$$\frac{d}{dt}\begin{vmatrix}M_x\\M_y\\M_z\end{vmatrix} = \begin{vmatrix}-R_2 & \Delta & 0\\ -\Delta & -R_2 & H\\ 0 & -H & -R_1\end{vmatrix}\begin{vmatrix}M_x\\M_y\\M_z\end{vmatrix} + \begin{vmatrix}0 & 0 & 0\\ 0 & 0 & 0\\ 0 & 0 & R_1\end{vmatrix}\begin{vmatrix}0\\0\\M_0\end{vmatrix} \quad (A-1)$$

where $\mathbf{M}_x$, $\mathbf{M}_y$, and $\mathbf{M}_z$ are each column vectors of length n representing the value for the two transverse and the longitudinal magnetizations of each spin, respectively. M_0 represents the equilibrium longitudinal component of each spin. The matrix Δ contains the offset parameters $(\omega_n - \omega)$ for each spin along its diagonal, where ω_n is the resonance frequency of spin n. $\mathbf{H}$ is a diagonal matrix of "driving terms" with the values $\gamma_n H_1$ along the diagonal, with γ_n being the gyromagnetic ratio of spin n and H_1 the magnitude of the perturbing radio frequency field. The spin-lattice relaxation matrix $\mathbf{R}_1$ is given by eq A-2. For the

$$\begin{vmatrix}\rho_1 & \sigma_{12} & \sigma_{13} & \sigma_{14} & \cdots & \sigma_{1n}\\ \sigma_{12} & \rho_2 & \sigma_{23} & \sigma_{24} & \cdots & \sigma_{2n}\\ \cdot & \cdot & \cdot & \cdot & & \cdot\\ \cdot & \cdot & \cdot & \cdot & & \cdot\\ \cdot & \cdot & \cdot & \cdot & & \cdot\end{vmatrix} \quad (A-2)$$

present work the F-F and H-F dipolar contributions to the ρ_i and σ_{ij}, as well as the chemical shift anisotropy contributions to the ρ for the fluorine nuclei, were computed as described in ref 2 and 3. The dipolar terms depend on the various internuclear distances and thus are a function of the Cartesian coordinates for each of the n nuclei. The matrix for spin-spin relaxation (R_2) was set up in a similar way, using the equations given by Solomon.[11] Solution of eq A-1 is straightforward[14] and in our implementation used the EISPAC routines.[15]

During the recovery phase of the experiment the driving field is absent, and the calculated behavior utilized only the equation along the bottom line of eq A-1. The initial values for each variable during the recovery phase were calculated by solution of the full equations parameterized according to our experimental conditions.

It will be recognized that the above description of relaxation and excitation in a complex spin system is extremely crude. Groups of spins with identical chemical shifts such as the three protons of a methyl group are treated as single, composite spins, spin coupling between proton spins is neglected, and all cross-correlation effects are neglected. More realistic theoretical approaches to describing intramolecular dipolar relaxation in multispin systems are available[16] but, given the current lack of *detailed* knowledge of protein dynamics in solution, we decided not to persue these more complicated descriptions at this time.

(14) Heading, J. "Ordinary Differential Equations"; George, Allen and Univin, Ltd.: London, 1971; p 49.
(15) Garbow, B. S.; Boyle, J. M.; Dongarra, J. J.; Moler, C. B. "Matrix Eigensystem Routines"; Springer-Verlag: New York, 1977.
(16) Werbelow, L. G.; Grant, D. M. *Adv. Magn. Reson.* **1977**, *9*, 198-299.

Reduction of Flavins by Thiols. 1. Reaction Mechanism from the Kinetics of the Attack and Breakdown Steps[1]

Edward L. Loechler*† and Thomas C. Hollocher*

Contribution from the Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. *Received November 29, 1979*

Abstract: In the reduction of 3-methylriboflavin (**3**) by dithiothreitol (DTT) and dithioerythritol (DTE) at constant pH, a change in rate-determining step was observed with increasing [buffer] from one which involves buffer catalysis to another which does not. The buffer catalyzed step followed the rate law $(k_{a_1}[HSS^-] + k_{a_2}[SS^{2-}])[HA][Fl]$, while the associated solvent terms obeyed $(k_{a_0}[HSSH] + k_{a_1}[HSS^-] + k_{a_2}[SS^{2-}])[Fl]$. The Brønsted plot for k_{a_1} gave $\alpha = 0.58$, and all general acids including hydronium ion and water lay on a single straight line. The step which is not buffer catalyzed obeyed $k_{b_1}[HSS^-][Fl]$. The corresponding reduction of **3** by mercaptoethanol (ME) obeyed $(k_{m_0}[RS^-] + k_{m_1}[RSH] + k_{m_m}[RS^-][RSH])[Fl]$ and showed no buffer catalysis. The first-order terms were small compared to the second-order term, and their cause is unknown. The second-order term in the monothiol reaction and the results from the dithiol reaction establish that the redox reaction between flavin and thiols proceeds entirely or very largely by way of a covalent adduct which then breaks down to product disulfide and reduced flavin. It is argued that buffer catalysis must occur as general-acid catalysis at N(5) in a step other than breakdown. Mechanisms thus ruled out include hydride transfer, general-base-catalyzed thiol deprotonation in attack, general-acid catalysis at N(1), and attack by thiolate at C(1a), C(2), C(4), N(5), C(6), and C(8). Reduced **3** is oxidized by bis(2,2′-dithio-4,4′-dinitrobenzoic acid) (DTNB) and obeyed the rate law $(k_{r_0}[FlH_2] + k_{r_1}[FlH^-])[DTNB]$. The rate constant for reaction of hydrazine with DTNB was more than 10^4 times smaller than k_{r_1} in spite of the greater basicity of hydrazine relative to N(1) of reduced flavin. Mechanisms involving adduct formation at N(1) are thus ruled out. Calculations show that mechanisms involving thiol and flavin radicals are too slow to account for the rate of the overall reaction. A mechanism fully consistent with the data is attack of thiolate at C(4a) with general-acid catalysis at N(5) followed by breakdown of the C(4a) adduct by displacement of reduced flavin anion upon attack of thiolate on sulfur.

Introduction

In this and the following two papers,[2,3] we consider the mechanism of reduction of flavins by thiols. The reaction is a model for the action of flavin-containing enzymes such as glutathione reductase[4] and lipoamide dehydrogenase,[5] which catalyze a redox exchange between thiols or dithiols and pyridine nucleotides.

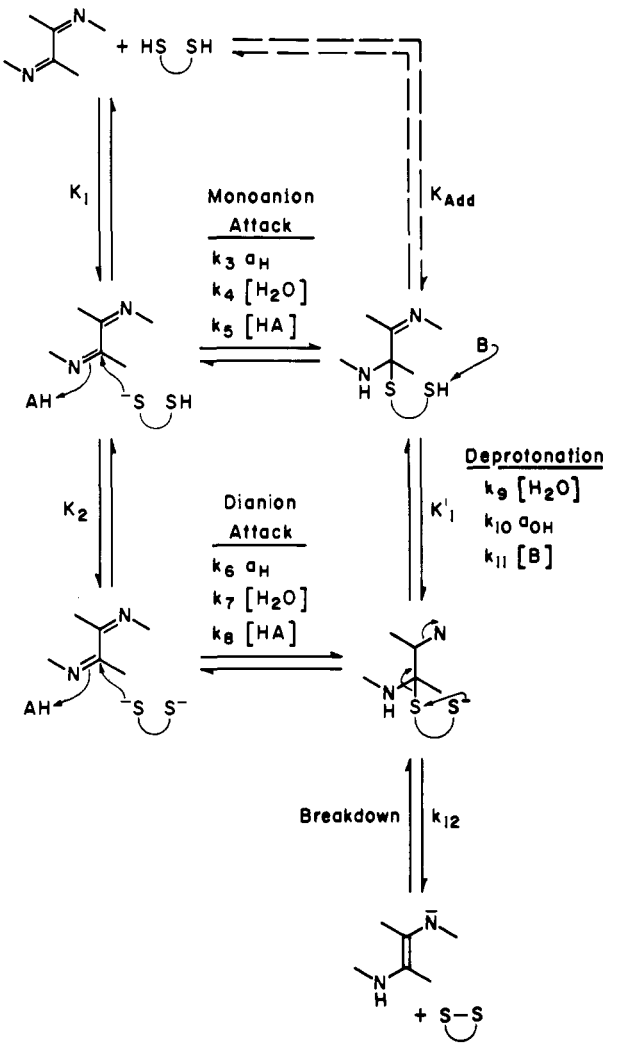
Gascoigne and Radda[6] first studied the thiol-flavin reaction by using several flavins with lipoic acid and found the reaction to show buffer catalysis and to be first order in dithiol and flavin. Gibian and co-workers[7] later showed that the reaction of flavins

† Address correspondence to E.L.L. at the Biology Department, Massachusetts Institute of Technology, Cambridge, MA 02139.

(1) Supported by grants from the National Science Foundation (Grants GB-29337A1, PCM74-04834, PCM76-21678) and by a training grant from the National Institutes of Health (Grant GM-212). We thank John Lambooy, Peter Hemmerich and Franz Müller for the flavin derivatives used in this study. We also thank William Jencks for many useful discussions.
(2) Part II: Loechler, E. L.; Hollocher, T. C., accompanying paper in this issue.
(3) Part III: Loechler, E. L.; Hollocher, T. C., accompanying paper in this issue.
(4) Staal, G. E. J.; Veeger, C. *Biochim. Biophys. Acta* **1969**, *185*, 49. Massey, V.; Williams, C. H. *J. Biol. Chem.* **1965**, *240*, 4470.

0002-7863/80/1502-7312$01.00/0

Scheme I. Reaction of Dithiols with Flavins[a]

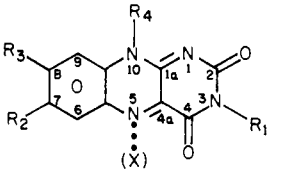


[a] Correspondence between rate constants in Scheme I and the rate constants observed kinetically is as follows: $k_{ao} = k_3K_1$; $k_{a1} = (k_4[H_2O] + k_6K_2)$; $k_{a2} = k_7[H_2O]$; $k_{ac1} = k_5$; $k_{ac2} = k_8$; $k_{do} = k_9K_{Add}[H_2O]$; $k_{d1} = k_{10}K_{Add}K_w/K_1$; $k_{dco} = k_{11}K_{Add}$; $k_{b1} = k_{12}K_{Add}K_1'/K_1$. The subscripts a (or A), b (or B), and d (or D) refer to attack, breakdown, and deprotonation steps, respectively. The subscript c (or C) designates buffer-catalyzed terms, while 0, 1, and 2 refer to the charge of the dithiol in the rate law.

with various thiols was a general one which stoichiometrically yielded the expected disulfide.

In the reaction of dithiothreitol (DTT) and 3-(carboxymethyl)lumiflavin (**1**) we observed[8] a change in rate-determining step and attributed it to a change from thiol attack at C(4a) with buffer catalysis at N(5) to uncatalyzed breakdown as the buffer concentration was increased (Scheme I). This interpretation was supported by the pH rate profile for the reaction. At about the same time, Yokoe and Bruice[9] studied the reaction of thiophenol and 3,10-dimethyl-8-isoalloxazine (**2**) and came to similar conclusions regarding mechanism. Nucleophilic addition to flavins is well established and has been observed or deduced to occur at N(5) with sulfite,[10-13] phosphines,[14] and nitroalkane carbanions,[9,15]

Chart I. Structural Designations



no.	flavins used	R_1	R_2	R_3	R_4	X
1	3-(carboxymethyl)-lumiflavin	$CH_2CO_2^-$	CH_3	CH_3	CH_3	
3	3-methylriboflavin	CH_3	CH_3	CH_3	ribotol	
4	7-chlororiboflavin	H	Cl	CH_3	ribotol	
5	8-chlororiboflavin	H	CH_3	Cl	ribotol	
6	7,8-dichlororiboflavin	H	Cl	Cl	ribotol	
7	3-methyl-5-ethyllumiflavonium perchlorate	CH_3	CH_3	CH_3	CH_3	C_2H_5

at C(4a) with sulfite,[12,13] at C(1a)[16,17] and C(4)[18] with hydroxide, and at C(6) and C(8) with sulfite.[11-13] In the reduction of 1,3-dimethyl-5-((*p*-nitrophenyl)imino)barbituric acid by thiols, Sayer et al.[86] demonstrated that thiolate adds to the imino carbon with general-acid catalysis ($\alpha \leq 0.05$) but that breakdown of the adduct shows no buffer catalysis. This reaction with an activated nonplanar analogue of a flavin corresponds to flavin reduction via a thiol–C(4a) flavin adduct and confirms the possibility of such a reaction.

In this paper we confirm Scheme I and rule out two mechanisms not previously excluded: thiol attack at N(1) and mechanisms involving radicals. The one-electron reduced flavin radical is rather stable and has been implicated in the reaction of reduced flavin with oxygen,[19] quinones,[7,20] and carbonyls.[21-24] The following paper[2] provides spectrophotometric evidence for the formation of a C(4a) adduct and considers the reaction under conditions in which deprotonation of the adduct becomes partially rate determining. The third paper considers structure-reactivity effects and transition-state structures for attack and breakdown steps.

Experimental Section

Materials. Most flavin derivatives used in this and the following two papers were generously supplied by John Lambooy (**3**, **4**, **5**, **6**), by Peter Hemmerich (**1**), and by Franz Müller (**7**). Each flavin derivative was judged to be >90% pure by paper chromatography, thin-layer chromatography, high-pressure liquid chromatography, and NMR. DTT and dithioerythritol (DTE) (mp $40^1/_2$–42 and 81–$82^1/_2$ °C, respectively, from diethyl ether) were stored under argon in the cold to prevent oxidation and were weighed out just prior to use. Concentrations based on weight and determined by assay with bis(2,2′-dithio-4,4′-dinitrobenzoic acid) (DTNB) agreed within 1.5%. Other chemicals used were purchased at the highest purities available and when appropriate were recrystallized or redistilled before use. Deionized glass-distilled water was used for all solutions.

Titrations were carried out between 24 and 26 °C, ionic strength 1.0 M (KCl). Dithiols were titrated anaerobically under argon. The pK values of the diastereomers DTT and DTE were found to be the same, 9.14 and 9.94.[25]

(5) Massey, V.; Palmer, G. *J. Biol. Chem.* **1962**, *237*, 2347. Massey, V.; Veeger, C. *Biochim. Biophys. Acta* **1961**, *48*, 33.
(6) Gascoigne, I. M.; Radda, G. K. *Biochim. Biophys. Acta* **1967**, *131*, 498.
(7) Gibian, M. J.; Elliot, D. L.; Kelly, C.; Borge, B.; Kupecz, K. *Z. Naturforsch. B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol.* **1972**, *27B*, 1016.
(8) Loechler, E. L.; Hollocher, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 3235.
(9) Yokoe, I.; Bruice, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 450.
(10) Michaels, G. B.; Davidson, J. T.; Peck, H. D. *Biochem. Biophys. Res. Commun.* **1970**, *39*, 321.
(11) Müller, F.; Massey, V. *J. Biol. Chem.* **1969**, *244*, 4007.
(12) Hevesi, L.; Bruice, T. C. *Biochemistry* **1973**, *12*, 290.
(13) Bruice, T. C., Hevesi, L.; Shinkai, S. *Biochemistry* **1973**, *12*, 2083.
(14) Müller, F. *Z. Naturforsch., B: Anorg. Chem. Org. Chem., Biochem., Biophys., Biol.* **1972**, *27B*, 1023.
(15) Porter, D. J. T.; Voet, J. G.; Bright, H. J. *J. Biol. Chem.* **1973**, *248*, 4400.
(16) Wadke, D. A.; Guttman, D. E. *J. Pharm. Sci.* **1966**, *55*, 1088, 1363.
(17) Guttman, D. E.; Platek, T. E. *J. Pharm. Sci.* **1967**, *56*, 1432.
(18) Smith, S. B.; Bruice, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 2875.
(19) Massey, V.; Palmer, G.; Ballow, D. "Oxidases and Related Systems"; King, T. E., Mason, H. S., Morrison, M., Eds.; University Park Press: Baltimore, MD, 1973; p 25. Ballow, D. *Biochem. Biophys. Res. Commun.* **1969**, *36*, 891, 898. Gibson, Q. H.; Hastings, J. W. *Biochem. J.* **1962**, *83*, 368.
(20) Gibian, M. J.; Rynd, J. A. *Biochem. Biophys. Res. Commun.* **1969**, *34*, 594.
(21) Williams, R. F.; Shinkai, S. S.; Bruice, T. C. *J. Am. Chem. Soc.* **1977**, *99*, 921.
(22) Bruice, T. C.; Yano, Y. *J. Am. Chem. Soc.* **1975**, *97*, 5763.
(23) Williams, R. F.; Bruice, T. C. *J. Am. Chem. Soc.* **1976**, *98*, 7752.
(24) Bruice, T. C.; Taulane, J. P. *J. Am. Chem. Soc.* **1976**, *98*, 7769.

Kinetics. Kinetic data were obtained at 25 ± 0.5 °C by using a Zeiss spectrophotometer PMQII. Hydrogen ion activity was determined using a Radiometer pH meter equipped with a GK2321C glass electrode.

Argon was used to establish anaerobic conditions, and reactions were run in 1-cm, 3-mL curvettes capped with fold-down rubber stoppers designed for serum bottles. Quick curing silicon rubber was coated over the stoppers after all injections had been made and the argon purging needles withdrawn in order to retard oxygen leakage. All transfers of anaerobic solutions were accomplished by using gas-tight syringes previously purged with argon.

The decrease in optical density of oxidized **3** was followed at λ_{max} = 448 nm. Under pseudo-first-order conditions the rate law was

$$-\frac{d[Fl]}{dt} = k_2[\text{thiol}][Fl] = k_{obsd}[Fl]$$

and $k_2 = k_{obsd}/[\text{thiol}]$. Pseudo-first-order plots were linear for 60–95% of reaction, but thereafter upward curvature was generally detectable. Deviation from linearity was less pronounced at lower initial concentrations of flavin. This effect did not depend on pH, buffer concentration, or erroneous OD_∞ values and is ascribed no mechanistic importance. The initial linear portion of pseudo-first-order plots was used to calculate k_{obsd}.

Reaction of Dithiol with Flavins. Under most conditions, the attack and breakdown steps of Scheme I were both partially rate determining. At constant pH with changing [buffer], k_2 obeyed

$$1/k_2 = 1/(k_A + k_{AC}[\text{buffer}]) + 1/k_B \quad (1)$$

where the subscript C refers to buffer catalysis. This equation is derived in the following paper (Appendix I)[2] from a more general case in which the deprotonation step is also considered to be partially rate determining.

Equation 1 rearranges to

$$k_{cor} = (1/k_2 - 1/k_B)^{-1} = k_A + k_{AC}[\text{buffer}] \quad (2)$$

and

$$1/k_2 = 1/k_B + \frac{1/k_{AC}}{k_A/k_{AC} + [\text{buffer}]} \quad (3)$$

A trial value of k_B in eq 2 graphically gave approximate values of k_A and k_{AC}. A plot of $1/k_2$ vs. $(k_A/k_{AC} + [\text{buffer}])^{-1}$ from eq 3 gave improved values of k_B and k_{AC}. This k_B was then used as input in eq 2. Iteration between eq 2 and eq 3 was terminated when values for k_{AC} agreed to within 2%. Whenever k_B could not be accurately determined from an experiment, it was calculated from its pH rate profile (Figure 2). In such cases, k_A and k_{AC} were calculated from eq 2 by using this value of k_B.

The rate law for breakdown was

$$k_B[\text{thiol}][Fl] = k_{b1}[HSS^-][Fl]$$

and as a function of pH

$$k_B = k_{b1}F_{HSS^-} = \frac{k_{b1}}{(1 + a_H/K_1 + K_2/a_H)} \quad (4)$$

where F_{HSS^-} refers to the mole fraction of DTT monoanion.

Equation 4 rearranges to eq 5 which was solved by iteration to provide values of k_{b1}, K_1, and K_2.

$$1/k_B = 1/k_{b1} + a_H/K_1k_{b1} + K_2/a_Hk_{b1} \quad (5)$$

The rate law for solvent attack was

$$k_A[\text{thiol}][Fl] = (k_{a0}[HSSH] + k_{a1}[HSS^-] + k_{a2}[SS^{2-}])[Fl]$$

and as a function of pH

$$k_A/F_{HSS^-} = k_A(1 + a_H/K_1 + K_2/a_H) = k_{a0}(a_H/K_1) + k_{a1} + k_{a2}(K_2/a_H) \quad (6)$$

Utilizing titrimetrically determined values of K_1 and K_2, we obtained the rate parameters k_{a0}, k_{a1}, and k_{a2} similarly by iteration.

The rate law for buffer-catalyzed attack was

$$k_{AC}[\text{buffer}][\text{thiol}][Fl] = (k_{ac1}[HA][HSS^-] + k_{ac2}[HA][SS^{2-}])[Fl]$$

and as a function of pH

$$k_{AC}/F_{HA}F_{HSS^-} = k_{ac1} + k_{ac2}(K_2/a_H) \quad (7)$$

(25) The pK_1 and pK_2 for DTT and DTE have been reported to be 8.3, 9.5 and 9.0, 9.9, respectively (Zahler, W. L.; Cleland, W. W. *J. Biol. Chem.* **1968**, *243*, 716). The values agree with ours for DTE but differ greatly for DTT. We do not expect these compounds to have different pK values and did not observe a difference. The values we reported previously, 9.12 and 10.15, for DTT (ref 8) were determined by less rigorous methods than those used in this study and are less accurate than the present values of 9.14 and 9.94.

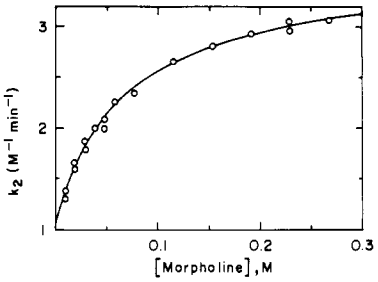


Figure 1. Dependence of the second-order rate constant $k_2(M^{-1}\ min^{-1})$ on morpholine concentration for the reduction of **3** by DTT at pH 7.92, 25 °C, and ionic strength 1.0 M (KCl). The line was fit to the data by using eq 1 with k_A = 1.5 M^{-1} min^{-1}, k_{AC} = 76.8 M^{-2} min^{-1}, and k_B = 3.57 M^{-1} min^{-1}.

A plot of $k_{AC}/F_{HA}F_{HSS^-}$ vs. K_2/a_H has a slope of k_{ac2} and an intercept of k_{ac1}.

Reaction of Monothiol with Flavin. The reaction of mercaptoethanol (ME) or mercaptopropionic acid (MP) with **3** at fixed pH obeyed eq 8.

$$k_{obsd}/[\text{thiol}] = k_M + k_{MM}[\text{thiol}] \quad (8)$$

The rate law for the k_{MM} term was

$$k_{MM}[\text{thiol}]^2[Fl] = k_{mm}[RS^-][RSH][Fl]$$

and as a function of pH

$$k_{mm} = k_{MM}/F_{RSH}F_{RS^-} \quad (9)$$

The value of k_{mm} used was the average of the different values obtained at different pH.

The rate law for the k_M term appeared to be

$$k_M[\text{thiol}][Fl] = (k_{m0}[RSH] + k_{m1}[RS^-])[Fl]$$

which rearranges to eq 10. A plot of k_M vs. F_{RS^-} was linear, and k_M reduces to k_{m0} and k_{m1} when F_{RS^-} equals 0.0 and 1.0, respectively.

$$k_M = k_{m0}F_{RSH} + k_{m1}F_{RS^-} \quad (10)$$

Reaction of Reduced 3 or Hydrazine with DTNB. **3** was reduced by the method of Bruice and Taulane.[24] Its reaction with excess DTNB (followed at 412 nm) was pseudo-first-order and obeyed the rate law

$$k_{obsd}[Fl_R] = k_R[DTNB][Fl_R] = (k_{r0}[FlH_2] + k_{r1}[FlH^-])[DTNB]$$

which rearranges to eq 11, where K_r is the ionization at N(1) of reduced flavin (pK_r = 6.45).[26]

$$k_R/F_{FlH_2} = k_{r0} + k_{r1}(K_r/a_H) \quad (11)$$

Pseudo-first-order plots of the reaction of hydrazine with DTNB were linear for 1–2 halftimes but showed downward or upward curvature thereafter with, respectively, high or low concentration of hydrazine. A straight line was drawn through the initial linear portion of the data, and k_{obsd} was determined from $t_{1/2}$. See Discussion and Appendix I for further discussion of this reaction.

Results

Reaction of 3 with DTT. Figure 1 shows the dependence of k_2, the apparent second-order rate constant, on [buffer] at constant pH. The data can be described by eq 1. The rate constant k_B predominates at high [buffer] and its pH rate profile is shown in Figure 2 (open circles). The data fit eq 4 which was used to determine k_{b1}, K_1, and K_2. These parameters were used to draw the line through the open circles of Figure 2. pK_1 and pK_2 were determined in this way to be 9.14 and 10.12, which can be compared to the titrimetric values of 9.14 and 9.94. The small discrepancy in pK_2 is not ascribed any mechanistic significance.

The pH rate profile for k_A is shown in Figure 2 (solid circles). Equation 6 was used to determine k_{a0}, k_{a1}, and k_{a2}, and these parameters were used to draw the line through the closed circles.

The open squares in Figure 2 were obtained by using DTT as the sole buffer at relatively high [DTT] (>20 mM) where deprotonation of the flavin–DTT adduct (see following paper[2]) was less than 5% rate limiting. The line through these points was generated by interpolating k_A and k_B values at a particular pH

(26) We assume that the pK of N(1) in reduced 3-methylriboflavin is the same as that reported for riboflavin (6.45) in ref 63.

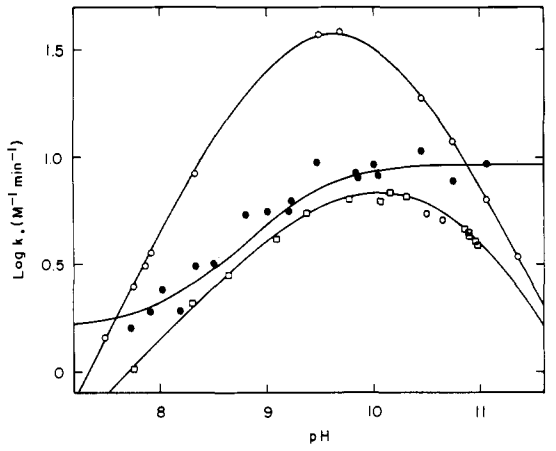


Figure 2. pH rate profile for the solvent steps associated with reduction of **3** by DTT at 25 °C and ionic strength 1.0 M (KCl). Open circles (k_B) were fit by the line by using eq 4 with k_{b1} = 63.2 M^{-1} min^{-1}, pK_1 = 9.14, and pK_2 = 10.12. Solid circles (k_A) were fit by using eq 6 with k_{a0}, k_{a1}, k_{a2}, pK_1, and pK_2 equal to 1.6 M^{-1} min^{-1}, 8.8 M^{-1} min^{-1}, 9.4 M^{-1} min^{-1}, 9.14, and 9.94, respectively. Open squares (k_2) were determined by using DTT as sole buffer at concentrations such that deprotonation was less than 5% rate determining (see following paper[2]) and fit as described in the text.

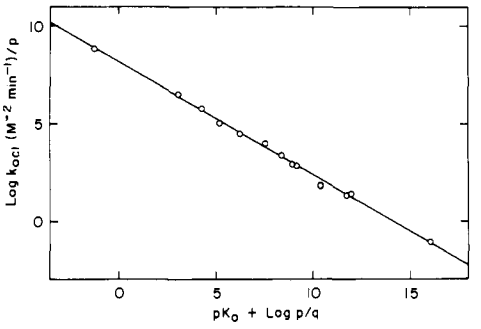


Figure 3. Brønsted plot for the general-acid-catalyzed attack of DTT monoanion on **3** at 25 °C and ionic strength 1.0 M (KCl). Statistical corrections have been applied to the catalytic constants (Table II) and pK values of the catalysts.[73] α is 0.58.

from k_A (solid circles) and k_B (open circles) and then calculating an apparent k_2 from $k_{2app} = (1/k_A + 1/k_B)^{-1}$.

The buffer catalyzed terms, k_{AC}, in eq 1 were determined at several pH values with several buffers (Table I) and analyzed according to eq 7 to determine k_{ac1} and k_{ac2} (catalysis rate constants for the mono- and dithiolate, respectively). Plots of $k_{AC}/F_{HA}F_{HSS^-}$ vs. K_2/a_H gave good straight lines. Methoxyacetic acid, methoxyamine hydrochloride, potassium phosphate, imidazole hydrochloride, glycine ethyl ester hydrochloride, and pyrrolidine hydrochloride were studied at a single pH. The pH chosen was in a range where the DTT monoanionic rate term, k_{ac1}[HA][HSS^-][Fl], dominated in buffer catalysis (>85%). Values of k_{ac1} for these compounds were calculated from the observed buffer rates by assuming no contribution from k_{ac2}. Table II is a compilation of the rate constants k_{ac1} and k_{ac2} for the buffers used in the reaction of DTT with **3** and includes values for hydronium ion and water acting as general acids on the basis of the solvent data (Figure 2, solid circles). Table II was used to construct the Brønsted plot in Figure 3 for the monoanion of DTT as the nucleophile. α is 0.58.

Table III compares rate parameters for DTT and DTE under comparable conditions.

Reaction of 3 with ME and MP. These reactions gave data that conformed to eq 8 as shown in Figure 4. The values of k_M (intercepts) and k_{MM} (slopes) are given in Table IV for ME and MP.

k_M in eq 8 obeyed eq 10, and a plot of k_M vs. F_{RS^-} is given in Figure 5. Values for k_{m0} and k_{m1} were determined by least squares. What appeared to be a weak buffer-catalyzed term was observed with morpholine, but tetramethylammonium chloride showed a similar effect. The effect is thus interpreted as a salt effect rather than as buffer catalysis. It could not in any case correspond to the strong buffer catalysis observed with DTT.

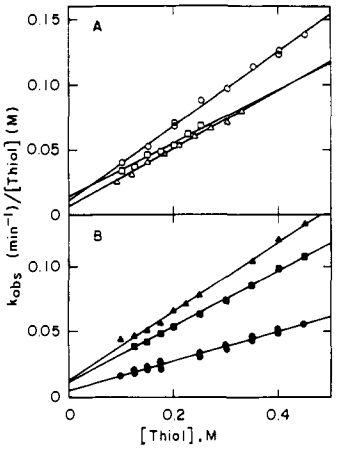


Figure 4. Plots of k_{obsd}/[thiol] vs. [thiol] for the reduction of **3** by monothiols at 25 °C and ionic strength 1.0 M (KCl). ○, □, ▲, ■, and ● refer to mercaptoethanol at pH 9.32, 10.12, 9.60, 9.09, and 8.61, respectively. Values of k_M (intercept) and k_{MM} (slope) are tabulated in Table IV. △ refers to mercaptopropionate at pH 9.63.

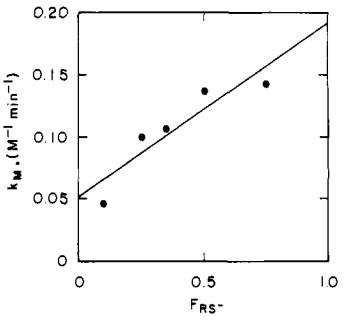


Figure 5. Dependence of k_M on the fraction of mercaptoethanol anion (F_{RS^-}) according to eq 10 in the reduction of **3** at 25 °C and ionic strength 1.0 M (KCl). The least-squares linear line gave k_{m0} = 0.0051 M^{-1} min^{-1} and k_{m1} = 0.019 M^{-1} min^{-1}.

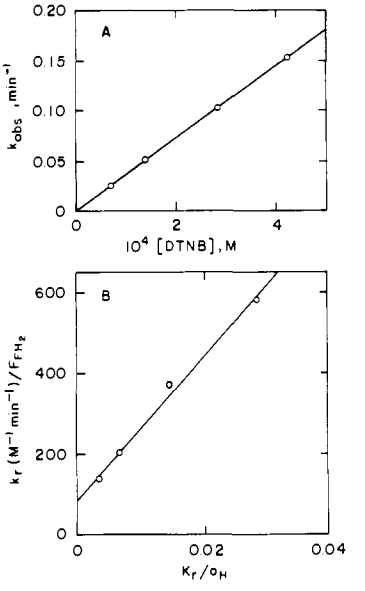


Figure 6. (A) Dependence of k_{obsd} on [DTNB] for the oxidation of 8 μM reduced **3** by DTNB at pH 4.61, 25 °C, and ionic strength 1.0 M (KCl). k_R (slope) = 366 M^{-1} min^{-1}. (B) Plot of k_R/F_{FH_2} vs. K_r/a_H. k_{r0}(intercept) = 8.5 × 10^1 M^{-1} min^{-1} and k_{r1}(slope) = 1.8 × 10^4 M^{-1} min^{-1}.

Reaction of Reduced 3 with DTNB. This reaction showed pseudo-first-order kinetics with excess DTNB. Figure 6A shows

Table I. General-Acid-Catalyzed Rate Constants (k_{AC}) at Various pH Values for the Reaction of DTT with 3 at 25 °C and Ionic Strength 1.0 M (KCl)

general acid	pK^a	total buffer, M	pH	k_{AC}, M^{-2} min^{-1}	$10^{-4}k_{AC}'$,[g] M^{-2} min^{-1}
methoxyacetic acid	3.39	0–0.5	8.38	5.14	339
acetic acid	4.59	0–0.4	8.29[e]	18.0	72.9
		0–0.4	8.36[f]	16.7	69.6
		0–0.5	9.59[e]	11.1	223
		0–0.5	9.85[e]	9.0	332
		0–0.5	10.00[e]	7.36	434
methoxyammonium chloride	4.72[b]	0–0.5	8.19	11.1	32.6
potassium phosphate	6.46	0.1–0.18	8.22[e]	119	6.51
imidazole hydrochloride	7.24[c]	0.004–0.10	8.03	219	2.13
glycine ethyl ester hydrochloride	7.95	0.02–0.07	8.20	269	0.729
morpholine hydrochloride	8.87	0–0.05	7.74	54.4	0.152
		0.01–0.23	7.92	76.8	0.152
		0.02–0.12	9.22	270	0.176
		0.02–0.12	9.69	242	0.337
		0.02–0.12	9.88	176	0.387
triethylenediamine hydrochloride	9.21	0.02–0.12	8.51	130	0.0826
		0.02–0.08	9.02	216	0.0867
		0.02–0.12	9.49	313	0.164
		0.02–0.18	10.06	175	0.344
3-quinuclidinol hydrochloride	10.11	0.02–0.12	9.25	111	0.0249
		0.02–0.12	10.02	153	0.0646
		0.02–0.12	10.47	132	0.197
		0.04–0.24	10.75	90.5	0.362
piperidine hydrochloride	11.45[d]	0–0.20	9.09[e]	21.6	0.00491
		0–0.2	9.28[e]	28.6	0.00560
		0–0.2	9.78[e]	45.6	0.00894
		0.04–0.24	10.23	48.7	0.0157
		0.04–0.24	10.56	57.0	0.00342
pyrolidine hydrochloride	11.65[d]	0.02–0.12	8.99[f]	21.5	0.00544

[a] The pK values were determined by titration unless noted otherwise. [b] Reference 73. [c] Reference 74. [d] pK determined from the pH of a solution of known ratio of acid to conjugate base corrected for water titration. [e] pH maintained with triethylenediamine. [f] pH maintained with morpholine. [g] $k_{AC}' = k_{AC}/(F_{HA}F_{HSS^-})$.

Table II. General-Acid-Catalyzed Rate Constants for the Reaction of the Mono- and Dianions of DTT with 3 at 25 °C and Ionic Strength 1.0 M (KCl)

general acid	pK^a	monoanion k_{ac_1}, M^{-2} min^{-1}	dianion k_{ac_2}, M^{-2} min^{-1}
hydronium ion	−1.74	2.19×10^9	[b]
methoxyacetic acid	3.39	3.39×10^6	
acetic acid	4.59	6.50×10^5	3.30×10^6
methoxyammonium chloride	4.72	3.26×10^5	
potassium phosphate	6.46	6.51×10^4	
imidazole hydrochloride	7.24	2.13×10^4	
glycine ethyl ester hydrochloride	7.95	7290	
morpholine hydrochloride	8.87	1496	3260
triethylenediamine hydrochloride	9.21	747	2070
3-quinuclidinol hydrochloride	10.11	150	540
piperidine hydrochloride	11.45	40.0	69.4
pyrolidine hydrochloride	11.65	54.4	
water	15.74	0.160	0.169

[a] See Table I for pK origins. [b] If the solvent rate term k_{a_1}-$[HSS^-][Fl]$ represented $k_{ac_2}[H^+][SS^{2-}][Fl]$, k_{ac_2} would be 1.5×10^{11} M^{-2} min^{-1}. It is more likely that this term arises from water catalysis with DTT monoanion.

Table III. Comparison of the Rate Constants k_A, k_{AC}, and k_B for the Reaction of DTT and DTE with 3 at 25 °C and Ionic Strength 1.0 M (KCl)

thiol	pH	k_A, M^{-1} min^{-1}	k_{AC},[a] M^{-2} min^{-1}	k_B, M^{-1} min^{-1}
DTT	9.69	5.6	242	38.8
DTE	9.64	6.3	179	16.0
DTT/DTE		0.89	1.35	2.43

[a] Morpholine buffer.

a plot of k_{obsd} vs. [DTNB] and demonstrates that the reaction is also first order in DTNB. The data were analyzed according to eq 11, and a plot of k_R/F_{FH_2} vs. K_r/a_H is shown in Figure 6B. The values calculated for k_{r0} and k_{r1} were 85 M^{-1} min^{-1} and 1.8×10^4 M^{-1} min^{-1}, respectively.

Table IV. Values of k_M and k_{MM} in the Reaction of ME and MP with 3 at 25 °C and Ionic Strength 1.0 M (KCl)

pH	$F_{(RS^-)}$	k_M, M^{-1} min^{-1}	k_{MM}, M^{-2} min^{-1}	k_{mm},[a] M^{-2} min^{-1}
		Mercaptoethanol		
8.61	0.10	0.0045	0.114	1.27
9.09	0.25	0.0100	0.218	1.16
9.32	0.35	0.0106	0.270	1.19
9.60	0.50	0.0137	0.278	1.11
10.12	0.75	0.0142	0.238	1.27
		Mercaptopropionate		
9.63	0.20	0.0066	0.208	1.30

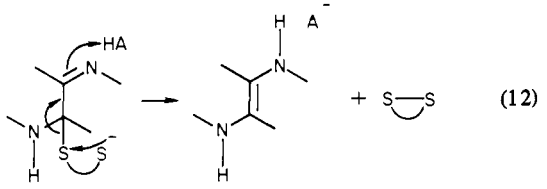
[a] $k_{mm} = k_{MM}/(F_{RSH}F_{RS^-})$. The average value for k_{mm} is 1.20 M^{-2} min^{-1}.

Discussion

The arguments presented below support the mechanism depicted in Scheme I of the Introduction. The observed leveling off in rate as buffer concentration was increased (Figure 1) is evidence for a change in rate-determining step from a step involving buffer catalysis to one which does not.[27] This implies the existence of at least one intermediate. Any mechanism proposed to explain the results must therefore include an attack step and a breakdown step. All one-step mechanisms such as hydride transfer[28] are thus ruled out.

Assignment of Catalysis at N(5). (1) Fixing the Proton.[29] Catalysis cannot occur at N(1) or -SH in attack. Catalysis at N(1), e.g., in breakdown of a C(4a) adduct (eq 12), could only

(27) Jencks, W. P. "Catalysis in Chemistry and Enzymology"; McGraw-Hill: New York, 1969; p 571.
(28) Reference 27, p 170.
(29) Reference 27, p 187.



(12)

occur at pH values below the reduced flavin N(1) pK (6.45)[26] where the protonated species is thermodynamically favored. Because buffer catalysis was observed at pH considerably above this pK, catalysis cannot occur at N(1). If buffer catalysis resulted from general-base catalyzed sulfur deprotonation during attack on flavin, the observed rate that might be reached by adding buffer could never exceed the rate of fully ionized sulfur, because HS–S$^-$ would always be a better nucleophile than HS–SH···B. This is a restatement of a rule referred to by Jencks as *fixing the proton.*[29] At pH 9.49 with 60 mM triethylenediamine buffer k_2 was 21.5 M^{-1} min^{-1} which exceeds k_{a1} (8.8 M^{-1} min^{-1}), the rate constant which would represent uncatalyzed attack of HS–S$^-$ on flavin. Others have also failed to detect catalysis of this type in reactions of thiols with various electrophiles.[30-32]

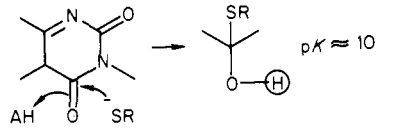
(2) Brønsted Plot. Assume, as will be shown, that the Brønsted plot of Figure 3 represents an attack step. The DTT solvent attack term, k_{a1}[HSS$^-$][Fl], is kinetically ambiguous and could represent hydronium ion catalyzed attack of SS^{2-}, k_{a1}/K_2[H_3O^+][SS^{2-}][Fl] with $k_{a1}/K_2 = 1.25 \times 10^9$ M^{-2} s^{-1}, rather than water-catalyzed attack of HSS$^-$. The former is ruled out by comparison with the k_{a0} attack term written as hydronium ion catalyzed attack of HSS$^-$, k_{a0}/K_1[H_3O^+][HSS$^-$][Fl] where $k_{a0}/K_1 = 3.7 \times 10^7$ M^{-2} s^{-1}. The 17-fold difference in apparent rate constants after statistical correction is unreasonable, inasmuch as it implies $\beta_{nuc} = 6.2$ for thiolate in a compound having two thiols differing by 1.6-fold in basicity. This conclusion is also supported by an argument based on diffusion-controlled rates.[87]

An α value of 0.58 is usually associated with concerted catalysis. Because all general acids studied, including hydronium ion and water, lie on a single Brønsted line, it is likely that they all involve catalysis by the same mechanism.

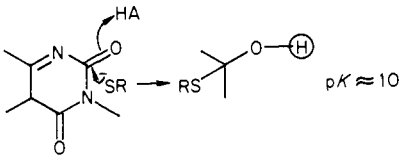
$k_{ac2}/k_{ac1} = 2.57$ was determined from data on morpholine, triethylenediamine, 3-quinuclidinol, and piperidine (Table II). After statistical correction this converts to a β_{nuc} of 0.55 to suggest the occurrence of carbon–sulfur bond making in the transition state.

As the pK of the general acid increases, the Brønsted curve for general-acid-catalyzed addition must show a break to a slope of −1 when out diffusion[35] becomes rate determining. Sayer and Jencks[36,37] have argued that the break must occur at a pK less than or equal to the pK of the adduct. Because we observe no break or curvature below the pK of water, $pK_{HOH} < pK_{break} \leq pK_{Add}$. Similarly the "Libido Rule"[38,39] for concerted catalysis requires the pK of any general acid to lie between the pK values of the site on reactant and product involved in proton transfer. By this rule, addition at flavin C(4a) may be catalyzed by general acids with pK between −6.6 and 22, the pK of N(5) in flavin and adduct, respectively.[3] Catalysis by buffers with pK between −1.75 and 15.75 is thus consistent with concerted catalysis with proton transfer to N(5).

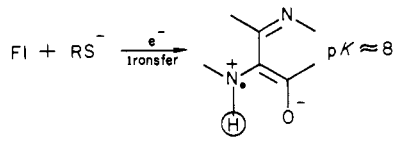
Reactions 13–15 cannot be involved in buffer catalysis, because the pK (encircled proton) of the atoms receiving catalysis are all



(13)



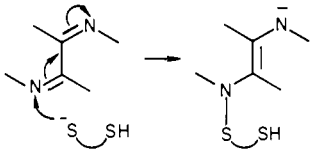
(14)



(15)

less than that of water. Note that these reactions are kinetically ambiguous. The *only* heteroatom which could exhibit a pK greater than that of water is the N(5) aniline-like nitrogen in reduced flavin or in certain flavin adducts. Although this locates the site of catalysis, there are several mechanisms that could give rise to species which have an N(5) proton with a pK greater than 16.

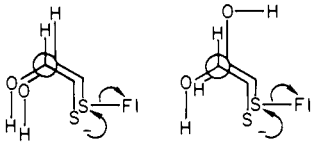
Absence of Catalysis in Breakdown. (1) Arguments Based on the pH Rate Profile of k_{b1}. If the step not catalyzed by buffer (k_{b1}, Figure 2, open circles) was due to an attack step (e.g., attack at N(5)), the rate constant for DTT monoanion attack would be $k_{b1} = 63$ M^{-1} min^{-1} (see eq 16). One would expect an analogous



(16)

attack step, k_{b2}, with the DTT dianion. There is no evidence for the dianion term in Figure 2 to pH 11.36. The value of k_B at this pH (3.4 M^{-1} min^{-1}) was more than 17 times smaller than k_{b1}. But due to the greater basicity of dithiolate, k_{b2} is expected to be greater than k_{b1}, if k_{b1} refers to attack. Therefore k_{b1} cannot refer to attack and must refer to a breakdown mechanism not subject to buffer catalysis.

(2) Stereochemical Effects. In the reaction of DTE with **3** (Table III), the rate constant most affected by the change from DTT to DTE as reductant was the k_B term. The attack steps for these two diastereomers are expected to be similar, because they have virtually identical values of pK_1 and pK_2. While ring closure in the breakdown reaction can occur without steric hindrance from the boat and chair forms of DTT and the chair form of DTE, steric hindrance occurs between eclipsing hydroxyls in the boat form of DTE. Steric hindrance is expected to make breakdown slower for DTE than for DTT and is consistent with assigning k_B to breakdown.



DTE-Fl Boat Form DTT-Fl Boat Form

(3) Reaction of 3 with ME. The reaction of monothiols and flavins was studied by Yokoe and Bruice[9] for the reaction of thiophenol with 3,10-dimethyl-8-cyanoisoalloxazine. They found the reaction to be first order in flavin, to be second order in thiol, to obey the rate law k[RSH][RS$^-$][Fl], and to show no buffer catalysis. The results of Holden and Main[40] with riboflavin and ME confirmed those of Yokoe and Bruice and showed in addition that the reaction of riboflavin ionized at N(3) (pK = 9.8) was undetectable to pH 10.5.

(30) Lienhard, G. E.; Jencks, W. P. *J. Am. Chem. Soc.* **1966**, *88*, 3982.
(31) Reference 27, p 500.
(32) Kallen, R. G. *J. Am. Chem. Soc.* **1971**, *93*, 6227, 6236.
(33) Scott, R. L. *J. Phys. Chem.* **1971**, *75*, 3843. Sayer, J. M.; Jencks, W. P. *J. Am. Chem. Soc.* **1973**, *95*, 5637. Hand, E. S.; Jencks, W. P. *Ibid.* **1975**, *97*, 6221. Estimates of K_e vary from 0.017–0.3 M^{-1}.
(34) Eigen, M. *Angew. Chem., Int. Ed. Engl.* **1964**, *3*, 1.
(35) The term "out diffusion" is taken to mean the diffusion apart of two species from an encounter complex; "in diffusion" means the diffusion together of two species to form an encounter complex.
(36) Sayer, J. M.; Jencks, W. P. *J. Am. Chem. Soc.* **1973**, *95*, 5637.
(37) Sayer, J. M.; Jencks, W. P. *J. Am. Chem. Soc.* **1974**, *96*, 7998.
(38) Jencks, W. P. *J. Am. Chem. Soc.* **1972**, *94*, 4731.
(39) Jencks, W. P. *Chem. Rev.* **1972**, *72*, 705.
(40) Holden, J. W.; Main, L. *Aust. J. Chem.* **1977**, *30*, 1387.

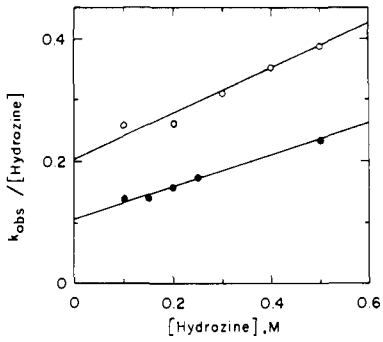
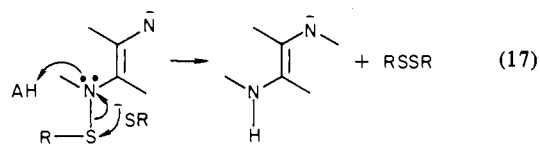


Figure 7. Dependence of k_{obsd}/[hydrazine] on [hydrazine] in its reaction with DTNB at pH 8.62 (○) and pH 8.20 (●). The buffer-catalyzed rate constants k_{HC}(slopes) are tabulated in Table V. The solvent rate constants k_H(intercepts) = 0.015 and 0.023 M^{-1} min^{-1}.

Our data with **3** and ME were similar, except for our having observed a small first-order term in ME, which is discussed in Appendix II. The possibility that the first- and second-order ME terms (Figure 4) were from attack (e.g., where the former might be solvent-catalyzed RS^- attack and the latter RSH-catalyzed RS^- attack) can be ruled out because the rate constant for the ME monoanion term (k_{m1} = 0.019 M^{-1} min^{-1}) was much smaller than the corresponding DTT monoanion term (k_{a1} = 8.8 M^{-1} min^{-1}). Because of similar pK values these two nucleophiles must have similar attack rate constants. We also found no evidence for buffer catalysis in the reactions of monothiols with flavins.

Because the term k_{mm}[RSH][RS^-][Fl] was second order in ME, it must refer to breakdown, most likely to disulfide formation from a C(4a) flavin–thiol adduct in a step analogous to the k_{12} step shown in Scheme I.

The k_{mm} term cannot arise from breakdown of an N(5) adduct (eq 17) because such a reaction would be buffer catalyzed and



(17)

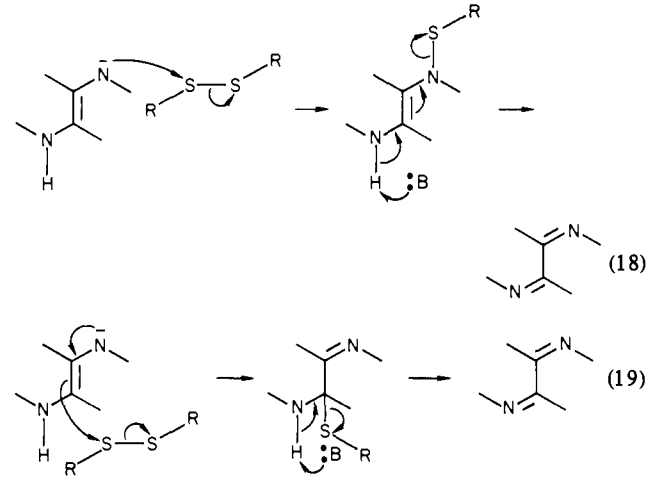
ought to exhibit three solvent terms by analogy with the DTT solvent terms (k_{a0}, k_{a1}, and k_{a2}). Analogous terms were not seen with ME. The term k_{mm}[RS^-][RSH] resembles therefore the term k_{b1}[HSS^-] for DTT, and both must represent breakdown terms.

The above arguments establish that buffer catalysis does not occur in a breakdown step and require that catalysis must occur at N(5). Attacks at C(1a), C(2), C(4), N(5), C(6), and C(8) all require catalysis at N(5) in breakdown and are thus ruled out. One mechanism that satisfies these criteria involves thiol attack at N(1) with acid catalysis at N(5).

Attack Cannot Occur at N(1). N(1) adducts are not generally considered in flavin reactions, because this nitrogen seems not to be electron deficient.[41] While N(5) adducts may be the more stable, *it is not clear that N(1) adducts are so unstable that they could not be kinetically significant.* Additions to N(5) alkylflavonium ions are assumed to occur at C(4a)[42,43] but 1,4 (Michael) addition to form N(1) adducts is not unreasonable. Chan and Bruice recently showed that nitromethane carbanion adds at C(4a) and not at N(1) to an N(5) alkylflavonium ion.[44]

Although Bruice ruled out dithiol attack at C(1a) based on the reaction of a sterically hindered flavin (10-(2,6-dimethylphenyl)-3-methylisoalloxazine),[45] molecular models show that sulfur could still bond at N(1) in this flavin.

The steps involved in the *reverse direction* for mechanisms involving N(1) and C(4a) attack are respectively eq 18 and 19.

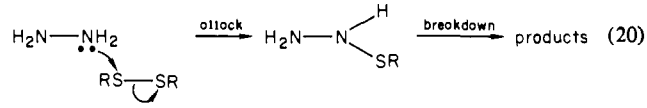

(18)

(19)

The first step involves either the enamine nitrogen anion (N(1) reaction) or the imine α-carbanion (C(4a) reaction). In the former one would expect any N anion of basicity similar to N(1) in reduced flavin (pK = 6.45) to react with a disulfide at a rate similar to that of reduced flavin.[46] Figure 6 shows results for DTNB reduction by reduced **3**. The first step of eq 18 or 19 must be rate determining from the principle of microscopic reversibility.[88]

Reduced flavin anion reacts with DTNB as if it were a strong nucleophile. From the linear free energy relationship of Hupe and co-workers,[50] we calculate that attack on DTNB by a thiol of pK similar to that of reduced flavin (pK = 6.45) should show a rate constant only 37 times k_{r1}. From the data of Wright and co-workers[51] attack by cyanide (pK = 8.9) should show a rate constant only about 14 times k_{r1}.

The reaction of hydrazine (pK = 8.26)[75] with DTNB was chosen as a model for possible flavin N(1) attack on a disulfide.[89] With excess hydrazine two moles of thiol anion were formed per mole of DTNB as determined spectrophotometrically in what was apparently a two-step process (see eq 20). Only the first (attack)



(20)

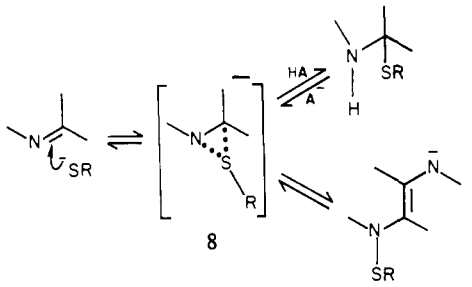
step was studied. Figure 7 indicates that the reaction at constant pH obeyed

$$k_{obsd}/[\text{hydrazine}] = k_H + k_{HC}[\text{hydrazine}]$$

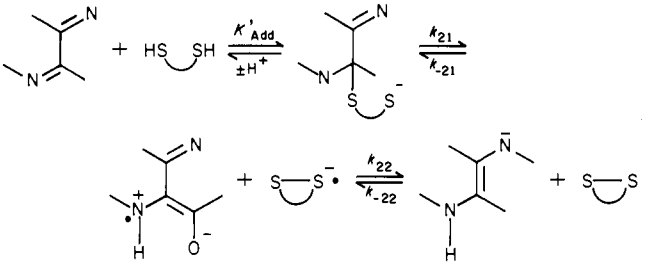
The second-order hydrazine term suggests that hydrazine acted both as a nucleophile and general catalyst. Because the slope and intercept values were greater the greater the fraction of hydrazine free base, it is likely that the base was the reactive species for both the catalyzed and solvent terms. Buffer catalysis was confirmed with morpholine and acetate. A table of buffer-catalyzed rate constants, a Brønsted plot, and a more complete discussion of the hydrazine–DTNB reaction appear in Appendix I. The rate

(41) Sun, M.; Song, P.-S. *Biochemistry* **1973**, *12*, 4667.
(42) Hemmerich, P.; Ghisla, S.; Hartmann, U.; Müller, F. "Flavins and Flavoproteins"; Kamin, H. Ed.; University Park Press: Baltimore, MD, 1971; p 83.
(43) Kemal, C.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 995.
(44) Chan, T. W.; Bruice, T. C. *Biochemistry* **1978**, *17*, 4784.
(45) Bruice, T. C.; Main, L.; Smith, S.; Bruice, P. Y. *J. Am. Chem. Soc.* **1971**, *93*, 7327.
(46) This method of distinguishing between kinetically ambiguous mechanisms is referred to as "model compounds" by Jencks (ref 27, p 188). The most frequent example is the replacement of a proton by a methyl group.
(47) Because the σ_m for carboxyl is small (−0.1), the pK of 3-carboxy-4-nitrothiophenol (CNP) should be similar to that of 4-nitrothiophenol (pK = 4.50) reported in ref 48. A plot of pK values for ArOH vs. ArSH gave a slope of 1.20. If ρ = 2.23 for phenol ionization (ref 49), then ρ = 1.86 for thiophenol ionization and the pK of CNP is about 4.31.
(48) Jencks, W. P.; Salvesen, K. *J. Am. Chem. Soc.* **1971**, *93*, 4433.
(49) Exner, O. "Advances in Free Energy Relationships"; Chapman, N. B., Shorter, J., Eds.; Plenum Press: New York, 1972; p 1.
(50) Wilson, J. M.; Bayer, R. J.; Hupe, D. J. *J. Am. Chem. Soc.* **1977**, *99*, 7922.
(51) Happer, D. A. R.; Mitchell, J. W.; Wright, G. J. *Aust. J. Chem.* **1973**, *26*, 121.

Scheme II

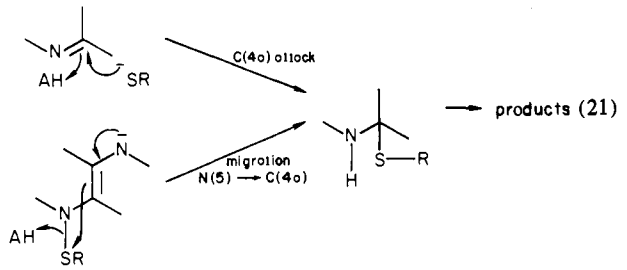


Scheme III



constant for hydrazine attack on DTNB with solvent catalysis must be $\leq 0.87\ M^{-1}\ min^{-1}$ (Appendix I) which is much smaller than $k_{r1} = 1.8 \times 10^4\ M^{-1}\ min^{-1}$ (Figure 6B) for reduced flavin reacting with DTNB. Even with strongly nucleophilic hydrazine the amine reaction is at least 4 orders of magnitude too slow to account for the flavin reaction. Thus mechanisms involving N(1) adducts cannot account for the flavin–thiol reaction.

Rate-Determining Step in Attack Must Form a C(4a) Adduct. Catalysis must occur at N(5) in attack, and thiol attack does not occur at N(1). Only two mechanisms fit these criteria: C(4a) attack and N(5) to C(4a) thiol migration (eq 21). Migration

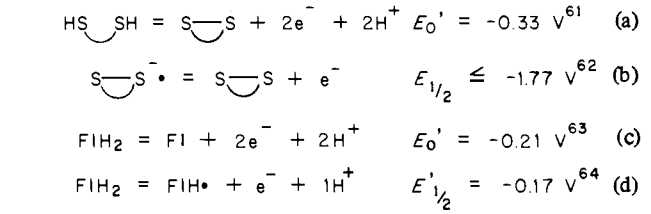


could occur only after initial rapid formation of the N(5) adduct. Some evidence for N(5) to C(4a) group migration has been obtained for both benzyl[52,53] and indole[54]–flavin adducts.

In the reaction of 10-(2,6-dimethylphenyl)-3-methylisoalloxazine-6,8-disulfonate with sulfite,[12,13] both the N(5) and C(4a) adducts were observed, and interconversion of these species by intramolecular rearrangement could not be ruled out.

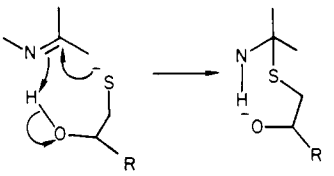
Bruice[55] has noted that migrations occur with adducts that have a stabilized carbonium ion migrating moiety. Although a migrating thiol cation is possible, a thiol anion is more appealing. Scheme II might apply to migration of thiol. Species like **8** (not necessarily a transition state) may exist by virtue of back-bonding into sulfur d orbitals and have been considered by others, including Lienhard and Jencks[30] and Friedman and co-workers,[56] to explain certain kinetic results such as the high nucleophilicity of sulfur relative to nitrogen. Because both the N(5) and C(4a) positions are known to be electron deficient, a sulfur anion approaching both in the transition state might be reasonable. **8** might partition to the N(5) or C(4a) adduct. In the absence of evidence for migration, direct C(4a) attack represents the simplest mechanism. Direct formation of a C(4a) flavin–thiol adduct (an N–S acetal) represents a class e–f reaction in the terminology proposed by Jencks.[57] There is ample precedent for the attack at C(4a), including the attack of thiols on imines[32,58] and carbonyls.[30,59,60] Of particular relevance is the formation of an adduct analogous

Scheme IV



to a C(4a) adduct between thiols and a nonplanar flavin analogue.[86]

Intramolecular general-acid catalysis might be expected to occur in the formation of the C(4a) adduct because of the adjacent hydroxyl groups of DTT. Its signature in the DTT–flavin reaction



would be a positive deviation of the water point in attack (k_{a1} monoanion term) from the Brønsted line. No such deviation was observed. Because the effective molarity for concerted proton transfer reactions is not expected to exceed 50 M,[69–72] the DTT–hydroxyl group ($pK \simeq pK_{H_2O}$) could react at a rate equivalent to that of 50 M water at most. But water is already 55.5 M. Thus intramolecular catalysis could not more than double the observed solvent term relative to that expected from the Brønsted line. An effect this small would not be readily apparent.

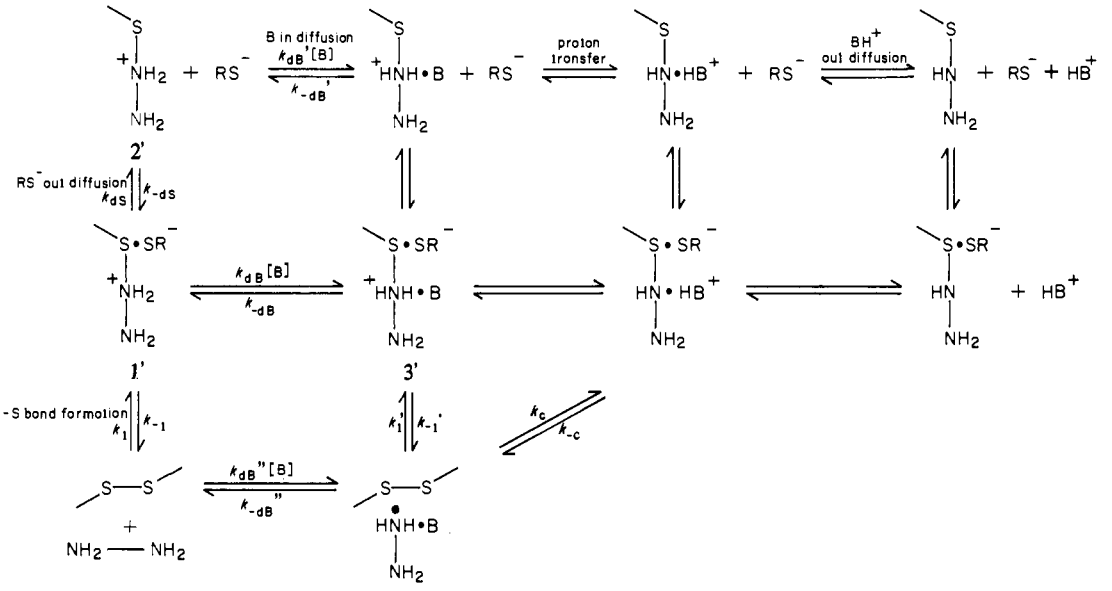
Breakdown Reaction Does Not Proceed by a Radical Mechanism. The breakdown step is written as an S_N2 reaction (Scheme I) which, when written in the reverse direction, is analogous to attack of cyanide ion on disulfides.[51] However, a radical mechanism can be written leading to the formation of a disulfide radical anion and neutral flavin radical. This mechanism must be considered because of the stability of flavin radicals.[52] Either step k_{21} or k_{22} of Scheme III could be rate determining. Based on the redox potentials (Scheme IV), one can calculate whether radical intermediates can lie on the reaction path.

The potential of (b) applies to the disulfide of 1,3-dithiopropane in acetonitrile as measured by polarography at a gold electrode. It was noted that in water this reaction must have an even lower potential. The redox potential for radical formation is [(a) + (d)] *minus* [(b) + (c)] or $>+1.5$ V, which at 25 °C and pH 7 would give

$$K'_{Add}k_{21}/k_{-21} \leq 10^{-25}$$

(52) Walker, W. H.; Hemmerich, P.; Massey, V. *Eur. J. Biochem.* **1970**, *13*, 258.
(53) Hemmerich, P.; Haas, W. "Reactivity of Flavins"; Yagi, K., Ed.; University Park Press: Baltimore, MD, 1975; p 1.
(54) Clerin, D.; Bruice, T. C. *J. Am. Chem. Soc.* **1974**, *96*, 5571.
(55) Bruice, T. C. "Progress in Bioorganic Chemistry"; Kaiser, E. T., Kézdy, F. J., Eds.; Wiley: New York, 1976; p 1.
(56) Friedman, M.; Cavins, J. F.; Wall, J. S. *J. Am. Chem. Soc.* **1965**, *87*, 3672.
(57) Jencks, W. P. *Acc. Chem. Res.* **1976**, *9*, 425.
(58) Ogata, Y.; Kawasaki, A. *J. Chem. Soc., Perkin Trans. 2* **1975**, 134.
(59) Barnett, R. E.; Jencks, W. P. *J. Am. Chem. Soc.* **1969**, *91*, 6758.
(60) Gilbert, H. F.; Jencks, W. P. *J. Am. Chem. Soc.* **1977**, *99*, 7931.
(61) Cleland, W. W. *Biochemistry*, **1964**, *3*, 480.
(62) Howie, J. K.; Houts, J. J.; Sawyer, D. T. *J. Am. Chem. Soc.* **1977**, *99*, 6323. Although the polarographic potential ($E_{1/2}' \leq 1.77$ V) given by Howie and co-workers is for two-electron reduction of 1,3-dithiopropane to dithiol dianion, the potential actually applies to disulfide anion radical formation which then rapidly captures a second electron, because of the higher redox potential of this second step.
(63) Walsh, C.; Fisher, J.; Spencer, R.; Graham, D. W.; Ashton, W. T.; Brown, J. E.; Brown, R. D.; Rogers, E. F. *Biochemistry* **1978**, *17*, 1942.
(64) Rao, P. S.; Hayon, E. *J. Am. Chem. Soc.* **1974**, *96*, 1287; **1975**, *97*, 2986.

Scheme V



Step k_{22} could be diffusion controlled (10^9 M^{-1} s^{-1})[64] so that the maximum rate constant for breakdown by this mechanism ($k_{22}k_{21}K'_{Add}/k_{-21}$) would be 10^{-16} M^{-1} s^{-1}, which is much smaller than the calculated rate constant at pH 7 of 7.6×10^{-3} M^{-1} s^{-1}. Thus Scheme III cannot represent the reaction mechanism.[65] The radical mechanism is discouraged chiefly by the instability and strong reducing power of the disulfide anion radical (Scheme IVb). Hoffman and Hayon[66] have studied these radicals in water by generating them from disulfides and hydrated electrons. For the scission of the sulfur–sulfur bond, they determined $k_f = 3.5 \times 10^5$ s^{-1} for

$$RS{-}\dot{S}R^- \underset{k_r}{\overset{k_f}{\rightleftharpoons}} RS^- + RS\cdot$$

cystamine anion radical at pH 7.7, while Adams and co-workers[67] measured $k_r = 4.9 \times 10^9$ M^{-1} s^{-1} at pH 7.4. When the small differences in pH are ignored, k_f/k_r would be 0.7×10^{-4} M, thus indicating that the thiyl radical (RS·) is also quite unstable.

In the reaction of reduced flavin with oxygen, there is evidence for the formation of superoxide (O_2^-·) during flavin oxidation.[19] Formation of a radical anion species during oxygen reduction but not during disulfide reduction may be related to the more positive (less strongly reducing) potential of superoxide ($E_{1/2} = -0.33$ V; pH 7).[68]

Appendix I. Mechanism of the Reaction between Hydrazine and DTNB

Table V summarizes data on buffer catalysis and Figure 8 is a Brønsted plot of these data. An "Eigen curve" was fitted to the points, but it is also possible that the data define a straight line with $\beta = 0.17$. The following discussion is not intended to establish the mechanism of the hydrazine–DTNB reaction uniquely but rather to provide a framework to calculate an upper limit for a possible reaction between reduced flavin N(1) and DTNB.

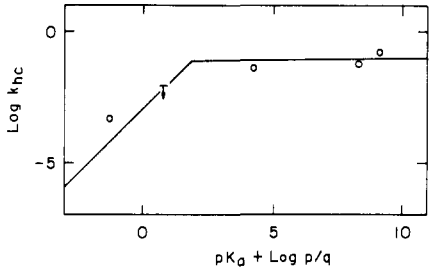


Figure 8. Brønsted plot for the general-base-catalyzed reaction of hydrazine with DTNB at 25 °C and ionic strength 1.0 M (KCl). The horizontal line is the average k_{hc} for acetate, hydrazine and morpholine. The line of slope 1 is drawn, as usual, one log unit below the point for water to account for the greater facilitated rate of diffusion of water relative to other bases.[34]

Table V. General-Base-Catalyzed Rate Constants (k_{hc}) for the Reaction of Hydrazine with DTNB at 25 °C and Ionic Strength 1.0 M (KCl)

general base	pK^a	total buffer, M	pH	k_{hc},[d] M^{-2} min^{-1}
water	−1.74			0.00045[e]
potassium dichloroacetate	0.92[b]	0–0.75	8.72	⩽0.0075
potassium acetate	4.59	0.2–0.8	8.85	0.065
hydrazine	8.26[c]	0.1–0.5	8.62	0.102
			8.20	0.116
morpholine	8.92	0–0.4	8.58	0.088

[a] pK values refer to the conjugate acid of the base. Values are from Table I unless noted otherwise. [b] Determined by measuring the pH of a solution to which a known amount of potassium hydroxide was added. [c] Reference 75. [d] $k_{hc} = k_{HC}/F_B$, where k_{HC} is the observed buffer-catalyzed rate constant at a particular pH. [e] This rate constant was determined from the rate constant first order in hydrazine (determined from the intercept values in Figure 7) divided by 55.5 M water.

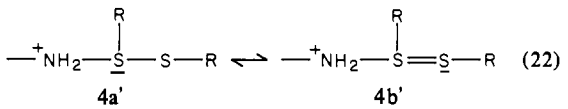
All of the possible steps in the formation of the sulfenamide between hydrazine and DTNB are shown in Scheme V. Four steps in this scheme might give rise to the buffer catalysis summarized in Table V: k_{dB}, k_{dB}', k_1', and k_C. k_{dB}' cannot be rate determining because k_{dB}'[B] must be larger than k_{-dS}[RS^-]. This is because k_{dB}' and k_{-dS} are both diffusion controlled and [B] is always >[RS^-].

If it is assumed that k_{dB} is rate determining (trapping mechanism) and equal to 10^9 M^{-1} s^{-1}, then K_1 must equal 1.3×10^{-12}

(65) Similarly, an intermediate caged flavin–disulfide radical pair is ruled out because it could not be 10^{12} times more stable than the separated pair.
(66) Hoffman, M. Z.; Hayon, E. *J. Am. Chem. Soc.* 1972, *94*, 7950.
(67) Adams, G. E.; Armstrong, R. C.; Charlesby, A.; Michael, B. D.; Willson, R. L. *Trans. Faraday Soc.* **1969**, *65*, 732.
(68) Sawada, Y.; Iyanagi, T.; Yamazaki, I. *Biochemistry* **1975**, *14*, 3761. Sayer, D. T. *Acc. Chem. Res.* **1979**, *12*, 105.
(69) Kayser, R. H.; Pollack, R. M. *J. Am. Chem. Soc.* **1977**, *99*, 3379.
(70) St. Pierre, T.; Jencks, W. P. *J. Am. Chem. Soc.* **1968**, *90*, 3817.
(71) Page, M. I.; Jencks, W. P. *J. Am. Chem. Soc.* **1972**, *94*, 8818.
(72) Harper, E. T.; Bender, M. L. *J. Am. Chem. Soc.* **1965**, *87*, 5625.
(73) Fox, J. P.; Jencks, W. P. *J. Am. Chem. Soc.* **1974**, *96*, 1436.
(74) Sayer, J. M.; Peskin, M.; Jencks, W. P. *J. Am. Chem. Soc.* **1973**, *95*, 4277.
(75) Bell, R. P.; Evans, P. G. *Proc. R. Soc. London, Ser. A* **1966**, *291A*, 297.

M^{-1} to give the observed buffer rate constant (0.087 M^{-1} min^{-1}) for the horizontal portion of the "Eigen curve". As [buffer] increases, a leveling off in k_{obsd} must occur as k_1 becomes rate determining. No leveling off occurred below 0.077 M^{-1} min^{-1}, so that $k_1 \geq 0.077$ M^{-1} min^{-1} and k_{-1} ($=k_1/K_1$) $\geq 10^9$ s^{-1}. Because k_{-1} cannot be larger than 10^{10} s^{-1}, k_1 can be no larger than 0.87 M^{-1} min^{-1}. The k_{dS} step can be no faster than the solvent reaction (0.025 M^{-1} min^{-1}), thus $k_{dS} \leq 3 \times 10^8$ s^{-1}. In summary $K_1 = 1.3 \times 10^{-12}$ M^{-1}, 0.87 M^{-1} $min^{-1} \geq k_1 \geq 0.077$ M^{-1} min^{-1}, 10^{10} $s^{-1} \geq k_{-1} \geq 10^9$ s^{-1}, and $k_{dS} \leq 3 \times 10^8$ s^{-1}. The limiting value of k_{dS} establishes that the out diffusion of RS^- from **1'** is unusually slow if trapping applies (maximum $\sim 10^{10}$ s^{-1}). The limiting value for k_{-1} is such that **3'** might be expected to partition by k_{-1}' faster than by k_{-dB}. If this were true, then k_1' would be rate determining in a preassociation mechanism. These considerations do not allow a decision between k_1' and k_{dB} as the rate-determining step. The break in the Brønsted slope in Figure 8 from a slope of 1.0 to 0 occurs at p$K \approx 1.7$. If catalysis occurred by the trapping mechanism (k_{dB}), this should be the pK of **1'**; if it occurred by the preassociation mechanism, the pK of **1'** would be lower. Estimates of the pK of protonated **2'** ($-1.2 \leq pK_{2'} \leq +0.7$)[76] argue that the reaction proceeds by the preassociation mechanism. The preassociation mechanism also does not require that k_{dS} be unusually small because this step is avoided. The alternative interpretation of Figure 8 as a straight line with $\beta = 0.17$ is more consistent with a preassociation mechanism with hydrogen bonding than with concerted catalysis (k_c).

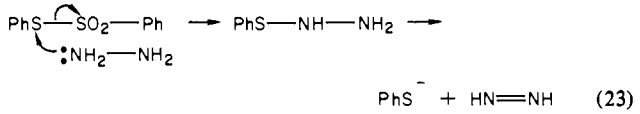
The resonance stabilized species **4'** (see eq 22) is probably not



4a' **4b'** (22)

involved because its pK should be greater than the pK of **2'**. The pK of **4b'** should be greater by about 5 units, as expected for an increase of one negative charge two atoms from an ionizing group (e.g., ΔpK between H_3PO_4 and $H_2PO_4^-$).[79] The pK difference between several tetrahedral intermediate pairs (i.e., (pK_{T-}) − ($pK_{T\pm}$)) is also about 5.[36,47,73] We assign p$K \simeq 5$ to **4b'** and p$K > 5$ to **4a'** on the basis of estimates of the pK of **2'**.

The formation of sulfenamides from the attack of amines on sulfenyl chlorides[80] and sulfenyl thiosulfonates[81] and disulfides[82] is known. Kice[83] has studied the reaction of hydrazine with benzene phenylthiosulfonate and has shown that eq 23 applies in



(23)

60% dioxane at 25 °C. The kinetics was similar to ours, showing a gradual increase in rate. In the first step phenylsulfenylhydrazine was formed, which then reacted to thiolate and diimine. In considering some of their data it appeared that the first step of this reaction had terms first and second order in hydrazine, the latter probably being a buffer-catalyzed term.

In summary, the preassociation mechanism (k_1') is favored. If this (or the concerted mechanism) applied, then the solvent term (k_H) in the hydrazine reaction is not a perfect analogy for attack of reduced flavin N(1) anion on DTNB, because general-base catalysis cannot occur in the latter. But without catalysis this reaction could not exceed the hydrazine solvent reaction, $k_H = 0.025$ M^{-1} min^{-1}. If the trapping mechanism applies, then comparison to $k_1 \leq 0.87$ M^{-1} min^{-1} is appropriate. This value is a limit for the attack of both hydrazine and reduced flavin N(1) on DTNB.

Appendix II. First-Order Terms in the Reaction of 3 with ME

These terms ($k_{m0} = 0.0051$ M^{-1} min^{-1}, $k_{m1} = 0.019$ M^{-1} min^{-1}) are probably not due to an impurity, because they do not diminish upon repeated distillation of ME. Nor is the oxidation of the alcohol moiety of mercaptoethanol to an aldehyde involved,[84] because 3-mercaptopropionate (MP) also showed these terms and because propanol showed no reaction. The results with MP also probably rule out an intramolecular reaction to form an intramolecular sulfenic ester.

These terms also cannot represent the formation of a sulfenic acid by hydroxide ion attack at the sulfur of a flavin–thiol adduct. Reaction by this mechanism would require a rate constant of 1.5×10^4 M^{-2} min^{-1}, which can be compared with breakdown through thiolate attack, $k_{mm} = 1.2$ M^{-2} min^{-1}, or a rate ratio of $\sim 10^4$. The corresponding values for hydroxide and thiolate attack on DTNB, 35[85] and 1.7×10^8 M^{-1} min^{-1},[50] respectively, give a rate ratio of $\sim 2 \times 10^{-7}$. The disparity of 10^{11} in this comparison rules out sulfenic acid formation. A related mechanism would involve thiolate attack on the oxygen of a flavin C(4a)–hydroxy adduct, which is expected to be 10^2–10^4 times less stable than the corresponding thiol adduct (Appendix I of ref 3). This would require that the thiolate reaction with the O adduct be as fast or faster than that with the S adduct. We know of no data that directly relate to this question.

The sulfur-containing product(s) of the ME reaction were not characterized, but because the k_{mm} term dominated, the major product was surely the disulfide, as has been shown by others for monothiol oxidation.[7,9] The flavin product was reduced flavin, as judged by its spectrum and the fact that oxygen completely regenerated oxidized flavin. The cause of the first-order monothiol term remains unknown.

(76) The pK of peroxyacetic acid (8.2) is 7.5 pK units below that of water. Because the pK of acetic acid (4.59) is about the same as that of thiol in 3-carboxy-4-nitrothiophenol (CNP),[47] it is expected that the pK of the hydrazine–CNP sulfenamide would be 7.5 pK units below the pK of hydrazine (8.26) or pK = 0.8. The pK of 1-methyluracil-4-sulfenic acid (MUSA) (6.3) is 9.4 pK units below that of water. A similar drop in pK for the hydrazine–CNP sulfenamide would give a pK of −1.2. This is a lower limit because the pK of the parent thiol of MUSA should be lower than that of CNP on the basis of two model compounds with lower pK values: thioacetic acid (pK = 3.62, ref 77) and 2-thiopyridine (estimated pK = −0.3). The latter was estimated from the difference in pK of 3.85 units between 2-hydroxypyridine (0.75, ref 78) and acetic acid (4.59) and the pK of thioacetic acid (3.62, ref 77).

(77) Irving, J.; Nelander, L.; Wadsö, I. *Acta Chem. Scand.* **1964**, *18*, 769.

(78) Rony, P. R.; Neff, R. O. *J. Am. Chem. Soc.* **1973**, *95*, 2896.

(79) Bjerrum, J.; Schwarzenbach, G.; Sillen, L. G. "Stability Constants of Metal-Ion Complexes", Chemical Society: London, England, 1957; Part II.

(80) Heimer, N. E.; Field, L. *J. Org. Chem.* **1970**, *35*, 3012.

(81) Dunbar, J. E.; Rogers, J. H. *J. Org. Chem.* **1968**, *31*, 2842.

(82) Bentley, M. D.; Douglass, I. B.; Lacadie, J. A.; Weaver, D. C. *J. Chem. Soc. D* **1971**, 1625.

(83) Kice, J. L.; Rogers, T. E.; Warheit, A. C. *J. Am. Chem. Soc.* **1974**, *96*, 8020.

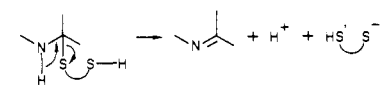
(84) Alcohol reduction of flavin is the reverse of the reaction extensively studied by Bruice, aldehyde oxidation of reduced flavin (see ref 21, 22, 23, 24).

(85) Determined from data at 1, 2, 5, and 10 mM KOH at 25 °C and ionic strength 1.0 M (KCl).

(86) Sayer, J. M.; Conlon, P.; Hupp, J.; Fancher, J.; Belanger, R.; White, E. J. *J. Am. Chem. Soc.* **1979**, *101*, 1890.

(87) If K_e for the formation of an encounter complex were 10^{-1} M^{-1},[33] then k_{a1}/K_2K_e, the rate constant for the second encounter to form the termolecular complex, becomes 1.3×10^{10} M^{-1} s^{-1}. This equals or exceeds proton diffusion ($\sim 10^{10}$ M^{-1} s^{-1})[34] and implies that subsequent bond-forming steps must exceed proton out diffusion[35] ($\sim 10^{11}$ s^{-1})[34] from the termolecular complex.

(88) In the attack of DTT on **3** $k_{a0} = 1.6$ M^{-1} min^{-1} (Figure 2) and $K_{Add} \approx 10^{-5}$ (following paper[2]). Therefore k_{-a0} is about 1.6×10^5 min^{-1} and refers to the second step in the reverse reaction.



Because the 3-carboxy-4-nitrothiophenolate group of DTNB should be a better leaving group than DTT monoanion,[47] its rate of elimination should be even faster. The second step rate constant of $>1.6 \times 10^5$ min^{-1} greatly exceeds the largest k_{obsd} (0.16 min^{-1}), and so the first step must be rate determining.

(89) Hydrazine is expected to give an upper limit to the rate at which N(1) of reduced flavin might react with DTNB. Hydrazine is a strong α-effect nucleophile 40 times more basic and less sterically hindered than reduced flavin N(1). Alloxanic acid (pK = 8.17), a structural analogue of reduced flavin N(1), was observed to react with DTNB more slowly than did hydrazine.