Rate Constants and Equilibrium Constants for Thiol–Disulfide Interchange Reactions Involving Oxidized Glutathione[1]

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Abstract: The rate of reduction of oxidized glutathione (GSSG) to glutathione (GSH) by thiolate anions (RS^-) follows a Brønsted relation in pK_as of the conjugate thiols (RSH): $\beta_{nuc} \simeq 0.5$. This value is similar to that for reduction of Ellman's reagent: $\beta_{nuc} \simeq 0.4$–0.5. Analysis of a number of rate and equilibrium data, taken both from this work and from the literature, indicates that rate constants, k, for a range of thiolate–disulfide interchange reactions are correlated well by equations of the form $\log k = C + \beta_{nuc}pK_a^{nuc} + \beta_c pK_a^c + \beta_{lg}pK_a^{lg}$ (nuc = nucleophile, c = central, and lg = leaving group sulfur): eq 36–38 give representative values of the Brønsted coefficients. The values of these Brønsted coefficients are not sharply defined by the available experimental data, although eq 36–38 provide useful kinetic models for rates of thiolate–disulfide interchange reactions. The uncertainty in these parameters is such that their detailed mechanistic interpretation is not worthwhile, but their qualitative interpretation—that all three sulfur atoms experience a significant effective negative charge in the transition state, but that the charge is concentrated on the terminal sulfurs—is justified. Equilibrium constants for reduction of GSSG using α,ω-dithiols have been measured. The reducing potential of the dithiol is strongly influenced by the size of the cyclic disulfide formed on its oxidation: the most strongly reducing dithiols are those which can form six-membered cyclic disulfides. Separate equilibrium constants for thiolate anion–disulfide interchange (K^{S^-}) and for thiol–disulfide interchange (K^{SH}) have been estimated from literature data: K^{S^-} is roughly proportional to $2\Delta pK_a$ is the difference between the pK_as of the two thiols involved in the interchange. The contributions of thiol pK_a values to the observed equilibrium constants for reduction of GSSG with α,ω-dithiols appear to be much smaller than those ascribable to the influence of structure on intramolecular ring formation. These equilibrium and rate constants are helpful in choosing dithiols for use as antioxidants in solutions containing proteins: dithiothreitol (DTT), 1,3-dimercapto-2-propanol (DMP), and 2-mercaptoethanol have especially useful properties.

Introduction

Oxidation of catalytically or structurally essential cysteine thiol groups can be an important mechanism for enzyme deactivation.[3] It is impractical to exclude oxygen completely from solutions of proteins during their manipulation, and the strategy normally followed to minimize the influence of autoxidation on enzymatic activity is based on the protection afforded by organic thiols (especially 2-mercaptoethanol and dithiothreitol[4,5]) present in excess. These added thiols probably inhibit the irreversible oxidative deactivation of proteins by several mechanisms. They reverse the initial stages of the protein autoxidation reactions by reduction of protein disulfides and sulfenic acids[6] to thiols. They may, by coordination, modify the catalytic activity of transition-metal ions in thiol autoxidation.[3,7] Their own oxidation consumes dioxygen and hydrogen peroxide[8] present in solution.

As part of a project aimed at the development of techniques to permit the use of enzymes as practical catalysts in the synthesis of organic and biochemicals,[9] we have begun to study the relation between the structures of organic thiols and their ability to prevent or reverse the autoxidation of proteins. The work described in this paper is focused on one aspect of this problem, viz., rate- and equilibrium-structure relations for the reduction of a model cystine-containing peptide (oxidized glutathione, GSSG, γ-glutamylcysteinylglycine) by base-catalyzed thiol–disulfide interchange. Oxidized glutathione was selected as substrate in this investigation for three reasons: it is readily available in pure form; its presence, as well as that of reduced glutathione (GSH), can be monitored in the presence of other thiols and disulfides using enzymatic assays;[10] and, because it occurs in biological systems,[3,11] information about its reactions with various thiols should prove generally useful. We[12] and Hupe et al.[13] have already reported rates of thiol–disulfide interchange reactions involving Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) and found them to follow a Brønsted correlation ($\beta_{nuc} \simeq 0.5$). Cleland et al. have carried out related studies with 4,4′-dipyridyl disulfide ($\beta_{nuc} = 0.34$), and Brocklehurst et al. have examined reductions of 2,2-dipyridyl disulfide ($\beta_{nuc} = 0.23$).[12,13] These studies indicate that thiolate–disulfide interchange is a mechanistically simple S_N2 displacement reaction, but suggest that the values of the characteristic Brønsted coefficients are sensitive to the particular set of reactants chosen. The extension of these model studies to GSSG was carried out for two reasons. First, the equilibria for reduction of diaryl disulfides with typical aliphatic thiols lie so far toward aryl thiol that equilibrium constants could not be measured conveniently. It was thus not possible to explore the influence of the structure of the reducing thiol on the thiol–disulfide interchange equilibria. Second, aryl thiols are arguably poor models for a protein cystine group.

Results

Rates of Reduction of Oxidized Glutathione by Mono- and Dithiols. The rate of release of GSH from GSSG was determined using a fast enzymatic following reaction at pH 7.0 (0.06 M phosphate buffer) and 30.0 ± 0.5 °C under argon. GSH was converted to *S*-lactoyl glutathione (GS-lac) by reaction with methylglyoxal in the presence of glyoxalase-I (GX-I), and the concentration of GS-lac was monitored spectrophotometrically at 240 nm, ϵ_0 3.37 mM^{-1} cm^{-1}.[14] Equations 1–6 list the reactions pertinent to this assay. In these equations, RSH is a monothiol reducing agent. Reaction 6, the enzymatic conversion of hemithioacetal to GS-lac, is essentially irreversible.[14]

$$RSH \overset{K_a^{RSH}}{\rightleftharpoons} RS^- + H^+ \quad (1)$$

$$RS^- + GSSG \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} GSSR + GS^- \quad (2)$$

$$RS^- + GSSR \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RSSR + GS^- \quad (3)$$

$$GSH \overset{pK_a^{GSH} = 8.72^{15}}{\rightleftharpoons} GS^- + H^+ \quad (4)$$

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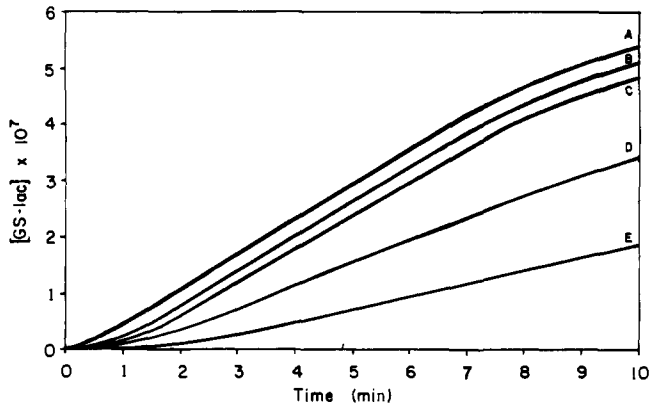


Figure 1. Formation of *S*-lactoyl glutathione (GS-lac, M) with time at 30.0 ± 0.5 °C, under argon in a pH 7.0 phosphate buffer (0.066 M) containing oxidized glutathione (GSSG, 0.35 mM), dithiothreitol (5.07 mM), and methylglyoxal (CH_3COCHO, 0.77 mM), and using (A) 24, (B) 2.4, (C) 1.8, (D) 1.2, (E) 0.3 U/mL, respectively, of glyoxalase-I (GX-I). The concentration of GS-lac was determined spectrophotometrically at 240 nm using ϵ_0 3.37 mM^{-1} cm^{-1}. There is no significant difference between d(GS-lac)/d*t* at enzyme concentrations of 24 and 1.8 U/mL; the concentration of enzyme used routinely in the assay was 2.4 U/mL.

$$GSCHOHCOCH_3 \overset{K_5 = 3\ mM^{14}}{\rightleftharpoons} GSH + CH_3COCHO \quad (5)$$

$$GSCHOHCOCH_3 + GX\text{-}I \xrightarrow{k_3} \underset{(GS\text{-}lac)}{GSCOCHOHCH_3} + GX\text{-}I \quad (6)$$

To derive rate expressions for k_1 from these equations, we require that the overall rate-limiting step in conversion of GSSG to GS-lac be the initial thiolate-disulfide interchange reaction (eq 2). We will assume that thiolate anion is the only nucleophile participating in the thiol-disulfide interchange reaction,[17] that reduced glutathione (GSH) is present only at a low, steady-state concentration, and that glyoxalase-I follows typical Michaelis-Menten kinetics with the hemithioacetal $GSCHOHCOCH_3$ as its only substrate (eq 7a).[14,16,18,19]

$$d(GS\text{-}lac)/dt = k_3(GX\text{-}I)\left[1 + \frac{K_m{}^{GSCHOHCOCH_3}}{(GSCHOHCOCH_3)}\right]^{-1} \quad (7a)$$

$$= k_3(GX\text{-}I)(GSH)\left[(GSH) + \frac{K_5K_m{}^{GSCHOHCOCH_3}}{(CH_3COCHO)}\right]^{-1} \quad (7b)$$

$$= [k_3(GX\text{-}I)(CH_3COCHO)/3\ mM^2](GSH) \quad (7c)$$

Substitution of the equilibrium expression for K_5 (eq 5) into eq 7a followed by rearrangement gives eq 7b. At a low steady-state concentration of GSH, eq 7b reduces to eq 7c for $K_5 \sim 3$ mM and $K_m{}^{GSCHOHCOCH_3} \sim 1$ mM.[20] The bracketed portion of eq 7c may, in principle, be made arbitrarily large and constant by adjusting the concentrations of either GX-I or CH_3COCHO; in practice, the quantity of GX-I used is the more easily varied. At the pH used for the assay (pH 7.0), the majority of the reduced glutathione is present as GSH rather than GS^-. Assuming these conditions and a steady-state concentration for GSH, we relate the rate of production of GS-lac to k_1 by the equation

$$0 = d(GSH)/dt = k_1(RS^-)(GSSG) + k_2(RS^-)(GSSR) - k_{-1}(GSSR)(GS^-) - k_{-2}(RSSR)(GS^-) - k_3[(GX\text{-}I)(CH_3COCHO)/3\ mM^2](GSH) \quad (8)$$

We ensure that kinetics observations extend only over the initial stages of the reaction, in which regime (GSSG) > (GSSR) > (RSSR): since the rate constants k_1, k_2, k_{-1}, and k_{-2} are all of the same magnitude, the second, third, and fourth terms of eq 8 can be neglected, and this expression may be combined with eq 7c and rewritten as eq 9a. For convenience, this equation was used in a form (eq 9b) involving an observed rate constant, $k_1{}^{obsd}$, based on the total concentration of thiol and thiolate anion in solution. The rate constants k_1 and $k_1{}^{obsd}$ are related by eq 10.

$$d(GS\text{-}lac)/dt = k_1(RS^-)(GSSG) \quad (9a)$$

$$= k_1{}^{obsd}[(RS^-) + (RSH)](GSSG) \quad (9b)$$

$$k_1 = k_1{}^{obsd}(1 + 10^{pK_a{}^{RSH} - pH}) \quad (10)$$

To ensure the accuracy of the assumptions and approximations made to generate eq 9, it is necessary to adjust the relative concentrations of the various species present in solution. Reactions were carried out using the following initial concentrations: $(RSH)_0 \simeq 0.77$ mM, $(GSSG) \simeq 0.35$ mM, and $(GX\text{-}I) \simeq 2.5 \times 10^{-4}$ mM (2.4 U/mL).[16] Because the starting concentration of thiol is much greater than any of the other solution components, this concentration is not significantly perturbed by hemithioacetal formation and remains essentially constant throughout the course of a kinetic run. The concentration of CH_3COCHO under these conditions is, of course, unknown; although a significant fraction of this material is probably converted to hemithioacetal, the velocity of equilibration of the hemithioacetal with aldehyde and hemihydrate is fast compared to the enzymatic reactions which follow.[14] Regardless of the equilibrium concentration of hemithioacetal, $(CH_3COCHO)_0 > (GSH)$ in the initial stages of the reaction. For small extents of reaction (in these experiments typically 10–20%) the concentration of CH_3COCHO can be considered constant. Thus, with the assumptions indicated, the rate of appearance of GS-lac will be directly proportional to the concentration of GSH. For the steady-state approximation to hold for GSH, it is necessary that it be converted to GS-lac much more rapidly than it is formed from GSSG. The rate of conversion of GSH to GS-lac can be adjusted over a wide margin by simply changing the concentration of glyoxalase-I or CH_3COCHO. The concentration of enzyme required to achieve steady-state conditions in GSH, at a convenient CH_3COCHO concentration, was established experimentally. The concentration (activity) of enzyme in solution was varied, and the value of (GS-lac) measured, holding all other parameters constant. At values of enzymatic activity greater than ~2 U/mL, increasing the activity of enzyme in solution shortened the duration of a non-steady-state interval at the beginning of the reaction, but did not influence the value of d(GS-lac)/d*t* once the steady-state region had been reached (Figure 1). More generally, the validity of eq 9 requires that d(GS-lac)/d*t* be zero order in GX-I and CH_3COCHO and first order in RSH and GSSG. Table I, which lists $k_1{}^{obsd}$ and velocities [d(GS-lac)/d*t*] for formation of GSH from GSSG using 2-mercaptoethanol as reducing agent, establishes that these conditions are fulfilled. The data in the first line of this table were obtained using the standard conditions employed routinely in this work; the other runs were performed on solutions in which these conditions were systematically varied. Analysis of the velocity data and extraction of the rate constant $k_1{}^{obsd}$ from these data are discussed later in the text. Under the conditions used, the assumptions made in obtaining eq 9 seem to be justified, since large deviations from the standard conditions have no influence on $k_1{}^{obsd}$.

Equation 9 applies to monothiol reducing agents. In this work, we were particularly interested in dithiols. In treating reducing agents, HSRSH, containing two identical thiol groups, we used an entirely analogous procedure, with two additional assumptions. First, we assumed that the nucleophilicities of the mono- and dianions, $HSRS^-$ and $^-SRS^-$,

Table I. Rate Constants (k_1^{obsd}, M^{-1} min^{-1}) for Reduction of Oxidized Glutathione (GSSG) by 2-Mercaptoethanol[a]

k_1^{obsd}	d(GS-lac)/dt[b]	$[HOCH_2CH_2SH]$[c]	$[CH_3COCHO]$[c]	[GSSG][c]	[GS-I][d]	comments[e]
8.8	14.4	5.07	0.76	0.32	2.4	standard
9.5	15.6	5.10	0.77	0.32	24.2	×10 GX-I
8.2	13.2	5.10	0.77	0.32	0.48	×0.2 GX-I
9.1	14.9	5.09	0.77	0.32	1.2	×0.5 GX-I
7.3	39.4	14.5	0.76	0.32	2.4	×3 RSH
9.3	1.5	0.49	0.75	0.31	2.4	×0.1 RSH
8.9	14.7	5.12	0.31	0.33	2.4	×0.5 CH_3COCHO
8.0	12.6	4.99	1.50	0.32	2.4	×2 CH_3COCHO
8.8	28.3	5.05	0.76	0.64	2.4	×2 GSSG
9.2	2.5	5.07	0.76	0.054	2.4	×0.17 GSSG

[a] All kinetics were run in 0.066 M phosphate buffer, pH 7.0, 30.0 ± 0.5 °C, under argon, were followed at 240 nm using ϵ_0 3.37 mM^{-1} cm^{-1}, and were corrected for blank reactions, if any. [b] Units of d(GS-lac)/dt are M^{-1} min^{-1} × 10^6. [c] Concentration units are mM. [d] In enzyme units (μmol/min) per mL. [e] This column indicates the major change from the standard conditions.

Table II. Rate Constants (k_1^{obsd}, M^{-1} min^{-1}) for Reduction of Oxidized Glutathione by Dithiothreitol[a]

k_1^{obsd}	d(GS-lac)/dt[b]	[DTT][c]	$[CH_3COCHO]$[c]	[GSSG][c]	[GS-I][d]	comments[e]
14.0	44.2	4.94	0.76	0.32	2.4	standard
14.6	47.0	4.97	0.77	0.32	24.2	×10 GX-I
8.4	26.9	4.97	0.77	0.32	0.48	×0.2 GX-I
13.1	41.3	4.94	0.77	0.32	1.8	×0.75 GX-I
13.0	81.6	9.94	0.76	0.32	2.4	×2 RSH
14.2	4.5	0.49	0.76	0.32	2.4	×0.1 RSH
9.8	31.9	5.01	0.39	0.33	2.4	×0.5 CH_3COCHO
12.1	37.1	4.86	1.50	0.32	2.4	×2 CH_3COCHO
12.7	80.1	4.93	0.76	0.64	2.4	×2 GSSG
14.0	5.8	4.94	0.76	0.042	2.4	×0.13 GSSG

[a] All kinetics were run in 0.06 M phosphate buffer, pH 7.0, 30.0 ± 0.5 °C, under argon. [b] Velocity of formation of GS-lac as determined spectrophotometrically at 240 nm using ϵ_0 3.37 mM^{-1} cm^{-1} and corrected for blank reactions, if any. Units of d(GS-lac)/dt are M min^{-1} × 10^6. [c] Concentration units are mM. [d] In units of enzyme activity per mL. [e] This column indicates the major deviation from standard conditions.

were indistinguishable. This assumption neglects electronic effects and a statistical factor of 2, both of which would make the dianion more reactive than the corresponding monoanion. The concentration of dianion will, however, be small relative to monoanion at pH 7 for the dithiols we have examined. Moreover, the observation (see below) that mono- and dithiols fall on the same Brønsted correlation line suggests that the cumulative error introduced into estimates of rate constants by this assumption is small. Second, we assume that, for the concentration of dithiol used, the rate of the initial intermolecular thiol-disulfide interchange is lower than that of a subsequent intramolecular reaction which releases a second equivalent of GSH (eq 11). A similar assumption, made in our previous treatment of the reduction of Ellman's reagent, could be justified experimentally because independent estimates of the rate constants for release of aryl thiols from diaryl disulfides and aryl alkyl disulfides indicated a sufficient difference to be detected.[12,13] Analysis of the equilibrium constants for reduction of GSSG by all of the dithiols examined below, except for glycol dimercaptoacetate, established a significant enhancement relative to analogous monothiols. If we assume that the rates of *inter*molecular reaction of mono- and dithiols having similar thiol pK_a values on GSSG will also be similar, the observed difference in equilibrium constants suggests that the rate of *intra*molecular thiol-disulfide interchange involving the second thiol group of an α,ω-dithiol is probably fast relative to the first intermolecular interchange (see below). Nonetheless, since we have not been able to measure rate constants for release of GSH from GSSG and GSSRSH by thiol-disulfide interchange experimentally, and since these values are probably similar for most of the thiols examined here,[21,22] we rely on analogy with this previous work to justify our assumption. Note, however, that, if this assumption is incorrect, it introduces an error of only a factor of 2 in the rate constants for dithiols.

$$HSRSH + GSSG \xrightarrow{slow} HSRSSG + GSH \quad (11a)$$

$$HSRSSG \xrightarrow{fast} \overline{SRS} + GSH \quad (11b)$$

With these assumptions, a treatment similar to that described previously leads to the expressions in eq 12 relating the observed rate of production of GS-lac to the rate constant of interest, k_1. Again k_1 and k_1^{obsd} are related by eq 10.

$$d(GS\text{-}lac)/dt = 2k_1[(HSRS^-) + (^-SRS^-)](GSSG) \quad (12a)$$

$$\cong 2k_1^{obsd}[(HSRSH) + (HSRS^-) + (^-SRS^-)](GSSG) \quad (12b)$$

Table II lists values of k_1^{obsd} and velocities [d(GS-lac)/dt] for formation of GSH from GSSG using dithiothreitol as reducing agent. The first line in the table reports data obtained using our standard conditions; the other runs were performed on solutions in which these standard parameters were varied. Again, under the conditions used, the assumptions made in obtaining eq 12 seem to be justified.

Straightforward consideration of material balance and integration of eq 9 (or eq 12) by standard methods gives[12,23]

$$k_1^{obsd}t = \frac{1}{(S)_0 - (GSSG)_0} \ln \frac{(GSSG)_0}{(S)_0} \times \left[\frac{(S)_0 - (GS\text{-}lac)/n}{(GSSG)_0 - (GS\text{-}lac)/n}\right] \quad (13)$$

The terms in the expression are defined as follows: for monothiols $S_0 \equiv [(RS^-) + (RSH)]$, $n = 1$; for dithiols $S_0 = [(^-SRS^-) + (^-SRSH) + (HSRSH)]$, $n = 2$. Figure 2 reproduces typical kinetic data for reduction of GSSG by several mono- and dithiols as derived using the integrated rate expression (eq 13). The crude absorbance data used to generate

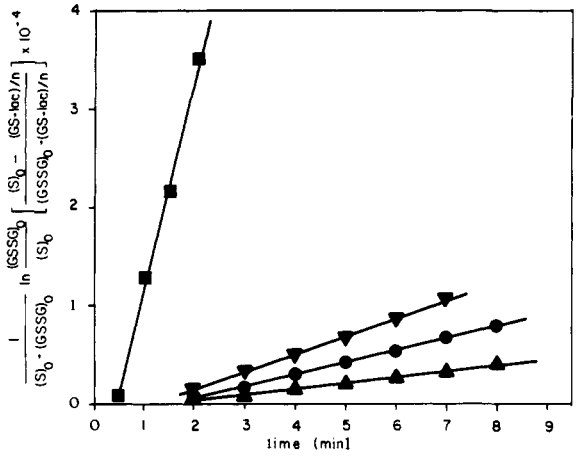


Figure 2. Rate-constant plots for the reaction of several mono- and dithiols with oxidized glutathione (GSSG) in pH 7.0 phosphate buffer (0.066 M) at 30.0 ± 0.5 °C under argon. The terms in the expression on the axis are defined as follows: for monothiols, $S_0 \equiv [(RS^-) + (RSH)]$, $n = 1$; for dithiols, $S_0 = [(^-SRS^-) + (HSRS^-) + (HSRSH)]$, $n = 2$ (cf. eq 13 of the text). Reducing agents: ■, glycol dimercaptoacetate; ▼, dithiothreitol; ●, 2-mercaptoethanol; ▲, thioglycolic acid. Kinetics are shown respectively to 60, 50, 20, and 20% of completion. The kinetic points shown here were obtained from the experimental A_{240} attributable to GS-lac and are corrected for the relatively slow blank reaction observed in these systems when using phosphate buffer (see ref 24).

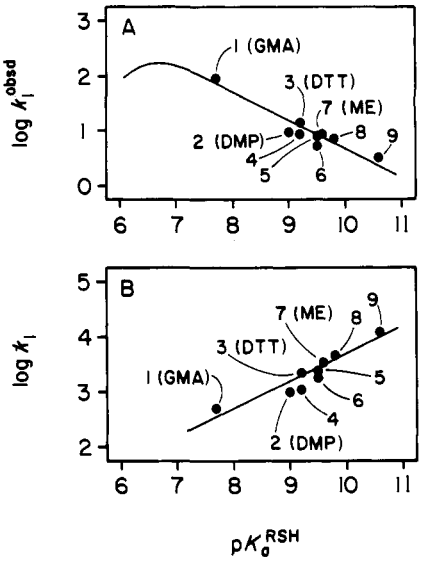


Figure 3. Plots of (A) log k_1^{obsd} and (B) log k_1 (M^{-1} min^{-1}) vs. thiol pK_a for the reduction of oxidized glutathione in pH 7.0 phosphate buffer at 30.0 ± 0.5 °C under argon by the mono- and dithiols listed in Table III. DTT is dithiothreitol, ME is 2-mercaptoethanol, DMP is 1,3-dimercaptopropanol, and GMA is glycol dimercaptoacetate. The least-squares slope for (B) is $\beta_{nuc} = 0.50$ ($r^2 = 0.89$). The equation used to generate the line in (A) is $\log k_1^{obsd} = -1.29 + 0.50pK_a - \log(10^{pK_a-7.0} + 1)$; the equation in (B) is $\log k_1 = -1.29 + 0.50pK_a$.

Tables I–III were corrected for the slow phosphate ion catalyzed reaction between methylglyoxal and thiols before analysis to obtain values of k_1^{obsd} using this equation.[24] Rate constants for reduction of GSSG by a series of mono- and dithiols under standard conditions are listed in Table III.[21,22] Figure 3 summarizes these data as plots of log k_1 and log k_1^{obsd} vs. the pK_a of the reducing thiol. The aromatic thiols which were used to obtain data for values of $pK_a^{RSH} < 8$ with Ellman's reagent[12,13] could not be used in studying the reduction of GSSG, because they absorbed strongly at the wavelength used to monitor the concentration of GS-lac (240 nm). For comparison, Figure 4 reproduces analogous data for reduction of Ellman's reagent; this plot includes data both from our work[12] and from the study of Hupe et al.[13]

The Brønsted coefficient ($\beta_{nuc} = 0.50$, coefficient of determination $\equiv r^2 = 0.89$) obtained by least-squares analysis of the rate constants for GSSG is similar to that obtained for reduction of Ellman's reagent by thiols. This latter system has now been the subject of two independent studies (Figure 4).[12,13] Our study analyzed a group of alkyl and aryl thiols together, and gave $\beta_{nuc} = 0.30$, $r^2 = 0.85$.[25] Hupe et al. analyzed data for a similar set of thiols, but suggested that alkyl and aryl thiols fell on separate correlation lines, with $\beta_{nuc}^{alkyl} = 0.49$ ($r^2 = 0.98$) and $\beta_{nuc}^{aryl} = 0.48$ ($r^2 = 0.96$). Analysis of our alkyl data alone yielded $\beta_{nuc}^{alkyl} = 0.41$ ($r^2 = 0.67$). Our data for aryl thiols cover a small range of pK_a values and are less accurate than those of Hupe.[26] The value of the Brønsted coefficient selected from these several analyses depends in part on subjective judgments concerning the choice of data to be included. We agree with Hupe et al. that the alkyl and aryl thiols should be analyzed separately,[27] and note that there is no independent evidence which can be used to select between values of β_{nuc}^{alkyl} lying between ~0.4 and 0.5. For simplicity, we will take $\beta \simeq 0.5$; arguments outlined later suggest that these β values may not, in any event, translate directly into fractional charges, and the symmetry and charge distribution of the transition state do not demand a specific value of β.[28,29] The value of $\beta_{nuc}^{alkyl} \simeq 0.5$ for reduction of GSSG determined in the present study thus seems physically reasonable, but, since the range of thiol pK_as covered is relatively small, since the scatter in the experimental points is significant, and since the assay system used to follow the reactions is complex, this value should not be considered highly precise. Qualitatively more important is the observation that reductions of GSSG and Ellman's reagent by thiol-disulfide interchange appear to be closely related reactions. Each is characterized by a Brønsted coefficient of similar size, and neither shows any evidence of curvature in the Brønsted plots suggesting a change in mechanism or an intermediate. Since Ellman's reagent and oxidized

Table III. Rate Constants (k, M^{-1} min^{-1}) for Reduction of Oxidized Glutathione (GSSG) by Mono- and Dithiols[a]

thiol	pK_a	k_a^{obsd}	k_1
1 $(HSCH_2CO_2CH_2-)_2$ (GMA)	7.7 (9.0)[b]	90	4.9×10^2
2 $HSCH_2CHOHCH_2SH$ (DMP)	9.0 (10.3)[b]	9.3	9.3×10^2
3 dithiothreitol (DTT)	9.2 (10.1)[b]	14.1	2.2×10^3
4 DTT/DTE mixture[c]	9.2 (10.1)[b]	8.4	1.1×10^3
5 $HOCH_2CHOHCH_2SH$	9.5[b]	7.9	2.5×10^3
6 $HSCH_2CH(NHCOCH_3)$-CO_2H	9.5[d]	5.2	1.8×10^3
7 $HOCH_2CH_2SH$ (ME)	9.6[e]	8.7	3.4×10^3
8 $HSCH_2CO_2H$	9.8[f]	7.3	4.6×10^3
9 $HSCH_2CH_2CO_2H$	10.6[g]	3.2	1.2×10^4

[a] All rates were determined in 0.066 M phosphate buffer, pH 7.0, 30.0 ± 0.5 °C, under argon. Data typically represent an average of three determinations. The rate constants are *not* corrected statistically for the presence of two equivalent sulfur atoms in GSSG. Reproducibility in these experiments was ±15%. Attempts to measure rates for several other representative thiols were unsuccessful. The rate of reduction of GSSG by CH_3COSH was not significantly faster than the rate of the blank reaction: k_1^{obsd} is smaller than 10^{-2} M^{-1} min^{-1}. Amino thiols (cysteine, $Et_2NCH_2CH_2SH$) react quickly with the methylglyoxal in the assay solutions to form species which absorb strongly at 240 nm, presumably *S*-lactoyl thioesters (see ref 24 for a discussion of the mechanism of these blank reactions). GX-I was inactivated by $HOCH_2CHSHCH_2SH$. [b] Sources of these pK_a values are listed in ref 12. These values for DTT have also been reported by E. L. Loechler and T. C. Hollocher, *J. Am. Chem. Soc.*, **97**, 3237 (1975). [c] Prepared as described in ref 12. [d] From M. Friedman, J. F. Cavins, and J. S. Wall, *J. Am. Chem. Soc.*, **87**, 3672 (1965). [e] This pK_a is taken from ref 13. [f] Reference 15. [g] From R. J. Irving, L. Nelander, and I. Wadso, *Acta Chem. Scand.*, **18**, 769 (1964).

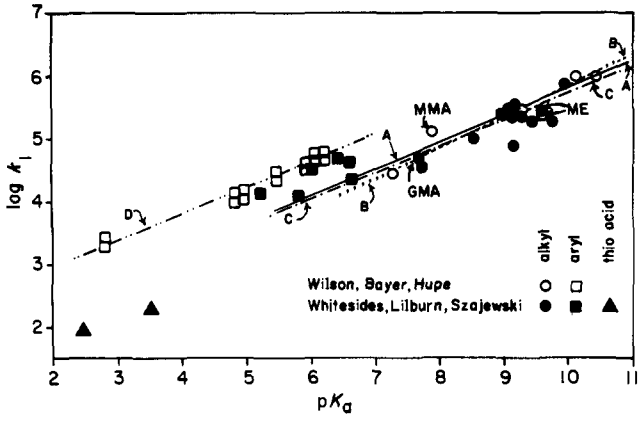


Figure 4. Collected rate constants (M^{-1} s^{-1}) for reduction of Ellman's reagent by thiols. Data from ref 12 are represented by solid points; data from ref 13 are represented by open points. Data for alkyl thiols are summarized by ● and ○; data for aryl thiols by ■ and □; data for thiol acids by ▲. ME is mercaptoethanol; GMA is glycol dimercaptoacetate; MMA is methyl mercaptoacetate. Other points are identified in ref 12 and 13. The lines represent least-squares fits to these sets of data: A (—), alkyls (○),[13] $\beta_{nuc} = 0.43$, $r^2 = 0.91$; B (- - - -), alkyls (○)[13] *excluding* methyl mercaptoacetate, $\beta_{nuc} = 0.49$, $r^2 = 0.97$; C (· – · –), alkyls (●),[12] $\beta_{nuc} = 0.41$, $r^2 = 0.67$; D (·· – ·· –), aryls (□),[13] $\beta_{nuc} = 0.44$, $r^2 = 0.97$.

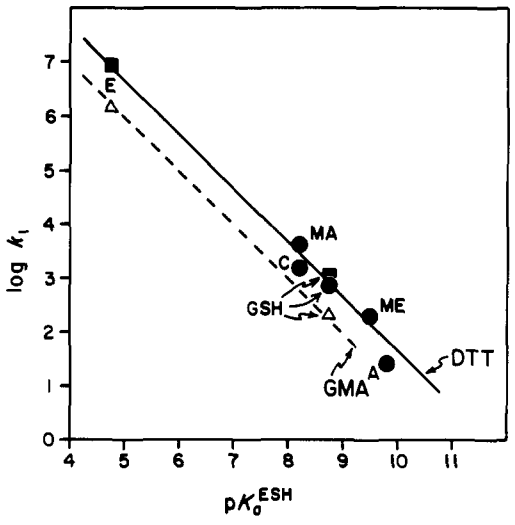


Figure 5. Rates of reduction k (M^{-1} min^{-1}) of symmetrical disulfides ESSE by thiolate anion, using dithiothreitol (DTT) or glycol dimercaptoacetate (GMA) as reducing agent. The lines have slope −1.0. Data for reduction using DTT are indicated by filled symbols, and are taken from this paper or ref 12 (■) or from Creighton (●);[22] data for reductions using GMA are indicated by △. The disulfides are identified by abbreviations for the corresponding thiols: E, Ellman's anion; MA, 2-mercaptoethylamine; C, cysteine; GSH, glutathione; ME, 2-mercaptoethanol; A, mercaptoacetic acid.

gluathione represent very different types of disulfides, their kinetic similarities encourage the conclusion that thiol-disulfide interchange is, in most instances, a mechanistically uncomplicated reaction.

Although we have not explicitly determined Brønsted coefficients for the leaving (β_{lg}) and central (β_c) thiol groups in the thiol-disulfide interchange, examination of our data, and those of Creighton,[22] yields a value for the sum of $\beta_c + \beta_{lg}$. We have determined the rates of reduction of GSSG and Ellman's reagent with a number of thiols; Creighton established the rate of oxidation of DTT in the presence of several symmetric disulfides. Representative data are summarized in Figure 5. It is evident that, for this heterogeneous group of disulfides, $\beta_c + \beta_{lg} = -1.0$. Creighton has reached the same conclusion.

Equilibrium Constants for Thiol–Disulfide Interchange Reactions. The measurement and interpretation of experimental values for equilibrium constants for thiol-disulfide interchange reactions in aqueous solutions are complicated by two considerations. First, the simplest type of equilibrium reaction to interpret—complete reduction of a symmetrical disulfide ESSE to a thiol ESH with concomitant oxidation of the reducing thiol RSH to a disulfide RSSR (eq 14)—is achieved only in two steps by way of an intermediate unsymmetrical disulfide (eq 15, 16). Second, both thiol and thiolate species

$$2(RSH + RS^-) + ESSE \overset{K^{obsd}}{\rightleftharpoons} RSSR + 2(ESH + ES^-) \quad (14)$$

$$(RSH + RS^-) + ESSE \overset{K_1^{obsd}}{\rightleftharpoons} RSSE + (ESH + ES^-) \quad (15)$$

$$(RSH + RS^-) + RSSE \overset{K_2^{obsd}}{\rightleftharpoons} RSSR + (ESH + ES^-) \quad (16)$$

$$K^{obsd} = K_1^{obsd}K_2^{obsd} = \frac{[(ESH) + (ES^-)]^2(RSSR)}{(ESSE)[(RSH) + (RS^-)]^2} \quad (17)$$

may be present in appreciable concentration in solution, and measured equilibrium constants ordinarily contain terms in the sum of the concentrations of thiol and thiolate species (eq 14–17). These equilibrium constants should thus, in principle, depend both on thiol and disulfide structures and on solution pH. In these equations, and subsequently, an equilibrium constant referring to a disulfide interchange involving a mixture of thiol and thiolate species will be denoted by the superscript "obsd". The influence of the organic groups R and E on the position of equilibrium in interchange reactions between disulfides and thiolate ions would not necessarily be expected to be parallel: reaction involving only thiolate anions (K^{S^-}, eq 18) should be strongly influenced by the pK_as of RSH and

$$\begin{array}{ccc} 2RS^- + ESSE & \overset{K^{S^-}}{\rightleftharpoons} & RSSR + 2ES^- \\ K_a^{RSH}\Big\| & & \Big\|K_a^{ESH} \\ 2RSH + ESSE & \overset{K^{SH}}{\rightleftharpoons} & RSSR + 2ESH \end{array} \quad (18)$$

ESH, while reaction with thiols (K^{SH}) should be much less dependent on these acidities. Qualitative analyses of equilibrium data obtained in nonaqueous media in which the equilibrium concentration of thiolate anion is very small have suggested that thiol-disulfide interchange is relatively insensitive to organic structure.[30,31] Independent examinations of K^{SH} and K^{S^-} in aqueous media have not been reported.

Our primary concern in the work reported here is the influence of ring size on equilibrium constants for interchange reactions which involve cyclic disulfides. We have approached this problem by measuring equilibrium constants for reduction of a standard disulfide (oxidized lipoamide) by a number of α,ω-dithiols having different separations between the thiol groups. Since these thiols have different pK_as, it is important, in trying to understand the influence of ring size in the cyclic disulfides on the measured equilibrium constants, to identify the contributions to these equilibrium constants which reflect the relative acidities of the thiols.

Separation of a value for K^{obsd} (eq 17) measured at a known pH into values of K^{SH} and K^{S^-} requires only knowledge of the pK_as for the two thiols ESH and RSH. The fact that only one experimental equilibrium measurement is required to obtain values of K^{SH} and K^{S^-} reflects the fact that these equilibrium constants are not independent: the relative concentrations of

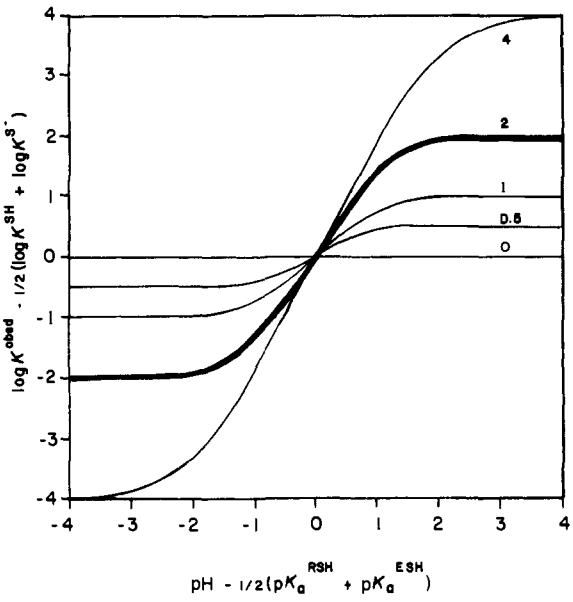


Figure 6. Schematic representation of the response of log K^{obsd} (defined by eq 17) for selected values of the parameter $pK_a^{RSH} - pK_a^{ESH}$ to changes in the solution pH; these curves were generated from eq 25. For convenience in plotting, the zero of the log K^{obsd} axis is taken to be the mean of the logarithm of the equilibrium for thiol and thiolate ion, $\frac{1}{2}(\log K^{SH} + \log K^{S^-})$, and the zero of the pH scale is taken to be mean of the pK_a values, $\frac{1}{2}(pK_a^{RSH} + pK_a^{ESH})$. Each curve is labeled with a value of $pK_a^{RSH} - pK_a^{ESH}$. The boldface curve is for $pK_a^{RSH} - pK_a^{ESH} = 2.0$; note that the observed equilibrium constant K^{obsd} for this curve increases by 10^4 as the pH is changed from values sufficiently acidic that both RSH and ESH are essentially completely protonated to values sufficiently basic that both components are essentially completely ionized.

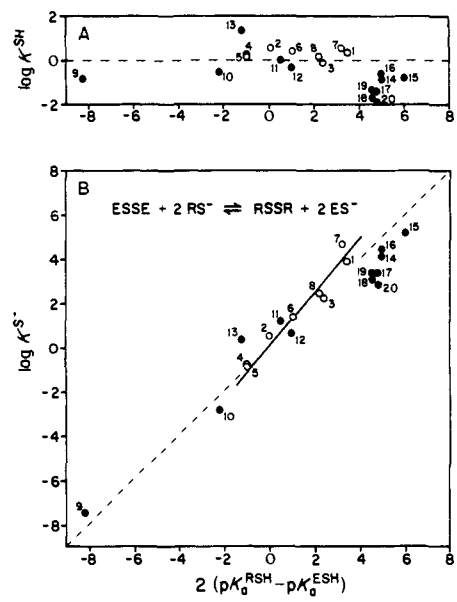


Figure 7. Plots of (A) log K^{SH} and (B) log K^{S^-} vs. $2(pK_a^{RSH} - pK_a^{ESH})$. The numbers refer to Table IV. (B) includes the line log $K^{S^-} = 2.42 \cdot (pK_a^{RSH} - pK_a^{ESH})$, which represents the best least-squares line through the points 1-8 (involving alkyl thiols) which passes through (0,0). The dashed lines are included for reference: (A) log $K^{SH} = 0$; (B) log $K^{S^-} = 2(pK_a^{RSH} - pK_a^{ESH})$.

thiol and thiolate anions are fixed by the known values of pH, pK_a^{RSH}, and pK_a^{ESH}. To derive K^{SH} and K^{S^-} from K^{obsd}, several equations are useful. Expressing the ratio of thiolate to thiol by eq 19 and 20, and substituting into eq 17, one obtains

$$(ES^-)/(ESH) = 10^{pH-pK_a^{ESH}} = \delta \quad (19)$$

$$(RS^-)/(RSH) = 10^{pH-pK_a^{RSH}} = \epsilon \quad (20)$$

$$K^{SH} = \frac{(1+\epsilon)^2}{(1+\delta)^2} K^{obsd} \quad (21)$$

$$K^{S^-} = \frac{(1+\epsilon^{-1})^2}{(1+\delta^{-1})^2} K^{obsd} \quad (22)$$

$$K^{S^-} = \frac{\delta^2}{\epsilon^2} K^{SH} \quad (23)$$

$$\log K^{S^-} = 2(pK_a^{RSH} - pK_a^{ESH}) + \log K^{SH} \quad (24)$$

$$\log K^{obsd} - 1/2[\log K^{SH} + \log K^{S^-}] = \log \frac{(1+\delta)(1+\delta^{-1})}{(1+\epsilon)(1+\epsilon^{-1})}$$

$$= (pK_a^{ESH} - pK_a^{RSH}) + 2 \log \frac{[1 + 10^{pH-1/2(pK_a^{RSH}+pK_a^{ESH})-1/2(pK_a^{ESH}-pK_a^{RSH})}]}{[1 + 10^{pH-1/2(pK_a^{RSH}+pK_a^{ESH})+1/2(pK_a^{ESH}-pK_a^{RSH})}]} \quad (25)$$

the relationships between K^{obsd}, K^{SH}, and K^{S^-} summarized by eq 21-23. Taking the logarithm of both sides of eq 23 and expanding yields eq 24. Multiplying of eq 21 and 22, taking the square root and the logarithm of the resulting expression, and expanding generates eq 25. This last equation, although cumbersome, describes the functional relations between log K^{obsd}, pK_a^{RSH}, pK_a^{ESH}, and pH in a form that is easily plotted (Figure 6). The particular functional form of eq 25 was chosen so that the shape of the curves generated from it would depend only on the differences in the characteristics (pK_a, K^{SH}, K^{S^-}) of the thiols involved, rather than on their absolute values. The thiols with which we are concerned in the following have pK_a values between 7.5 and 11.0, and experimental measurements were made at pH 7. Thus, typically, $pH - 1/2(pK_a^{RSH} + pK_a^{ESH})$ had values from −3.0 to −0.5, and $pK_a^{RSH} - pK_a^{ESH}$ had values between 0 and 2.5. Under these circumstances, Figure 6 indicates that K^{obsd} could contain significant contributions from both thiol- and thiolate-disulfide interchange equilibria.

To check the physical reasonableness and consistency of the values of K^{SH} and K^{S^-} obtained by dissection of K^{obsd}, we have examined a number of literature equilibrium constants. Figure 7 shows separate plots of log K^{SH} and log K^{S^-} vs. $2(pK_a^{RSH} - pK_a^{ESH})$ derived from the data summarized in Table IV. The equilibrium constants in the upper plot shows no obvious correlation with pK_as, but are influenced by steric effects: sterically hindered thiols give low values of K^{SH}. We note, however, that the data in this figure have been drawn from several sources, and differences in experimental conditions between sets of data, and inaccuracies in individual data, may disguise a weak dependence of log K^{SH} on ΔpK_a. The approximate linearity of the plot of log K^{S^-} vs. $2(pK_a^{RSH} - pK_a^{ESH})$ indicates that K^{S^-} is strongly influenced by the acidities of the participating conjugate thiols, and that the data are adequately rationalized by the scheme of eq 18. In principle, the slope of a plot of log K^{SH} vs. $2\Delta pK_a$ should be $(\beta_{nuc} - \beta_{lg} = 1)$ and that of a plot of log K^{SH} vs. $2\Delta pK_a$ should be $(\beta_{nuc} - \beta_{lg})$ (see below); it should therefore be possible to determine $\beta_{nuc} - \beta_{lg}$ experimentally from these plots. Figure 7B includes for reference the least-squares line drawn through the set of aliphatic thiols, and suggests that $\beta_{nuc} - \beta_{lg} \sim 1.2$. We reiterate that the quality of the data makes this estimate uncertain by at least ±20%.

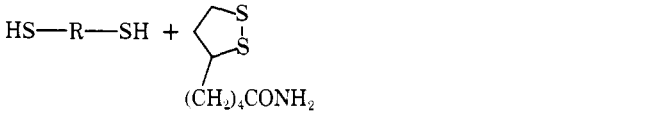
With this background, we can interpret equilibrium constants for reduction of oxidized lipoamide by a series of α,ω-dithiols characterized both by different separations between the thiol groups and by different values of thiol pK_a in a way that separates the influence of ring size in the product disulfide from that of the acidity of the starting dithiol. The equilibrium constants K_{lipo}^{obsd} for reduction of oxidized li-

Table IV. Separation of Literature Values for K^{obsd} into K^{S^-} and K^{SH} Using Equations 21 and 22

no.	thiol (RSH)	disulfide (ESSE)	pH	K^{obsd} [a]	K^{S^-} [a]	K^{SH} [a]	ref
1	$CHCONHCH_2CH_2SH$	cystine	7.4	3.1	8.0×10^3	2.4	*b*
2	$NH_2CH_2CH_2SH$	cystine	7.4	3.6	3.6	3.6	*b*
3	$CH_3CONHCH_2CH_2SH$	glutathione	7.3	0.80	1.9×10^2	0.75	*b*
4	$NH_2CH_2CH_2SH$	glutathione	7.4	1.7	0.18	2.1	*b*
5	$NH_2CH_2CH_2SH$	glutathione	6.5	1.4	0.15	1.4	*c*
6	glutathione-SH	cystine	6.0	3.0	28.	3.0	*d*
7	$HSCH_2CO_2H$	cystine	6.0	3.3	5.6×10^4	3.3	*e*
8	$HSCH_2CO_2H$	glutathione	6.0	1.7	2.7×10^2	1.7	*e*
9	C_6H_5SH	$(CH_3(CH_2)_5S)_2$	*f*	0.16	$(4.4 \times 10^{-8}$	0.16)	*g*
10	$HOCH_2CH_2SH$	$(CH_3(CH_2)_5S)_2$	*f*	0.28	$(1.4 \times 10^{-3}$	0.28)	*g*
11	$CH_3CH_2CH(CH_3)SH$	$(CH_3(CH_2)_2S)_2$	*f*	0.98	(19.	0.98)	*g*
12	$(CH_3)_3CSH$	$(CH_3(CH_2)_3S)_2$	*f*	0.54	(4.0	0.54)	*g*
13	dithiothreitol	lipoamide	7.0	31.	3.0	31.	*h*
14	cysteine	BSSB[i]	6.0	0.98	1.2×10^4	0.12	*j*
15	glutathione-SH	BSSB	6.0	1.1	1.3×10^5	0.14	*j*
16	$NH_2CH_2CH_2SH$	BSSB	6.0	2.1	2.6×10^4	0.26	*j*
17	$NH_2CH_2C(CH_3)_2SH$	BSSB	6.0	0.33	2.2×10^3	0.04	*j*
18	$NH_2CH(CH_3)CH(C_3H_7)SH$	BSSB	6.0	0.14	1.1×10^3	0.02	*j*
19	$NH_2CH_2CH(CH_3)SH$	BSSB	6.0	0.36	2.3×10^3	0.05	*j*
20	$NH_2CH(CH_3)C(CH_3)_2SH$	BSSB	6.0	0.09	6.2×10^2	0.01	*j*

[a] Equilibrium constants are dimensionless. [b] L. Eldjarn and A. Pihl, *J. Am. Chem. Soc.*, **79**, 4589 (1957). [c] L. Eldjarn and A. Pihl, *J. Biol. Chem.*, **225**, 499 (1957). [d] G. Gorin and G. Doughty, *Arch. Biochem. Biophys.*, **126**, 547 (1968). [e] I. M. Kolthoff, W. Stricks, and R. C. Kapoor, *J. Am. Chem. Soc.*, **77**, 4733 (1955). [f] These equilibrations were performed in hydrocarbon solvents and are included for reference. For each, it was assumed that K^{SH} in water would equal the listed value of K^{obsd}. [g] Reference 31. [h] Reference 4. [i] BSSB is 4,4'-dithiobis(benzenesulfonic acid). [j] G. Gorin, G. Doughty, and R. Gideon, *J. Chem. Soc. B*, 729 (1967).

poamide by several mono- and dithiols were determined experimentally using a procedure developed by Cleland;[4] details of this procedure are summarized in the Experimental Section. In the simplest case, represented by eq 26 and exemplified by

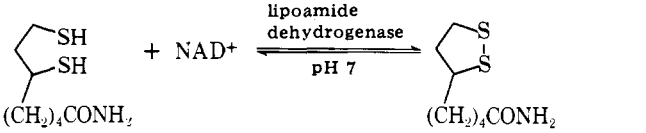


(26a)

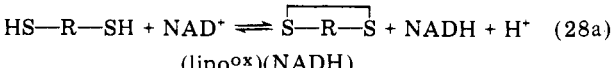
$$K_{lipo}^{obsd} = \frac{(\overline{S—R—S})(lipo^{red})}{(HS—R—SH)(lipo^{ox})} \quad (26b)$$

HS-R-SH = DTT (see below), reaction of 1 equiv of α,ω-dithiol with oxidized lipoamide generated reduced lipoamide and the cyclic disulfide formed by intramolecular oxidative coupling of the α and ω thiol groups. This equilibration was carried out in the presence of NAD^+ and lipoamide dehydrogenase (eq 27). The concentration of NADH at equilibrium was measured, and straightforward calculations based on known initial concentrations of α,ω-dithiol, oxidized lipoamide, and NAD^+ yielded the concentrations required to calculate K_{lipo}^{obsd} (eq 26b). Multiplication of K_{lipo}^{obsd} by K_{27} gives the biochemically relevant quantity K_{NAD}^{obsd} (eq 27, 28). In eq 27b, ($lipo^{ox}$) and ($lipo^{red}$) are respectively the concentrations of oxidized and reduced lipoamide. By convention, K_{27} and K_{NAD}^{obsd} are defined for a particular value of solution pH (here, pH 7) and are dimensionless (eq 27b and 28b). The balanced equations to which they refer (eq 27a and 28a) involve protons; thus K_{27} and K_{NAD}^{obsd} implicitly incorporate a term in hydrogen ion concentration, and depend on solution pH. K_{lipo}^{obsd}, K_{27}, K_{NAD}^{obsd}, and K_{GSSG}^{obsd} (see below) are all based on calculations which utilize the *total* concentrations of thiol and thiolate species derived from each SH-containing compound. We take the value of K_{27} to be 0.0858.[32]

The numerical value of the equilibrium constant calculated



(27a)



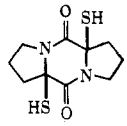
(28a)

$$K_{27} = \frac{(lipo^{ox})(NADH)}{(lipo^{red})(NAD^+)} = 0.0858 \quad (27b)$$

$$K_{NAD}^{obsd} = \frac{(\overline{S—R—S})(NADH)}{(HS—R—SH)(NAD^+)} = K_{lipo}^{obsd} K_{27} \quad (28b)$$

for K_{lipo}^{obsd} from a particular set of starting and observed concentrations depends on the choice of equilibrium equation. Equation 26 assumes that the product of oxidation of an α,ω-dithiol HS-R-SH is the corresponding cyclic disulfide. In principle, this reaction might also generate dimeric or higher oligomeric cyclic poly(disulfides), or linear polymeric disulfides. The experimental procedure used here measures directly only the concentration of NADH (and by inference that of reduced lipoamide); it does not identify the structures or concentrations of the disulfides present. We have not attempted to resolve the ambiguity concerning the structures of the disulfides produced in these thiol-disulfide interchange equilibria by their isolation: since all of the plausible types of products can interconvert, there is no guarantee that any species identified in concentrated, isolated form would be that present at equilibrium under the conditions used to measure K_{lipo}^{obsd}. Instead, we have examined the concentration dependence (or independence) of equilibrium constants calculated from experimental data measured at different concentrations of dithiols, for three equilibrium expressions: eq 26 (for formation of a cyclic monomeric disulfide), 29 (for reaction generating a cyclic oligimer of n members), and 30 (for linear polymerization to a higher molecular weight α,ω-dithiol). The second part of eq 29c is derived from eq 29b by assuming that $(\overline{SR[SSR]_{n-1}S}) = 1/n(lipo^{red})$ (that is, in a system originally containing only α,ω-dithiol and oxidized lipoamide, the

Table V. Equilibrium Constants for Thiol-Disulfide Interchange Reaction and Derived Constants[a]

compd	chain length	thiol	pK_a	K_{lipo}^{obsd} [b]	disulfide form	K_{NAD}^{obsd} [b]	K_{GSSG}^{obsd} [c]	E_0' [d]	$K_{GSSG}^{S^-}$ [c]	K_{GSSG}^{SH} [c]
1	4	$HOCH_2CHSHCH_2SH$ (BAL)	8.6 (10.5)[e]	5.8	cyclic dimer	4.2×10^{-2}	2.9×10^{2}	−0.298	1.0×10^{3}	5.8×10^{2}
2	5	$HSCH_2CHOHCH_2SH$ (DMP)	9.0 (10.3)[e]	0.51	cyclic monomer	4.4×10^{-2}	3.1×10^{2}	−0.279	3.8×10^{3}	6.2×10^{2}
3	5	$HSCH_2CH_2CH_2SH$	9.8 (10.9[f]	0.12	cyclic monomer	1.6×10^{-2}	1.1×10^{3}	−0.296	7.8×10^{5}	2.0×10^{3}
4	5	lipoamide ($lipo^{red}$)	10.7 (10.7)[g]	1	cyclic monomer	8.6×10^{-2}[h]	5.9×10^{2}	−0.288[h]	1.1×10^{7}	1.3×10^{3}
5	6	dithiothreitol (DTT)	9.2 (10.1)[e]	15	cyclic monomer	1.3	8.8×10^{3}	−0.323	6.0×10^{5}	1.8×10^{4}
6	6	dithioerythreitol (DTE)	9.2 (10.1)[e]	11	cyclic monomer	0.95	6.6×10^{3}	−0.319	5.2×10^{5}	1.4×10^{4}
7	6	$HS(CH_2)_4SH$	10.0 (10.7)[e]	22	cyclic monomer	1.9	1.3×10^{4}	−0.328	9.6×10^{6}	2.6×10^{4}
8	7	$(HSCH_2CH_2)_2O$	9.2 (9.9)[e]	7.5	cyclic monomer	0.64	4.4×10^{3}	−0.314	8.0×10^{4}	8.8×10^{3}
9	7	$(HSCH_2CHOH)_2CH_2$	9.1 (10.6)[f]	0.68	cyclic monomer	5.8×10^{-2}	4.0×10^{2}	−0.283	4.6×10^{3}	7.8×10^{2}
10	8	$(HSCH_2CONH)_2$[i]	8.0 (9.6[f]	0.88	cyclic monomer	7.6×10^{-2}	5.2×10^{2}	−0.286	4.4×10^{1}	1.2×10^{3}
11	8	$(HSCH_2CHOHCH_2)_2CH_2$	9.3 (11.1)[f]	0.50	cyclic monomer	4.3×10^{-2}	3.0×10^{2}	−0.279	8.4×10^{1}	6.0×10^{2}
12	10	$(HSCH_2CO_2CH_2)_2$ (GMA)	7.7 (8.9)[e]	9.2×10^{-3}	polymer	7.9×10^{-4}	5.4	−0.227	4.0×10^{2}	3.9
13	monothiol	$HSCH_2CH_2OH$ (ME)	9.6[j]	2.0×10^{-3}	dimer	1.7×10^{-4}	1.2	−0.207	7.1×10^{1}	1.2
14	monothiol	glutathione (GSH)	8.7[k]	1.8×10^{-3}	dimer	1.5×10^{-4}	1	−0.205	1	1
15	6				cyclic monomer	6.4		−0.344		8.6×10^{4} [l]

[a] All equilibrations were carried out in 0.05–0.02 M phosphate buffer at pH 7.0, 30.0 ± 0.5 °C, under argon using the lipoamide–lipoamide dehydrogenase couple. [b] K_{lipo}^{obsd} and K_{NAD}^{obsd} have units of M^{-1} for entries **1, 12, 13,** and **14;** for all of the other thiols, they are dimensionless. [c] K_{GSSG}^{obsd}, $K_{GSSG}^{S^-}$, and K_{GSSG}^{SH} for entries 1, 12, 13, and 14 are dimensionless; for all the other thiols they have units of M. Reference 36 describes the calculation of $K_{GSSG}^{S^-}$ and K_{GSSG}^{SH} from K_{GSSG}^{obsd}. [d] E_0 (V) values vs. standard hydrogen electrode at pH 7.0 and 30.0 °C (see ref 32). [e] These pK_a values are taken from ref 12. [f] Determined by the methods described in ref 12. [g] I. M. Gascoigne and G. K. Radda, *Biochim. Biophys. Acta,* **131,** 498 (1967). [h] Standard value; see ref 32. [i] Synthesis of this material is described in ref 41. [j] This pK_a value is taken from ref 13. [k] Reference 15. [l] Oxidized dithiodiketopiperazine was equilibrated against $HS(CH_2)_4SH$ in $CHCl_3$, and an equilibrium of $K = 3.3$ determined by ^1H NMR spectroscopy. This value corresponds to $K_{GSSG}^{SH} = 8.6 \times 10^4$ M.

$$n\text{HSRSH} + n\text{lipo}^{ox} \rightleftharpoons \overline{\text{SR[RSSR]}_{n-1}\text{S}} + n\text{lipo}^{red} \quad (29a)$$

$$K_{cyclic}^{obsd} = \frac{(\overline{\text{SR[SSR]}_{n-1}\text{S}})(\text{lipo}^{red})^n}{(\text{HSRSH})^n(\text{lipo}^{ox})^n} \quad (29b)$$

$$(K_{cyclic}^{obsd})^{1/n} = \frac{(\overline{\text{SR[SSR]}_{n-1}\text{S}})^{1/n}(\text{lipo}^{red})}{(\text{HSRSH})(\text{lipo}^{ox})} \simeq \frac{[1/n]^{1/n}(\text{lipo}^{red})^{1+1/n}}{(\text{HSRSH})(\text{lipo}^{ox})} \quad (29c)$$

$$\text{R'SH} + \text{R''SH} + \text{lipo}^{ox} \rightleftharpoons \text{R'SSR''} + \text{lipo}^{red} \quad (30a)$$

$$K_{poly}^{obsd} = \frac{(\text{R'SSR''})(\text{lipo}^{red})}{(\text{R'SH})(\text{R''SH})(\text{lipo}^{ox})} = \frac{(\text{R'SSR''})(\text{lipo}^{red})}{[2(\text{HSR''SH})]^2(\text{lipo}^{ox})} \quad (30b)$$

number of disulfide bonds formed from the former must equal the number of lipoamide disulfide bonds reduced). Taking the nth root of eq 29b and converting the numerator by substitution to an expression containing only the concentration of reduced lipoamide generates the final expression in eq 29c.

Equation 30 assumes that all SH groups present in the solution are equally reactive in each thiol–disulfide interchange; that is, it assumes that the thiol groups of an α,ω-dithiol react completely independently, and that intramolecular interchange is neither favored nor disfavored relative to intermolecular interchange. In this equation, R'SH and R''SH are thus arbitrary SH groups in the reaction mixture, either from the original dithiol or from some oligomer, and (HSR''' SH) refers to the total concentration of all the α,ω-dithiols present in the system. If intramolecular reactions are excluded and only intermolecular reactions occur, the factor of 4 in the denominator of eq 30b disappears. The concentrations of the species derived from the α,ω-dithiol in eq 29 and 30 are easily estimated in these systems by balancing redox equations, following procedures analogous to those outlined in the Experimental Section for calculation of K_{lipo}^{obsd}.

The purpose of this analysis is to distinguish between the equilibria represented by eq 26, 29, and 30. If the equilibrium expression correctly represents the reaction occurring in solution, the derived equilibrium constant should be invariant with changes in the starting concentration of dithiol. Examination of the form of eq 29b and 29c reveals one expected shortcoming of this form of analysis. If K_{cyclic}^{obsd} is invariant with concentration, $(K_{cyclic}^{obsd})^{1/n}$ is also invariant. Equation 29c differs from that for K_{lipo}^{obsd} (eq 26b) only in the exponent of the term ($lipo^{red}$) and a constant factor: for K_{lipo}^{obsd} this exponent is 2; for formation of a cyclic dimer (eq 29c, $n = 2$), it is 3/2. Thus, equilibrium expressions for generation of monomeric and dimeric disulfides (and for higher cyclic disulfides) on oxidation of α,ω-dithiols are expected to be experimentally difficult to distinguish.

Figure 8 plots calculated values of log K_{lipo}^{obsd} and log K_{poly}^{obsd} as a function of the concentration of starting α,ω-dithiol for three dithiols spanning the range of intrathiol separations examined: 2,3-dimercaptopropanol (BAL), DTT, and glycol dimercaptoacetate (GMA). For comparison, this figure includes calculated values for log K_{cyclic}^{obsd} ($n = 2$) for BAL. As expected, this figure confirms that DTT forms a cyclic monomer, and not a linear polymer. The data for GMA indicate that this material forms a linear polymer. The range of usable thiol concentrations in this latter case is limited on the low end by the limited reducing ability of GMA and on the high end by its low solubility. The data for BAL are not compatible with formation of a linear polymer (eq 30). They do not, however, clearly distinguish between equilibration to a cyclic monomer, dimer, or possibly higher cyclic oligomer, although the slight slope observed for K_{lipo}^{obsd} suggests that a dimer or oligomer might be the preferred species. Efforts to resolve this ambiguity using other techniques indicate that a cyclic monomer is not formed and suggest that a cyclic dimer is the probable oxidation product under these conditions.[33-35]

The studies summarized in Figure 8 are compatible with the hypotheses that oxidation of DTT yields a cyclic monomeric disulfide, that oxidation of BAL yields a cyclic dimer, and that oxidation of GMA yields a linear polymer. Table V lists K_{lipo}^{obsd} and K_{NAD}^{obsd} for these reactions, and corresponding data for other mono- and dithiols. All of the other α,ω-dithiols were assumed to form cyclic monomeric disulfides on oxidation; the justification for this assumption is discussed below. Table V also contains several other useful constants: K_{GSSG}^{obsd}

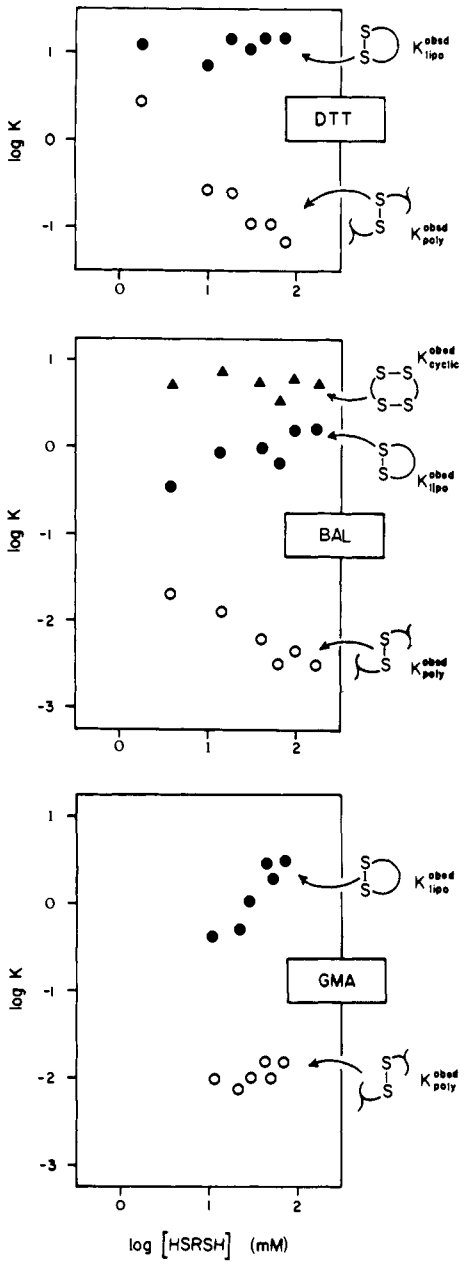


Figure 8. Plots of log K as a function of the concentration of reducing thiol [HSRSH] calculated on the assumption that oxidation of the dithiols yields (●) cyclic monomeric disulfides (K_{lipo}^{obsd}, eq 26); (▲) cyclic dimeric disulfides (K_{cyclic}^{obsd}, eq 29); or (○) polymeric disulfides (K_{poly}^{obsd}, eq 30). K_{cyclic}^{obsd} and K_{poly}^{obsd} have units of M; K_{lipo}^{obsd} is dimensionless. All equilibrations were carried out in 0.05–0.2 M phosphate buffer, pH 7.0 at 30.0 ± 0.5 °C under argon. Data are shown for (A) dithiothreitol (DTT, **5**, Table V); (B) 2,3-dimercapto-1-propanol (BAL, **1**, Table V); (C) glycol dimercaptoacetate (GMA, **12**, Table V).

is the equilibrium constant for eq 31, and is obtained by dividing K_{NAD}^{obsd} by 1.5×10^{-4} M^{-1} (the value of K_{NAD}^{obsd} for glutathione); E_0 for each of the thiols is related to the half-cell potential for the $NAD^+/NADH$ couple (eq 32) by eq 33; $K_{GSSG}^{S^-}$ and K_{GSSG}^{SH} are calculated from K_{GSSG}^{obsd} and the thiol pK_a values using eq 21 and 22.[36]

$$HS\text{-}R\text{-}SH + GSSG \underset{pH\ 7}{\overset{K_{GSSG}^{obsd}}{\rightleftharpoons}} \overline{S\text{-}R\text{-}S} + 2GSH \quad (31)$$

$$2e^- + NAD^+ + H^+ \xrightarrow[\text{vs. SCE, pH 7}]{E_0^{NAD} = 0.320\ V^{32}} NADH \quad (32)$$

$$E_0^{RSH} - E_0^{NAD} = \frac{RT}{nF} \log K_{NAD}^{obsd} \quad (33)$$

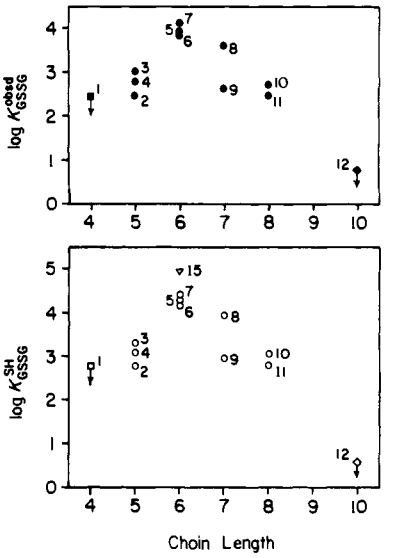


Figure 9. Plots of log K_{GSSG}^{obsd} and log K_{GSSG}^{SH} vs. chain length for the reduction of oxidized glutathione (GSSG) by a series of dithiols at 30.0 ± 0.5 °C in pH 7.0 phosphate buffer under argon. The numbers refer to Table V. All dithiols are believed to form cyclic disulfides on oxidation, with the exception of 2,3-dimercaptopropanol (□, ■, 1), which may form a cyclic dimer (see the text), and glycol dimercaptoacetate (◇, ◆, 12), which forms a linear polymer. The point for the dithioketopiperazine (▽, 15) was obtained in an organic solvent. The units of K_{GSSG}^{obsd} and K_{GSSG}^{SH} are M. The equilibrium constants for these dithiols cannot be contrasted directly for that with monothiols, since the units are different. For comparison, however, addition of a small quantity of GSSG to a 1 M solution of 2-mercaptoethanol, a 0.22 M solution of glycol dimercaptoacetate, and a 1.4×10^{-4} M solution of DTT would produce the same ratio of (GSH)/(GSSG).

For comparison, Table V includes a value for a conformationally rigid dithiodiketopiperazine which forms a six-membered cyclic disulfide on oxidation. This compound is not soluble in water, and its equilibrium constant was measured in chloroform by equilibration of the diketopiperazine disulfide against butane-1,4-dithiol. The derived constant K_{GSSG}^{SH} should, however, be comparable to those obtained in aqueous solutions, since these constants are probably relatively insensitive to medium.[31]

Figure 9 illustrates the variation in K_{GSSG}^{obsd} and K_{GSSG}^{SH} (from Table V) with intrathiol chain length. The former data (pH 7.0) are useful in the practical problem of choosing a reducing agent for pH 7 aqueous solution. The plot gives only upper limits for α,ω-dithiols connected by chains of two (BAL) and eight (GMA) atoms. Since these compounds appeared to form respectively a cyclic dimer and polymer, it is not possible to establish equilibrium constants for intramolecular disulfide formation. It is, however, possible to estimate what these equilibrium constants would have had to have been for the product of intramolecular coupling to have been predominant under the reaction conditions. These constants are indicated on the figure as upper limits. The plot of log K_{GSSG}^{SH} vs. chain length is useful in establishing the structural basis of the reducing ability of the dithiols. These data contain no contribution from the relative acidities of the thiol groups. The close qualitative similarity between the plots of log K^{obsd} and log K^{SH} vs. chain length suggests that differences in the acidities of the thiol groups are relatively unimportant in determining the reducing ability of these dithiols.

Several features of the data summarized in Figure 9 warrant discussion. First, the equilibrium constant $K_{GSSG}^{SH} = 3.9$ for glycol dimercaptoacetate (GMA) is approximately that for monothiols (for example, $K_{GSSG}^{SH} = 1$ for glutathione, and $K_{GSSG}^{SH} = 1.2$ for mercaptoethanol). The fact that a solution of GMA is not significantly more reducing than a solution containing an equal concentration of monothiol SH groups is

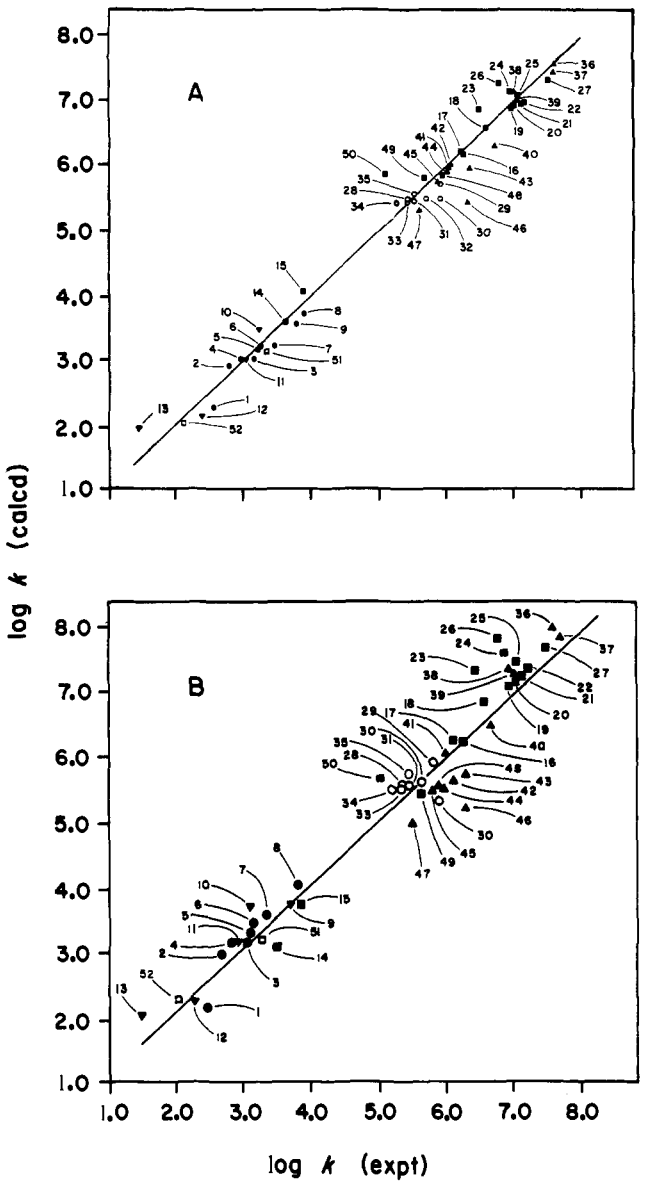
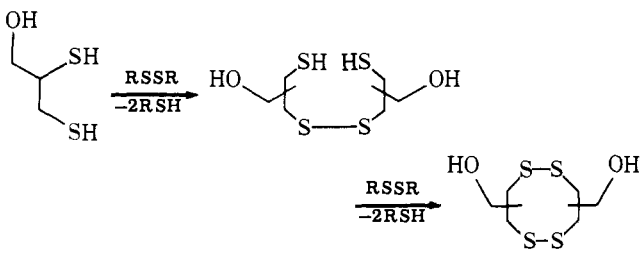


Figure 10. (A) Plot of rate constants for thiol-disulfide interchange (k) calculated using eq 36 against corresponding experimental rate constants: units for rate constants are M^{-1} min^{-1}. Points 1-8 (●) are taken from this work; 9-13 (▼) from ref 22; 14-27 and 48-50 (■) from ref 12; 28-35 (○) from Table IV, footnote *j* of G. Gorin, G. Doughty, and R. Gideon, *J. Chem. Soc. B*, 729 (1967); 36-47 (▼) from ref 13; 51-52 (□) from Weber, Hartter, and Flohe (ref 21). Points 42-52 refer to unsymmetrical disulfides; all others are symmetrical. The least-squares correlation line shown has $r^2 = 0.98$, slope = 0.98, and intercept = 0.11. (B) Similar plot for eq 38. Detailed identification of the points is given in supplementary material in the microfilm edition (see paragraph at end of paper).

compatible with our contention that the thiols of GMA act independently and that GMA forms polymers on oxidation. If oxidation of GMA generated a cyclic disulfide, we would expect it to be a stronger reducing agent than a monothiol. Second, α,ω-dithiols capable of forming five-, six-, seven-, and eight-membered rings *are* stronger reducing agents than monothiols. The stability of many cyclic five- and six-membered disulfides is well known. Although we have not explicitly established the form of the equilibrium reactions involving seven- and eight-membered disulfide-containing rings, examination of the magnitudes of equilibrium constants calculated using the various equilibrium expressions (eq 26, 29, and 30) suggests that intramolecular disulfide formation is occurring. In particular, equilibrium constants calculated for compound **8, 9, 10,** and **11** (Table V) assuming that oxidation yields a polymeric disulfide are K_{poly}^{obsd} (**8**) = 26 M^{-1}; (**9**) ≃ 28 M^{-1}; (**10**) ≃ 16 M^{-1}; (**11**) ≃ 15 M^{-1}. These constants are ca. 10^4 higher than those for other thiols which form intramolecular dimers (GSH, ME) or polymer (GMA). Since all of the evidence from the work suggests that this constant is not very sensitive to structural variations in the starting thiol, we conclude either that dithiols having seven or eight bonds separating the thiol groups show anomalously high reactivity in polymer formation or that they form cyclic disulfides. The latter alternative seems the more probable. It is more difficult to distinguish intramolecular disulfide formation from formation of a cyclic dimer for reasons discussed above. Third, the observation that a solution of BAL is much more strongly reducing than a solution of a monothiol containing an equal concentration of thiol groups supports the hypothesis that BAL forms a cyclic dimeric disulfide: an initially formed, half-oxidized dimer from BAL enjoys the same advantage in closing to an eight-membered ring as does an α,ω-dithiol of similar structure, but the initial formation of the half-oxidized dimer requires a bimolecular step.



Discussion

The rate of reduction of oxidized glutathione, GSSG, by thiol-disulfide interchange in aqueous solution follows a Brønsted relation. The analytical method used to follow these rates was less general than that employed in our previous study of the rates of reduction of Ellman's reagent, and fewer thiols were examined as reducing agents. Nonetheless, the close parallel between the results of these two studies, and the similarity of the β_{nuc} values derived from them suggests that a correlation between the proton basicity of the thiolate nucleophile and the rate of its attack on a sterically unemcumbered sulfur-sulfur bond is characteristic of thiol-disulfide interchange. A picture of thiol-disulfide interchange as a mechanistically uncomplicated reaction is in accord with theoretical results, which suggest a S_N2 transition state involving little d-orbital participation by the central sulfur,[37] and with X-ray studies, which indicate that the preferred direction for approach of nucleophiles to a disulfide bond is along the sulfur-sulfur axis.[38]

The two Brønsted coefficients determined experimentally in this work ($\beta_{nuc} \simeq 0.5$; $\beta_c + \beta_{lg} \simeq -1.0$) differ in their accuracy. The first is derived from a large number of data, and seems reliable. The second is based on a smaller set, and depends heavily on examples involving Ellman's reagent; the generality of this coefficient is not yet defined. We have not explicitly determined separate values for β_c and β_{lg}. We have, however, estimated values for these parameters, using rate constants from this work and from literature sources for reactions involving unsymmetrical disulfides having both $R^{nuc}SH$ and R^cSH aliphatic. Assuming that these rates are described by an equation of the form of eq 35, imposing the conditions that $\beta_{nuc} = +0.5$ and $\beta_c = -1.0 - \beta_{lg}$, and solving for the unknown C and β_{lg} by least-squares analysis we estimate that $\beta_{lg} \simeq -0.72$, $\beta_c = -0.27$, and $C = 7.0$. The ability of the resulting equation (eq 36, k = M^{-1} min^{-1}) to fit the available data is summarized by Figure 10A as a plot of log k(exptl) vs. log k(calcd).

$$R'^{nuc}S^- + R^cSSR^{lg} \xrightarrow{k} R^{nuc}SSR^c + {}^-SR^{lg} \qquad (34)$$

$$\log k + C + \beta_{nuc}pK_a^{nuc} + \beta_c pK_a^c + \beta_{lg}pK_a^{lg} \quad (35)$$

$$\log k = 7.0 + 0.50pK_a^{nuc} - 0.27pK_a - 0.73pK_a^{lg} \quad (36)$$

$$\log k = 5.5 + 0.63pK_a^{nuc} - 0.30pK_a^c - 0.63pK_a^{lg} \quad (37)$$

$$\log k = 6.3 + 0.59pK_a^{nuc} - 0.40pK_a^c - 0.59pK_a^{lg} \quad (38)$$

$$\log k^{obsd} = \log k - (1 + 10^{pK_a^{nuc}-pH}) \quad (39)$$

If the attacking thiolate anion and the leaving thiolate anion are both derived from aromatic thiols, the only change in eq 36 is that the constant C has the value 7.9.

Figure 10A establishes that eq 36 provides a good model for a large number of rates. In fact, the quality of the correlation is probably higher than indicated by the coefficient of determination ($r^2 = 0.98$), because most of the points which fall far off the line (points 13, 26, 40, 46, and 50) involve either thioglycolic acid (or its anion) or methyl thioglycolate ($ROCOCH_2SH$, R = H, CH_3). Hupe et al. have summarized evidence that these compounds behave anomalously in other Brønsted correlations.[13]

Although eq 36 fits the experimental data well, there remains some ambiguity concerning the mechanistic significance of the value of β_{lg} used in it. This equation implies that $\beta_{nuc} - \beta_{lg} = 0.50 + 0.73 \simeq 1.2$, and both the inequality in the magnitudes of these Brønsted coefficients and the fact that their difference is greater than 1.0 are reasons to consider the possibility that they might reflect some element of experimental or analytical artifact. In principle, it should be possible to estimate the parameter $\beta_{nuc} - \beta_{lg}$ independently from the slopes of Figure 7,[39] but, for reasons discussed previously, experimental estimates based on these slopes are too uncertain to be of great value. Further, it is possible to find other combinations of Brønsted β's which give good correlations. For example, if we solve for the set of β's which best fit the data summarized in Figure 10 under the imposed conditions that $\beta_c = -0.30$ and $\beta_{nuc} = -\beta_{lg}$, we obtain the parameters summarized in eq 37 ($r^2 = 0.96$). We have also explored solutions to eq 35 in which $\beta_{nuc} = -\beta_{lg}$ and in which β_c is allowed to assume values other than 0.3. The best solution[40] with these constraints is given by eq 38 ($r^2 = 0.96$); the agreement between experimental rates and those calculated using this equation is also summarized in Figure 10.

The observation that all three of these correlations give similar coefficients of determination indicates that the Brønsted coefficients are not sharply defined by the available data. Further, the population of thiols and disulfides represented by the experimental rates is sufficiently heterogeneous that great precision is not expected. For a more limited set of thiols and disulfides, it would probably be possible to develop a Brønsted equation which would correlate experimental rates more closely than eq 36-38, but for semiquantitative estimation of rate constants for thiol-disulfide interchange given known values for the pK_as of the participating thiols, or for kinetic measurements of thiol pK_as using rate constants for thiol-disulfide interchange, any one of these equations should prove equally useful (and equally accurate).[41] We routinely use eq 36 and 38, and find little difference between the two. We emphasize, however, that these equations should be considered primarily as kinetic *models* for thiol-disulfide interchange, and that detailed mechanistic interpretation of the β coefficients should wait on simultaneous, direct, experimental determination of these coefficients using a carefully limited set of thiols and disulfides.

Although quantitative discussion of the charge distribution in the transition state for thiol-disulfide interchange based on the coefficients in eq 36-38 is not justified, qualitative discussion certainly is. Hydrogen and sulfur have similar electronegatives ($X_H = 2.20$, $X_S = 2.44$),[42] and there is no evidence of curvature in the Brønsted plots suggesting an intermediate or change in rate-limiting step. Thus the transition state appears to be symmetrical, with significant effective negative charge on all three sulfurs, but concentrated on the terminal positions.[43] Hupe et al. have reached similar conclusions.[13]

An important objective of this work, unrelated to questions of transition-state structure, was that of trying to understand the relationships between the structures of the thiols and their reducing potentials in thiol-disulfide interchange reactions: especially, what structural features characterize strongly reducing thiols? Four useful conclusions emerge from this work. First, as anticipated, many α,ω-dithiols capable of forming cyclic disulfides are more strongly reducing than the corresponding monothiols. This effect is attributable in major part

$$\text{HS-R-SH} + \text{ESSE} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{HS-R-SSE} + \text{HSE} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \overset{\frown}{\text{S-R-S}} + 2\text{HSE} \quad (40)$$

$$2\text{RSH} + \text{ESSE} \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} \text{RSH} + \text{RSSE} + \text{HSE} \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} \text{RSSR} + 2\text{HSE} \quad (41)$$

to a much higher forward rate for the second step of eq 40 than for that of eq 41, reflecting the high effective concentration of thiol groups in the intramolecular thiol-disulfide interchange step.[45] We have not been able to compare the rate constants k_2 and k'_2 directly, so that we cannot presently judge whether other structural features of the intermediate mixed disulfide of eq 41 might contribute to its rapid conversion to products. Second, there is a clear correlation between the reducing potential of an α,ω-dithiol and the size of the cyclic disulfide formed on its oxidation. The maximum equilibrium advantage is observed for dithiols capable of forming six-membered rings, although seven-membered and five-membered rings can also have good stability. All-carbon systems seem to favor six-membered rings in a wide variety of cases.[46] We were surprised to find that the same apparently holds true for cyclic disulfides, since a CSSC dihedral angle of 90° is favored both experimentally[46] and theoretically,[47] and elemental sulfur itself forms eight-membered rings. The relative stabilities of six- and seven-membered cyclic disulfides seems to represent a delicate balance between CSSC angle strain and entropy of ring closure.[47] Third, a large contribution to thiol-disulfide interchange equilibrium constants can, in principle, arise from the relative pK_as of the reducing thiol and the thiol derived from the disulfide. This contribution is proportional to the difference between the thiol pK_as, and is largest when both thiols are present entirely as thiolate anions (i.e., when the equilibrium considered is a thiolate-disulfide interchange). For the systems considered here—aqueous solution, pH 7, thiols having pK_a values between 8 and 10—contributions to K^{obsd} from this source are less important than those due to intramolecular disulfide bond formation. Fourth, the *rate* of reduction of a disulfide by a thiol does not necessarily correlate with the *equilibrium constant* for the reduction. The former is determined by the relation of thiol pK_a to solution pH, with the most rapidly reacting thiols normally being those having pK_a values close to the solution pH;[12] the latter is determined by factors unrelated to thiol pK_a, except in solutions sufficiently basic that the interconverting thiols are significantly ionized. Dithiols are ordinarily more rapidly reducing than monothiols of the same pK_a, especially in dilute solution: although the initial conversion of starting disulfide to an intermediate mixed disulfide proceeds equally rapidly with either, the conversion of the mixed disulfide to products is more rapid with the dithiol. Within the group of dithiols, the selection of rate and equilibrium constant can usually be made on the basis of the thiol

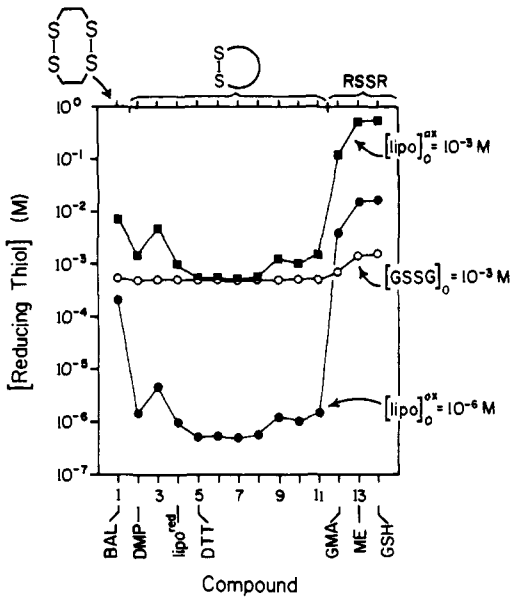


Figure 11. Concentrations of thiols required to reduce by half the concentration of the indicated disulfide group in solutions originally containing oxidized lipoamide (10^{-3} M, ■) oxidized glutathione (10^{-3} M, ○), and oxidized lipoamide (10^{-6} M, ●). Compound numbers refer to Table V. The schematic structures at the top of the figure represent the type of disulfide believed to be formed from the thiol reducing agent (cyclic dimeric, cyclic monomeric, and intermolecular, respectively).

pK_{a1} and of the ring size that would be formed on oxidation of the dithiol to a disulfide. Thus, DTT (pK_{a1} = 9.2) reacts with GSSG approximately a factor of 6 more slowly than glycol dimercaptoacetate (pK_{a1} = 7.7) in pH 7 aqueous solution, but DTT is a much stronger reducing agent for GSSG than is GMA, especially in low concentrations.

A major practical objective of this work is to provide data which would make it possible to select a thiol reducing agent having a reduction potential appropriate for a particular application. It requires some effort to compare all of the thiols examined on the basis of their equilibrium constants, since these constants have different units. To assist in the use of these thiols, we have calculated from the data in Table V the concentrations of thiols required to reduce by half the concentration of the indicated disulfide groups present in three solutions: $(lipo^{ox})_0 = 10^{-6}$ M, $(lipo^{red})_0 = 0$; $(lipo^{ox})_0 = 10^{-3}$ M, $(lipo^{red})_0 = 0$; $(GSSG)_0 = 10^{-3}$ M, $(GSH)_0 = 0$ (Figure 11). These disulfides and concentrations were chosen to suggest the conditions which might be required to reduce a stable, intramolecular, cystine linkage of a protein in dilute solution ($(lipo^{ox})_0 = 10^{-6}$ M), and stable intramolecular ($(lipo^{ox})_0 = 10^{-3}$ M) and less stable intermolecular ($(GSSG)_0 = 10^{-3}$ M) disulfide linkages at concentrations representative of those encountered in solutions of low molecular weight disulfides. This figure suggests that, for general work in enzymology, three thiols have useful characteristics. 2-Mercaptoethanol is inexpensive, but is a weak reducing agent. Dithiothreitol, Cleland's reagent, is the strongest reducing agent among the thiols examined. It is, however, expensive (although a mixture of diastereomeric 1,4-dithiobutane-2,3-diols having equivalent properties is readily available by synthesis).[12] Compound **2** (1,3-dimercaptopropanol, DMP) is a compromise: it is almost as strong a reducing agent as DTT, but it is less expensive. All three have good water solubility and tolerable odor. We now use DMP in place of DTT for much of our work in enzymology: as a general-purpose antioxidant, it so far appears to be interchangeable with DTT.

A long-range concern of this and related work in our laboratory is to understand the mechanistic and structural characteristics of thiol-disulfide interchange in sufficient detail to be able to rationalize rates and equilibria of redox reactions involving protein cysteine and cystine groups. This area is relevant not only to mechanistic biochemistry, but also to the practical problem of stabilizing the sensitive cysteine-containing proteins important in enzyme-catalyzed organic synthesis against autoxidation.[9] Studies of rates and equilibrium constants for thiol-disulfide interchange involving structurally simple thiols and disulfides provide background work for studies of similar reactions with proteins, but it is important not to try to infer the behavior of the latter from that of the former. Our work with proteins is incomplete, but two qualitative conclusions are evident which are relevant to the subject of this paper. First, the rates of reduction of certain protein disulfide groups by thiols also follow Brønsted correlations. Second, the fact that a thiol is a strong reducing agent does not guarantee that it is an effective antioxidant. Protein autoxidation is a complex process, and the antioxidant activity of added thiols is only partly due to thiol-disulfide interchange. We have, for example, encountered one instance in which DTT is a less effective antioxidant (although a much stronger reducing agent) than 2-mercaptoethanol, presumably because DTT is more rapidly autoxidized than 2-mercaptoethanol. The H_2O_2 produced in these autoxidations is itself an efficient enzyme oxidant.[8]

Experimental Section

Methods and Materials. pH was determined using a Radiometer Model 28 pH meter, and UV spectra were measured using a Gilford Model 240 spectrometer equipped with a sample chamber thermostated at 30.0 ± 0.5 °C and a strip chart recorder. Distilled water was passed through an ion-exchange column, redistilled using a Corning AG-1B still, and purged with argon prior to use. All reactions and measurements were carried out under an argon atmosphere. Dithiothreitol, NAD^+, NADH, GSSG, GSH, methylglyoxal solution, lipoamide, lipoamide dehydrogenase (yeast) (E.C. 1.6.4.3), glutathione reductase (E.C. 1.6.4.2), and glyoxalase-I (E.C. 4.4.1.5) were obtained from Sigma Chemical Co. Thiolacetic acid (97%), thioglycollic acid (98%), 2,3-dimercapto-1-propanol (95%), 2-mercaptoethanol (98%), 1-thioglycerol (97%), 1,3-dimercapto-2-propanol (technical), 3-mercaptopropionic acid (99%), Ellman's reagent (99+%), 2,2′-oxydiethanethiol (technical), 1,5-hexadiene (98%), *m*-chloroperoxybenzoic acid (85%), 1,3-propanedithiol (97%), and 1,4-butanedithiol (99%) were obtained from Aldrich Chemical Co. 1,4-Pentadiene was obtained from Chemical Samples Co. Glycol dimercaptoacetate was a gift of Evans Chemetics, Darien, Conn. All other chemicals used were AR grade. Organic thiols were distilled under argon before use. Enzymatic activity is defined in units having dimensions (μmol product produced)(min^{-1}).

***N,N′*-Dimercaptoacetylhydrazide** was prepared following a literature procedure, mp 154–156 °C (lit. mp[48] 156–158 °C). The pK_as of the thiol groups in this material were measured as previously described:[12] pK_{a1} = 8.0, pK_{a2} = 9.6.

1,6-Dimercapto-2,5-hexanediol. To 4.1 g (50 mmol) of 1,5-hexadiene in 400 mL of CH_2Cl_2 at 0 °C was slowly added 20.0 g (100 mmol) of finely divided *m*-chloroperoxybenzoic acid with stirring. The reaction mixture was allowed to come to room temperature and after 14 h gave only a weak positive reaction to starch–iodide test paper. Peroxides were removed by addition of 5 mL of water and 1 g of $Na_2S_2O_3$ and stirring until the reaction mixture was negative to starch–iodide test paper (about 10 min). The reaction mixture was diluted with 20 mL of water and slowly added with stirring to 100 g of Na_2CO_3. After frothing had subsided, the reaction mixture was filtered through fritted glass and the CH_2Cl_2 removed by distillation at room temperature and reduced pressure to give the crude diepoxide as a clear oil: IR (neat) 3050, 2990, 2920, 2860, 1260, 830, and 740 cm^{-1}; NMR ($CDCl_3$) δ 3.0–2.8 (m, 2), 2.5–2.3 (m, 2), 1.7–1.4 (m, 4). *Caution. This diepoxide and that used in the preparation of 1,5-dimercapto-2,4-pentanediol are probably mutagenic. They should be handled only with caution in a good hood, and care taken to avoid contact with liquid or vapor.* The diepoxide was treated without further purification under argon with 11.6 g (110 mmol) of thiolacetic acid. After 48 h all volatiles were removed at room tem-

perature under water aspirator vacuum. Argon-degassed methanol (30 mL) and 2 mL of a 1 N solution of HCl in diethyl ether were added, the mixture was refluxed under argon for 5 h and cooled, and ether, methyl acetate, and methanol were removed by distillation at atmospheric pressure. The residual oil was distilled bulb to bulb at 130–150 °C (0.05–0.1 Torr) to give 1.86 g (10.2 mmol) of 1,6-dimercapto-2,5-hexanediol as a clear, foul-smelling liquid: IR (neat) 3450, 2920, 2860, 1050 cm^{-1}; NMR (CCl_4) δ 4.1–3.9 (m, 2), 3.5–3.3 (m, 2), 2.8–2.4 (m, 4), 2.4–1.8 (m, 4), 1.6 (t, 2, J = 9 Hz). The epimeric composition of this material was not determined. The pK_as of the thiol groups in this material were measured as previously described:[12] pK_{a1} = 9.3, pK_{a2} = 11.1.

Anal. Calcd for $C_6H_{14}O_2S_2$: C, 39.56; H, 7.75. Found: C, 39.75; H, 7.63.

1,5-Dimercapto-2,4-pentanediol. Application of the epoxidation, thioacetylation, and transesterification sequence as described above to 3.4 g (50 mmol) of 1,4-pentadiene gave 3.16 g (19 mmol) of 1,5-dimercapto-2,4-pentanediol as a clear, odiferous oil: IR (neat) 3400–3200, 2920, 2550, 1420, 1050 cm^{-1}; NMR (CCl_4) δ 3.8–3.2 (m, 2), 2.8–2.2 (m, 6), 2.2–1.8 (m, 2), 1.4 (br t, 2, J = 9 Hz); pK_{a_1} = 9.1, pK_{a_2} = 10.6.

Anal. Calcd for $C_5H_{12}O_2S_2$: C, 35.72; H, 7.19. Found: C, 35.39; H, 6.91.

Kinetics of Reduction of Oxidized Glutathione (GSSG) by Thiols. One representative kinetic run using the coupled enzymatic indicator reaction will be described in detail as will the corresponding control experiments. Other runs were performed in an analogous manner. To 1000 μL of 0.2 M phosphate buffer, pH 7.0, containing 0.1% egg albumin, under argon at 30.0 ± 0.5 °C in cuvette 1 was added 2000 μL of a 7.6 mM solution (as determined by Ellman's assay[49]) of dithiothreitol prepared by dissolving 117 mg of that thiol in 100 mL of water. To this solution was added 50 μL of a freshly steam distilled solution of methylglyoxal (47 mM) in water. The concentration of the stock solution of methylglyoxal was determined by enzymatic conversion to *S*-lactoyl glutathione using an excess of reduced glutathione and glyoxalase-I.[50] A blank solution, in cuvette 2, was prepared similarly. A slow blank reaction between methylglyoxal and added thiol was noted under these conditions. To cuvette 1 was added 20 μL of a glyoxalase-I solution exhibiting 0.37 U/μL activity. The activity of stock glyoxalase-I was determined in a separate experiment utilizing reduced glutathione and methylglyoxal following a literature procedure.[50] Comparison of the blank reactions in cuvettes 1 and 2 over a 5-min period showed no difference, thus establishing that, under these conditions, glyoxalase-I did not mediate the redox coupling of the added thiol with methylglyoxal. To both cuvettes were simultaneously added 10-μL aliquots of a 99 mM stock solution of GSSG prepared by dissolving 68.3 mg of GSSG in 1000 μL of 0.2 M phosphate buffer, pH 7.0. The end point of the kinetic run, after correction for the slow blank reaction, indicated a concentration of *S*-lactoyl glutathione of 0.69 mM in the assay. This value corresponds to a concentration of 106 mM GSSG in the stock solution prepared as above. The concentration could be further checked by oxidation of NADPH by GSSG utilizing the enzyme glutathione reductase.[10] The absorbance in both cuvettes was followed simultaneously by appropriate repositioning of the cuvette holder (at 240 nm, the λ_{max} for GS-lac[14]) until the slopes of both curves were again parallel. As a further control, the above assay was repeated, the only difference being in the use of 100 μL of the glyoxalase-I solution. The absorbance curves produced were essentially identical. Figure 12 illustrates this experiment. The quantities added correspond to the following initial assay conditions: 66 mM phosphate, 5.07 mM dithiothreitol, 0.77 mM methylglyoxal, 2.4 U/mL glyoxalase-I, 0.35 mM GSSG. The final pH of the assay solution was 7.02. The raw data were refined by subtraction of the blank from the reaction curve, determination of the GS-lac concentration using ϵ_0^{240nm} = 3.37 mM^{-1} cm^{-1},[14] and substitution into the integrated form of the appropriate rate equation (eq 13). These data are illustrated in Figure 2. Alternatively, subtraction of the blank from the reaction curve, extrapolation to time 0 (past the short initial log phase), determination of the initial slope, and division by ϵ_0 gave an approximation of the initial velocity, d(GS-lac)/dt, of the reaction in units of mM min^{-1}. Division of this velocity by the initial GSSG and RSH concentrations gives a value of k_1^{obsd} indistinguishable from that obtained using eq 13.

Determination of Relative Reducing Ability of Mono- and Dithiols toward NAD^+ Utilizing a Coupled Enzymatic Assay. One representative equilibration using the coupled enzymatic indicator reaction

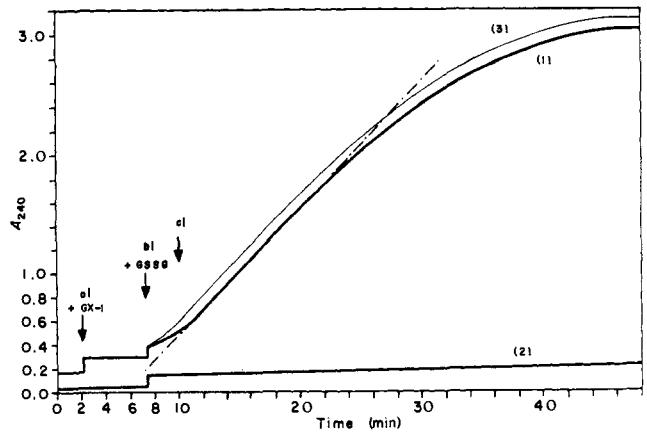


Figure 12. Plot of A_{240} vs. time in 0.066 M phosphate buffer, pH 7.0, at 30.0 °C under argon with 5.07 mM dithiothreitol as reducing agent and 0.77 mM methylglyoxal as enzyme cosubstrate. Curve 1 is the reaction mixture; curve 2 is a blank. The arrows indicate (a) addition of glyoxalase-I (2.4 U/mL) to cuvette 1; (b) simultaneous addition of oxidized glutathione (GSSG, 0.32 mM) to cuvettes 1 and 2; (c) attainment of steady-state concentrations of reduced glutathione in cuvette 1. Use of larger amounts of glyoxalase-I (12 U/mL) gave curve 3. The tangent used to calculate d(GS-lac)/dt is indicated by the broken line (· — · —).

with DTT as reducing thiol will be described as will the corresponding control experiments. Other runs were performed in an analogous manner. To 2900 μL of 0.2 M phosphate buffer, pH 7.0, containing 0.1% egg albumin, at 30.0 ± 0.5 °C under argon, was added 10 μL of a 48 mM NAD^+ stock solution prepared by dissolving 32.4 mg of NAD^+ in 1000 μL of 0.2 M phosphate buffer, pH 7.0. To the resulting solution were added 10 μL of 9.0 mM lipoamide solution (prepared by dissolving 3.68 mg of oxidized lipoamide in 2000 μL of degassed methanol) and 50 μL of a 105 mM dithiothreitol stock solution in 0.2 M phosphate buffer, pH 7.0. The initial concentration of the dithiothreitol stock solution was checked by Ellman's method,[48] and the initial concentration of NAD^+ could be checked by total conversion of NADH using a large excess of thiol. The absorbance at 340 nm was noted and 30 μL of solution containing approximately 1 U/μL of lipoamide dehydrogenase was added. The absorbance at 340 nm was followed until it became constant. Controls indicated that no formation of NADH occurred when lipoamide, lipoamide dehydrogenase, or organic thiol was absent. From the absorbance at 340 nm, the concentration of NADH was obtained. The concentration of NAD^+ is given by $(NAD^+) = (NAD^+)_0 - (NADH)$, where $(NAD^+)_0$ is the concentration of NAD^+ present before adding the lipoamide dehydrogenase. The ratio of oxidized to reduced lipoamide is then given by inserting values for (NAD^+) and (NADH) into eq 27:

$$\frac{(\text{lipo}^{ox})}{(\text{lipo}^{red})} = \frac{(NAD^+)_0 - (NADH)}{(NADH)} K_{27} = \gamma K_{27} \qquad (42a)$$

$$(\text{lipo}^{red}) = [1 + \gamma K_{27}]^{-1}(\text{lipo})_0 \qquad (42b)$$

$$(\text{lipo}^{ox}) = \gamma K_{27}(\text{lipo}^{red}) \qquad (42c)$$

This equation yields values for (lipo^{ox}) and (lipo^{red}), since their sum is $(\text{lipo}^{ox})_0$, the known concentration of oxidized lipoamide before addition of lipoamide dehydrogenase. The sum of (NADH) and (lipo^{red}) equals (DTT^{ox}), the concentration of oxidized DTT present at equilibrium. Since the original concentration of DTT before addition of lipoamide dehydrogenase is known, $(DTT)_0$, the concentration of (DTT) at equilibrium, is easily calculated. Combination of these concentrations yields eq 43 and 44 which directly relate K_{lipo}^{obsd} and K_{NADH}^{obsd} to known quantities.

$$K_{\text{lipo}}^{obsd} = \frac{1}{\gamma K_{27}} \frac{(NADH)(1 + \gamma K_{27}) + (\text{lipo})_0}{[(DTT)_0 - (NADH)](1 + \gamma K_{27}) - (\text{lipo})_0} \qquad (43)$$

$$K_{NADH}^{obsd,\ DTT} = K_{27} K_{\text{lipo}}^{obsd} \qquad (44)$$

In the particular experiment used here for illustration, the initial concentrations of materials were 0.2 M phosphate, pH 7.0; 0.166 mM

NAD^+; 0.030 mM lipoamide; 1.68 mM dithiothreitol; 10 U/mL lipoamide dehydrogenase. The concentrations of species present at equilibrium were 0.0146 mM NAD^+; 0.153 mM NADH; 1.49 mM DTT^{red}; 0.183 mM DTT^{ox}; 0.0297 mM $lipo^{red}$; $\sim 3 \times 10^{-3}$ mM $lipo^{ox}$. In other runs, higher concentrations of organic thiols were necessary to achieve comparable reductions of NAD^+. In the least favorable case, 540 mM 2-mercaptoethanol was required. For several runs it proved more convenient to start with known mixtures of oxidized and reduced thiol. Thus, a stock solution prepared by dissolving 23.7 mg ($\sim$0.132 mmol) of a mixture of oxidized and reduced dimercaptoacetylhydrazide (**10**, Table V) in 2.0 mL of 0.2 M phosphate buffer, pH 7.0, showed a dithiol concentration of 44.5 mM when assayed by Ellman's method.[49] The composition of the mixed stock solution is then 44.5 mM in dimercaptoacetylhydrazide^{red} and 21.3 mM in dimercaptoacetylhydrazide^{ox}. The remainder of the equilibration was carried out as described above. Initial concentrations of materials were 0.2 M phosphate, pH 7.0; 0.162 mM NAD^+; 14.8 mM dimercaptoacetylhydrazide^{red}; 6.98 mM dimercaptoacetylhydrazide^{ox}; 0.00367 mM lipoamide^{ox}; 10 U/mL lipoamide dehydrogenase. The concentrations of species present at equilibrium were 0.139 mM NAD^+; 0.0223 mM NADH; 14.7 mM dimercaptoacetylhydrazide^{red}; 7.01 mM dimercaptoacetylhydrazide^{ox}; 0.00128 mM lipoamide^{ox}; 0.00239 mM lipoamide^{red}.

Equilibration of 1,4-Dimercaptobutane and Dithiodiketopiperazine. Easily monitored physical properties of 1,4-dimercaptobutane and 1,2-dithiane are their ^1H NMR spectra which are, for 1,4-dimercaptobutane ($CDCl_3$), δ 2.6-2.4 (m, 4, CCH_2S), 1.8-1.6 (m, 4, CCH_2C), 1.3 (t, 2, J = 8 Hz, -SH), and for 1,2-dithiane ($CDCl_3$), δ 3.0-2.8 (m, 4, CCH_2S), 2.1-1.9 (m, 4, CCH_2C).[52] Oxidized dithiodiketopiperazine shows ($CDCl_3$) δ 4.1-3.5 (br m, 4), 3.1-2.6 (br m, 2), 2.5-2.0 (br m, 6). This latter NMR spectrum is little changed on reduction to the disulfide. A mixture of 47.5 mg (0.185 mmol) of oxidized dithiodiketopiperazine and 29.0 mg (0.238 mmol) of 1,4-dimercaptobutane in 1.0 mL of $CDCl_3$ under argon, at 30.0 $\pm$ 0.5 °C, showed initially a ^1H NMR spectrum which was just the superimposition of the spectra of the constituents. The spectrum of this mixture was monitored over a period of 7 days during which signals, δ 3.0-2.8, characteristic of 1,2-dithiane appeared while signals, δ 1.8-1.6, characteristic of 1,4-dimercaptobutane disappeared in equal amounts as determined by integration. After 5 days, the spectrum became static. The integrated ratio of signal intensity at δ 1.8-1.6 to that at δ 3.0-2.8 is equivalent to $(HS(CH_2)_4SH)/(\overline{S(CH_2)_4S})$. The ratio of oxidized to reduced diketopiperazine may be calculated by the law of mass balance since the initial quantities of both 1,4-dimercaptobutane and dithiodiketopiperazine are known. Multiplication of these ratios gives a value of 3.3 as the equilibrium constant for reduction of 1,2-dithiane by dithiodiketopiperazine; this value corresponds to $K_{NAD}^{obsd} = 7.1 \times 10^{-2}$ and $K_{GSSG}^{SH} = 4.6 \times 10^5$ mM. The definitions and interconversions of these constants appear in Table V and in the text.

Experimental Checks of Calculated Data in Figure 11. Four equilibrations were carried out to verify representative calculations included in Figure 11. In each instance, a quantity of thiol calculated to be sufficient to reduce 50% of GSSG to GSH (or $lipo^{ox}$ to $lipo^{red}$) was added to a solution containing GSSG (or $lipo^{ox}$) and the final concentrations of thiols and disulfides were measured. These experiments were semiquantitative: the difficulties in measuring small quantities of air-sensitive thiols, and the low solubility of oxidized lipoamide in water, precluded high precision. They nonetheless served to establish that the data in Figure 11 were not grossly in error. Representative experimental details follow, and results are summarized in Table VI.

The absorbance of a solution of 0.003 84 g of DTT in 50.0 mL of 0.02 M phosphate at pH 7.0, 30 $\pm$ 0.5 °C, was monitored at 290 nm, the λ_{max} for 1,2-dithianes,[34] before and after the addition of 0.0308 g of oxidized glutathione. After equilibration, 0.0304 g of oxidized glutathione was added and the absorbance again measured to establish a blank correction for that quantity of glutathione. Initial concentrations used in this experiment were 0.497 mM DTT and 1.007 mM GSSG. The absorbance change at 290 nm indicated the amount of oxidized DTT present at equilibrium. From this value concentrations of GSH, GSSG, and DTT at equilibrium could be calculated. The concentrations achieved at equilibrium are listed in Table VI.

Equilibrations against lipoamide were performed by adding known quantitities of oxidized lipoamide to solutions of BAL, DTT, or ME in 1:1 MeOH-H_2O buffer and measuring the absorbance at 330 nm before the addition of lipoamide and after equilibrium had been obtained. Extinction coefficients $\epsilon_{290} \simeq 290$ M^{-1} cm^{-1} for 1,2-dithianes (DTT^{ox}) and $\epsilon_{330} \simeq 147$ M^{-1} cm^{-1} for 1,2-ditholanes (oxidized lipoamide) were used in the work.[34]

Table VI. Initial and Equilibrium Concentrations (mM) in Thiol-Disulfide Interchange Reactions

expt	initial	equilibrium predicted	equilibrium found
1[a]	GSSG 1.01	0.513	0.514
	DTT^{red} 0.497	0.000	0.004
	GSH	0.994	0.986
	DTT^{ox}	0.497	0.493
2[b]	$lipo^{ox}$ 1.04	0.497	0.231
	BAL^{red} 7.73	7.22	6.92
	$lipo^{red}$	0.507	0.809
	BAL^{ox}	0.254	0.405
3[a]	$lipo^{ox}$ 1.04	0.540	0.538
	DTT^{red} 0.531	0.031	0.028
	$lipo^{red}$	0.500	0.503
	DTT^{ox}	0.500	0.503
4[a]	$lipo^{ox}$ 1.04	0.538	0.497
	ME^{red} 502.	501	500.
	$lipo^{red}$	0.502	0.545
	ME^{ox}	0.502	0.545

[a] Conducted in aqueous buffer solution. [b] Conducted in 1:1 methanol-aqueous buffer solution.

Acknowledgment. Our colleagues John Deutch and James Barber provided us with most useful discussions of the separation of K^{obsd} into K^{SH} and K^{S-}, and of the compatibility of the kinetic and thermodynamic equations. We also thank William Rastetter and Robert Williams for the gift of the dithiodiketopiperazine used in these studies, and Edward Solomon and Al Hepp for determination of Raman spectra. Ze'ev Shaked provided data concerning rates of thiol-disulfide interchange reactions involving proteins used to test some of the Brønsted equations summarized here. We thank especially Professor W. W. Cleland for detailed readings of several versions of this paper and for exceptionally useful suggestions and criticisms. Professors W. Jencks and D. Hupe also contributed substantially in correcting errors in these early versions.

Supplementary Material Available: Identification and sources of the data in Figure 10 (4 pages). Ordering information is given on any current masthead page.

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(26) The data of Hupe et al. also show some small sensitivity to the particular data included in the analysis. Two sets of experiments were carried out in obtaining rate constants for reductions with aryl thiols: one used the reducing agent in excess, one had Ellman's reagent in excess. Least-squares analysis of the first set of data yielded $\beta_{nuc}^{aryl} = 0.48$ ($r^2 = 0.96$); analysis of the second set of data yielded $\beta_{nuc}^{aryl} = 0.41$ ($r^2 = 1.0$); analysis of both together yielded $\beta_{nuc}^{aryl} = 0.44$ ($r^2 = 0.97$). Inclusion of a point for methyl mercaptoacetate in Hupe's analysis changes β_{nuc}^{alkyl} to 0.43 ($r^2 = 0.91$).

(27) Although the data for alkyl thiols in these studies are in good agreement, there is a small but real difference in the data for the aryl thiols. The similarity in the alkyl thiol rate constants suggests that no consistent experimental difference accounts for this difference. All of our data were obtained using the same procedure, although their experimental precision was lower for the more rapidly reacting thiols. Hupe et al. used slightly different procedures for alkyl and aryl thiols. Either this difference in procedure, or differences in the compositions of solutions (especially ionic strength and buffer composition), might contribute to the consistent factor of ca. 2 which separates these sets of rate constants.

(28) We note that the values of β_{nuc} derived from these two independent investigations are, in fact, experimentally indistinguishable ($\beta_{nuc}^{alkyl} = 0.41$[12] vs. 0.43[13]) if the datum for methyl mercaptoacetate is included in the calculation of the latter point. Hupe et al. point out that this compound shows deviations in analyses of rate constants derived from both thioesters and disulfides, and exclude it on that basis.

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(33) UV spectra of solutions obtained by mixing BAL and Ellman's reagent showed only those features attributable to Ellman's anion and to acyclic S–S bonds (Ellman's reagent). However, consideration of the trends observed in the UV of various disulfides[34] (acyclic, ϵ_{max} 480 at λ 250 nm; 1,2-dithiepane, ϵ_{max} 444 at λ_{max} 258 nm; 1,2-dithiane, ϵ_{max} 290 at λ_{max} 290 nm; 1,2-dithiolane, ϵ_{max} 147 at λ_{max} 330) suggest that the ϵ_{max} for a four-membered cyclic disulfide might be vanishingly small. Raman spectra of an identical mixture of BAL and Ellman's reagent show, in addition to the expected absorptions, a new band at 510 cm^{-1}. This position is consistent with there being no special strain in the S–S bond and is substantially different from the ν 500 cm^{-1} predicted for a 1,2-dithietane.[35] Thus, these spectral data are compatible with formation of either a cyclic dimer or higher oligomer or a polymer on oxidation of BAL. The isolation of a dithietane has not been reported, but stable disulfide-containing rings containing seven or more members are well established: A. E. Asato and R. E. Moore, *Tetrahedron Lett.*, 4841 (1973); G. H. Wahl, J. Bordner, D. N. Harpp, and J. G. Gleason, *J. Chem. Soc. Chem. Commun.*, 985 (1972); T. C. Owen, J. M. Fayach, and J. S. Chen, *J. Org. Chem.*, **38**, 937 (1973).

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$$K^{S^-} = 2(\beta_{nuc} - \beta_{lg})(pK_a^{RSH} - pK_a^{ESH})$$

The data in Figure 7 cover both alkyl and aryl thiols and disulfides, and trying to fit them all to a single equation may not lead to useful parameters. If we analyze, however, only the group of points 1–8 corresponding to thiol–disulfide interchange involving aliphatic thiols, and force the least-squares line to go through (0,0), we estimate that $\beta_{nuc} - \beta_{lg} \simeq 1.21$, or $\beta_{lg} = -0.71$. This value is in qualitative agreement with the value of $\beta_{lg} = -0.73$ generated by analyzing rates. R. Freter, E. R. Pohl, J. M. Wilson, and D. J. Hupe, *J. Org. Chem.*, **44**, 1770 (1979), have independently estimated $\beta_c \simeq -0.3$ for displacement of Ellman's anion from mixed disulfides of the form RSS–Ellman's.

(40) Values of $\beta_c = -0.30$ ($\beta_{nuc} = -\beta_{lg} = 0.63$, $C = 5.54$), $\beta_c = -0.35$ ($\beta_{nuc} = -\beta_{lg} = 0.62$, $C = 5.91$), and $\beta_c = 0.40$ ($\beta_{nuc} = -\beta_{lg} = 0.61$, $C = 6.29$) all have $r^2 = 0.96$ for plots of the type shown in Figure 10. The values corresponding to $\beta_c = -0.40$ were chosen as "best" because the slope a of the correlation line and the intercept b in the equation $\log k^{calcd} = a \log k^{expt} + b$ were closest to the values of $a = 1.0$, $b = 0.0$ expected for a perfect correlation: $\beta_c = -0.30$ ($a = 0.93$, $b = 0.34$); $\beta_c = -0.35$ ($a = 0.96$, $b = 0.20$); $\beta_c = -0.40$ ($a = 0.98$, $b = 0.09$).

(41) In practice, in applications of these equations to the determination of the pK_as of protein thiol groups using thiol–disulfide interchange rates, we find that the difference between the pK_as estimated using eq 36–38 is less than the uncertainty from other aspects of the experimental work.

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Solvolysis of D-Glucopyranosyl Derivatives in Mixtures of Ethanol and 2,2,2-Trifluoroethanol[1]

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Abstract: The products of solvolysis of α- and β-D-glucopyranosyl fluorides, 2,4-dinitrophenyl β-D-glucopyranoside, and the trifluoromethanesulfonates of the β-D-glucopyranosyl 3-bromopyridinium and α-D-glucopyranosyl 4-methylpyridinium ions in an equimolar mixture of ethanol and trifluoroethanol buffered with ~2 equiv of 2,6-lutidine have been examined by GLC of their trimethylsilyl ethers. The initial products of the solvolyses of phenyl α- and β-D-glucopyranosides catalyzed by trifluoromethanesulfonic acid in an equimolar mixture of ethanol and trifluoroethanol, and the products of uncatalyzed solvolysis of β-D-glucopyranosyl-*p*-nitrophenyltriazene, have been likewise examined. The composition of the medium for solvolysis of the glucosyl fluorides has also been systematically varied from pure ethanol to pure trifluoroethanol. The percentage of products with the same anomeric configuration as the starting material is in the range 8.1–88.5%; change of leaving group, at constant anomeric configuration, or of anomeric configuration, at constant leaving group, yields different product distributions. Therefore the transition state for the product-determining step contains the leaving group. The preference for attack by ethanol as compared with trifluoroethanol varies from 0.9 to 20 in a way which shows no general systematic distinction between pathways for retention or inversion. The nucleophilic selectivity for retention is lowered by anionic leaving groups, especially fluoride, which preferentially stabilize the transition state containing trifluoroethanol by hydrogen bonding. Nucleophilic attack at the α face is preferred over nucleophilic attack at the β face, and exhibits a lower selectivity: this is ascribed to hydrogen bonding between the oxygen atom of the 2-hydroxyl group and the hydroxyl group of the approaching alcohol. A model for solvolysis involving a reversibly formed ion pair or encounter complex is incompatible with the selectivities still observed with leaving groups less nucleophilic than the solvent components: a model involving selection between the components of a pool of solvent molecules by an irreversibly formed ion pair or encounter complex requires an implausibly large pool to explain observed specificities. It is therefore concluded that the observed selectivities are a consequence of the facilitation of the departure of the leaving group by the solvent, from either side of the reaction center.

Widely accepted mechanistic descriptions of nucleophilic substitutions at C-1 of pyranose rings have commonly invoked aldopyranosyl cations as intermediates in reactions in neutral and acidic media.[3] In aqueous solution product formation was usually held to take place from the free cations, but in less polar solvents the predominance of products in which the configuration of the reaction center was inverted led to the proposal that the species whence products are formed still contained the leaving group, but only in the capacity of a steric obstruction.[4,5] A direct, S_N2 reaction has also been observed.[4]

For the purposes of this discussion we define an intermediate as a species with a lifetime longer than a molecular vibration. A more stringent and experimentally accessible criterion for an intermediate is a lifetime which is long enough that the various fragments from its precursor are not still surrounded by a common solvent shell when the products are formed. In water at 25 °C this requires a lifetime of ~10 ps or more.

It has recently proved possible to estimate the lifetimes of oxocarbonium ions of the type $ArCMe{=}O^+Me$ by using the diffusion-controlled reaction of these ions with SO_3^{2-} (to give $ArC(Me)(OMe)SO_3^-$) as a "clock" with which to time their reactions with solvent water.[6] The lifetimes so estimated are surprisingly short (e.g., 10 ns for $PhCMe{=}O^+Me$), but extrapolation of the results of direct measurements in aqueous sulfuric acid to pure water give essentially the same results.[7]

A linear free energy relationship exists between the rate of reaction of $ArCMe{=}O^+Me$ with solvent water and the rate of reaction of the parent ketone ArMeCO with bisulfite. If this relationship is extrapolated to $CH_2{=}O$ and $CH_2{=}O^+Me$, the predicted lifetime of the latter is $\sim10^{-15}$ s, below the period of even the fastest bond vibration. The methoxymethyl cation is thus too unstable to exist as a free solvent-equilibrated intermediate in aqueous solution. The solvent molecule or another nucleophile with which a methoxymethyl center reacts must therefore be present in the transition state in which the leaving group departs. Second-order reactions with nucleophiles have been observed with both methoxymethyl 2,4-dinitrophenolate[8] and methoxymethyl *N,N*-dimethylanilinium ions.[9] These reactions, even though bimolecular, are very S_N1-like in the high degree of positive charge on the central atom in the transition state, as shown by high-β_{lg} and low β_{nuc} values. The presence of a nucleophile in the transition state is enforced by the instability of the potential intermediate, just as the presence of proton donors and acceptors in the transition states of certain acyl transfer reactions is enforced by the short lifetime of the tetrahedral intermediate.[10]

The available evidence is that aldopyranosyl cations are yet more unstable than $CH_2{=}O^+Me$. The acid-catalyzed hydrolysis of formaldehyde dimethyl acetal[11] is about 10^4 times faster than that of methyl β-D-glucopyranoside,[12] and likewise the spontaneous hydrolysis of methoxymethyl 2,4-dinitrophenolate[8] is ca. 10^2 faster than that of 2,4-dinitrophenyl β-D-glucopyranoside.[12] The spontaneous hydrolysis of $MeOCH_2F$[13] can be estimated to be a similar factor of 10^2 faster than that of the β-glucopyranosyl compound, if this is estimated to react more slowly than its C-4 epimer[14] by the usual[3] factor of 3.

In the light of this instability of aldopyranosyl cations, and the consequent enforced participation of a nucleophile, the fact

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