concentration on the rate of exchange. In the presence of increasing concentrations of acetate ion, B, the rate law can be expanded[4] to include a term for general-base catalysis:

$$k_{obsd} = k_{OD}[OD^-] + k_B[B] \quad (5)$$

Catalysis is quite weak so a substantial change in the buffer concentration from 0.05 to 1.0 M is required to see an increase in the rate. Plots of $k_{obsd} - k_{OD}[OD^-]$ against [B] should be linear with a slope k_B. Second-order rate constants k_B for substituted acetate catalysts have been reported (*4*).

Students can also be introduced to the concept of deriving

[3] Students are required to obtain the value of the ion product of deuterium oxide at 30 °C (pK_w = 14.70) from the *CRC Handbook of Chemistry and Physics*. A more rigorous derivation requires an activity coefficient correction (*10*).

[4] There is no evidence for general acid catalysis, but in very strong acid solutions there is evidence for a water-catalyzed rate that is independent of lyonium ion concentration (*4*).

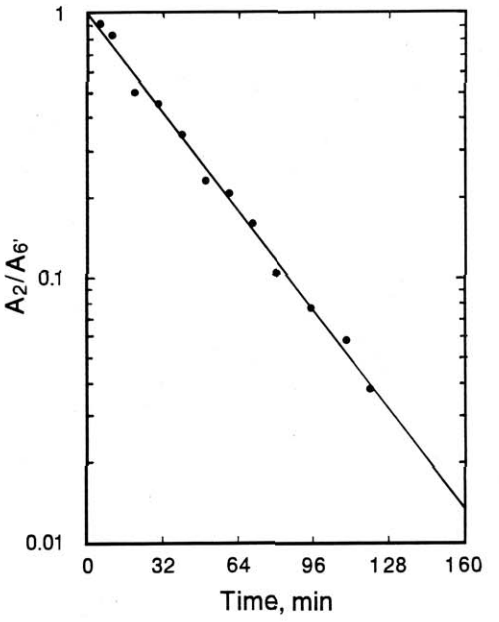
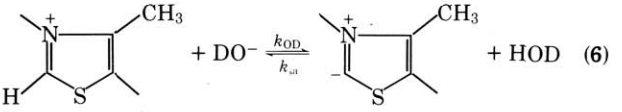


Figure 2. Semilogarithmic plot of the relative integrated areas of the C(2)–H (A_2) and C(6′)–H ($A_{6'}$) signals as a function of time.

an equilibrium constant from a ratio of rate constants to obtain an approximate pK_a for thiamine in D_2O (eqs 6 and 7).



(6)

$$K_{eq} = k_{OD}/k_{-a} = K_a^{CH}/K_a^{D_2O} \quad (7)$$

The derivation is based on several simplifying assumptions. The acidity constant $K_a^{D_2O}$ is calculated from the ion product K_w according to $K_a^{D_2O} = K_w/[D_2O]$ (*11*), and the reverse reaction in eq 6 is assumed to be diffusion-controlled with a rate constant $k_{-a} = 10^{10}$ $M^{-1}s^{-1}$. The pK_a of thiamine in D_2O equal to 19.7 estimated using eq 7 is similar to estimates of the pK_a = 18–20 in water (*4*, *5*).

Conclusion

Rate and estimated equilibrium constants for catalysis by OD^- of C(2)-proton exchange for thiamine can be obtained by ^{1}H NMR spectroscopy. The students enjoy the kinetic and synthetic experiments on thiamine catalysis (*1*, *2*) because they combine several experimental techniques. The experimental protocols can be readily adjusted for either CW or FT-NMR spectrometers. Finally, from a teaching standpoint, the laboratory serves the important function of linking both kinetic and mechanistic insight into organic reaction mechanisms.

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