

MERCAPTAN-DISULFIDE INTERCHANGE REACTIONS

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CHAPTER I

INTRODUCTION

The purpose of this thesis is to report an investigation of mercaptan-disulfide interchange reactions. More particularly, the thesis deals with mercaptans and disulfides of biological interest; namely, cystine, cysteine, oxidized and reduced glutathione, 2-aminoethanethiol and some of its congeners. These compounds are more or less effective as protectors against radiation injury (4,33,94,104), one purpose of the present work was to elucidate the mechanism of this action.

The investigation is divided into three parts. In the first part, the relationships between the initial concentrations of reactants, the equilibrium constants, and the equilibrium concentrations of the products were examined. This examination led to the realization that the mercaptan-disulfide interchange equilibrium constants can be calculated if the equilibrium concentrations of one mercaptan in each of two different reaction mixtures can be determined; the appropriate algebraic expressions were derived and a program devised for solving them by computer.

The aforementioned procedure and expressions were used in the second part of the investigation, which concerns the reaction of 4,4'-dithiobis(benzenesulfonic acid) with 2-aminoethanethiol and some congeners. According to one hypothesis, 2-aminoethanethiol and related mercaptans protect organisms from ionizing radiation by forming mixed

disulfides with sulfur-containing constituents of the organism (32,33, 47). It was therefore hoped that radioprotective ability might be correlated with the ability to react with a given disulfide. The reaction of various mercaptans with 4,4'-dithiobis(benzenesulfonic acid) can be followed easily because the mercaptide anion released in the reaction has a characteristic ultraviolet spectrum that can be measured. Unfortunately, the results of the work did not conform to expectations; the rates and extents of reaction do not correlate in a straightforward way with the radioprotective ability.

The third part of the investigation is concerned with the biologically important reaction of cystine with glutathione. Conflicting reports have appeared in the literature concerning the equilibrium constants for this reaction (59,77). In this work, all the components of the reaction present at equilibrium were determined by ion-exchange chromatography. This procedure affords better precision than the methods previously employed.

The results obtained in these three parts have been described in a form suitable for publication in Chapters III, IV and V, respectively. In writing for publication it is desirable to omit many details, which nevertheless might be of some interest. These have been described in the Appendix.

CHAPTER II

GENERAL DISCUSSION AND REVIEW OF THE LITERATURE

Scope of Search for Pertinent Literature

A number of reviews and papers concerning mercaptan-disulfide interchange have been consulted and in addition, Chemical Abstracts has been searched from Volume 1 through Volume 66 (June-January 1967). Chemical Abstracts provides a comprehensive coverage of the recent literature, but, unfortunately, the nature of the subject and the indexing procedure employed by CA make it difficult to retrieve all the information. An attempt will therefore be made to delineate the exact scope of the search that was made; this could provide a point of departure for anyone wishing to extend the coverage further.

One difficulty in searching the indices of CA is that entries regarding interchange reactions are often indexed under the names of the compounds involved rather than under general descriptive terms. Several compounds names of special interest (vide infra) were used as search words, but no attempt was made to be complete. Information regarding interchange reactions might also be found under the names of individual mixed disulfides, that are indexed under "Disulfide", e.g., "Disulfide, ethyl n-propyl". However, so many such entries are to be found that it was not deemed profitable to search this topic thoroughly. Another feature of the CA indexing procedure gives rise to difficulties, namely, the "order of precedence" of functions used in naming. "Thiol" and

especially, "disulfide" have a low precedence, which means that many compounds containing these groups might be indexed under names that refer to other functions.

Table II-1 lists the subject headings which were scanned for references to mercaptan-disulfide interchange reactions and related topics. It will be noted that slightly different search words were used for volumes 64-66 because of changes made in indexing and naming after volume 63.

TABLE II-1
SUBJECT HEADINGS USED IN LITERATURE SEARCH

Cystamine	disulfide ¹	mercaptan	sulfhydryl ¹
cysteamine	disulfide group ²	mercapto-	sulfide, di- ²
	glutamine, N,N'-dithiobis{1-[(carboxymethyl)carbamoyl]ethylene}di- ²		
cysteine	glutathione	mercapto compounds ²	thio-
cystine		mercapto group ²	thiol

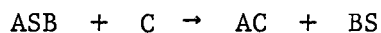
¹Used only for the Keyword Index of Volumes 64-66.

²Used only for the Subject Indexes of Volumes 1-63.

Nucleophilic Displacement at the Sulfur-Sulfur Bond

Mercaptan-disulfide interchange reactions are a special case of nucleophilic displacement at the sulfur-sulfur bond. This section will consider briefly reactions of this type involving compounds in which at least one of the sulfur atoms is divalent, except mercaptan-disulfide interchange that is the subject of a later section.

Parker and Kharash (75) have written a comprehensive review. Their list of the classes of compounds pertinent to the present discussion includes: disulfides; polysulfides (RS_nR, S_8); thiocyanates (RSCN); sulfenyl derivatives (thiolsulfonate esters, $RSSO_2R'$; Bunte salts, $RSSO_3^-$; sulphenyl monothiophosphates, $RSSPO(OR)_2$; sulfenyl thiosulfates, $RSS_2O_3^-$; and sulphenic anhydrides, $RS(O)SR$); dialkylsulfonyl disulfides (RSO_2SSO_2R); and polythionates ($^-O_3SS_nSO_3^-$). A nucleophilic reagent C (54) would attack any of the above, which can be represented by ASB (only one divalent sulfur is specifically shown and group B would also contain sulfur) according to the schematic equations (in which electrical charges are not shown):



There are also other recent reviews that cover various aspects of this subject (26,40,90).

Quite a few studies have been made of the reaction of disulfide with sulfite and with cyanide ion (98,99). Actually, these reagents are rather weak nucleophiles and do not react easily with simple aliphatic disulfides. However, the presence of electron-withdrawing groups close to the disulfide bonds, neighboring-group participation by suitably placed carboxyl or amino groups, or the possibility of cyclization of the product may accelerate the reaction. Specific cases that have been studied are the reactions with thiocyanogen (97), elemental sulfur (39), 2,4-dinitrodiphenyl disulfide (76), cystine (20,90,91,92), oxidized glutathione (20,99) and related disulfides (71,92).

A mechanism in which displacement occurs by a concerted bimolecular reaction is usually referred to as S_N2 . Available kinetic evidence indicates that this mechanism operates in most if not all the

cases mentioned above. For example, the reaction of cystine and its congeners with cyanide and sulfite proceed by second order kinetics, with the additional complication that they are reversible (20,71,99). Recently, Wagner and Davis (106) have suggested that cyanide may not attack the sulfur-sulfur bond in cystine directly. Their experiments indicate that cyanide reacts with the amino group of cystine to form the carbanion $-C=NH$ which then displaces an RS moiety and forms the thiazolidine ring.

Mercaptan-Disulfide and Disulfide-Disulfide Interchange Reactions

Mercaptan-disulfide interchange may be represented by the equations:



The corresponding equilibrium expressions are:

$$K_1 = (XSSY)(YSH) / (XSH)(YSSY) \quad (II-3)$$

$$K_2 = (XSSX)(YSH) / (XSH)(XSSY) \quad (II-4)$$

Dividing II-4 into II-3 gives the expression

$$K_3 = (XSSY)^2 / (XSSX)(YSSY) \quad (II-5)$$

which is the equilibrium constant for the disulfide interchange reaction



Considerable work has been done on these reactions and several reviews of this work have been published (13,40,69,75,86).

The formation of mixed disulfides was first reported by Otto and Rössing in 1886 (74). Lecher (65) did not succeed in forming a mixed

disulfide by heating together the symmetrical phenyl and o-nitrophenyl disulfides. He then tried reacting sodium, thiophenol and o-nitrophenylsulfenyl chloride; this gave him a mixture of mixed and symmetrical disulfides (66,67). He postulated that the mixed disulfide formed first, and that this exchanged with the thiophenol; he confirmed this by reacting the mixed disulfide with thiophenol, which indeed gave a mixture of all three disulfides. In 1938, Toennies (102) studied the reduction of cystine to cysteine by thiourea. He suggested that the reaction takes place in two steps to form S-(guanylthio)cysteine as an intermediate and subsequently formamidinium disulfide and cysteine. The reverse reaction also was reported by him (103).

Although by 1951 several mixed disulfides had been prepared, it was not generally understood how their stability compares to that of the symmetrical disulfides; the view seems to have been held, at least in some quarters, that the mixed disulfides were intrinsically more unstable. The work of Birch et al. (10) was important in dispelling this view; they showed that mixed disulfides would be formed from mixtures of simple symmetrical disulfides at equilibrium. A large number of unsymmetrical disulfides have now been prepared and characterized; an extensive list of them is given in a book by Reid (82).

Bersin and Steudel (8) made a pioneering study of the equilibria in a mercaptan-disulfide system. They measured the reaction of cystine with thioglycolic acid and observed that an equilibrium would be reached from either direction. They assumed that the mixed disulfide was unstable and not present at equilibrium to an appreciable extent, and on this basis they calculated an equilibrium constant of about 1. They found that the rate increased with pH, indicating that it depends on

the extent of ionization of the thiol. In later work, Kolthoff et al. (59) showed that mixed disulfide was in fact present at equilibrium and derived values of 0.8 and 4.1 for K_1 and K_2 , respectively.

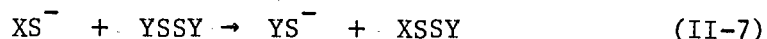
The equilibrium constants of many other mercaptan-disulfide reactions have also been reported. Three groups of investigators determined the constants for some simple alkyl mercaptans and disulfides (10,24,51), and many papers give the results obtained with cysteamine, cysteine, and several congeners (30,31,46,59,68,78,88,96). Many of the results obtained in these experiments have been tabulated by Smith et al. (96). In addition, Eldjarn and Pihl (31) have reported equilibrium constants for the reaction of cystamine and N,N'-diacetylcystamine with glutathione. Schöberl and Grafje (88) have determined the interchange constants for the equilibria involving thioglycolic acid, cysteine, and homocysteine; the constants were found to range between 1.2 and 2.1. Kapoor (55) extended the work of Kolthoff et al. (59) by determining the equilibrium constants for the reaction of cystine with glutathione at 50°C. From these values and those found earlier he was able to calculate the heats of reaction for these interchanges.

The equilibrium constants which have been determined for primary and secondary mercaptans indicate that the interchange equilibria are not much affected by the nature of the substituent on sulfur. That is, the vast majority of constants are close to the values that would be expected on the basis of probability considerations, $K_1 = 2$ (Equation II-3) and $K_2 = 0.5$ (Equation II-4). The situation is quite different, however, for the tertiary mercaptans (24,35,51). In this case, K_1 is quite large and K_2 quite small, so that the equilibrium concentration of mixed disulfide is large.

Smith et al. (96) studied the reaction of disodium 4,4'-dithiobis-(benzenesulfonic acid) (BSSB) with cysteine. This involves a situation not present in the other investigations, namely, the derivative mercaptan BSH has a comparatively low pK_a (SH). Thus, at pH 7 BSH is almost completely ionized, whereas cysteine is not; although the values of the equilibrium constants are by no means unusual, the equilibrium is in these conditions almost completely displaced in favor of cystine and BS^- . In this and similar cases, one can alter the point of equilibrium by varying the pH.

A considerable number of kinetic and other studies have been made in an effort to determine the mechanism of mercaptan-disulfide interchange. By means of polarimetry, Fredga measured the rate of reaction of (+)-dithiodilactic acid and thiolactic acid (41); he found that the reaction was of second order and that the rate increased with pH, indicating that mercaptide ion probably is the reactive species. As has already been stated, quite similar results were found by Bersin and Steudel (8) in their investigation of the reaction of cystine with thio-glycolic acid. Fava et al. (35) measured the rate of interchange for n-butyl, n-hexyl, t-butyl and phenyl disulfides, labeled with S^{35} , with the corresponding mercaptans in butanol-water and methanol-water. In the absence of a catalyst, reaction was very slow and kinetic studies were made only in the presence of added sodium hydroxide, which would react with mercaptan to give the mercaptide ion. The rate was proportional to the disulfide and the mercaptide concentrations. Mondovi et al. (73) found only a very slow reaction between cystine and cystamine at pH 3.8. Parker and Kharasch (76) investigated the scission of mixed disulfides and arranged a series of ions, including mercaptides,

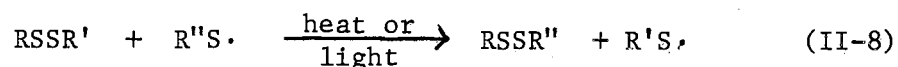
in order of their nucleophilicity. It is clearly indicated by the reaction proceeds via mercaptide ion:



Phil et al. (78) measured the initial rates of reaction of several mercaptans with cystine at pH 7.4 and found that the rates varied little despite the large differences in the pK_a values of the mercaptans used, and hence in the concentration of mercaptide ion present in the reactions. This was rationalized as being due to the compensation of two opposing effects: a mercaptan with a relatively high pK_a will be less ionized but the derived ion will be more strongly nucleophilic.

In their study of the reaction of 3,5-diimino-1,2,4-dithiazoline, a cyclic disulfide, with cysteine, glutathione, and 2-aminoethanethiol, Roesler et al. (83) found that the reaction was overall of third-order; the rate was proportional to the concentration of disulfide and to the concentration of mercaptan squared. The rates were determined in the vicinity of pH 4 and an inverse relationship was found between the rate and the hydrogen ion concentration. These results were interpreted in terms of a mechanism that involves reaction of the unionized mercaptan with a protonated form of the disulfide.

According to Gur'yanova and Vasil'eva (50), the rate of the interchange between ditolyl disulfide and thiocresol was independent of mercaptan concentration and proportional to the square root of the disulfide concentration. It was also found that the reaction was greatly accelerated by ultraviolet light. Similar results were reported by Cavallini et al. (19) on irradiating cystamine and cysteine. These results strongly suggest the occurrence of homolytic fission and a free-radical mechanism for the interchange:



It seems reasonable to conclude that the mechanism involving mercaptide ion is favored in most circumstances, but that a free-radical mechanism may occur in special conditions, i.e., with aromatic disulfides, in strictly nonpolar media, at high temperature, or under ultraviolet irradiation.

The interchange of disulfides is usually slow but may be speeded by various means. In 1953, Leandri demonstrated that unsymmetrical diaryl disulfides would disproportionate to symmetrical disulfides when subjected to u.v. light or when heated to 170°C (64). In the same year, Sanger (86) demonstrated that disulfide interchange might also be catalyzed by strong acid. Benesch and Benesch (7) made a more extensive study of the reaction between cystine and bis(2,4-dinitrophenyl)cystine and found that the rate decreased rapidly when the concentration of hydrochloric acid was less than 9 M. They suggested that the intermediate could be the sulfenium ion. Schöberl and Bauer (87) and Glazer and Smith (46) also have studied disulfide interchange in concentrated acid solution.

Schöberl and Grafje (88) found that the interchange of disulfides derived from thioglycolic acid, cysteine and homocysteine was catalyzed by u.v. light, strong acid or by mercaptide ions (RS^-). Eager and Savage (28) reported that practically no disulfide interchange took place with cystine and related disulfides at pH 1-6, but that small amounts of a thiol, hydrogen sulfide, or thiourea would initiate the reaction. This observation concurs with that of Field *et al.* (38) who recently noted that, for the alkyl-aryl mixed disulfides they studied, thermally induced (reportedly largely heterolytic) disproportionation

was accelerated by strong acid, alkali, or thiol, but was inhibited by dilute acids.

Methods Used in Studies of Mercaptan-Disulfide Interchanges
and Problems Associated with Them

In mercaptan-disulfide interchanges the total moles of mercaptan and of disulfide remain unchanged, and five components may be present at equilibrium. This makes determination of rates and equilibria more difficult than in most other cases. In general, at least one disulfide and one mercaptan, or two disulfides, must be determined in the presence of the others; this requires the application of specific analytical methods, prior separation of the reaction mixture, or a combination of both. An alternative, which makes possible calculation of the constants from determination of only one component in two different mixtures, will be described in Chapter IV. Although several attempts have been made to determine rates and equilibria from measurements of an appropriate physical property, this can be done correctly only in special cases.

Fava et al. (35) and Gur'yanova and Vasil'eva (49,50) labeled a mercaptan or disulfide with radioactive S^{35} , after separation of the mercaptan and disulfide fractions, the amount of exchange could be determined by radioactivity measurements. Phil et al. (30,31,77,78) also used radioactively-labeled compounds; furthermore they separated the reaction mixtures by paper electrophoresis. Both rates and equilibria were determined. Paper electrophoresis was also used by van Rensburg (105). Schöberl (88) and Sluyterman (95) applied paper chromatography to the determination of disulfide equilibrium constants and

Koshlánd (61) used ion-exchange chromatography to investigate disulfide equilibria in proteins.

McAllan et al. (10,70) separated mixtures of mercaptans and disulfides by distillation. This was before the (widespread) development of gas chromatography, and the latter is certainly to be preferred. It has been employed by Haraldson et al. (51) and by Dalman et al. (24).

Kolthoff et al. (59) have studied equilibria involving cystine, which is sparingly soluble, by measuring the increase of solubility in solutions of disulfides and of mercaptans. The total mercaptan and disulfide concentrations were determined by mercurimetric titration. Polarography was also used for confirmatory analyses.

Spectrophotometry has been used by some workers. Fava et al. (35) determined rates and equilibria for the reaction of trimethylene disulfide with thiols. This cyclic disulfide has an absorption maximum at 334 m μ which disappears when the ring is opened and it was further assumed that only the mixed disulfide is formed in the reaction.

Lamfrom and Nielsen (62) studied the reaction of cystine with thio-glycolic acid in the following way: the mixed disulfide was prepared and found to have a spectrum somewhat different from that of the other disulfides; initial rates of reaction were determined spectrophotometrically for the appropriate combination of starting reagents; and equilibria were calculated by taking the ratio of the appropriate rate constants. Their results are not in good agreement with those of Kolthoff et al. (59) and the method used by the latter inspired more confidence. Smith et al. (96) measured the reaction of 4,4'-dithiobis-(benzenesulfonic acid) with cysteine by measuring the absorption of the aromatic mercaptide ion formed in the reaction; K_1 , the constant for

formation of the mixed disulfide, was determined first in reaction mixtures that contained little or no cystine and, knowing K_1 , it was then possible to calculate K_2 .

In connection with the use of spectrophotometry, it may be useful to mention the studies of Parker and Kharasch (76) and of Campaine (15,16), who prepared and measured the spectra of aryl-alkyl disulfides; they found that the absorption maximum was near that of the symmetric aromatic disulfide and that the absorption was near half.

The specific determination of oxidized glutathione by its reaction with NADPH in the presence of glutathione reductase was used by Hird (52) in a study of the reaction of glutathione with the disulfide groups of bovine serum albumin, insulin, and several low molecular weight disulfides.

Polarimetry was used by Fredga (41) in following the reaction of (+)-dithiodilactic acid (-)-thiolactic acid. The reaction with (-)-thiolactic acid would of course give the optically inactive meso compound, and the decrease in optical rotation was formulated as a second-order reversible reaction. This possibly is an oversimplification, because it neglects the formation of (+)-, or (-)-dithio acid from the meso, but the error might be negligible. This is not the case for the reaction of thioglycolic acid and cystine, studied by Bersin and Steudel, which has already been discussed (see p. 7).

Applications of Mercaptan-Disulfide Interchange Reactions

This section lists references that deal with applications of mercaptan-disulfide interchanges. This list is not exhaustive but comprises the most informative references found in the literature search.

The largest number of applications occur in biological systems. Several reviews have appeared concerning this subject (21,27,69,77). Other reviews concern more specific topics: radioprotection (4,33,47), wool chemistry (108,109), protein structure determination (100), cancer therapy (29), and enzymic catalysis (12,25).

Interchange reactions also are important in the chemistry and technology of petroleum (82, p. 16-18) and of rubber (36).

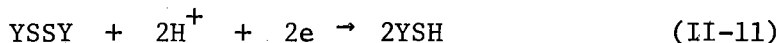
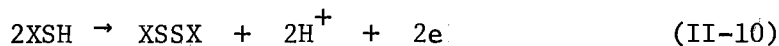
Interchange reactions have found utility in the preparation of new mercaptans and disulfides (57), in the structure determination of diallyl disulfide (22), and in the cleavage of disulfide polymers (37).

Oxidation-Reduction Potentials of Mercaptans and Disulfides

The reaction:



involves oxidation-reduction. It may be considered as the sum of the two partial equations:



for which "half-cell" potentials might be defined relative to a standard. The equilibrium constant K_4 for reaction II-9 is related to the potentials by the equation

$$\begin{aligned} \ln K_4 &= \ln (XSSX)(YSH)^2 / (YSSY)(XSH)^2 \\ &= (nF/RT) (E_{XSH/XSSX} - E_{YSH/YSSY}) \end{aligned} \quad (\text{II-12})$$

In what follows, potentials refer to the standard hydrogen electrode as is customary; the potentials of the mercaptan-disulfide complex refer to pH 7. The values of the potentials are of only limited significance with respect to mercaptan-disulfide interchange, because they

do not define the amount of mixed disulfide that might be formed. However, the potentials would measure the extent of reaction with other oxidizing and reducing agents.

A considerable amount of work has been done to establish the values of the potentials experimentally. There has been an unusual amount of disagreement and controversy concerning the results, so that there is still some uncertainty concerning the merits of the respective investigations. An extensive critical review of the subject has been given by Clark (23), and the matter has also been considered by Cecil and McPhee (21). The present review will be brief, and limited to cysteine-cystine and to oxidized and reduced glutathione.

Many attempts have been made to determine the potential of the cysteine-cystine system by measuring the potentials of galvanic cells, one half of which contained cystine, cysteine and a (presumably) inert electrode. The potential of such a cell should be described by equation II-12, but in all cases save one the potential in fact did not show the expected dependence on the cystine concentration. This indicates that equations II-10 and II-11 do not describe the reactions taking place at the electrode and/or that a reversible equilibrium is not established at the electrodes. The exception referred to is the work of Ghosh and Ganguli (45). In their galvanic cell the electrode was a mercury pool. Immediately prior to making potential measurements, the mercury electrode was subjected to reduction; the authors believed that this treatment removed a layer of oxide, after which equilibrium could be established. The results of Ghosh and Ganguli were in accordance with equation II-12, $E = -0.33$. Freedman and Corwin (42) have questioned the validity of Ghosh and Ganguli's results, which,

the former investigators believed, are due to mercury-cysteine complexes.

Many attempts also were made to determine the potential of cysteine-cystine by polarography. Most results have been considered invalid. However, Kolthoff et al. (60) in 1955 obtained results which they considered reliable: $E = -0.34$.

The same value for the potential was obtained by Tanaka et al. (101) from measurements of equilibria involving cysteine and cystine complexes with ferrous and ferric ions.

Borsook et al. (11) determined the potential of cysteine-cystine in an entirely different way, from calorimetric measurements. The result was $E = -0.39$. Although unexceptionable in principle, this work suffers from the disadvantage that the potential depends critically on the difference between the heats of combustion of cysteine and cystine, a small difference between large numbers. Holtmeyer (53) recently attempted to improve this measurement by directly determining the heat of the reaction of cysteine with ferricyanide and with iodine. This work did not effect the expected improvement, because inconsistencies in the thermodynamic properties of the oxidizing agents left about as great a margin of uncertainty as had existed before; Holtmeyer's data indicate a potential between -0.332 and -0.375 .

Two additional results should be mentioned, that differ more markedly. One is that of Fruton and Clarke (43), who found $E = -0.222$. This value was determined by equilibration with some oxidation-reduction dyes of known potential. The other investigation is by Ryklan and Schmidt (84), who report a value of -0.14 . Freedman and Corwin (42) could not repeat Ryklan and Schmidt's results.

As far as glutathione is concerned, three values have been

reported: Ghosh and Ganguli, $E = 0.34$ (45); Fruton and Clarke, $E = -0.23$ (43); and Rykman and Schmidt $E = +.04$ (84). The determinations were done in the same way as for cysteine.

CHAPTER III

(As stated in the Introduction, this chapter is written
in a form suitable for publication)

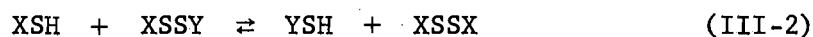
STOICHIOMETRIC RELATIONS AT EQUILIBRIUM

Summary

Mercaptan-disulfide interchange reactions generally involve two consecutive, competitive equilibria. Equations have been developed that relate the composition of the equilibrium mixture to the equilibrium constants and the initial concentrations. The equilibrium compositions corresponding to selected values of the constants have been calculated and certain relationships made evident. In order to deduce the values of the constants from measurement of one equilibrium mixture, the concentration of at least two disulfides or of a disulfide and a mercaptan must be directly determined. It is, however, possible to obtain the constants from the concentration of a single mercaptan species in two equilibrium mixtures, obtained from different initial concentrations. The applications and limitations of the latter approach are discussed.

Introduction

Mercaptan-disulfide interchange reactions occur in diverse chemical systems and may have consequences of considerable importance; some examples have been mentioned in earlier papers of this series^(24,96) and the relation of these reactions to the problem of radioprotection will also be discussed in Chapter IV which follows. The present paper deals with the problem of determining the equilibrium constants for such reactions. Usually, two consecutive, competitive equilibria are operative, which can be represented by the equations:



The corresponding equilibrium expressions are:

$$K_1 = (\text{YSH}) (\text{XSSY}) / (\text{XSH}) (\text{YSSY}) \quad (\text{III-3})$$

$$K_2 = (\text{YSH}) (\text{XSSX}) / (\text{XSH}) (\text{XSSY}) \quad (\text{III-4})$$

The stoichiometric relationships that result from these equilibria are quite complex and the effect of varying the values of the constants upon the relative amounts of products is not obvious. This will be illustrated in what follows.

In order to analyze the equilibrium mixture, one must either have specific methods for determining a sufficient number of components (see below) or else separate the components without disturbing the equilibrium. The latter approach is exemplified in a paper by Dalman *et al.*,⁽²⁴⁾ in which vapor-phase chromatography was used to separate the components. Unfortunately, most mercaptans and disulfides of biological interest cannot be analyzed in the vapor phase. Most studies of the latter

compounds have accordingly been done by liquid-phase chromatography (see Smith et al., (96) for references); this method of analysis, however, is in general more laborious and of limited precision.

The alternative approach, that of using a specific analytical method, is illustrated by a study of the reaction of cysteine with 4,4'-dithiobis(benzenesulfonic acid) by Smith et al., (96); in this study the extent of reaction was determined from the ultraviolet absorption of the corresponding mercaptan. The ionization equilibrium of the mercaptan had to be considered in addition to reactions (III-1) and (III-2), and discussion of this case is remanded to Chapter IV. In the present paper, two problems will be examined as they pertain to reactions (III-1) and (III-2) only: (a) the concentrations that will obtain at equilibrium, given certain initial concentrations and values of \underline{K}_1 and \underline{K}_2 ; and (b) the calculation of \underline{K}_1 and \underline{K}_2 given certain initial concentrations and the concentrations obtaining at equilibrium.

Composition of Equilibrium Mixtures in Mercaptan-Disulfide

Interchange Reactions

If one takes some specified amounts of mercaptan and disulfide and allows them to react until equilibrium is established, a unique composition will result, which depends on the amounts taken and the values of \underline{K}_1 and \underline{K}_2 . The equilibrium composition can accordingly be calculated in a straightforward way although the ensuing algebraic expressions are complex. Let \underline{M} and \underline{D} represent the concentrations of mercaptan and disulfide taken, respectively. In an interchange reaction, the total amounts of mercaptan and of disulfide do not change and one can accordingly write the expressions:

$$\underline{M} = (\text{XSH}) + (\text{YSH}) \quad (\text{III-5})$$

$$\underline{D} = (\text{XSSX}) + (\text{XSSY}) + (\text{YSSY}) \quad (\text{III-6})$$

One also has the relation:

$$\underline{Y} = 2(\text{YSSY}) + (\text{XSSY}) + (\text{YSH}) \quad (\text{III-7})$$

and a corresponding expression can be written for the \underline{X} groups which, however, is not independent. If only one disulfide, YSSY, is taken initially, $\underline{Y} = 2\underline{D}$, but this is not a necessary restriction; one might start with a mixture of disulfides.

Since there are five unknown quantities, (XSH), (YSH), (XSSX), (XSSY), and (YSSY), and five independent equations, a unique solution can be obtained. To find the value of (YSH), one can combine equations III-3 to III-7 to obtain the following cubic equation:

$$\alpha(\text{YSH})^3 + \beta(\text{YSH})^2 + \gamma(\text{YSH}) + \delta = 0 \quad (\text{III-8})$$

in which

$$\alpha = 1 + \frac{K_1 K_2}{K_1 - K_2} - \frac{K_1}{K_1} \quad (\text{III-9})$$

$$\beta = -\frac{K_1 K_2}{K_1 - K_2} (\underline{Y} + 2\underline{M}) + \frac{K_1}{K_1} (\underline{M} - \underline{D} + \underline{Y}) + 2\underline{D} - \underline{Y} \quad (\text{III-10})$$

$$\gamma = \frac{MK_1}{K_1} [\frac{K_2}{K_2} (2\underline{Y} + \underline{M}) + (\underline{D} - \underline{Y})] \quad (\text{III-11})$$

$$\delta = -\frac{K_1 K_2 M^2 \underline{Y}}{K_1 - K_2} \quad (\text{III-12})$$

Equation III-8 of course has three roots but only one of them will be physically significant ($0 < (\text{YSH}) < \underline{M}$ or \underline{Y} , whichever is smaller).

After obtaining the value of (YSH), that of (XSH) can be obtained from equation III-5. (YSH) can be substituted into equation III-7 and this combined with equation III-1 and (XSH) to give the following expression for (YSSY):

$$(\text{YSSY}) = (\text{YSH}) \frac{[\underline{Y} - (\text{YSH})]}{[2(\text{YSH}) + \frac{K_1}{K_1} (\text{XSH})]} \quad (\text{III-13})$$

With this value, (XSSY) and (XSSX) can finally be obtained from equations III-7 and III-6, respectively. It must be noted that combining equations III-3 and III-4 with III-5, III-6, and III-7 equates activities with concentrations; this of course is an approximation which is implied both in the results derived above and in those that follow.

A program has been developed for solving the foregoing system of equations with an IBM 1410 computer (see Appendix) and several hundred solutions have been worked out for selected values of \underline{K}_1 and \underline{K}_2 at various initial mercaptan-disulfide ratios. Table III-1 reports a few of the results, chosen to illustrate certain relationships. All the reported solutions refer to equilibria that would be reached by mixing XSH and YSSY; under these conditions it will be seen that $\underline{Y} = 2\underline{D}$.

First, let us consider the case (I) in which $\underline{K}_1 = 2$ and $\underline{K}_2 = 0.5$. These are the values of the constants which would obtain if the interchange were determined solely by probability considerations. For this case, and for $\underline{R} = 1$, the composition of the equilibrium mixture may be easily deduced on the basis that (XSH) and (YSH) must be equal and (XSSY) twice as large as either (XSSX) or (YSSY). Equation III-8, which in this special case reduces to a linear equation since $\alpha = 0$ and $\beta = 0$, gives the expected result. At other values of \underline{R} the interesting result will be noted that ϕ has the same values at $\underline{R} = \underline{m}$ and $\underline{R} = 1/\underline{m}$, i.e., as \underline{R} is increased or decreased by an equal factor relative to $\underline{R} = 1$, the equivalence ratio. The last two results calculated for case I indicate how large an excess of XSH must be taken in order to reduce YSSY to a small fraction, 2% and 0.1%, respectively, of its original value. As can be seen, the excess required to attain the last result is fairly large.

TABLE III-1
EQUILIBRIUM RELATIONSHIPS IN SOME MERCAPTAN-
DISULFIDE INTERCHANGE REACTIONS

Initial Ratio $R = \frac{M}{2D}$	Relative Equilibrium Concentrations					ϕ Per Cent Reaction ^a
	YSH	YSH	XSSX	XSSY	YSSY	
<u>Case I:</u> $K_1 = 2, K_2 = 0.5$						
1/4	0.1000	0.4000	0.0400	0.3200	0.6400	80.00
1/2	0.3333	0.6666	0.1111	0.4444	0.4444	66.66
1/1	1.0000	1.0000	0.2500	0.5000	0.2500	50.00
2/1	2.6666	1.3333	0.4444	0.4444	0.1111	66.66
4/1	6.4000	1.6000	0.6400	0.3200	0.0400	80.00
6.065/1	10.4130	1.7169	0.7369	0.2430	0.0200	85.85
30/1	58.0645	1.9344	0.9365	0.0624	0.0010	96.77
<u>Case II:</u> $K_1 = 4, K_2 = 1$						
1/4	0.0615	0.4384	0.0480	0.3423	0.6096	87.69
1/2	0.2360	0.7639	0.1458	0.4721	0.3819	76.39
1/1	0.8284	1.1715	0.3431	0.4852	0.1715	58.58
2/1	2.4721	1.5278	0.5835	0.3606	0.0557	76.39
4/1	6.2462	1.7537	0.7689	0.2158	0.0151	87.69
<u>Case III:</u> $K_1 = 0.40, K_2 = 0.10$						
1/4	0.2228	0.2771	0.0191	0.2387	0.7420	55.42
1/2	0.5746	0.4253	0.0452	0.3349	0.6198	42.54
1/1	1.3819	0.6180	0.0954	0.4270	0.4774	30.90
2/1	3.1492	0.8507	0.1809	0.4888	0.3301	42.54
4/1	6.8915	1.1084	0.3071	0.4941	0.1986	55.42
<u>Case IV:</u> $K_1 = 1, K_2 = 1$						
1/4	0.1231	0.3768	0.0744	0.2279	0.6976	75.37
1/2	0.3611	0.6388	0.1695	0.2998	0.5306	63.89
1/1	1.0000	1.0000	0.3333	0.3333	0.3333	50.00
2/1	2.6072	1.3927	0.5495	0.2935	0.1568	69.64
4/1	6.3076	1.6923	0.7461	0.2001	0.0537	84.62
<u>Case V:</u> $K_1 = 8, K_2 = 0.125$						
1/4	0.0456	0.4543	0.0055	0.4432	0.5512	90.87
1/2	0.2435	0.7564	0.0281	0.7000	0.2717	75.64
1/1	1.0000	1.0000	0.1000	0.8000	0.1000	50.00
2/1	2.8181	1.1818	0.2207	0.7407	0.0388	59.09
4/1	6.6421	1.3578	0.3735	0.6108	0.0156	67.89

^a ϕ equals 100 (YSH) divided by $\frac{M}{2}$ or Y , whichever is smaller.

In Case II, both constants have been taken larger than the statistical values, $\underline{K}_1 = 4$ and $\underline{K}_2 = 1$. The values of ϕ are naturally greater, i.e., the reaction is shifted in favor of the products, but the change is not large, the twofold increase in each constant giving only an increase from 50% to 58% at $\underline{R} = 1$. In case III, both constants are smaller than the statistical values and the values of ϕ are smaller. It will be further noted that in both cases the values of ϕ are the same for $\underline{R} = \underline{m}$ and for $\underline{R} = 1/\underline{m}$, as in case I. These and other results which are not reported in detail have led to the generalization that the values of ϕ will be equal at $\underline{R} = \underline{m}$ and $\underline{R} = 1/\underline{m}$ whenever the ratio $\underline{K}_1/\underline{K}_2 = 4$. The ratio $\underline{K}_1/\underline{K}_2$ is the constant for the disulfide interchange reaction:



$$\underline{K}_3 = \underline{K}_1/\underline{K}_2 = (\text{XSSY})^2/(\text{XSSX})(\text{YSSY}) \quad (\text{III-15})$$

In the next two sets of results, case IV with $\underline{K}_1 = 8$ and $\underline{K}_2 = 0.125$ (1/8) and case V with $\underline{K}_1 = 1$ and $\underline{K}_2 = 1$, it can be seen that $\phi = 50\%$ at $\underline{R} = 1$, the same as in case I. This leads to a second generalization, which also has been tested in many other cases not reported here, that $\phi = 50\%$ at $\underline{R} = 1$ whenever $\underline{K}_1/\underline{K}_2 = 1$. Cases IV and V do not have the symmetry exhibited by cases I-III at $\underline{R} = \underline{m}$ and $\underline{R} = 1/\underline{m}$, in accordance with the generalization that this kind of symmetry is associated with $\underline{K}_1/\underline{K}_2$ being equal to 4.

Calculation of the Equilibrium Constants from the Composition of Equilibrium Mixtures

For a simple equilibrium reaction, it is usually possible to calculate the equilibrium constant from the initial concentrations of

reactants and the concentration of any one component at equilibrium, inasmuch as the stoichiometric conservation equations give the concentration of all other components. This is not the case for mercaptan-disulfide interchange equilibria. If one mercaptan is determined, for instance, but none of the disulfides, it is not possible to deduce the distribution of mixed and symmetrical disulfides and the equilibrium constants accordingly cannot be determined. The same conclusion is reached by a more formal analysis of equations III-5 to III-7. If only one mercaptan is determined, say (YSH), four unknowns remain, but there are only three equations, which cannot therefore be solved. Since K_1 and K_2 are not known, equations III-3 and III-4 are not usable in this context. On the other hand, if one mercaptan and one disulfide are determined, or two disulfides, three unknowns remain and the equations can be solved.

This conclusion is exemplified by some of the results in Table III-1. It has been seen that the same amounts of (XSH) and (YSH) are obtained at $R = 1$ in cases I, IV, and V; clearly, if this result had been obtained experimentally, the separate constants could not be deduced from this datum alone. It can also be seen that these cases could be distinguished if one also knew one of the disulfide concentrations.

Another possible solution to the problem is to do two experiments at different initial concentrations. It can be seen from Table III-1 that a unique result is obtained for each case, say $R = 1$ and $R = 2$. This is a general result, as can be shown by the following analysis. If two experiments are done at different initial concentrations, M' and M'' , D' and D'' , and Y' and Y'' , six independent conservation equations can be written. Furthermore, the equilibrium expressions can be written,

even though K_1 and K_2 are unknown, and the two expressions for the same constant can be set equal to one another. One thus obtains the equations:

$$(YSH)'(XSSY)'/(XSH)'(YSSY)' = (YSH)''(XSSY)''/(XSH)''(YSSY)'' \quad (III-16)$$

$$(YSH)'(XSSX)'/(XSH)'(XSSY)' = (YSH)''(XSSX)''/(XSH)''(XSSY)'' \quad (III-17)$$

If the concentrations of one mercaptan in each equilibrium mixture, say $(YSH)'$ and $(YSH)''$, are determined by analysis, eight unknowns remain. Since there are eight independent equations, the system can be solved. The solution to the foregoing set of equations is the following:

$$K_2 = (b''c' - b'c'')/(a'c'' - a''c') \quad (III-18)$$

$$K_1 = -c'/(a'K_2 + b') \quad (III-19)$$

in which

$$\begin{aligned} a' = & [(YSH)']^3 - (Y' + 2M')[(YSH)']^2 + M'(2Y' + M')(YSH)' \\ & - Y'[M']^2 \end{aligned} \quad (III-20)$$

$$\begin{aligned} b' = & - [(YSH)']^3 + (Y' + M' - D')[(YSH)']^2 \\ & + M'(D' - Y')(YSH)' \end{aligned} \quad (III-21)$$

$$c' = [(YSH)']^3 + (2D' - Y')[(YSH)']^2; \quad (III-22)$$

a'' , b'' , and c'' are given by corresponding equations involving $(YSH)''$, M'' , D'' , and Y'' . A program has been written for the IBM 1410 computer which calculates the values of K_1 and K_2 given the initial concentrations and the values of ϕ' and ϕ'' (see Appendix). This program has been applied to the output of the program described in the previous section, exemplified by the data in Table III-1. In some two hundred cases examined, the values of K_1 and K_2 originally assumed were exactly regenerated. It is believed that this constitutes an adequate test of the mathematical validity of the procedure and of the computer programs.

Unfortunately, application of the method in practice involved

additional, and quite grave difficulties. These arise from the fact that in solving the system of equations one subtracts terms that may be of nearly equal magnitude. If these terms are quantities with finite experimental uncertainties, much larger uncertainties will be generated in the derived results. Consequently, the constants calculated from equations III-18 and III-19 will always be less accurate than the analytical data from which they are derived and in many cases the possible errors may be so large as to render the results completely unreliable.

In order to illustrate the nature of the difficulty, it may be well to consider a few examples, which are summarized in Table III-2. Let us suppose, for instance, that two experiments, done with the initial ratios $\underline{R}' = 0.5$ and $\underline{R}'' = 2.0$, had given values of $\underline{\phi}' = \underline{\phi}'' = 66.66\%$; application of equations III-18 and III-19 would lead to values of $\underline{K}_1 = 2.0$ and $\underline{K}_2 = 0.5$ (cf. Table III-1, Case I). Now let us suppose that the value of $\underline{\phi}'$ were changed by +1% and the value of $\underline{\phi}''$ by -1%, or vice versa; it can be seen that the values of the derived constants, listed in Table III-2, Case Ia, would differ by some 15%. In Case Ib and Case II one sees that the variations could be larger, depending on the values of the constants and of \underline{R} ; 1% change in $\underline{\phi}$ can in these cases change the constants by a factor of two.

Systematic analysis of the problem has led to the conclusion that the greatest accuracy can be achieved by choosing \underline{R}' and \underline{R}'' so the values of $\underline{\phi}$ will be about 75%. This is illustrated in Table III-2, Case Ic; with the values of \underline{R} chosen to give this extent of reaction, it can be seen that 1% change in $\underline{\phi}$ causes about 10% change in the constants. This cannot be improved, except by reducing the variance in $\underline{\phi}$.

Translated into practice, the foregoing discussion indicates that the extent of a mercaptan-disulfide interchange reaction should first be studied at $R = 1$; the result gives the relative magnitude of $(\frac{K_1}{K_2})$. Then, R should be increased and decreased until the respective values of ϕ increase midway to completion. The precision of the derived constants can, of course, also be improved by doing additional measurements; once approximate values of the constants have been obtained, the computational methods described above can be used to determine optimal initial conditions.

In Chapter IV, the relations described here will be utilized to obtain semi-quantitative estimates of the constants for the interchange of several mercaptans with 4,4'-dithiobis(benzenesulfonic acid).

Conclusions

It emerges from the foregoing discussion that the precise determination of the equilibrium constants in mercaptan-disulfide interchange reactions is a difficult problem from the experimental point of view. Doubtless this is in part responsible for the fact that the results reported by various investigators are not in good agreement (96).

The best prospect for obtaining precise values of the equilibrium constants seems to lie in the development of techniques that will separate all the components present at equilibrium and determine each precisely.

If fewer components are determined, precision will be diminished, although determination of the constants remains possible as long as at least two disulfides, or one disulfide and one mercaptan, are determined, and the initial concentrations are known. This is because the

TABLE III-2
PRECISION OF CALCULATED EQUILIBRIUM CONSTANTS

Initial Ratios		Extent of Reaction (%)		Derived Constants	
\underline{R}_1	\underline{R}_2	ϕ'	ϕ''	\underline{K}_1	\underline{K}_2
<u>Case Ia</u>					
0.500	2.000	66.667	66.667	2.000	0.500
		67.333	66.000	2.280	0.438
		66.000	67.333	1.739	0.575
<u>Case Ib</u>					
0.250	0.500	80.000	66.667	2.000	0.500
		80.800	66.000	2.435	0.270
		79.200	67.333	1.536	0.896
<u>Case Ic</u>					
0.300	3.600	76.923	78.261	2.000	0.500
		77.692	77.478	2.216	0.451
		76.153	79.043	1.798	0.556
<u>Case II</u>					
0.400	1.300	85.741	80.497	2.000	8.000
		86.598	79.692	3.203	5.390
		84.883	81.301	0.983	15.118

concentrations of all components can then be calculated from the stoichiometric conservation equations. In order to use these equations properly, it is of course necessary to insure that they will in fact hold, that is, that there will be no loss of reagents by way of side reactions such as oxidation of the mercaptans by oxygen.

If only one component can be measured, determination of the constants is in principle still possible, by combining the results of two experiments. The necessary equations have been developed. However, the results depend very critically on the precision of the analytical data. Care and caution should be therefore applied in estimating the precision of the derived constants.

Acknowledgment

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CHAPTER IV

(As stated in the Introduction, this chapter is written
in a form suitable for publication)

REACTION OF 4,4'-DITHIOBIS(BENZENESULFONIC ACID) WITH SOME 2-AMINOETHANETHIOLS

Summary

4,4'-Dithiobis(benzenesulfonate) (BSSB) has been reacted with several mercaptans (XSH). The amount of BSH formed at equilibrium was determined for initial (XSH)/(BSSB) molar ratios of 2 and 4 at 25^o, pH 6.0 and r=0.1. The treatment of mercaptan-disulfide interchange equilibria given in Chapter III has been extended to include the ionization of BSH and applied to the interpretation of the results. Approximate equilibrium constants have been estimated for the interchange reactions. Measurements of the rate of reaction also are presented.

The injury inflicted by ionizing radiation to living organisms can be moderated by the timely administration of certain chemical substances. Among these, 2-aminoethanethiol (caSH), 2-mercaptoethylguanidine (geSH) and some related mercaptans and disulfides offer some promise of potential utility as palliatives in case of unavoidable exposure to radiation (3,33,94,104). Consequently, the aforementioned compounds have been,

and are at present, the subject of considerable study. When caSH, geSH or the derivative disulfides are administered to rats, it has been demonstrated that a large proportion of the protective compound becomes bound to tissue proteins, and it is generally believed that the bonds linking protector and proteins are disulfide bonds (33,94). These bonds could be formed by oxidative coupling of the protective mercaptan with protein mercapto groups or by disulfide-mercaptan interchange. Although the mechanism of protection is still a matter of conjecture, there can be little doubt that, directly or indirectly, mercaptan-disulfide interchange has an important role in the protection afforded by caSH and its congeners (33,47).

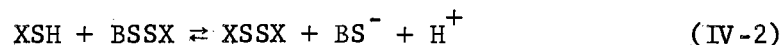
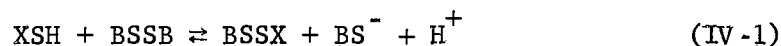
Knowledge concerning mercaptan-disulfide interchange reactions is at present rather limited. Equilibrium constants which had been reported before the submission of Papers II and III of this series (24,96) are summarized in those papers and in Chapter II, and no additions have been noted since that time. The present paper presents several new results, obtained in a study of the interchange reaction between 4,4'-dithiobis-(benzenesulfonate)(BSSB) and 2-aminoethanethiol as well as eight of its derivatives. The thiophenolate ion produced in the reaction, BS^- , has a characteristic spectrum; this makes possible the determination of the extent of reaction by means of spectrophotometric measurements without separating the reaction mixture. Unfortunately, determination of the equilibrium constants for the interchange reactions from measurements of only one product at equilibrium is subject to difficulties, which have been discussed in the preceding Chapter. In the present case, the stoichiometric relationships at equilibrium are even more complicated, owing to the partial ionization of BSH.

In the first part of this paper, the stoichiometric relationships

existing among the products at equilibrium will be considered; secondly, the experimental results will be given for the equilibria in the reaction of BSSB with the aminoethanethiols; thirdly, some measurements of the rates of reaction will be presented; and finally, the results will be discussed.

Stoichiometric Relationships at Equilibrium in Mercaptan-Disulfide Interchange Reactions Involving One Ionizing Mercaptan

The ionizing mercaptan will be denoted by BSH and the corresponding disulfide by BSSB. The following equations can then be written (cf. Chapter III and (96)):



$$\underline{K}_1^* = (\text{BSSX})(\text{BS}^-)(\text{H}^+) / (\text{BSSB})(\text{XSH}) \quad (\text{IV-3})$$

$$\underline{K}_2^* = (\text{XSSX})(\text{BS}^-)(\text{H}^+) / (\text{BSSX})(\text{XSH}) \quad (\text{IV-4})$$

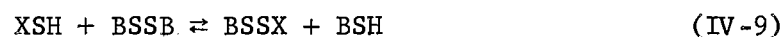
$$\underline{K}_i = (\text{H}^+)(\text{BS}^-) / (\text{BSH}) \quad (\text{IV-5})$$

$$\underline{M} = (\text{XSH}) + (\text{BSH}) + (\text{BS}^-) \quad (\text{IV-6})$$

$$\underline{D} = (\text{XSSX}) + (\text{BSSX}) + (\text{BSSB}) \quad (\text{IV-7})$$

$$\underline{B} = 2(\text{BSSB}) + (\text{BSSX}) + (\text{BSH}) + (\text{BS}^-) \quad (\text{IV-8})$$

\underline{K}_1^* , defined in equation IV-3, is related to the constant that can be written for the equation involving no ionization in the following way:



$$\underline{K}_1 = (\text{BSSX})(\text{BSH}) / (\text{BSSB})(\text{XSH}) = \underline{K}_1^* / \underline{K}_i; \quad (\text{IV-10})$$

a corresponding expression relates \underline{K}_2^* and \underline{K}_2 .

Equations IV-3 to IV-8 given above involve one more unknown, (BS^-) , than the corresponding set of equations in Chapter III, but there is also one additional independent equation, namely IV-5, the expression

for \underline{K}_i . Given the values of this constant, \underline{K}_1 , \underline{K}_2 , \underline{M} , \underline{D} , and \underline{B} , it is therefore possible to obtain a general solution to the equations, analogous to the one given in Chapter III for the non-ionizing case. The solution to the equation is, of course, dependent on pH, since the term (H^+) appears in equation IV-5. If, however, the interchange reaction is conducted in a buffer solution, the pH will be held constant as well as the ratio $(\text{BS}^-)/(\text{BSH})$. It is convenient to define the quantity \underline{n} :

$$\underline{n} = 1 + [\underline{K}_i/(\text{H}^+)]$$

which, when combined with equation IV-5, gives the expression:

$$(\text{BS}^-) + (\text{BSH}) = [(\text{BSH})\underline{K}_i/(\text{H}^+)] + (\text{BSH}) = \underline{n}(\text{BSH}) \quad (\text{IV-11})$$

Substituting equation IV-11 into IV-6 and IV-8 eliminates (BS^-) . The equations and their solution are now analogous to those in Paper V. The solution considered in that paper is for the non-ionized case, where $\underline{n} = 1$. The general solution for the equations (at a particular pH) is:

$$\alpha(\text{BSH})^3 + \beta(\text{BSH})^2 + \gamma(\text{BSH}) + \delta = 0 \quad (\text{IV-12})$$

in which:

$$\alpha = \underline{n}(1 + \underline{K}_1\underline{K}_2\underline{n}^2 - \underline{K}_1\underline{n}) \quad (\text{IV-13})$$

$$\beta = \underline{K}_1\underline{n} [\underline{M} + \underline{B} - \underline{D} - \underline{K}_2\underline{n}(2\underline{M} + \underline{B})] + 2\underline{D} - \underline{B} \quad (\text{IV-14})$$

$$\gamma = \underline{K}_1\underline{M}[\underline{D} - \underline{B} + \underline{K}_2\underline{n}(\underline{M} + 2\underline{B})] \quad (\text{IV-15})$$

$$\delta = -\underline{K}_1\underline{K}_2\underline{B}\underline{M}^2 \quad (\text{IV-16})$$

A slight modification of the computer program employed to calculate the results for Chapter III permits the introduction of the value of \underline{n} and calculation of the composition of equilibrium mixtures. (see Appendix).

Table IV-1 gives the results of a few illustrative calculations

TABLE IV-1
EQUILIBRIUM RELATIONSHIPS IN SOME MERCAPTAN-DISULFIDE
INTERCHANGE REACTIONS

\underline{R}^a	\underline{n}^b	Relative Equilibrium Concentrations						ϕ^c
		(XSH)	(BSH)	(BS)	(BSSB)	(BSSX)	(XSSX)	
<u>Case I:</u> $\underline{K}_1 = 2, \underline{K}_2 = 0.5$								
1/4	2	0.0615	0.2192	0.2192	0.6096	0.3423	0.0480	87.69
1/2	2	0.2360	0.3819	0.3819	0.3819	0.4721	0.1458	76.39
1/1	2	0.8284	0.5858	0.5858	0.1715	0.4852	0.3431	58.58
2/1	2	2.4721	0.7639	0.7639	0.0557	0.3606	0.5845	76.39
4/1	2	6.2462	0.8769	0.8769	0.0151	0.2159	0.7689	87.69
1/1	5	0.6180	0.2763	1.1056	0.0954	0.4270	0.4774	69.10
1/1	10	0.4805	0.1519	1.3675	0.0577	0.3650	0.5772	75.97
1/1	100	0.1818	0.0181	1.8000	0.0082	0.1652	0.8264	90.91
<u>Case II:</u> $\underline{K}_1 = 1, \underline{K}_2 = 1$								
1/4	2	0.0773	0.2113	0.2113	0.6666	0.2440	0.0893	84.53
1/2	2	0.2530	0.3734	0.3734	0.4679	0.3171	0.2148	74.69
1/1	2	0.8046	0.5976	0.5976	0.2404	0.3237	0.4358	59.77
2/1	2	2.3890	0.8054	0.8054	0.0783	0.2323	0.6892	80.55
4/1	2	6.1649	0.9175	0.9175	0.0189	0.1271	0.8539	91.75

$\underline{R}^a = \underline{M}/2\underline{D}$; see text.

$\underline{n}^b = (\underline{K}_1/[H^+] + 1)$; see text.

ϕ^c is defined as $n(\text{BSH})$ divided by \underline{M} or \underline{B} , whichever is smaller.

done for selected values of the equilibrium constants, of the initial concentrations, which are specified by the ratio $\underline{R} = \underline{M}/2\underline{D}$, and of \underline{n} . This table is analogous to Table III-1 in Chapter III, and comparison of the two tables therefore shows the effect of the ionization. Qualitatively, it is obvious that as \underline{n} increases the reaction will be driven more and more toward completion, and the magnitude of the effect can be seen by comparing the corresponding values of ϕ ; e.g., when the constants \underline{K}_1 and \underline{K}_2 have the statistical values (Case I) and $\underline{R} = 1$, ϕ increases from 50% for $\underline{n} = 1$ to 58.6% for $\underline{n} = 2$ (50% ionization) to 90.9% for $\underline{n} = 100$ (99% ionization). It should be noted also that (BSSB) and (BSSX) decrease as \underline{n} is increased; as the ionization of BSH approaches 100%, ϕ does also, XSSX approaches 1 and BSSB and BSSX approach 0. In other words, the interchange reaction can be driven toward completion by employing a mercaptan BSH which is much more acidic than RSH and by operating at a sufficiently high pH so BSH will be completely ionized.

It is possible to obtain the values of \underline{K}_1 and \underline{K}_2 from the values of $(\text{BSH} + \text{BS}^-)$ obtained in two equilibrium experiments involving different initial concentrations of XSH and BSSB, in the same manner as described in Chapter III. The expressions are:

$$\underline{K}_2 = (\underline{c}'\underline{b}'' - \underline{c}''\underline{b}') / (\underline{a}'\underline{c}'' - \underline{a}''\underline{c}') \quad (\text{IV-17})$$

$$\underline{K}_1 = -\underline{c}' / (\underline{a}'\underline{K}_2 + \underline{b}') \quad (\text{IV-18})$$

where

$$\underline{a}' = (\underline{n}')^3 (\text{BSH}')^3 - (\underline{B}' + 2\underline{M}') (\underline{n}')^2 (\text{BSH}')^2 + (2\underline{B}' + \underline{M}') \underline{M}' \underline{n}' (\text{BSH}') - \underline{B}' (\underline{M}')^2$$

$$\underline{b}' = -(\underline{n}')^2 (\text{BSH}')^3 + (\underline{B}' + \underline{M}' - \underline{D}') \underline{n}' (\text{BSH}')^2 + (\underline{D}' - \underline{B}') \underline{M}' (\text{BSH}')^2$$

$$\underline{c}' = \underline{n}' (\text{BSH}')^3 + (2\underline{D}' - \underline{B}') (\text{BSH}')^2$$

The terms \underline{a}'' , \underline{b}'' , and \underline{c}'' are given by corresponding expressions

involving (BSH)"', \underline{n} '', \underline{M} '', \underline{D} '', and \underline{B} ''. A program was devised for calculating the values of \underline{K}_1 and \underline{K}_2 with an IBM 1410 computer; (see Appendix); application of equations IV-17 and IV-18 to some actual cases will be considered in the next section.

Equilibria in the Interchange Reactions of BSSB

with caSH and Some Congeners

The design of the experiments was essentially as follows. An aliquot portion of a solution of BSSB in buffer was mixed with a considerable excess of cysteine (equivalent ratio $\underline{R} = \underline{M}/2\underline{D} = 10$), while two other aliquots were mixed with cysteine at $\underline{R} = 2$ and $\underline{R} = 1$, respectively. The absorbances at 284 μ after attainment of the equilibrium were determined in each case. Corrections were made for the appreciable initial absorbance of BSSB and the very slight absorbance of XSH. Provisional values of the extent of reaction, $\underline{\phi}$, were calculated on the basis that the absorbance obtained in the first instance corresponded to complete reaction, i.e., $\underline{\phi}_{10} = 100\%$; \underline{K}_1 and \underline{K}_2 were then computed. With these values of the constants, it could be calculated that the reaction at $\underline{R} = 10$ had in fact been only 98.8% complete, and second-approximation values of $\underline{\phi}$ and \underline{K} were computed on this basis. A third cycle of approximation yielded the result $\underline{\phi}_{10} = 98.8\%$, i.e. only 0.3% different from the result of the second approximation. The second approximation values were taken as the final ones. Measurements with other mercaptans were done in the same way, the extents of reaction being calculated in each case by proportion to the value obtained with cysteine at $\underline{R} = 10$ (see Experimental Section for sample calculation).

Each set of three experiments was made with the same buffer, thus avoiding the effects of possible small differences between one buffer and the next (since the buffers had to be made air-free, it was not considered desirable to do all the measurements with a single buffer, which would have to be stored for a long time). The three reaction mixtures differed in the concentrations of aminothiols hydrochloride present, and although these concentrations were small the effect on the pH and on the absorbancy was appreciable. It was difficult to determine the differences accurately, because they were barely greater than the precision of the pH meter. However, systematic and indeterminate errors were minimized by making a large number of comparative measurements; in this way the following average values were found for the pH of the solutions: reaction mixture of $\underline{R} = 1$, 5.97; mixture of $\underline{R} = 2$, 5.95; mixture of $\underline{R} = 10$, 5.92. It was furthermore ascertained that the change in absorbance of (BSH + BS^-) was 6.0% per 0.1 pH unit (the value calculated from the absorbancy index and \underline{K}_1 (96) is 7.4%). This coefficient was used to adjust the absorbances to the values they would have at pH 6.00, and these adjusted values were used for subsequent calculations.

Two other experimental details require comment before the results themselves are considered: (a) the possible effect of air oxidation; and (b) the presence of impurities in the mercaptan samples. The first problem is one that needs always to be considered in work with mercaptans and is particularly important in this context, because if mercaptan were converted to disulfide by air in the course of the reaction, neither \underline{M} nor \underline{D} would remain constant, as required for the correct solution of the equations. It is believed that no appreciable

amount of air-oxidation occurred in the experiments because the absorbance values, due to BS^- , remained constant for a period many times longer than that required for the value to be first reached. It may be well to recall at this point that the value of the pH was chosen so the reaction would be fast, that the solutions themselves were made air-free, and that the buffer contained $10^{-3} M$ ethylenedinitrilotetraacetate (EDTA). The latter constituent was essential, since in its absence no constant value of the absorbance would be obtained; instead this would rise to a maximum and then decrease slowly (see (96), Fig. 1).

The susceptibility of mercaptans to oxidation creates a problem also with respect to the purity of the samples; it is very difficult to prepare and to keep samples of mercaptans free of disulfide. The hydrochloride salts of the aminothiols are much less susceptible to oxidation, but on the other hand they are hygroscopic, so that absorbed water becomes a problem. The samples used in this work were very carefully stored and handled, to minimize their exposure to air and moisture; nevertheless, they could not reasonably be considered pure, and it was difficult, if not impossible, to weigh small quantities of them with satisfactory accuracy. To circumvent these difficulties, a direct determination of mercaptan content was made upon an aliquot portion of the solution which was used in the interchange measurements. All the solutions were prepared shortly before use and only a short time was allowed to elapse between the assay and the interchange-reaction measurements; in this period of time, it was ascertained by direct measurement that the titer did not change appreciably. The analytical method used was the reaction with N-ethylmaleimide; this method has been critically tested with both cysteine (1) and glutathione (56), and

it has been assumed that all the other mercaptans would be determined with the same accuracy, about 1%. The precision of the assay generally was better than this (for details, see Experimental Section).

Table III-2 reports the data that were obtained with 2-aminoethanethiol, cysteine, glutathione, and six other aminothiols. The table also gives the purity of the compounds as determined by the N-ethylmaleimide procedure, expressed as a percentage of the nominal titer calculated from the weight taken (the difference between the values quoted and 100% was mostly water, as could be demonstrated by loss of weight on drying; however, the samples used for the measurements were not dried, since they could not be weighed accurately in any case; it must be expected that some disulfide was present, likely less than 3%). The significance of these data will be considered in the Discussion Section.

Rates of Mercaptan-Disulfide Interchange

The rate of the mercaptan-disulfide interchange reaction could easily be determined by measuring the absorbance at 285 m μ in the period before equilibrium was reached.

The interpretation of such rate data is, however, a more difficult problem, since four reactions are involved. The problem is not insoluble, but the actual determination of reasonably accurate constants would require a large number of data and great amount of computation. In the present work, no attempt has been made to attain this goal.

A much simpler problem is to estimate the constant for the forward reaction in equation IV-1, k_1 . Since in general the values of K_1 are near the statistical value and those of K_2 generally smaller, it may be surmised that the other rate constants would be about the same as, or

smaller than, k_1 . In this case, the rate of reaction to about 20% of the equilibrium value essentially measures k_1 . The eighth and ninth columns in Table IV-2 give the average rates of reaction determined from zero time to the aforementioned point, 20% of the equilibrium absorbance value. An approximate specific reaction-rate constant was calculated, on the assumption that the kinetics would be of second order, by dividing the rates by the concentrations of the reactants at the midpoint of the reaction period. The values given in the last column of the Table are the averages of the results obtained at $R = 1$ and $R = 2$, and the deviation indicates the degree of agreement between the two results.

Discussion

The values for the extents of reaction ϕ given in Table IV-2 show good precision, mostly less than $\pm 1\%$ and slightly more than $\pm 1\%$ in a few cases. In view of the experimental difficulties which have been mentioned, this precision must be considered satisfactory. It is difficult to estimate the absolute accuracy of the values, which, however, must be less than the precision, perhaps $\pm 2\%$; the relative accuracy is perhaps a little better.

Unfortunately, this degree of accuracy is not sufficient to give satisfactory derived values of the constants K_1 and K_2 . The difficulty, which has already been alluded to in Chapter III, comes from the fact that the algebraic operations necessary to solve the equations involve some subtractions of terms of nearly equal magnitude. In these cases, moderate uncertainties in the original data can give rise to very large uncertainties in the results. The nature of the difficulty can be

TABLE IV-2

INTERCHANGE OF SEVERAL MERCAPTANS WITH BSSB

Mercaptan	NEM Assay (%)	No.	% Reaction at Equilibrium		Equilibrium Constants		Average Initial Rate (10^8 x mole/ liter-sec)		Rate Constant (liter/ mole-sec)
			$\underline{R} = 1$	$\underline{R} = 2$	\underline{K}_1	\underline{K}_2	$\underline{R} = 1$	$\underline{R} = 2$	
I Cysteine	97.2	14	64.8 \pm 0.8	86.2 \pm 0.5	0.77	1.27	5.37	10.2	48 \pm 2
II Glutathione	99.3	3	62.4 \pm 0.8	79.4 \pm 0.9	2.58	0.41	4.69	9.36	42 \pm 1
III 2-Aminoethanethiol	98.6	3	68.1 \pm 0.3	86.8 \pm 0.5	2.21	0.96	12.5	26.5	115 \pm 2
IV N-tris(hydroxymethyl) methyl-2-aminoethanethiol	90.3	4	70.1 \pm 0.1	88.7 \pm 0.4	2.40	1.20	46.2	----	400 --
V 1-Methyl- 2-aminoethanethiol	94.7	6	55.4 \pm 0.5	71.1 \pm 1.5	1.62	0.20	7.61	19.4	77 \pm 8
VI 2-Methyl-1-(propyl)- 2-aminoethanethiol	98.5	6	51.5 \pm 0.5	66.7 \pm 0.8	1.12	0.14	8.50	24.5	90 \pm 20
VII 1,1-Dimethyl- 2-aminoethanethiol	97.5	6	51.1 \pm 0.3	54.1 \pm 0.2	36.0	0.01	8.42	11.1	60 \pm 15
VIII 1,1,2-Trimethyl- 2-aminoethanethiol	94.8	6	49.7 \pm 0.3	53.3 \pm 0.5	9.0	0.01	4.55	10.4	44 \pm 2
IX 3-Aminopropanethiol	94.6	4	68.9 \pm 0.5	86.9 \pm 0.9	2.97	0.92	4.55	8.21	39 \pm 3

illustrated for the case of cysteine, by considering what effect a change of 1% in the values of ϕ has on the values of the constants. Let it accordingly be assumed that the values of ϕ_1 and ϕ_2 would be 65.45% and 85.34%, respectively, instead of the values listed in the table; the corresponding values of K_1 would be 1.32 and K_2 0.93. If, alternatively, it be assumed that ϕ_1 were 64.15% and ϕ_2 87.06%, K_1 would be 0.41, K_2 1.98. In other words, 1% change in the value of ϕ can change the constants by a factor of 2. Clearly, then, no quantitative significance can be attached to the constants listed in the table. Their values have been given to illustrate the fact that they can be calculated, in principle, from the values of ϕ_1 and ϕ_2 , but only if the latter values are extremely precise; in the present instance, only the order of magnitude can be considered significant.

A measure of confidence that the constants for cysteine are approximately correct may be derived from the fact that they agree approximately with the values obtained in Paper III (96) by a different method of calculation. It seems reasonably well established, therefore, that the constants for cysteine are both approximately 1, i.e., K_1 is smaller, and K_2 larger, than the "statistical" values.

The other three primary mercaptans, III, IV, and IX, have values of K_2 near the statistical value, K_1 is somewhat larger. For glutathione, which has a more complex molecule and a primary mercapto group, both constants are near the statistical values. For the secondary mercaptans, V and VI, both constants appear to be smaller, but again the difference is not large. On the other hand, the two tertiary mercaptans VII and VIII stand apart in that ϕ_2 is much smaller than for the other mercaptans; K_2 is correspondingly small, K_1 apparently large. The

uncertainty in the constants for the last two compounds is large, but there can be little doubt that these compounds behave differently from the rest. These results agree with the generalization formulated earlier with respect to the interchange of simple mercaptans and disulfides (24), namely, that the equilibrium constants of primary and secondary mercaptans have values fairly close to those expected from probability considerations and are not much affected by differences in structure. Tertiary mercaptans show distinctly lower values of K_2 , which likely is due to the steric crowding of groups that would occur in the derived disulfides (51).

The reaction rates do not follow the same pattern. As can be seen, the N-trimethylolmethyl derivative of 2-aminoethanethiol (IV) reacts much faster than the rest, 2-aminoethanethiol is second fastest, and all the other rates vary by less than a factor of two. The dependence of the rate on the pH indicates that the mechanism of the reaction proceeds by way of the mercaptide ion (78,96). Attempts to interpret the rates should accordingly take into account at least two effects, the acidity, which determines how much mercaptide ion will be present at a specified pH, and the nucleophilic reactivity of this anion (78). In the case of 2-aminoethanethiol, cysteine, and glutathione, the rates are in the same order as the ionization constants, but not proportional to them. For the other mercaptans, ionization constants have not yet been determined and an interpretation of the results cannot therefore be made at the present time.

It should be added that the extent of ionization is small in any case, e.g., 0.5% for 2-aminoethanethiol, so that it could be neglected for the purpose of the equilibrium calculations. This would not be the

case at substantially higher pH, say 7.4. To calculate true interchange reaction constants at this pH, one must include in the stoichiometric equations all the ionized species present, and the ionization constants of both mercaptans would have to be known.

Data on the radioprotective ability of most of the compounds studied have been reported. 2-Aminoethanethiol has long been recognized as a protective substance (3). Recently, van Bekkum and Nieuwekerk (104) have reported that the maximal protection afforded by 1-methyl-2-aminoethanethiol is about as great as that by CaSH and that the therapeutic range of the former is much greater. Shapira, Doherty, and Burnett (93) found that 3-aminopropanethiol protected mice about two and one-half times as effectively as 2-aminoethanethiol on a molar basis. These authors obtained moderate protection with cysteine, insignificant protection with glutathione. 1,1-Dimethyl-2-aminoethanethiol and 2-methyl-1-propyl-2-aminoethanethiol have been reported to be inactive (17,18).

At the outset of this work, hope was entertained that the reaction of mercapto compounds with BSSB might serve as a convenient diagnostic test of their potential as radioprotectors. This anticipation has not been satisfactorily realized, although there is a slight degree of correlation between the radioprotective ability and the rate and equilibrium constants. All the compounds tested do take part in mercaptan-disulfide interchange and the reactions are quite fast in all cases. The results by no means controvert the possibility that interchange reactions have a definite role in the radioprotection by these substances, but it does appear that the chemical reactivity of these compounds toward the interchange reaction is not of determining importance.

The data presented in this paper are admittedly approximate. More accurate results might have been obtained by doing more experiments, by employing more favorable values of R or a lower pH (which would reduce n and ξ) and by refining the experimental procedure. It seems doubtful, however, that the increased accuracy attainable in this way would justify the effort required. If more accurate values of the constants are desired, the considerations expounded in this paper and in the preceding one (Chapter III) indicate that it would be more rewarding to study a reaction in which all the components present at equilibrium could be separated and separately determined.

Experimental

Materials.--Disodium 4,4'-dithiobis(benzenesulfonate) was prepared in this laboratory (96). N-ethylmaleimide (NEM) and cysteine hydrochloride monohydrate were obtained from California Corporation for Biochemical Research, Los Angeles 63. Glutathione was obtained from Schwarz Bioresearch, Mount Vernon, N. Y. The other mercaptans were supplied by Dr. D. Jacobus and T. E. Sweeney, Department of Medical Chemistry, Walter Reed Army Institute of Research, to whom we are indebted. All other chemicals were of A.C.S. analytical-reagent grade.

The medium used for the reactions was 0.02 M phosphate buffer containing 1.5093 g. $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.9873 g. of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 11.695 g. of NaCl and 1.000 g. of EDTA per liter of solution. The water used in preparing this buffer was steam-condensate which had been passed through a mixed-bed anion-cation exchange resin; it was boiled and cooled under nitrogen to remove most of the air, and stored under nitrogen until used.

Apparatus.--Absorbance measurements were made in 1-cm. silica cells with a Beckman Model DU spectrophotometer. Measurements of pH were made with a Beckman Model G pH meter.

Assay of Mercaptans with N-Ethylmaleimide.--Mercaptan solutions were prepared by dissolving 1.4×10^{-4} moles of XSH in 100 ml. of the deaerated buffer (molarity = \underline{M}) and analyzed immediately after dissolution. The NEM solution was prepared by dissolving 0.025 g. in 100 ml. of buffer and filtering twice to remove any undissolved particles. Ten ml. of NEM solution were mixed with 10 ml. of the buffer, and the absorbance at 300 μ , \underline{A}_0 , was recorded. This procedure was repeated using NEM and 10 ml. of XSH solution and the absorbance \underline{A} was recorded at 300 μ until a constant value was attained. The mercapto-group titer was calculated using the value of 620 for the molar absorbance of NEM: $(\underline{A}_0 - \underline{A})/3.10\underline{M} = \% \text{ of theoretical SH titer.}$

Calculation of Φ from Absorbance.--The absorbance, \underline{A} , of an equilibrium mercaptan-disulfide mixture may be due to BSSB, BSSX, XSSX, BSH, BS^- , or XSH. It has been found that the absorbances of cysteine, cystine and the other mercaptans used in these experiments are negligible at 285 μ ; it has been assumed that the absorbance due to XSSX would also be negligible. It has been further assumed that the molar absorbance of BSSX is half that of BSSB (96), i.e., the absorbance of the (BS) moiety does not change as BSSB is converted to BSSX, and one can represent the combined absorbances of the symmetrical and mixed disulfides by the term $\underline{A}_{\text{BS}}$:

$$\underline{A}_i = \underline{A}_{(\text{BS}^- + \text{BSH})} + \underline{A}_{\text{BS}}; \quad (\text{IV-19})$$

\underline{i} is the index corresponding to the various values of \underline{R} ; 1, 2, or 10.

The absorbance corresponding to 100% reaction, A_{∞} , is calculated by successive approximations, as explained earlier. Then one can write:

$$\frac{A}{i} = \frac{A}{(BS^- + BSH)} + (1 - \frac{A}{(BS^- + BSH)}) / \frac{A_{\infty}}{A_{BS}^0} \quad (IV-21)$$

Calculation of Rates and Rate Constants.---The average absorbance values from a series of experiments were plotted vs. time and a smooth curve was drawn through the points. The absorbance corresponding to 20% of the equilibrium value is given by the expression:

$$\frac{A}{i, 0.2} = \frac{A_{BS}^0}{A_{BS}^0} + 0.002 \frac{A_{\infty}}{i} (A_{\infty} - \frac{A_{BS}^0}{A_{BS}^0}) \quad (IV-22)$$

and the time required to attain this value was obtained from the graph.

The average rate is given by:

$$\overline{(\text{Rate})}_i = \Delta(BSH)_i / \Delta t_i \quad (IV-23)$$

and the average rate constant by:

$$\overline{k}_i = \overline{(\text{Rate})}_i / (\overline{BSSB})_i (\overline{XSH})_i \quad (IV-24)$$

where \overline{BSSB} and \overline{XSH} are the average concentrations in the time interval Δt_i .

The calculations outlined in the previous sections can best be elucidated by means of a sample calculation, which is presented in the Appendix.

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CHAPTER V

EQUILIBRIUM CONSTANTS FOR THE REACTION OF CYSTINE WITH GLUTATHIONE AND THEIR RELATIVE OXIDATION-REDUCTION POTENTIALS

Summary

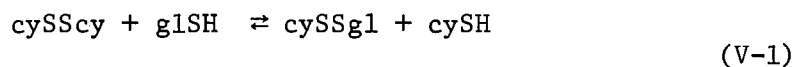
The reaction of cystine with glutathione has been studied because a rather large discrepancy existed in the values which had been reported in the literature for the equilibrium constants of this reaction. For the system containing cysteine (cySH), cystine (cySScy), glutathione (glSH), oxidized glutathione (glSSgl), and the mixed disulfide (cySSgl), the following equilibrium constants have been obtained at 25° and 37°C by means of the automatic amino acid analyzer: $\underline{K}_1 = \frac{(\text{cySSgl})}{(\text{cySH})(\text{cySScy})(\text{glSH})} = 3.74 \pm 0.09$, and $\underline{K}_2 = \frac{(\text{glSSgl})(\text{cySH})}{(\text{cySSgl})(\text{glSH})} = 0.79 \pm 0.01$. The method utilized permits the separation and determination of each component in the equilibrium mixture. From these data, the relative oxidation-reduction potential of the glSSgl-glSH couple with respect to the cySScy-cySH couple is -0.017 (E'_0 at pH 6.6, 25°C).

Introduction

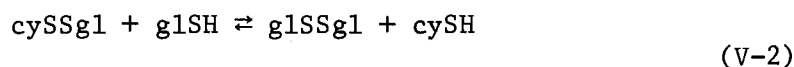
Mercaptan-disulfide interchange reactions have received considerable attention in recent years. Cysteine and other mercaptans have frequently been used to reduce protein disulfide bonds (69). The biological implications of mixed disulfide formation have been discussed

(69,77). Cysteine and similar substances protect living organisms to some extent from the harmful effects of ionizing radiation and Eldjarn and Pihl (33) have put forward the hypothesis that mixed disulfide formation is important in this connection. The reaction of cystine with glutathione is of particular interest because the former is present in many types of protein and the latter is a constituent of blood.

The reaction of cystine (cySScy) with glutathione (glSH) produces the mixed disulfide (cySSgl) and cysteine (cySH); subsequent reaction of the mixed disulfide with more glutathione produces the second symmetrical disulfide, oxidized glutathione (glSSgl). Equations V-1 and V-2 show these reactions and give the expressions for the equilibrium constants.



$$\underline{K}_1 = (\text{cySSgl})(\text{cySH})/(\text{cySScy})(\text{glSH})$$



$$\underline{K}_2 = (\text{glSSgl})(\text{cySH})/(\text{cySSgl})(\text{glSH})$$

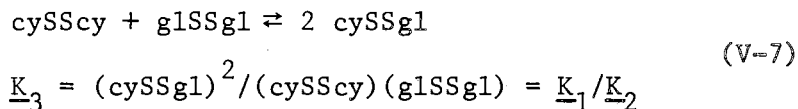
$$\underline{M} = (\text{cySH}) + (\text{glSH}) \quad (\text{V-3})$$

$$\underline{D} = (\text{cySScy}) + (\text{cySSgl}) + (\text{glSSgl}) \quad (\text{V-4})$$

$$\underline{C} = (\text{cySH}) + 2(\text{cySScy}) + (\text{cySSgl}) \quad (\text{V-5})$$

$$\underline{G} = (\text{glSH}) + 2(\text{glSSgl}) + (\text{cySSgl}) \quad (\text{V-6})$$

Here \underline{M} is the total concentration of mercaptan present, \underline{D} is the total disulfide, and \underline{C} and \underline{G} are the total cysteinyl and glutathiyyl moieties, respectively. One may also consider the disulfide equilibrium described by the following equations:



This reaction, between two disulfides, is known to be very slow, except in the presence of a mercaptan or other initiator (59,88). Equation V-7, however, can serve as a check on the values of \underline{K}_1 and \underline{K}_2 as determined with equations V-1 and V-2.

In their pioneering work with thioglycolic acid and cystine, Bersin and Steud el (8) assumed that the mixed disulfide was intrinsically much less stable than the symmetrical disulfides, and they neglected the equilibrium concentration of mixed disulfide in calculating the interchange constants. Subsequent investigation of this system by Kolthoff, Stricks, and Kapoor (59) revealed that this assumption is entirely incorrect, and that both thioglycolic acid and glutathione form substantial amounts of mixed disulfide with cystine. Pihl and Eldjarn (77) also investigated this reaction and reported quite different values for the interchange equilibrium constants (vide infra). Because of this disparity, it was deemed advisable to determine the constants by yet another method.

Experimental

Materials and Apparatus.--Oxidized glutathione and cysteine hydrochloride monohydrate were products of the California Corporation for Biochemical Research, Los Angeles 63. The former gave 84.3% of the nominal titer by alkalimetric titration of the carboxyl groups (56). The latter was assayed by the N-ethylmaleimide method (56) and found to have a titer of 97.2%. Glutathione was obtained from Schwartz Bio-research, Inc., Mount Vernon, New York; the titer was found to be 98.3%

using N-ethylmaleimide. Cystine was a product of the Fisher Scientific Company, New York, and was 96.6% pure when compared with the amino acid analyzer with a standard amino acid sample furnished by Beckman Instruments, Inc., Palo Alto, California. In each case, the appropriate correction, based on the titers, was applied to the weight of the substances taken. Water used in the experiments was passed through a mixed-bed ion exchange column and deaerated by boiling for at least 30 minutes and cooling and storing under nitrogen atmosphere. Other chemicals were of ACS-reagent grade. A phosphate buffer stock solution was prepared by dissolving 0.02 moles each of di- and monosodium phosphate, 0.18 moles of sodium chloride, and 2.0 g of disodium ethylenedinitrilotetraacetate (EDTA) in one liter of solution. Citrate buffer stock solution, which was used as the medium for application of the sample to the amino acid analyzer, was prepared according to the directions furnished with the analyzer (110), except that the concentration of each component was doubled to allow for dilution (vide infra). The amino acid analyzer has been described (110). The ion exchange resin used was PA-28, obtained from Beckman Instruments, Inc.; the chromatographic column was 0.9 cm in diameter, and the resin height was 58 cm. Measurements of pH were made with a Beckman Expandomatic pH meter.

Procedure.--Since the dissolution of cystine is quite slow in neutral solutions, the experiments in which cystine was a reactant were begun by dissolving the cystine in 1 ml of 1.0 M hydrochloric acid; this was made up by volume by adding 25 ml of buffer stock solution, 1 ml of 1.0 M sodium hydroxide, the other reactants and water to 50 ml. When cystine was not to be present initially, the acid and base were mixed before adding the phosphate buffer and reactants. The pH of the

resulting mixture was 6.6; higher pH values for a few reactions were obtained by adding sodium hydroxide. The reaction solutions were incubated at 25°C or 37°C until constant composition was attained. At the end of the reaction period, 5 ml of the solution was removed and the pH of this aliquot was lowered to about 2.8 by mixing with 5 ml of the pH 2.2 citrate buffer stock solution. This step arrests the reaction by removing the active mercaptide ions and prepares the sample for application to the ion exchange column. One milliliter of this solution was forced into the top of the column with nitrogen pressure and the sample was eluted with pH 3.25 citrate buffer at a column temperature of 45°C and with a flow rate of 50 ml/hr. Under these conditions, a separate peak was recorded for each of the five components of the equilibrium system.

Calculations were made by means of the formula $\mu = (\underline{H} \cdot \underline{W}) / \underline{C}$, where μ is the number of micromoles of the component in the sample, \underline{H} is the net height of the recorded peak, \underline{W} is the width of the peak (in recorder dots) at half the net height, and \underline{C} is the color constant for the particular constituent (110). The color constants for the two mercaptans and the symmetrical disulfides were determined by dissolving the pure substances singly or in pairs and subjecting them to chromatography immediately. The color constant for the mixed disulfide was found in two ways. A solution containing approximately $5 \times 10^{-4} \text{ M}$ cysteine and glutathione and $2 \times 10^{-5} \text{ M}$ iron(II) sulfate was adjusted to pH 8 with sodium hydroxide. Oxygen was bubbled through this solution until no mercaptan was left in the solution, and the resulting mixture of symmetrical and mixed disulfides was subjected to column chromatography. The concentrations of the symmetrical disulfides were found by means of

the previously determined color constants, and that of the mixed disulfide was calculated by subtraction from the total amount of disulfide possible. The color constants could then be calculated from the dimensions of the chromatographic peak. The second method was similar, but the mixed disulfide was formed by mercaptan-disulfide interchange over a period short enough that only a negligible amount of mercaptan oxidation could take place.

Results.--The color constants which were determined are given in Table V-1. As Wainer reported recently (107) the color yield for the reaction of cysteine with ninhydrin is quite low and the absorbance at 440 μ is greater than that at 570 μ . Table V-2 gives the values of the equilibrium constants which were calculated by inserting the concentrations found into equations V-1, V-2, and V-7. The first two lines of the table refer to reaction in which the equilibrium mercaptan concentrations were too low to permit accurate calculation of K_1 and K_2 . Control experiments showed that the reaction was indeed stopped in the citrate buffer medium and that prolonged exposure to this medium did not displace the reaction from equilibrium. In addition to the runs reported in Table V-2, the equilibrium was studied under slightly different conditions. After attaining the equilibrium state indicated in the last line of Table V-2, this reaction was re-equilibrated at 37°C; identical values for the interchange constants were found. On adjusting the pH of this mixture to 7.4 and once again allowing the reaction to come to equilibrium at 37°C, the equilibrium constants were found to be $K_1 = 3.46$ and $K_2 = 0.761$.

TABLE V-1
AMINO ACID ANALYZER COLOR CONSTANTS

Compound ^a	Color constants ^{b,c}
cySScy	97.3 ± 0.8
cySSgl	135.5 ± 3.3
glSSgl	168.0 ± 1.6
cySH	15.31 ± 0.18
glSH	67.1 ± 0.6

^aSee text for abbreviations and conditions.

^bdots/micromole ± average deviation (see text).

^c570 mμ except cySH at 440 mμ.

TABLE V-2
EQUILIBRIUM CONSTANTS FOR THE REACTION OF CYSTINE WITH GLUTATHIONE
pH 6.6, 25°C

Initial Concentrations (μmole/ml)				\underline{K}_1	\underline{K}_2	\underline{K}_3
cySScy	glSH	glSSgl	cySH			
0.432	-----	0.592	0.112	----	-----	4.70
0.812	-----	0.826	0.216	----	-----	4.69
0.806	1.188	-----	-----	3.74	0.798	4.69
1.237	1.692	----	-----	3.73	0.794	4.75
0.420	1.340	----	-----	3.53	0.758	4.65
-----	-----	0.618	2.572	3.81	0.797	4.79
0.784	0.668	0.450	1.678	3.88	0.816	4.75
		Average		3.74	0.791	4.72
		Av. Dev.		0.09	0.017	0.04

The rates of the interchange reactions in the conditions used are indicated by an experiment in which the concentrations of cySScy and glSSgl were each approximately 0.8 μ moles/ml and that of cySH was about 10 mole per cent of the total disulfide concentration. The formation of mixed disulfide was clearly evident after a reaction period of 10 min. Chromatography of the mixture after incubation for 6 hr indicated that the concentrations had attained about 90% of the equilibrium values; for reaction periods exceeding 16 hours, constant values were obtained for the equilibrium constants. For a similar reaction in which the mercaptan concentration was one mole per cent of the total disulfide concentration, the reaction attained about 10% of the equilibrium extent of reaction in 6 hours, and was not at equilibrium after 6 days; likely, the conversion of the mercaptan to disulfide by oxidation slowed the reactions.

Throughout the equilibrium determinations, equations V-5 and V-6 were used to check on the constancy of the quantities \underline{C} and \underline{G} ; they were found to average $97 \pm 1.3\%$ and $101 \pm 1.7\%$ of the theoretical values, respectively. During the course of the reactions, \underline{M} was found to decrease slowly and \underline{D} was found to increase slowly. These observations indicate that although there was a slow oxidation of mercaptan to disulfide, other side reactions could be neglected. Since the actual concentrations were substituted into the equilibrium expressions the oxidation did not affect the results.

Discussion

Calculations have recently been performed which show that large errors may arise in the determination of mercaptan-disulfide interchange

constants when some concentrations are determined from the conservation equations by subtraction (Chapters III and IV). Direct analytical determination of the components at equilibrium gives better precision. The procedure used in these experiments allows the determination of four of the five equilibrium species.

Two groups of investigators have determined interchange constants for the reaction of cystine with glutathione, as mentioned above. Kolthoff, Stricks, and Kapoor (59) utilized the low solubility of cystine in solutions of near-neutral pH. They determined the increase in total disulfide dissolved when glSSgl or cySH was added and from this they derived the following equilibrium constants at 25° , as defined in Equations V-1, V-2 and V-7: $\underline{K}_1 = 2.8$, $\underline{K}_2 = 1.0$, and $\underline{K}_3 = 3.0$. A second determination of these constants was made by Eldjarn and Pihl (77). Their method consisted of using one reactant labelled with ^{35}S . After the reaction was stopped by the addition of acid, the equilibrium mixture was subjected to electrophoresis on paper impregnated with mercuric acetate. The equilibria were established under physiological conditions, 37°C and pH 7.4. From their data, these authors calculated $\underline{K}_1 = 12.4$ and $\underline{K}_2 = 0.17$; substitution of these values into equation V-7 yields the results $\underline{K}_3 = 72.9$. The results of the present study (Table V-2) agree fairly well with those of the former investigators and not with the latter. Pihl and Eldjarn (77) rationalized the high value of \underline{K}_3 by postulating that the mixed disulfide is stabilized by two internal hydrogen bonds. However, the argument lacks quantitative force, since it is not known to what extent such structures, if they exist, would effect the pertinent constants.

The interchange reactions can be looked upon as oxidation-reductions, and the potentials of these reactions have long been of interest, particularly to biochemists (23). Eldjarn and Pihl (30) have given the equation which relates the equilibrium constants with these potentials:

$$\frac{E'}{E'_0} \text{glSSgl/glSH} - \frac{E'}{E'_0} \text{cySScy/cySH} = -0.0307 \log (\underline{K}_1 \underline{K}_2) \quad (\text{V-8})$$

The data of Kolthoff *et al.* (59), when substituted into equation V-8 give a relative potential of -0.0137 v; those of Eldjarn and Pihl (77) give -0.0099 v. The latter authors (30) also determined equilibrium constants for the reactions of several mercaptans with both cystine and oxidized glutathione and combined the results to calculate the relative potential of the cystine-glutathione reaction; their results give an average of -0.0101 v. The results of the present work yield the relative potential of -0.0174 at 25°C and pH 6.6. Thus, it is evident that the values of the relative oxidation-reduction potentials do not differ greatly, even though the individual equilibrium constants \underline{K}_1 , \underline{K}_2 , and, particularly, \underline{K}_3 , vary considerably. It will be noted that multiplying the two equilibrium expressions, equations V-1 and V-2, eliminates the mixed disulfide from the resulting expression.

The equilibrium constant expressions, equations V-1 and V-2, have been written in terms of the un-ionized mercaptans. At a pH of 6.6, only about one per cent of the total cysteine or glutathione is ionized, so the mercaptide ions have been neglected in the equilibrium expressions. At pH 7.4, however, approximately 7% of the cysteine mercapto groups are ionized, so this effect can no longer be neglected. It will be noted that concentrations rather than activities have been used in

the equilibrium expressions; the non-ideal effects are minimized by the comparatively high ionic strength of the sodium chloride and the rather low concentrations of the reactants.

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APPENDIX

Chapters III-V were written in a form that might be suitable for publication. For brevity some details were omitted. This appendix reports these additional details.

Computer Program for Calculating Equilibrium Concentrations

This program is written in FORTRAN IV, a computer language which is compatible, after minor changes, with the computing equipment of many manufacturers. The calculations reported in this thesis were done with an IBM Model 1410 Computer.

The program operates on (utilizes) the values of the equilibrium constants K_1 and K_2 , as defined in equations III-3 and III-4, the initial concentrations, given in equations IV-6 to IV-8, and the quantity n , which specifies the extent of ionization of the mercapto group, equation IV-11. These quantities are represented in the program by the mnemonic variable names AK1, AK2, AM, D1, B1, and AN, respectively. From the values of these variables, the program first calculates the coefficients of the cubic equation given in equation IV-12. The program continues with the appropriate solution: cubic, quadratic, or linear, depending on the values of the coefficients. Once all of the solutions have been found, the one physically significant root is selected and the other concentrations are determined from the appropriate equations. The extent of reaction, R , is calculated and the results, together with the input data, are printed. The program continues until no more input

data are available.

Table A-1 is a reproduction of pertinent computer output sheets.

Bard and King (J. Chem. Ed. 42, 127 (1965)) have recently presented an alternative program for calculating concentrations in consecutive, competitive equilibria. The procedure can solve n independent equations for n unknowns. In this program, one begins with guessed values of two variables; $(n - 2)$ equations are solved simultaneously for the other variables. The latter results are tested in the two remaining equations; if these equations are not satisfied, the initial values are systematically varied, until all the equations are satisfied. When this is attained, it is the solution to the equations.

This iterative procedure takes much more computer time than the algebraic solution program given in Table A-1. However, it was used in two calculations to check the results given by the first program. The first calculation was for Table III-1, $R = 1$, $n = 1$; the second one for $n = 2.86$. In both instances, the respective programs gave identical answers.

Results Supplementing Table III-1

Table III-1 gives 27 solutions to the cubic equation III-8. In this section are given 45 additional solutions, which support the generalizations made in Chapter III. These additional results are given in Table A-2.

Cases I and II support the generalization that the values of ϕ are the same for $R = m$ and $R = 1/m$ when $K_1/K_2 = 4$. In the next three cases, $K_1K_2 = 1$; as observed earlier, ϕ then has the value 50% when $R = 1/1$, i.e.,

TABLE A-1

COMPUTER PROGRAM TO CALCULATE EQUILIBRIUM CONCENTRATIONS

```

C          FORTRAN IV   IBM 1410
00001     FORMAT(6F6.3)
00002     FORMAT(1HL,6F8.3, //7X,1HA,12X,1HB,12X,1HC,12X,1HD,10X,6HREAL
          *1,7X,6HIMAG 1,7X,6HREAL 2,7X,6HIMAG 2,7X,6HREAL 3,7X,6HIMAG
          *3, /10E13.5)
00003     FORMAT(1HL,6F8.3, F12.4, 1X, 6F10.4)
00004     FORMAT(1HL,18X,14HIDENTIFICATION,56X,26HEQUILIBRIUM
          *CONCENTRATIONS, //5X,2HK1,6X,2HK2,7X,1HM,7X,1HD,7X,1HB,7X,
          *1HN,10X,1HR,9X,5H(BSH).5X,6HN(BSH),4X,5H(RSH),5X,6H(BSSB),
          *4X,6H(BSSR),4X,6H(RSSR) //)
00008     WRITE(3,4)
          DO181 I=1,15
00009     READ(1,1) AK1,AK2,AM,D1,B1,AN
          P=1.+AK1*AK2*AN*AN-AK1*AN
          Q=AK1*AM-2.*AK1*AK2*AM*AN
          R=AK1*AK2*AM*AM
          S=2.-AK1*AN
          T=AK1*AM
          A=AN*P
          B=D1*S+AN*Q-B1*P
          C=D1*T+AN*R-B1*Q
          D=-B1*R
          R1=0.0
          R2=0.0
          R3=0.0
          R4=0.0
          R5=0.0
          R6=0.0
          IF(A.EQ.0.0)GOTO15
          P=(C/A)-(B*B/(3.0*A*A))
00012     Q=(2.0*B*B*B/(27.0*A*A*A))-(B*C/(3.0*A*A))+D/A
00101     RADL=(Q*Q/4.0)+(P*P*P/27.0)
00102     SRADL=(ABS(RADL))**0.5)
00103     IF(RADL.GE.0.0)GOTO116
00104     UI=SRADL
00105     UR=-Q/2.0
00106     IF(UR.NE.0.0)GOTO112
00107     IF(UI.GT.0.0)GOTO151
00150     ANGLE=4.712388979
          GOTO153
00151     ANGLE=1.570796326
          GOTO153
00112     ANGLE=0.0
00113     IF(UR.GT.0.0)GOTO115
00114     ANGLE=3.141592653
00115     ANGLE=ANGLE+ATAN*UI/UR)

```

TABLE A-1 (Continued)

```

00153  VR=(UR*UR+ABS(RADL))**(1.0/6.0)
        UR=COS(ANGLE/3.0)*VR
        UI=SIN(ANGLE/3.0)*VR
        VR=UR
        VI=-UI
        GOTO128
00015  IF(8.EQ.0.0)GOTO250
00200  DISC=(C*C-4.*B*D)
        IF(DISC.LT.0.0)GOTO201
        ROOT=SQRT(DISC)
        R1=(-C+ROOT)/(2.*B)
        R2=(-C-ROOT)/(2.*B)
        GOTO300
00201  R1=C/(2.*B)
        ABROOT=SQRT(ABS(DISC))
        R6=ABROOT
        R2=R1
        R4=-R6
        GOTO300
00250  R1=-D/C
        GOTO300
00116  UI=0.0
00117  VI=0.0
00118  UR=-(Q/2.0)+SRADL
00119  VR=-(Q/2.0)-SRADL
00120  IF(UR.GE.0.0)GOTO123
00121  UR=-((ABS(UR))**(1.0/3.0))
00122  GOTO124
00123  UR=UR**(1.0/3.0)
00124  IF(VR.GE.0.0)GOTO127
00125  VR=-((ABS(VR))**(1.0/3.0))
00126  GOTO128
00127  VR=VR**(1.0/3.0)
00128  R1=UR+VR-(B/(3.0*A))
        R2=-0.5*UR+0.8660254035*UI-0.5*VR-0.8660254035*VI-(B/(3.0*A))
00129  R3=-0.5*UR-0.8660254035*UI-0.5*VR+0.8660254035*VI-(B/(3.0*A))
00130  R4=-0.5*UI+0.8660254035*UR-0.5*VI-0.8660254035*VR
00131  R5=-R4
00300  T2=1.0E-4
        IF(AM.GE.B1)GOTO176
        T1=AM/AN
        GOTO177
00176  T1=B1/AN
00177  IF(R1.LE.0.)GOTO178
        IF(R1.GT.T1)GOTO178
        IF(ABS(R6).GT.T2)GOTO178
        X=R1
        GOTO301

```

TABLE A-1 (Continued)

```
00178  IF(R2.LE.0.)GOTO179
        IF(R2.GT.T1)GOTO179
        IF(ABS(R4).GT.T1)GOTO179
        X=R2
        GOTO301
00179  IF(R3.LE.0.)GOTO180
        IF(R3.GT.T1)GOTO180
        IF(R5.GT.T2)GOTO180
        X=R3
00301  ANBSH=AN*X
        BSSB=X*(B1-ANBSH)/(S*X+T)
        RSH=AM-ANBSH
        BSSR=B1-2.*BSSB-ANBSH
        RSSR=D1-BSSB-BSSR
        IF(AM.GT.B1)GOTO10
        R=(AN*X/AM)*100.
        GOTO11
00010  R=(AN*X/B1)*100.
00011  WRITE(3,3)AK1,AK2,AM,D1,B1,AN,R,X,ANBSH,RSH,BSSB,BSSR,RSSR
        GOTO181
00180  WRITE(3,2)AK1,AK2,AM,D1,B1,AN,A,B,C,D,R1,R6,R2,R4,R3,R5
00181  CONTINUE
        GOTO8
        END
```

TABLE A-2
EQUILIBRIUM RELATIONSHIPS IN SOME MERCAPTAN-
DISULFIDE INTERCHANGE REACTIONS

Initial Ratio R=M/Y	Relative Equilibrium Concentrations					ϕ Per Cent Reaction ^a
	XSH	YSH	XSSX	XSSY	YSSY	
Case I: $K_1 = 6, K_2 = 1.5$						
1/4	0.0447	0.4552	0.0518	0.3516	0.5965	91.05
1/2	0.1861	0.8138	0.1655	0.4826	0.3517	81.39
1/1	0.7320	1.2679	0.4019	0.4641	0.1339	63.40
2/1	2.3722	1.6277	0.6623	0.3029	0.0346	81.39
4/1	6.1789	1.8210	0.8290	0.1629	0.0080	91.05
Case II: $K_1 = 8, K_2 = 2$						
1/4	0.0351	0.4648	0.0540	0.3567	0.5891	92.96
1/2	0.1547	0.8452	0.1786	0.4880	0.3333	84.53
1/1	0.6666	1.3333	0.4444	0.4444	0.1111	66.67
2/1	2.3094	1.6905	0.7145	0.2615	0.0239	84.53
4/1	6.1407	1.8592	0.8642	0.1308	0.0049	92.96
Case III: $K_1 = 0.5, K_2 = 2$						
1/4	0.1383	0.3616	0.1093	0.1429	0.7476	72.34
1/2	0.3777	0.6222	0.2203	0.1815	0.5980	62.23
1/1	1.0000	1.0000	0.4000	0.2000	0.4000	50.00
2/1	2.5671	1.4328	0.6286	0.1754	0.1958	71.64
4/1	6.2447	1.7552	0.8199	0.1152	0.0647	87.76
Case IV: $K_1 = 5, K_2 = 0.2$						
1/4	0.0628	0.4371	0.0118	0.4133	0.5747	87.43
1/2	0.2776	0.7223	0.0481	0.6260	0.3257	72.24
1/1	1.0000	1.0000	0.1428	0.7142	0.1428	50.00
2/1	2.7663	1.2336	0.2916	0.6503	0.0580	61.68
4/1	6.5571	1.4428	0.4654	0.5120	0.0225	72.14
Case V: $K_1 = 10, K_2 = 0.1$						
1/4	0.0386	0.4613	0.0037	0.0438	0.5423	92.28
1/2	0.2270	0.7729	0.0214	0.7300	0.2485	77.29
1/1	1.0000	1.0000	0.0833	0.8333	0.0833	50.00
2/1	2.8410	1.1589	0.1906	0.7776	0.0317	57.95
4/1	6.6810	1.3189	0.3319	0.6551	0.0129	65.95

TABLE A-2 (Continued)

Initial Ratio R=M/Y	Relative Equilibrium Concentrations					ϕ
	XSH	YSH	XSSX	XSSY	YSSY	Per Cent Reaction ^a
Case VI: $K_1 = 2, K_2 = 1$						
1/4	0.0897	0.4102	0.0624	0.2853	0.6521	82.05
1/2	0.2955	0.7044	0.1607	0.3829	0.4563	70.44
1/1	0.9067	1.0932	0.3410	0.4111	0.2478	54.66
2/1	2.5265	1.4734	0.5703	0.3326	0.0969	73.67
4/1	6.2682	1.7317	0.7607	0.2101	0.0290	86.59
^b Case VII: $K_1 = 1, K_2 = 1$						
1/4	0.2714	0.2285	0.7842	0.6601	0.5556	45.70
1/2	0.5795	0.4204	0.8880	0.6443	0.4675	42.05
1/1	1.2777	0.7222	1.0612	0.5997	0.3390	36.11
2/1	2.8884	1.1115	1.3047	0.5020	0.1932	55.58
4/1	6.5174	1.4825	1.5634	0.3556	0.0809	74.13
^b Case VIII: $K_1 = 2, K_2 = 0.5$						
1/4	0.2777	0.2222	0.6172	0.9876	0.3950	44.44
1/2	0.6000	0.4000	0.7200	0.9600	0.3200	40.00
1/1	1.3333	0.6666	0.8888	0.8888	0.2222	33.33
2/1	3.0000	1.0000	1.1250	0.7500	0.1250	50.00
4/1	6.6666	1.3333	1.3888	0.5555	0.0555	66.67
^b Case IX: $K_1 = 2, K_2 = 0.5$						
1/4	0.4047	0.0952	3.2766	1.5419	0.1814	19.05
1/2	0.8181	0.1818	3.3471	1.4876	0.1652	18.18
1/1	1.6666	0.3333	3.4722	1.3888	0.1388	16.67
2/1	3.4285	0.5714	3.6734	1.2244	0.1020	28.57
4/1	7.1111	0.8888	3.9506	0.9876	0.0617	44.44

^aFor definition, see Table III-1. All calculations refer to $\underline{n} = 1$.

^bCases VIII-IX have initial concentrations of RSSR. See text.

initial mercaptan and disulfide are in stoichiometrically equivalent amounts. In Case VI, $\underline{K}_1/\underline{K}_2 \neq 4$ and $\underline{K}_1\underline{K}_2 \neq 1$; the solutions do not have the properties seen in Cases I-V, again supporting the above generalizations.

In all the above cases, as well as those reported in Chapters III and IV, the initial reagents are YSSY and XSH, i.e. $\underline{Y} = 2\underline{D}$. The last three cases in Table A-2 refer to equilibria attained after mixing these two reactants plus XSSX. Thus, in cases VII and VIII, \underline{D} and \underline{Y} both have a value of 2; equimolar amounts of XSSX and YSSY are taken initially. In the last case, $\underline{D} = 5$ and $\underline{Y} = 2$; i.e. XSSX and YSSY are taken initially in 4:1 molar ratio. As can be seen in the table, the expected happens; adding some of the product disulfide reduces the per cent reaction.

Program for Calculating Equilibrium Constants

This program was written to calculate the equilibrium constants \underline{K}_1 and \underline{K}_2 from the analytical results of two experiments (see Chapter IV). The constants were calculated by means of equations IV-17 and IV-18 by substituting the values of the quantities a' , b' , c' , a'' , b'' , and c'' found from the equations given in Chapter IV. The input data for this program are the initial concentration parameters \underline{M} , \underline{D} , and \underline{B} , the specification of ionization of BSH, \underline{n} , and the experimentally determined extent of reaction \underline{R} ; these quantities are specified for each of the two experiments and are all defined in Chapter IV. The last item in the input record is an identifier which may have 20 or fewer characters; this is printed with the input data and results at the conclusion of the calculation. The program continues until there is no

TABLE A-3

COMPUTER PROGRAM TO CALCULATE EQUILIBRIUM CONSTANTS

```

C      FORTRAN IV   IBM 1410
      DIMENSIONAM(2),D(2),B(2),AN(2),X(2),R(2),A1(2),B1(2),C1(2),
      *NAME(4)
00001  FORMAT(1H1,5X,1HM,7X,1HD,7X,1HB,7X,1HN,10X,1HR,25X,8HCOMPOUND,
      *27X,21HEQUILIBRIUM CONSTANTS,/)
00002  FORMAT(10F6.3,4A5)
00003  FORMAT(1HL,60X,4A5,/1X,4F8.3,F12.3,/100X,3HK1=,F8.4,3X,3HK2=,
      *F8.4,/1X,4F8.3,F12.3)
00010  WRITE(3,1)
      DO51=1,8
      READ(1,2)AM(1),D(1),B(1),AN(1),R(1),AM(2),D(2),B(2),AN(2),
      *R(2),NAME
      DO4J=1,2
      IF(AM(J).CT.B(J))GOTO15
      X(J)=(AM(J)*R(J))/(100.*AN(J))
      GOTO16
00015  X(J)=(B(J)*R(J))/(100.*AN(J))
00016  A1(J)=AN(J)**2*X(J)**2*(AN(J)*X(J)-B(J)-2.*AM(J))+AN(J)*AM(J)
      **X(J)*(2.*B(J)+AM(J))-B(J)*AM(J)**2
      B1(J)=AN(J)*X(J)**2*(B(J)+AM(J)-D(J)-AN(J)*X(J))+AM(J) X(J)*
      *(D(J)-B(J))
00004  C1(J)=X(J)**2*(AN(J)*X(J)+2.*D(J)-B(J))
      AK2=(C1(1)*B1(2)-C1(2)*B1(1))/(C1(2)*A1(1)-C1(1)*A1(2))
      AK1=(-C1(1))/(A1(1)*AK2+B1(1))
00005  WRITE(3,3)NAME,AM(1),D(1),B(1),AN(1),R(1),AK1,AK2,AM(2),D(2),
      *B(2),AN(2),R(2)
      GOTO10
      END

```

more input data.

Table A-3 is a facsimile reproduction of the pertinent computer-output sheets.

Chromatography of Cystine, Cysteine, and Oxidized
and Reduced Glutathione

In a representative experiment, a solution containing glSH, 1.080 $\mu\text{mole/ml}$; glSSgl, 0.742 $\mu\text{mole/ml}$; and cySScy, 1.108 $\mu\text{mole/ml}$ was prepared and immediately chromatographed as described in Chapter V. The parameters of the recorded chromatograms and the calculated values of the color constants are given in Table A-4 (note that the concentrations are reduced by a factor of two by dilution).

TABLE A-4
CHROMATOGRAPHY OF INTERCHANGE COMPONENTS

Compound	Elution Time ^a	H ^b	W ^b	C ^b (=HxW/u)
glSH	67 min	1.20	30.5	67.8
glSSgl	93	0.786	79.0	167.4
cySScy	193	0.900	60.4	98.12
cySH	117	0.470	36.3	15.40
cySSgl	134	0.900	71.6	130.5

^aTime from start to maximum of recorder trace; elution rate 50 ml/hr.

^bSee Chapter V for definitions. Table V-1 lists the average color constants determined from several experiments.

In a separate experiment, a solution of cysteine, 2.2658 $\mu\text{mole/ml}$ was prepared and immediately subjected to column chromatography as before. The chromatogram exhibited the characteristic peak for cySH with the absorbance at 440 $\text{m}\mu$ greater than that at 570 $\text{m}\mu$; the parameters of this peak are also given in Table A-4. In addition, a small cystine peak was found which corresponded to 0.0246 $\mu\text{moles/ml}$ of cystine. If the cySScy were formed by oxidation of cySH, the actual cysteine concentration would have been $2.2658 - 2(0.0252) = 2.2154$ $\mu\text{moles/ml}$; this concentration was used in calculating the color constant given in the table.

Oxidation of Mercaptan Mixtures: Determination
of the Mixed-Disulfide Color Constant

Cysteine, 1.7100 $\mu\text{moles/ml}$ and glSH, 1.918 $\mu\text{moles/ml}$ were dissolved in 50 ml of water and 3 drops of 5 N sodium hydroxide were added. Pure oxygen was slowly bubbled through the solution for a period of eight hours. The resulting solution was diluted with citrate buffer and subjected to chromatography as usual. No trace of either mercaptan was visible in the chromatogram, but three other peaks were recorded, two of which corresponded to those usually found for cySScy and glSSgl. From the previously determined color constants, it was determined that the concentrations of these substances in the citrate buffer solution were 0.1739 and 0.2393 $\mu\text{moles/ml}$, respectively. The third peak was identical with that found for the mixed disulfide in the interchange experiments (see Chapter V); the parameters recorded for this compound are listed in Table A-4. The total disulfide concentration in the solution was 0.907 $\mu\text{moles/ml}$, so that the mixed disulfide concentration

must have been $0.907 - 0.1739 - 0.2393 = 0.4938$ $\mu\text{moles/ml}$. The calculated color constant is listed in the table.

Interchange of Cystine with Glutathione

In a representative experiment, cystine, 1.237 $\mu\text{moles/ml}$, and glutathione, 1.692 $\mu\text{moles/ml}$ were dissolved and incubated for 32 hours as described in Chapter V. Column chromatography of the solution, after dilution with citrate buffer, gave the following results in $\mu\text{moles/ml}$ of the solution analyzed: cySScy, 0.190 ; glSH, 0.258 ; glSSgl, 0.131 ; cySH, 0.518 ; and cySSgl, 0.324 . Substituting the equilibrium concentrations into the equilibrium constant expressions V-1, V-2, and V-7 gives the results $K_1 = 3.61$, $K_2 = 0.769$, and $K_3 = 4.70$. After three days in the thermostat, the results given in Table V-2, line 4, were obtained.

Detailed Description of an Interchange Reaction of BSSB with Cysteine

This section supplements Chapter IV; it presents a detailed description and calculation for a representative experiment.

A solution of cysteine was prepared by diluting 0.02154 g to 100 ml with 0.02 \underline{M} phosphate buffer. Ten ml of this solution, mixed with an equal volume of N-ethylmaleimide solution (approx. 2×10^{-3} \underline{M}) gave an absorbance of 0.193 at 300 $\mu\mu$. When the mercaptan solution was replaced by 10 ml of phosphate buffer in a similar mixture, the absorbance was 0.587 . The concentration of the mercaptan solution was thus $(0.587 - 0.193)/310 = 1.27 \times 10^{-3}$ \underline{M} . Fifty ml of the solution were diluted with 40.78 ml of buffer to give a 7.0×10^{-4} \underline{M} solution of

cysteine; this stock solution was used in the interchange experiment described below.

The BSSB stock solution was prepared by dissolving 0.00802 g/100 ml of phosphate buffer to give a solution of 1.75×10^{-4} M. The stock solutions and phosphate buffer were used in the proportions given in Table A-5 to give the desired initial concentration ratios for the interchange experiments. In the same table are listed the pH values and equilibrium absorbances for the reactions.

In each case the BSSB concentration was initially 2.5×10^{-5} M; the absorbance of a buffer solution containing BSSB at this concentration and no cysteine was found to be 0.135. The absorbance of a 10^{-4} M solution of cysteine, corresponding to the initial concentration in a 2/1 reaction mixture, was found to be 0.002; this quantity was neglected in the calculations.

TABLE A-5
INTERCHANGE REACTION SOLUTIONS

Initial Ratio (=M/2D)	Stock Solutions		Phosphate Buffer	pH	Absorbance Attained ^a
	XSH	BSSB			
1/1	2 ml	4 ml	22 ml	5.97	0.499
2/1	5	5	25	5.95	0.616
10/1	25	5	5	5.92	0.696

^aCorrected to the value at pH 6.0 (see Chapter IV); the added corrections were: 1/1, 0.009; 2/1, 0.018; 10/1, 0.031.

One other correction, compensation for incomplete reaction at the 10/1 ratio, should be mentioned. The iterative procedure given in Chapter IV indicated that the extent of reaction for this case would be

98.5%; the absorbance of the 10/1 reaction mixture was therefore increased to the value which would obtain from 100% reaction, $A_F = 0.706$.

The calculations of ϕ , the per cent reaction, were made using equation IV-21:

$$\phi_1 = 100(0.499 - 0.135)/(0.706 - 0.135) = 63.7\%$$

$$\phi_2 = 100(0.616 - 0.135)/(0.706 - 0.135) = 84.2\%$$

The average kinetic parameters for the reactions were calculated from equations IV-22 to IV-24. The absorbance of the reaction mixtures at 20 per cent of the equilibrium value is given by the expressions:

$$A_{1,0.2} = 0.135 + 0.002 \times 63.7(0.706 - 0.135) = 0.208$$

$$A_{2,0.2} = 0.135 + 0.002 \times 84.2(0.706 - 0.135) = 0.231.$$

From graphs of the absorbances of the mixtures during the first part of the reactions, it was determined that 120 sec and 84 sec, respectively, were required for 20% reaction in the 1/1 and 2/1 cases. The average rates were therefore:

$$\overline{\text{Rate}}_1 = 0.002 \times 63.7 \times 5 \times 10^{-5} / 120 = 5.31 \times 10^{-8} \text{ moles liter}^{-1} \text{ sec}^{-1}$$

$$\overline{\text{Rate}}_2 = 0.002 \times 84.2 \times 5 \times 10^{-5} / 84 = 10.0 \times 10^{-8} \text{ moles liter}^{-1} \text{ sec}^{-1}$$

and the average rate constants for the reactions are:

$$k_1 = 5.31 \times 10^{-8} / (2.34 \times 10^{-5} \times 4.68 \times 10^{-5}) = 48.5 \text{ liter mole}^{-1} \text{ sec}^{-1}$$

$$k_2 = 1.00 \times 10^{-9} / (2.29 \times 10^{-5} \times 9.57 \times 10^{-5}) = 46.1 \text{ liter mole}^{-1} \text{ sec}^{-1}.$$

Additional Data on the Equilibria of BSSB with Mercaptans

Data on the assays, extents of reaction, and kinetic parameters for the reactions of nine mercaptans were presented in Table IV-2; Table A-6 gives the average values of the absorbances attained in the

TABLE A-6

ADDITIONAL DATA ON EQUILIBRIA OF BSSB WITH MERCAPTANS

Mercaptan	Absorbance Attained		20% Reaction Time ^a	
	R=1	R=2	R=1	R=2
I Cysteine	.502	.623	2.0	1.4
II Glutathione	.488	.584	2.2	1.4
III 2-Aminoethanethiol	.520	.626	0.9	0.5
IV N-tris(hydroxymethyl)- methyl-2-aminoethanethiol	.532	.637	0.3	---
V 1-Methyl- 2-aminoethanethiol	.449	.537	1.2	0.7
VI 2-Methyl-1-(propyl)- 2-aminoethanethiol	.426	.513	1.0	0.5
VII 1,1-Dimethyl- 2-aminoethanethiol	.424	.441	1.3	0.8
VIII 1,1,2-Trimethyl- 2-aminoethanethiol	.416	.437	1.8	0.9
IX 3-Aminoethanethiol	.525	.627	2.5	1.8

^aTime, in minutes, required to attain 20% of the equilibrium entent of reaction.

reactions and the time required for 20% of the reaction to occur.

The results reported in Table IV-2 are averages for several experiments which were performed on different days, sometimes months apart. Comparing each reaction with one in which cysteine was present in great excess ($R = 10$) eliminated much of the variability in the results and provided an internal check on the validity of the measurements. As a result of this situation, however, the calculation of parameters such as the initial rates and the rate constants were necessarily made using averaged values of $\frac{A}{BS}^0$ and $\frac{A}{\infty}$ found in equations IV-21 to IV-24; for a large number of measurements, these were found to be 0.135 and 0.701, respectively.

VITA

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