has been found to be catalyzed by primary amines (and to a lesser extent by secondary amines), and the proposed mechanism involves formation of a Schiff base intermediate (eq 23).[19]

Acknowledgement. The authors are extremely grateful to Susan Crase Wilson for her superior technical assistance. This work was supported by a grant from the National Institutes of Health.

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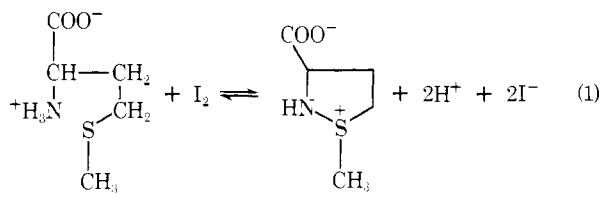
Reduction of Dehydromethionine by Thiols. Kinetics and Mechanism[1a]

David O. Lambeth[1b]

Contribution from the Department of Biochemistry, School of Medicine, The University of North Dakota, Grand Forks, North Dakota 58202. *Received January 16, 1978.*

Abstract: Dehydromethionine (*S*-methylisothiazolidine-3-carboxylate) reacts with dithiothreitol, 5-thio-2-nitrobenzoic acid, and 2-thio-5-nitropyridine to form methionine and the corresponding disulfides. The reaction is second order overall, being first order with respect to each reactant. The rate-limiting step is associated with the initial attack of thiol on the sulfur of dehydromethionine—completion of disulfide formation by the attack of the second thiol on the thiol–dehydromethionine addition intermediate occurs rapidly. The slopes of the pH-log rate profiles show breaks corresponding to the p*K*s of the thiols used and thus implicate the thiolate anion as the attacking nucleophile. The reactions are strongly catalyzed by the acid components of phosphate, imidazole, acetate, and formate buffers, the increase in rate being as much as 100- to 1000-fold with 0.1 M phosphate buffers. The rate-determining step is proposed to be a general-acid-catalyzed S_N2 displacement reaction in which thiolate anion attacks the sulfur of dehydromethionine concerted with protonation of the nitrogen of dehydromethionine resulting in its expulsion as a neutral amine.

Dehydromethionine (*S*-methylisothiazolidine-3-carboxylate) is a cyclic sulfonium derivative of methionine first prepared by Lavine.[2] The compound may be variously considered as an azasulfonium salt or a cyclic, N-protonated sulfilimine or sulfimide. Dehydromethionine is formed as the immediate product of the iodinic oxidation of methionine in neutral solution (eq 1), and the reaction is rapidly and quantitatively



(1)

reversed upon acidification of the solution. The structure of dehydromethionine has been verified by the recent X-ray diffraction study of Glass and Duchek.[3]

Iodinic oxidation of thioethers in aqueous solution normally results in the formation of sulfoxides. However, with methionine, formation of dehydromethionine rather than methionine sulfoxide occurs due to an approximation effect[4] resulting in a very favorable intramolecular attack of the amino group on an iodo-sulfonium intermediate. Dehydromethionine is stable indefinitely when stored over desiccant and its half-life in neutral aqueous solutions in the absence of buffer species is estimated to be 600 days.[5] Hydrolysis of dehydromethionine to form the sulfoxide is strongly catalyzed by a number of buffers, and the pH-log rate profile for the reaction shows a minimum near 7 with slopes of +1 and −1 above and below pH 7, respectively.[5]

Although dehydromethionine has been known for over 30 years, the literature on this substance is limited to the references cited above. In fact, apart from dehydromethionine, isothiazolidines as unicyclic structures appear to be unknown entities.

Lavine reported without supporting evidence that dehydromethionine is reduced by thiols.[2c] Since such a reaction would set one boundary condition for the existence of dehydromethionine-like (azasulfonium) compounds in biological systems, we undertook the study reported here to determine the characteristics of the reaction. The presence of the azasulfonium linkage in biochemical systems seems plausible in view of the ubiquity of amine and thioether functional groups and the ease with which the linkage can be formed.

We have found the reduction of dehydromethionine to be

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accelerated many-fold by buffers, and we believe this study provides a rare example of a concerted, general-acid-catalyzed displacement reaction. Similar examples known to us are an intramolecular transalkylation reaction[6a] and the cyclization of 4-chlorobutanol.[6b]

Recent reviews covering the chemistry of tricoordinate sulfur compounds in general[7a] and sulfilimines in particular[7b] are available.

Results

Reaction Order. The reactions of dehydromethionine with thiols were studied under pseudo-first-order conditions, wherein dehydromethionine was present in at least 20-fold excess over thiol. Formation of the disulfide form of dithiothreitol was followed by recording the increase in absorbance at 283 nm with time. The reactions of thionitrobenzoate and thionitropyridine with dehydromethionine were monitored at 412 and 396 nm, respectively, where decreases in absorbance due to the disappearance of thiolate were observed. In general, the reactions of each thiol conformed closely to first-order behavior for more than 90% of the reaction. However, with the aromatic thiols, slight deviation from first-order behavior was detected as an upward curvature of first-order plots under two conditions: (1) at the highest pH values shown in Figure 2 where significant rates of alkaline hydrolysis of the disulfide forms are observed and (2) when catalysis by formate buffers were being studied. Deviation in the latter case is probably due to a side reaction involving buffer-catalyzed dehydromethionine hydrolysis. At these times, data were analyzed for the first 50% of the reaction as this portion should reflect most accurately, the initial attack of thiol on dehydromethionine. Even when the deviations from first-order behavior were most noticeable, correlation coefficients were greater than 0.9995.

The dependence of k_{obsd} on the concentration of dehydromethionine was examined for each thiol. Representative results are shown in Figure 1 for the reaction of dehydromethionine with dithiothreitol in two different buffer systems. For each thiol, the plots were linear and extrapolated through the origin, indicating that the reaction rates were linearly dependent on dehydromethionine concentration and that the reactions were not reversible under the conditions used. Thus, the reaction of dehydromethionine with thiols is second order overall, being first order with respect to each reactant.

Verification of Products. The formation of methionine and disulfides in the reaction of dehydromethionine with thiols was verified by thin-layer chromatography on cellulose (see Experimental Section). Methionine sulfoxide, the principal contaminant in the preparations of dehydromethionine used, did not react at a significant rate with the thiols under the conditions of the kinetic experiments.

Additional experiments were conducted to make sure the products were methionine and disulfides and to look for possible buildup of a 1:1 adduct of dehydromethionine with thiol as a reaction intermediate. When dehydromethionine and thionitrobenzoate were mixed in a molar ratio of 1:2, a second-order plot of thiolate disappearance [as (absorbance of thiolate)$^{-1}$ vs. time] was linear for over 90% of the reaction, in sharp contrast to results obtained when the initial ratio was 1:1. These observations confirmed that dehydromethionine and thionitrobenzoate disappeared in a molar ratio of 1:2, which is consistent with disulfide formation.

Reaction mixtures of dehydromethionine and thionitrobenzoate at a variety of initial concentration ratios and pHs were repetitively scanned between 500 and 300 nm during the course of the reaction. The final spectra were the same as those of authentic samples of the disulfide, and the tightness of the isosbestic points observed during the course of the reactions showed that little, if any, buildup of intermediate occurred.

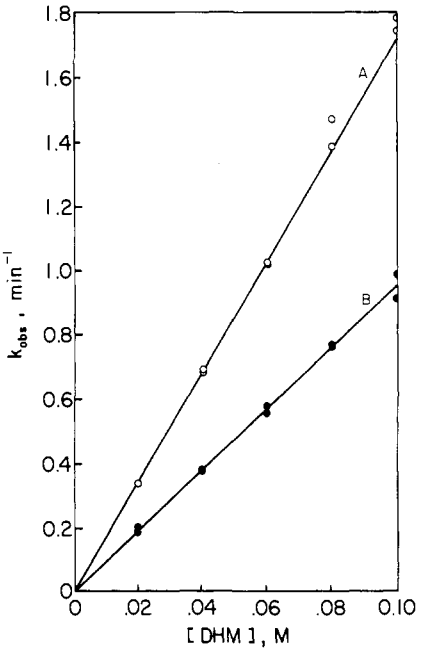


Figure 1. Dependence of k_{obsd} on the concentration of dehydromethionine for the reaction of dehydromethionine (DHM) with dithiothreitol: A, in phosphate buffer; B, in Tris-HCl buffer. Concentration of dithiothreitol was 1.5 mM. The total concentration of both buffers was 0.08 M and each contained equimolar concentrations of the acid and base species. Temperature was 25 °C and μ = 1.0 M with KCl.

Similar results were obtained with thionitropyridine. Spectral scans of reaction mixtures of dehydromethionine with dithiothreitol showed the appearance of a peak centered at 283 nm, consistent with formation of the disulfide.[8]

pH–Rate Profiles. The kinetic data to be presented are described by the following rate expression in which DHM represents zwitterionic dehydromethionine and RS^- is the thiolate anion:

$$-d[RSH]/dt = (k_{H_2O} + k_H[H^+] + k_{HA}[HA])[DHM][RS^-] \quad (2)$$

Replacing $[RS^-]$ by the equivalent expression incorporating the K_a and total thiol concentration, [RSH], gives

$$-d[RSH]/dt = \frac{(k_{H_2O} + k_H[H^+] + k_{HA}[HA])[DHM][RSH]}{([H^+]/K_a) + 1} = k_{obsd}[RSH] \quad (3)$$

Rearranging eq 3 gives

$$\frac{k_{obsd}}{[DHM]} = \frac{k_{H_2O} + k_H[H^+] + k_{HA}[HA]}{([H^+]/K_a) + 1} \quad (4)$$

The buffer-independent rates of reaction of dehydromethionine with thiol were determined for a series of pH values by the usual procedure of extrapolating to zero buffer concentration, plots of k_{obsd} vs. concentration of buffer. The pH–log rate profiles are presented in Figure 2 where the points represent the extrapolated values for the buffer-independent rates and the curves are calculated according to eq 4 (after setting the $k_{HA}[HA]$ term to zero) using rate constants presented in Table I.

For dithiothreitol, the best fit of the curve to the experimental points was obtained by assuming a pK_a of 9.57, which can be compared with the reported titrimetric values of pK_1 = 9.12 and pK_2 = 10.15.[9] The calculated curves for the reactions of the aromatic thiols with dehydromethionine were based on values for their pK_as determined spectrophotometrically

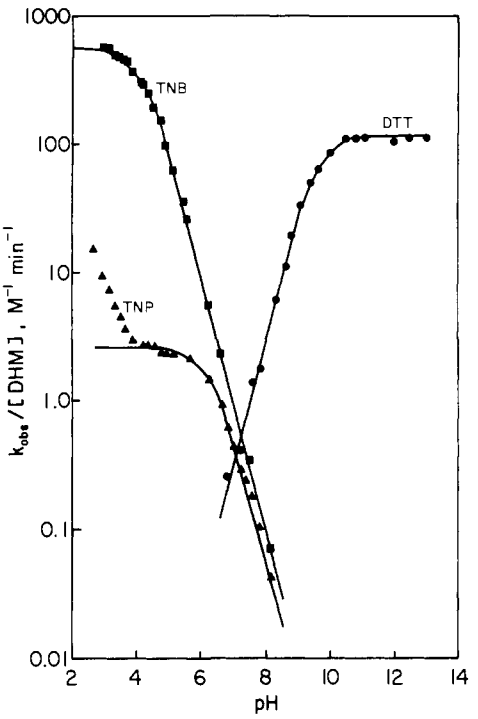
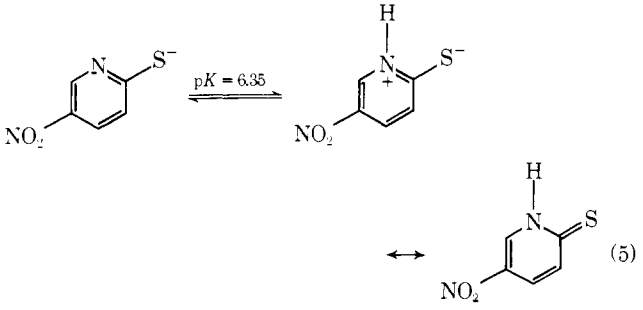


Figure 2. pH-log rate profiles for the reactions of dehydromethionine with dithiothreitol (DTT), thionitrobenzoate (TNB), and thionitropyridine (TNP) at 25 °C and μ = 1.0 M (with KCl). The points shown are values of k_{obsd} extrapolated to zero buffer concentration divided by the concentration of dehydromethionine. The lines shown are calculated from eq 4 and rate constants tabulated in Table I. Concentration of dithiothreitol was 1.5 mM and the concentrations of dehydromethionine for the DTT reactions were varied from 0.015 to 0.1 M. For the reactions with TNB and TNP, the concentration of dehydromethionine was always in at least 20-fold excess over aromatic thiol.

for the conditions of the kinetic experiments (25 °C and μ = 1.0). The spectrophotometric titration results are shown in Figure 3 where the experimental data are fitted by curves calculated by using pK_a = 4.25 and 6.35 for thionitrobenzoate and thionitropyridine, respectively.

Protonation of the thiolate chromophore of thionitrobenzoate prevented extension of the pH-log rate profile below pH 3. However, the protonation of thionitropyridine occurring with pK_a = 6.35 apparently does not involve the sulfur, but the neighboring pyridinum nitrogen (eq 5). Consequently, a



(5)

chromophore in the visible region is still present at low pH, and Figure 2 shows that, although the slope of the profile is zero between pH 4 and 6, it again increases at low pH. Determination of k_{obsd}[dehydromethionine] for the reaction of dehydromethionine with thionitropyridine in 0.1 M HCl, where the rate can only be approximated because the half-life of dehydromethionine is less than 2 min due to hydrolysis, gave a value near 300 M^{-1} min^{-1}. This indicates that the slope of the pH-log rate profile in the pH range of 1-4 probably assumes a value of −1 near pH 1.

For Figure 2, theoretical curves could be fitted to the data

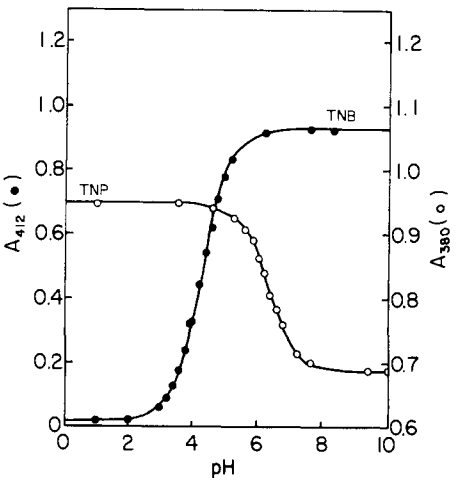


Figure 3. Spectrophotometric determinations of the pK_a of thionitrobenzoate (TNB) and of thionitropyridine (TNP) at 25.0 °C and μ = 1.0 M with KCl. The experimental points were obtained by diluting 1.0 mL of an aqueous solution of the aromatic thiol into 4.0 mL of buffer of known pH and reading the absorbance at the appropriate wavelength against a blank containing the same buffer. The lines shown are calculated from a modified form of the Henderson-Hasselbach equation using pK_a = 4.25 for TNB and pK_a = 6.35 for TNP.

for the aromatic thiols without a contribution by the k_{H_2O} term of eq 4. At higher values of pH where the term should be more readily observed, hydrolysis of the disulfide products prevented reliable values of k_{obsd} from being obtained. Conversely, the calculated curve for dithiothreitol in Figure 2 did not require a contribution by the k_H term of eq 4. Such a term may be measurable in a pH range lower than shown in Figure 2 for dithiothreitol.

Buffer Catalysis. The reaction of dehydromethionine with thiol is strongly catalyzed by phosphate, imidazole, and carboxylate buffers. A representative result is shown in Figure 4 where values for k_{obsd} for the reaction of dehydromethionine with dithiothreitol are plotted as a function of total imidazole concentration. The several lines correspond to buffers differing in the fraction of base present. From eq 4, the slopes of these buffer plots are as indicated in eq 6

$$\text{slope} = \frac{k_{obsd}}{[HA]_{tot}} = \frac{(k_{HA})_{app}[DHM]}{([H^+]/K_a) + 1} \quad (6)$$

where $(k_{HA})_{app}$ is the apparent third-order catalytic constant and $[HA]_{tot}$ is the total concentration of the buffer species present. Values of $(k_{HA})_{app}$ obtained by using eq 6 were plotted vs. the fraction of the buffer in the base form (f_B). The result for imidazole is shown as an inset in Figure 4 where the ordinal intercept of the plot gives k_{HA}. There is no detectable catalysis when f_B = 1 and, thus, catalysis is attributed solely to the imidazolium cation (acid form) of the buffer.

The values of k_{HA} determined for several buffers and the reactions of dehydromethionine with the three different thiols used in this study are presented in Table I. In all cases, plots of k_{obsd} vs. total buffer concentration were linear in the concentration ranges used, and plots of $(k_{HA})_{app}$ vs. f_B were linear with catalysis being attributed only to the acid form of the buffer. For each buffer reported in Table I, f_B was varied from 0.1 to 0.9 in increments of 0.1.

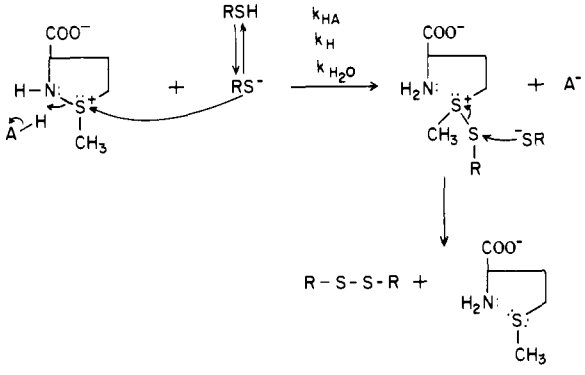
For the reaction of dehydromethionine with dithiothreitol at pH 11 where more than 80% of the dithiol is in the dianion form, the addition of 75 mM HPO_4^{2-} more than doubled the rate of the reaction (calculated $k_{HA} \approx 1.3 \times 10^3$ M^{-2} min^{-1}). This degree of catalysis, when dithiothreitol is almost fully ionized, makes it very improbable that the mechanism of phosphate catalysis is base-catalyzed ionization of thiol, as the latter makes an attack on dehydromethionine.

Table I. Summary of Derived Rate Constants for Equation 4 for the Reactions of Dehydromethionine with Thiols at 25 °C, Ionic Strength Maintained at 1.0 with KCl[a]

thiol	k_{H_2O} (M^{-1} min^{-1})	k_H (M^{-2} min^{-1})	k_{HA}, M^{-2} min^{-1}			
			$H_2PO_4^-$	Imid·H^+	CH_3COOH	HCOOH
DTT	117		5.2×10^5	2.64×10^4		
TNB[b]		9.95×10^6	2.42×10^3	2.68×10^1	7.55×10^3	3.95×10^4
TNP		5.82×10^6	4.05×10^2	4.05	1.95×10^3	1.1×10^4

[a] The pK_a values for the buffers are reported in the Experimental Section. The values of k_{HA} were determined as described in the text and illustrated in Figure 4 for catalysis of the reaction of dehydromethionine with dithiothreitol by imidazole buffers. For each buffer and each reaction, the fraction of buffer in the base form was varied in 0.1 increments from 0.1 to 0.9. The concentration ranges used were: phosphate, 0.025–0.10 M; imidazole, acetate, and formate, 0.1–0.4 M. [b] A statistically corrected Bronsted plot of the data for thionitrobenzoate has a slope of ~0.5, with imidazole deviating most from the line.

SCHEME I



Discussion

The reaction of dehydromethionine with thiols must occur in at least two steps, as indicated in Scheme I where the probable structure of the thiol–dehydromethionine addition intermediate is shown. However, below pH 9, the amine of both the intermediate and the product will become triprotonated subsequent to ring opening of dehydromethionine.

Under pseudo-first-order conditions achieved by keeping dehydromethionine in excess, appearance of disulfide and disappearance of thiol are both first-order processes, which indicates that the rate-limiting step is associated with the first, ring-opening step. In fact, no evidence could be obtained for the buildup of an appreciable concentration of addition intermediate during the course of the reaction. This is not surprising in the case of dithiothreitol where disulfide formation occurs by intramolecular attack of the second thiol, and is reasonable in the case of monothiols, since the thioether produced in the second step should be a far better leaving group than the intramolecularly located amine group displaced in the initial attack by thiol.

Although the pH–log rate profiles for the reactions of dehydromethionine with dithiothreitol and aromatic thiols are very different, the data can be rationalized by a single mechanism consisting of nucleophilic attack by thiolate anion on the sulfur of the zwitterionic form of dehydromethionine *concerted with* protonation of the departing nitrogen of dehydromethionine by general acids.

The proposition that it is the thiolate anion which makes a nucleophilic attack on the zwitterionic form of dehydromethionine is firmly supported by the occurrence of a change in slope in each pH–rate profile (Figure 2) which coincides with the pK of the thiol. In addition, the experimental data for the profiles are fitted by eq 4 in which the denominator of the right side reflects the fraction of thiol in the anion form. Studies of numerous reactions of thiols including addition to carbonyl compounds,[10] thiol–disulfide exchange reactions,[11] reactions of dithiols with flavins,[9a] and reactions of thiols with ethylene

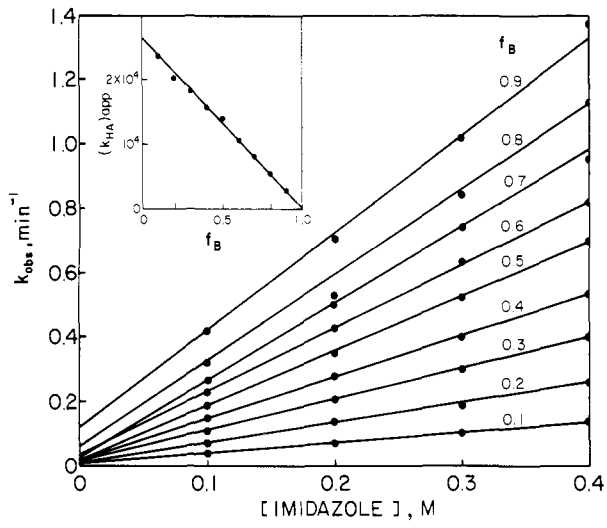


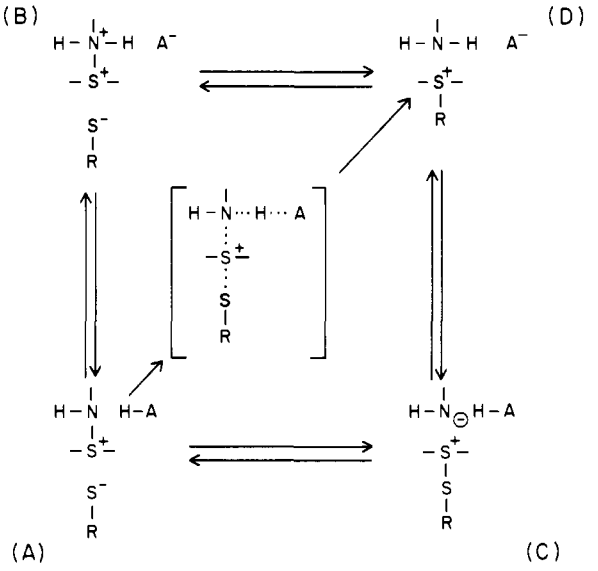
Figure 4. Catalysis of the reaction of 27.9 mM dehydromethionine with 1.5 mM dithiothreitol by imidazole buffers. The fraction of imidazole in the base form (f_B) is shown for the various lines. The inset shows a plot of $(k_{HA})_{app}$, calculated from the slopes of the buffer plots according to eq 6 vs. f_B. Calculated values based on K_a for ionization of imidazolium cation = 6.03×10^{-8} M. The ordinal intercept gives the third-order rate constant, k_{HA}, for catalysis of the reaction by the imidazolium cation.

oxide[12] and benzene oxide[13] have all shown the thiolate anion to be a much more active nucleophile than the protonated species.

The number of protons on the nitrogen of dehydromethionine is of particular concern in postulating mechanisms. The ground state for the structure of dehydromethionine has a monoprotonated nitrogen as shown in Scheme I. Diprotonation of nitrogen is expected to be very unfavorable, as a cationic center would be generated adjacent to the sulfonium center. Removal of the last proton from nitrogen to give a sulfilimine structure would stabilize the charge on sulfur. However, we have found no evidence by acid–base titrations for a pK for nitrogen in the pH range 3–11. Thus, it seems very improbable that any of the breaks in the pH–rate profiles is associated with an ionization of dehydromethionine.

The contrasting slopes for the pH–rate profiles for the aromatic thiols when compared to dithiothreitol are consequences of the very different acidities of the thiols used and the pH ranges in which the reactions could be conveniently followed. These consequences can be discerned by examination of the effects of pH on eq 2 as $[H^+]$ is varied above and below the pK of each thiol and by recalling that in the case of dithiothreitol the k_H term can be neglected (since it was too small to be measured in the pH range studied), whereas with the aromatic thiols the k_{H_2O} term was negligible in comparison with $k_H[H^+]$. Thus, by examining the behavior of eq 4 in the absence of buffers and with the restrictions mentioned above, it is seen that the slope of −1 seen in portions of the profiles for

SCHEME II



the aromatic thiols is still consistent with the thiolate anions being the active nucleophiles.

With thionitropyridine, the pH-rate profile again approaches a slope of −1 below pH 4. This can be explained most easily by assuming that the thione species (eq 5) is an active nucleophile. Thus, the general rate expression shown in eq 2 should have the additional term $k_H[H^+][\text{thione}]$ to describe the reaction involving thionitropyridine.

Scheme II shows alternate reaction routes for the formation of the thiol-dehydromethionine addition intermediate proceeding by nucleophilic displacement at the sulfonium sulfur of dehydromethionine. The species HA should be taken to represent water, hydronium ion, and acid components of buffers as appropriate for the solvent- and buffer-catalyzed reactions. The stepwise A → B → D route proceeds through intermediate B which is expected to be very unstable because of the adjacent cationic centers. The stepwise A → C → D route is even less attractive, as it requires the displacement of an anionic nitrogen, an extremely improbable event in any reaction and especially when such a group is intramolecularly located as in dehydromethionine. As a consequence of the instabilities of B and C, the conversion of A to D probably proceeds as a concerted reaction involving a transition state of the type shown in the center of Scheme II. Thus, the developing negative charge on the departing nitrogen is relieved by donation of a proton from a general acid. Because the aromatic thiolates are relatively poor nucleophiles, the absence of a detectable k_{H_2O} term (eq 4) may be an indication that a stronger acid than water is needed to effectively form the transition state.

Factors affecting stepwise vs. concerted routes for general acid-base catalysis have been discussed in detail by Bruice[14] and by Jencks[15] who employ three-dimensional reaction-coordinate contour diagrams as aids in rationalizing various mechanisms.

Two alternative mechanisms for the catalysis of the thiol-dehydromethionine reaction have been rejected by us. General-base-assisted ionization of the protonated thiol as it makes an attack on dehydromethionine, combined with specific-acid-catalyzed protonation of nitrogen, would be kinetically indistinguishable from the general-acid-catalysis mechanism shown in Scheme II. However, we know of no documented examples for general-base assistance of ionization of thiols and we found the rate of reaction of dithiothreitol with dehydromethionine to be more than doubled by 75 mM phosphate buffer at pH 11 where dithiothreitol is largely (80-90%) in the dianion form. However, general-acid catalysis could still occur at this pH by protonation of nitrogen by phosphate dianion. Even more convincing are observations of strong catalysis by phosphate and imidazole of the reaction of dehydromethionine with thionitrobenzoate at pH values 2-3 units above the pK of the thiol.

Nucleophilic catalysis has often been proposed for reactions involving sulfonium intermediates, for example, in the iodinic oxidation of sulfides.[16a,b] Oxidation of a variety of sulfides by halogens in nonaqueous solvents results in the formation of phosphate anhydrides when phosphate esters are present,[16c] and the oxidation of N-acetylmethionine by iodine in aqueous phosphate buffers results in transfer of ^{18}O from H_2O to phosphate.[16d] Gensch and Higuchi rationalized their observations of hydrolysis of dehydromethionine by buffers in terms of nucleophilic catalysis.[5] Despite these precedents, we believe general-acid catalysis provides a simple, more uniform explanation for buffer catalysis of the reaction of dehydromethionine with thiols. Furthermore, the water and hydronium-mediated reactions are explained by the same mechanism. In contrast, the solvent reaction proceeding by nucleophilic attack of water or hydroxide on the sulfonium sulfur would lead to formation of sulfoxide which is not readily reduced by thiols. Finally, we do not see catalysis by increasing concentrations of acetate, formate, or imidazole when the pH of the solution is maintained by the presence of a low concentration of another buffer at 2 or 3 units above the pKs of these substances.

An additional reaction pathway open to tricoordinate sulfur compounds is simple addition of a nucleophile to form a tetracovalent (sulfurane) intermediate. A recent review of this type of mechanism is available.[7a] Addition of thiolate to dehydromethionine would form an intermediate very unstable to dissociation back to the reactants but which could be trapped by water and acid-mediated protonation of nitrogen resulting in expulsion of the neutral amine and collapse of the sulfurane to a tricoordinate species. Such a trapping mechanism bears some similarities to the mechanism proposed for the attack of thiols on carbonyl compounds.[17] Criteria have been discussed for establishing a trapping mechanism,[18] and one of the more conclusive methods involves examination of the Bronsted relationship for a series of buffers. A Bronsted α of approximately 0.5 is obtained from the data for thionitrobenzoate listed in Table I. However, we feel this result cannot be used as evidence against a sulfurane mechanism, since the buffers used have divers charges and structure. A more critical investigation of the Bronsted relationship is underway.

Since these studies were completed, we have become aware of the work on the reduction of N-p-toluenesulfonylsulfilimines in methanol by derivatives of benzene thiols.[19] In agreement with our results, they found the initial attack by thiol to be rate limiting and the reaction was second order overall.

The ease with which dehydromethionine is produced by oxidation of methionine led Lavine[2b,c] to suggest that dehydromethionine may have biological importance. The ready reduction of dehydromethionine by thiols described in this paper suggests that the abundance of glutathione and cysteine in biological systems would reduce any dehydromethionine adventitiously produced. However, the nitrogen-sulfur interaction could exist at sites protected from thiols; e.g., in protein and peptide structures, or as transient intermediates in enzymatic reactions. For the organosulfur chemist, dehydromethionine constitutes a particularly simple structure for probing mechanisms of reaction and catalysis at tricoordinate, sulfonium centers.

Experimental Section

Materials. Fine chemicals were obtained from Sigma Chemical Co.

and used without further purification except for imidazole, which was recrystallized twice from benzene. Thin-layer cellulose chromatograms were from Eastman.

Preparation of Thionitrobenzoate. 5,5′-Dithiobis(2-nitrobenzoic acid) (0.20 mmol) was reduced at pH 7 by dithiothreitol (0.25 mmol). The sample was adsorbed onto a 20-mL bed of DEAE-cellulose in the chloride form. After washing the column with 0.2 M KCl, fraction eluted with 0.2 M KCl was retained and kept at 0–5 °C. Spectral measurements at 412 nm[20] before and after addition of dithiothreitol indicated the sample was completely reduced.

Preparation of Thionitropyridine. One gram of 2,2′-dithiobis(5-nitropyridine) in 50 mL of 0.5 M Tris-HCl, pH 8.2, was reduced with 70 mmol of 2-mercaptoethanol. After several minutes, the pH was decreased to about 1.5 and, upon cooling, orange crystals formed. A sample was recrystallized from aqueous methanol, dried, and sublimed at 80–85 °C and 0.25 Torr onto a cold finger at 0 °C. The absorbance of the product dissolved in phosphate buffer at pH 7.2 did not change upon addition of 2-mercaptoethanol. The spectrum of thionitropyridine is pH dependent with an apparent pK of 6.35 and an isosbestic point at 396 nm. All kinetic studies involving thionitropyridine were conducted at this wavelength.

Preparation of Dehydromethionine. Dehydromethionine was prepared by the I_2–Ag_2O method of Lavine.[2b] The resulting white material was stable for several months when stored over desiccant at 0–5 °C. The purity was at least 97% and the contaminants were methionine and methionine sulfoxide.

Iodometric Assay for Dehydromethionine. Dehydromethionine (50–75 mg) was dissolved in water (10 mL). Three milliliters of 5 M KI and 5 mL of 3 M HCl were added in that order, resulting in the immediate formation of iodine which was titrated immediately with a standardized solution of sodium thiosulfate.

Thin-layer chromatography on cellulose was used to determine the contaminants in dehydromethionine and to analyze product mixtures resulting from reaction of dehydromethionine with thiols. The solvent systems used were 1-butanol–pyridine–H_2O (7:8:6) and 1-butanol–acetic acid–H_2O (6:3:1). Detection systems used were ninhydrin spray for amino acids, a freshly prepared mixture of 1 M KI and 1 M HCl used as a spray to detect dehydromethionine, and iodine vapor to detect thiols and disulfides. Chromatograms, sprayed with ninhydrin and developed at room temperature, showed a purple spot for dehydromethionine only after several days. The KI–HCl spray reagent immediately reveals dehydromethionine as a brown spot, and a similar spot sometimes develops for methionine sulfoxide only after several hours. Dehydromethionine is partially hydrolyzed to sulfoxide during chromatography in the solvent system containing acetic acid.

Preparation of Buffers. Buffers of known acid–base composition were prepared in boiled deionized water from carefully dried and weighed samples of solid reagents and standaridized solutions of NaOH, KOH, or HCl as required. The pKs of buffers were obtained by extrapolating the pH readings of equimolar acid–base solutions of varying total concentration (μ = 1 M with KCl, 25 °C) to zero-buffer concentration. The determined pKs were: formic acid, 3.53; acetic acid, 4.55; imidazole, 7.22; 2-(N-morpholino)ethanesulfonic acid, 6.21; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 7.61; N,N-bis(2-hydroxyethyl)glycine, 8.33; 2-(N-cyclohexylamino)ethanesulfonic acid, 9.39; and cyclohexylaminopropanesulfonic acid, 10.48. A value of 6.49 reported for $H_2PO_4^{-1}$ was assumed.[21] The zwitterionic buffers listed above were very useful for determining the pH–rate profiles, since they did not strongly catalyze the reaction, thus giving better defined values for the buffer-independent rate.

Procedure for Kinetic Studies. Kinetic studies were performed using a Gilford 240 spectrophotometer thermostated at 25.0 °C with a Lauda K2R circulating water bath. Absorbances were recorded using a strip chart recorder.

Reactions were initiated by adding dehydromethionine and were routinely followed to at least 99% completion. Thiol and dehydromethionine solutions were prepared fresh daily, and the concentration of dehydromethionine was determined titrimetrically. Autoxidation of thiols was not a problem under the conditions of the experiment. Reactions were followed by monitoring the following wavelengths: formation of oxidized dithiothreitol (283 nm), disappearance of thionitropyridine (396 nm) and thionitrobenzoic acid (412 nm).

Spectral scans were recorded on a Varian 634S spectrophotometer equipped with a wavelength programmer and a Houston 2000 X-Y recorder.

Analysis of Kinetic Data. For each recorder trace of absorbance vs. time, absorbances were determined for ten evenly spaced time intervals covering the first 75% of the reaction. The observed first-order rate constant (k_{obsd}) was obtained with a computer program utilizing a linear, least-squares analysis of log ($A_\infty - A_t$) vs. time. The program also gave standard deviations of slopes and intercept, a plot of residuals, and the correlation coefficient.

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References and Notes

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