THE REDUCTION OF 3,5-DIIMINO-1,2,4-DITHIAZOLINE BY SOME MERCAPTANS: KINETICS AND OXIDATION POTENTIALS

By

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CHAPTER I

INTRODUCTION

Thiol and disulfide groups are present in all living matter and play an important role in many essential physiological processes[3,7]. These groups probably interact in the oxidative processes which continually take place in living cells, and may be involved in energy transfer as well as metabolic control. A detailed knowledge of these reactions would be desirable.

Interest in this matter has recently been stimulated by the discovery that some simple mercaptans afford protection against ionizing radiation. A reasonable hypothesis for the mechanism of protection involves the oxidation or reduction of the mercapto and disulfide groups[17]. For these reasons, a study was undertaken of the mercaptans; cysteine, glutathione and mercaptoethylamine.

Attempts to measure the oxidation potentials of these mercaptans have been made by numerous authors, but widely discrepant values have been estimated. It is still uncertain whether a reliable answer has been found. As far as the rate and mechanism of thiol-disulfide exchange are concerned very little experimental work has been done[4,18]. Most of the proposals concerning the role of this reaction

are speculative.

This thesis describes an investigation of the reaction of 3,5-diimino-1,2,4-dithiazoline (DDA) with cysteine, glutathione and mercaptoethylamine. The reaction can be readily followed since DDA and its reduction product, dithiobiuret (DTB), have strong absorption bands in the ultraviolet. A kinetic study was undertaken to determine the nature of the reaction. Attempts were also made to measure the equilibrium constants, from which the oxidation potentials of the mercaptans might be calculated, since that of DDA is known.

CHAPTER II

EXPERIMENTAL

Preparation and Purification of Compounds

Dithiobiuret (DTB) was obtained from the American Cyanamid Company. It was crystallized from hot 0.01M hydrochloric acid, filtered, washed with more solvents and with alcohol, and dried under vacuum[45].

3,5-Diimino-1,2,4-dithiazoline (DDA) and cystamine were prepared as the hydrochlorides by a modification of known methods[16,47]. A five per cent solution of the mercaptan was made up in 1M hydrochloric acid. Excess 30% hydrogen peroxide was added dropwise over a period of two hours while cooling with an ice bath and stirring the solution. The crystals which separated were filtered, washed with 1M hydrochloric acid, ethanol, and ether, and finally were dried under vacuum. DDA was purified by recrystallization from methyl alcohol and dried under vacuum.

Mercaptoethylamine (2-aminoethanethiol) was obtained from Evans Chemical Company, New York 17, New York (manufacturer's assay: 96.6% pure). It was purified by recrystallization from methyl alcohol and dried under

vacuum. L-Cysteine hydrochloride hydrate was purchased from the California Corporation for Biochemical Research, Los Angeles 63, California; L-cystine, glutathione and oxidized glutathione were purchased from Schwarz Laboratories, 230 Washington Street, Mt. Vernon, New York.

Four samples each of DDA and DTB were prepared. Mr. G. Baudo prepared the first samples of each. Samples 2 and 3 were prepared by the author and recrystallized once. Their spectra were identical. These samples were used for all the experimental work. Sample 4 was prepared from sample 3 and recrystallized twice. The spectrum showed little change attesting to the high purity of the samples. Sample 2 was analyzed by other techniques, the results of which are recorded in Table I.

Analysis of Mercaptans

Table I reports the results obtained upon analysis of the mercaptans.

The ferricyanide method of analysis for cysteine by Katyal and Gorin[29] was applied to mercaptoethylamine and cysteine. DTB in buffered solution with ferricyanide gave a turbid solution and was not further investigated.

The Kjeldahl determination of nitrogen for DTB and DDA was performed according to a standard procedure[44].

With the help of Mr. David Barnard the sulfur content was determined by the Carius method[56].

TABLE I

PURITY OF MERCAPTANS

Compound	Method of Analysis			
	Ferricyanide	Kjeldahl	Carius	Lavine
DTB		100.9% 101.3% 100.6%	100%	97.2% 99.6%
DDA·HCl		98.7% 99.1% 96.4%	92.3% 93.1%	
Mercaptoethylamine	93.1%			98.4% 96.0%
Cysteine	92.7% 94.9%			95.7%
Glutathione				102.5%

The SH-group content was also determined by the method of Lavine[36]. A known amount of iodine was added to oxidize the mercaptan in 1M hydrogen iodide medium. The excess iodine was titrated with sodium thiosulfate.

Each datum in Table I with the exception of the Kjeldahl represents an average of at least three values for the same stock solution.

Spectrophotometric Determinations

The extinction coefficients of DTB and DDA were

determined in 0.05 sodium acetate-0.05M acetic acid buffer of pH 4.6. All pH measurements were made using a Beckman model GS pH meter.

The spectrum of Figure 1 was obtained with a Beckman DK-1 spectrophotometer. Absorbance measurements at fixed wave lengths (282 mμ and 246 mμ) were obtained with a Beckman DU spectrophotometer. The extinction coefficients are listed below in Table II.

TABLE II

MOLAR EXTINCTION COEFFICIENTS OF DDA AND DTB

Wave Length mμ	Samples 2 & 3		Sample 4	
	DTB	DDA	DTB	DDA
246	4,300	16,400	4,290	16,800
282	20,500	5,800	20,520	5,875

Special Techniques

The water used in the preparation of all solutions was distilled once and then passed through a bed of ion-exchange resin, Amberlite MB-1. This water was then boiled 20-25 minutes and allowed to cool with a stream of nitrogen passing through it. The solution was stored in a syphon out of contact with atmospheric oxygen. Each time some water was removed the air replacing it was freed of oxygen by passing through alkaline pyrogallol. Even then, the solutions were discarded in a few days.

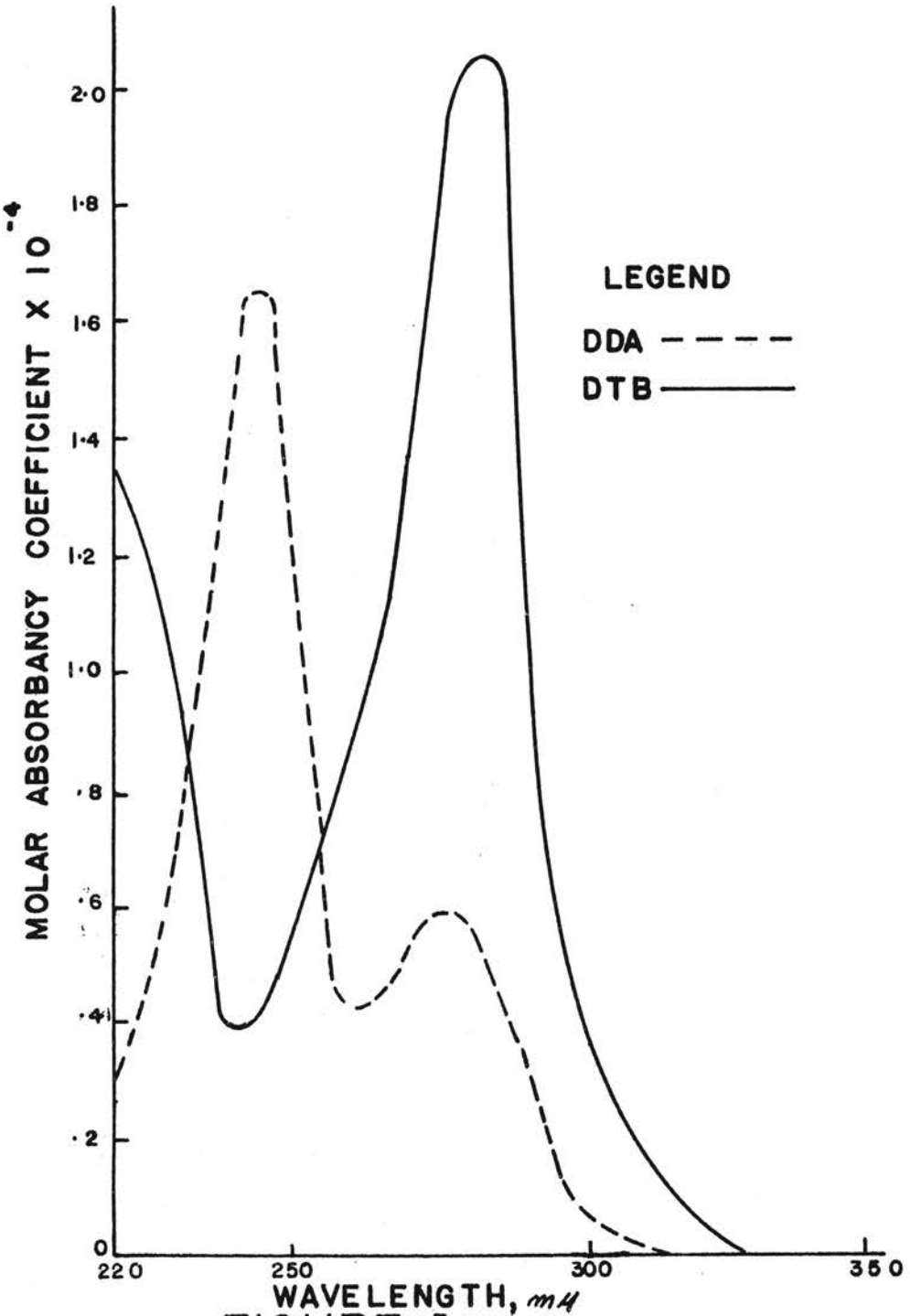

FIGURE I
DDA-DTB SPECTRUM

All containers were flushed with oxygen-free nitrogen before filling with solution. The nitrogen was passed through acid vanadate solution to remove oxygen[42]. Finally, each time an aliquot was removed from a container its volume was replaced by oxygen-free nitrogen.

CHAPTER III

KINETICS

Previous Studies

The kinetics of the reaction between thiols and disulfides have been studied by Bersin and Steudel[5] and by Fave et al.[18] Both investigations showed that the reaction took place through the mercaptide ion. An exchange can also occur in strongly acid solution; in this case it is suggested that the reaction proceeds through the sulfenium ion[4].

The investigation of this exchange reaction is complicated by the possible formation of two products: the mixed disulfides[1,16,18,28,48] and symmetrical disulfides derived from mercaptan. It is difficult to differentiate them, and the relative amounts of each in the final product have not been clearly established. In the reaction of butyl mercaptan with trimethylene disulfide Barltrop, Hayes and Calvin postulate (without proof) that the mixed disulfide was the only product, except for the possible formation of polymeric disulfides in small amounts. Eldjarn[16] claimed that in the exchange of several disulfides and mercaptans both mixed and symmetrical disulfides were formed, with the former generally predominating.

The reaction of a disulfide with water,

$$RSSR + H_2O \rightarrow RSH + RSOH$$

may interfere with the study of other reactions in aqueous solution. The reaction takes place in both acid [52] and base [49,50,51]; hydrogen sulfide is finally liberated and a sulfonic acid is formed. Since this reaction requires at least one day to produce a detectable amount of hydrogen sulfide it may be neglected in the kinetic studies to be reported, which were never carried out for prolonged periods of time.

Kinetic Study Techniques

The reaction of DDA with cysteine, mercaptoethylamine and glutathione was at first studied with nearly equivalent quantities in an attempt to evaluate the equilibrium constant. However, the data obtained were of questionable significance, as will be discussed in the subsequent chapter. The results indicated that the rate of reaction might be measured conveniently, and a kinetic study was accordingly undertaken.

The concentration of the mercaptan in the reaction medium was made 20, 35 and 50 times the concentration of DDA. This had a two-fold advantage. First, the reaction was accelerated, decreasing the possibility for side reactions such as hydrolysis and photolysis [1]. Secondly, first-order kinetics could be used for treating the data.

The reaction was carried out in a Beckman DU spectrophotometer so that the concentration of the DDA could be

easily and rapidly determined. Temperature was maintained constant at 30°C using Martin and Gorin's[41] thermostat. The buffer strength was 0.1M for all runs; the ionic strength was thus kept constant.

Varsene (10^{-3}M) was added to many of the solutions to prevent oxidation[53]. There was a substantial change in the rates observed with mercaptoethylamine. The reactions with glutathione and cysteine were apparently unaffected. The reaction was carried out to 1½-2 half-lives. No reaction was allowed to run for more than four hours.

The concentration of DDA present at any time in the reaction mixture was calculated on the assumption that the reaction was

$$DDA + 2RSH \rightleftharpoons DTB + RSSR$$

The absorbance of RSH and RSSR is negligible. Then,

1) $OD = DDA\ e_{DDA} + DTB\ e_{CTB}$

2) $DDA + DTB = In$

where OD = optical density

e = extinction coefficient

DTB = concentration of DTB

DDA = concentration of DDA

Equation (3), giving the concentration of DDA at any wavelength may be derived from equations (1) and (2).

3) $$DDA = \frac{OD - In\ e_{DTB}}{e_{DDA} - e_{DTB}}$$

Insertion of the extinction coefficents reported previously

gives

$$4) \quad DDA = \frac{OD_{282} - \text{In } 20{,}500}{14{,}700}$$

$$5) \quad DDA = \frac{OD_{246} - \text{In } 16{,}400}{12{,}100}$$

Kinetic Data

The results obtained at the two wavelengths, i.e. from equations (4) and (5), do not agree. The amount of reaction calculated from the data at 246 mμ was about 10% greater than that calculated from the data at 282 mμ. However, the 246 mμ data followed the first-order kinetic equations, i.e. a plot of -log DDA versus time gave a straight line (see Figure 2). The 282 mμ also gave a straight line in some cases although they showed a slight curvature in others. The rates of reaction calculated from the slope of the line were continually greater for the 246 mμ data.

The discrepancy indicates that equation (1) is not correct. The most likely explanation is that a third product was formed, having appreciable absorption at the wavelengths in question. This would invalidate equations (2) and (3) and their consequences. Despite this, the data do conform to a simple kinetic equation and it was thought worthwhile to calculate an apparent reaction rate constant from the data at 246 mμ, which must correspond closely to the rate of

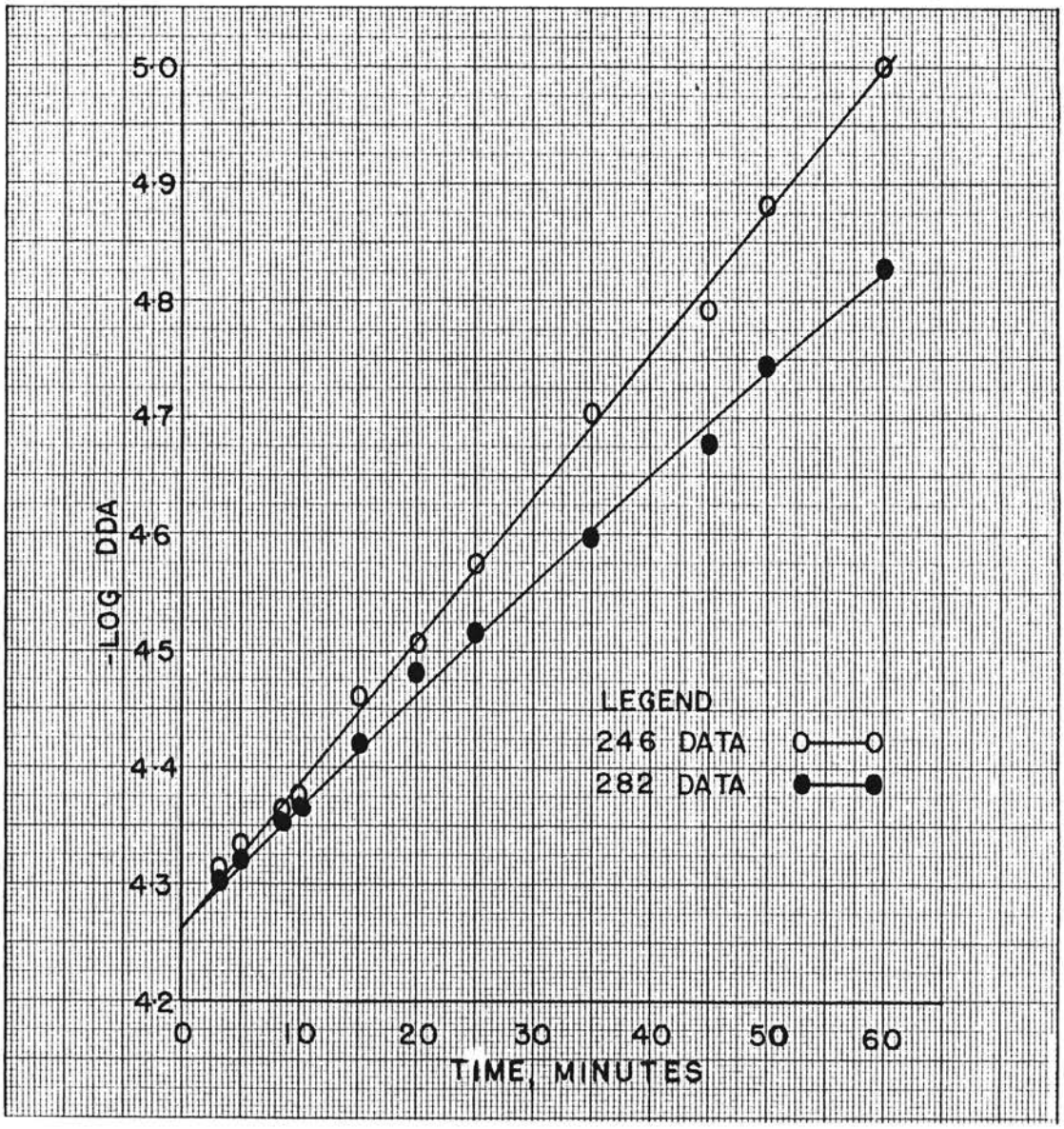

FIGURE 2

REDUCTION OF DDA PLOTTED AS A PSEUDO FIRST ORDER REACTION

disappearance of DDA. It must be stressed that the reaction does not correspond entirely to that represented by equation (1), although it may be so to the extent of about 90%. The pseudo first-order rate constants calculated on the basis just explained are listed in Table III.

TABLE III

PSEUDO FIRST ORDER RATE CONSTANTS (pH = 4.6)

Rates	Mercaptans		
	Cysteine	Mercaptoethylamine	Glutathione
	$k \times 10^3$ (l/min)		
20:1	4.14*	7.48*	3.05*
	3.86	7.51*	3.00
35:1	11.6	23.8*	9.09*
	12.1	22.5*	9.32
	12.3*	21.9	
50:1	26.7	45.2*	18.1*
	27.8	45.7*	18.8
	24.3		
	27.5*		

Note: * indicates Versene used

When these pseudo first-order rate constants are divided by the square of the concentration of the mercaptan a third-order rate constant is obtained. The values are reported in Table IV. A value is obtained for each mercaptan, and the agreement is good considering the large variation of concentrations.

TABLE IV

THIRD ORDER RATE CONSTANTS (pH = 4.6)

Ratio	Mercaptan		
	Cysteine	Mercaptoethylamine	Glutathione
	k $x10^{-3}$ ($l^2/moles^2 min$)		
20:1	4.14*	7.48*	3.05*
	3.86	7.51*	3.00
35:1	3.80	7.72*	2.97*
	3.96	7.34*	3.04
	4.00*	7.15	
50:1	4.27	7.23*	2.90*
	4.44	7.32	3.01
	3.89		
	4.40*		

Note: * indicates Versene used

A series of experiments was also done to determine the effect of pH on the rate constant. The measurements were carried out with cysteine at various pH values under similar conditions. The medium was prepared by titrating 0.05M sodium acetate-acetic acid (1:1 ratio) buffers with hydrochloric acid to the desired pH. It was found that the rate constant increases approximately linearly with $1/(H^+)$ as is shown in Figure 3.

Mechanism

Experimentally, it was found that at constant pH the rate expression is approximately,

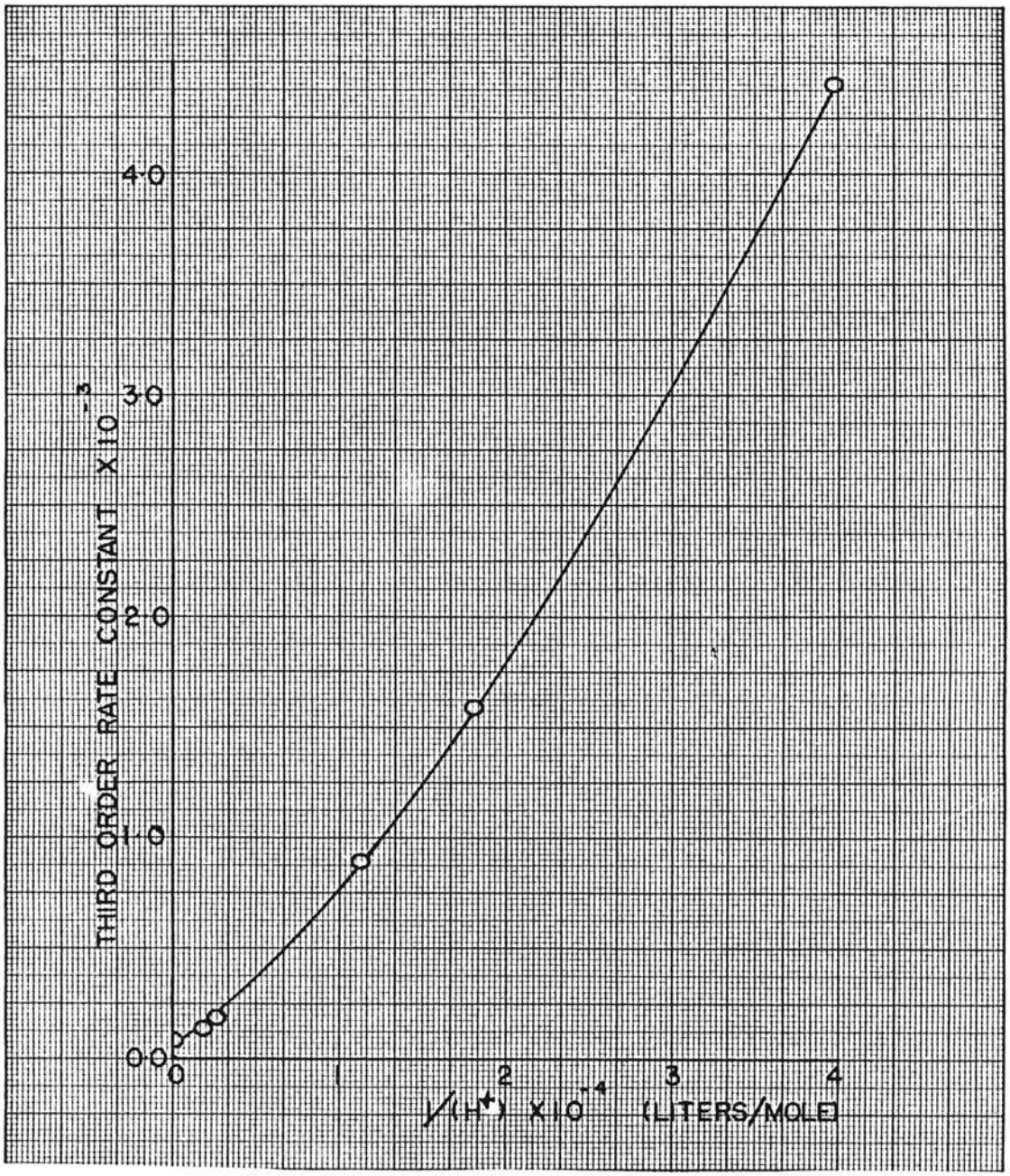

FIGURE 3

THE EFFECT OF HYDROGEN ION CONCENTRATION
ON KINETICS OF DDA REDUCTION

6) $-d(DDA)/dt = k(RSH)^2(DDA)$.

A possible mechanism which would fit expression (6) is represented by equations (7) and (8)

7) $DDA + RSH \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MTB$

8) $MTB + RSH \underset{k_4}{\overset{k_3}{\rightleftharpoons}} RSSR + DTB$

where MTB is the mixed disulfide. The rate of formation of the mixed disulfide is given by

9) $d(MTB)/dt = k_1(RSH)(DDA) - k_2(MTB) - k_3(RSH)(MTB) + k_4(DTB)(RSSR)$.

It is assumed that MTB is an unstable intermediate, whose concentration never builds up to a large value, so that $d(MTB)/dt \sim 0$ (steady-state approximation) then,

10) $$MTB = \frac{k_1(RSH)(DDA) + k_4(DTB)(RSSR)}{k_2 + k_3(RSH)}$$

since the rate constant k_4 for the reverse reaction is relatively small compared to k_1 and also since a large excess of RSH is used, the product $k_1(RSH)(DDA) \gg k_4(DTB)(RSSR)$, and the latter term may be neglected. If it is assumed that k_2 is substantially larger than $k_3(RSH)$ then $k_3(RSH)$ may also be neglected. Hence,

11) $$MTB = \frac{k_1(RSH)(DDA)}{k_2}$$

Also,

12) $-d(DDA)/dt = d(DTB)/dt = k_3(RSH)(MTB)$.

if (11) is substituted into (12), equation (13) is obtained

13) $$-d(DDA)/dt = \frac{k_3k_1}{k_2}(RSH)^2(DDA)$$

This is identical to equation (6) if $k_3k_1/k_2 = k$.

The reaction is also definitely pH dependent. The rate is approximately proportional to the reciprocal concentration of (H^+). DDA has two possible ionizations[45]. Its pK values are -1.0 and 7.4. Thus, in the pH range of 3 to 5 DDA is essentially monoprotonated, i.e. it exists as $DDAH^+$. Equation (14) describes the equilibrium constant.

14) $$K_1 = \frac{(DDA)(H^+)}{(DDAH^+)}$$

Solving equation (14) for DDA and substituting into equation (8) yields equation (15).

15) $$-d(DDA)/dt = kK_1(RSH)^2(DDAH^+)/(H^+)$$

which shows the reciprocal dependence of the rate on (H^+).

CHAPTER IV

OXIDATION AND REDUCTION POTENTIALS

Fundamental Conventions and Equations

Two different and opposite conventions have been used in connection with oxidation-reduction potentials, and confusion may result. In order to avoid it, the convention to be used will be stated here; it is accord with the recommendations recently made by the International Union of Pure and Applied Chemistry[39].

The electrode potential of half-cell, E_h, is defined by considering the half-cell in conjunction with a standard hydrogen half-cell. E_h is then given the sign of the terminal attached to the electrode in the system under consideration.

This use may be illustrated by an example. Consider the half-cells,

1) H_2 (1 atm) $\rightleftharpoons 2H^+$(activity = 1) $2e$ (in standard half-cell)

2) Oxidant + $2e \rightleftharpoons$ (Reductant)$^{-2}$ (in the other half-cell).

In all cases for which E_h is used the first of these half reactions is involved. If E_h has the orienting sign + it signifies that, were the cell to operate, the trend would be the reduction of the oxidant and the oxidation of H_2. If E_h had the sign - the converse would be true.

If equation (2) involves hydrogen ions, E_h will depend on pH. E_o is defined as the potential when the activity ratio is unity and the pH is zero. The relation between E_h and E_o may be become complicated when the oxidant and the reductant have acidic or basic properties and ionize as the pH is varied. The appropriate equations have been derived previously by several authors[9,10,11,45,47]. Only the final equations applicable in the cases of interest will be given here.

DDA ionizes with the loss of two hydrogen atoms. The pK values of the ionization are -1.0 and 7.4. Equation (3), derived by Preisler and Bateman[47], then holds for the pH range 0.7 to 5.2.

3) $E_h^{DDA} = E_o^{DDA} + 0.03 \log DDA/DTB - 0.03\ pH.$

The case of cysteine-cystine is more complicated since cysteine may exist in several ionizations forms, as +RSH, $\pm$RSH, -RSH, and -RSH-. Considering $\pm$RSH as the normal molecule then three ionization constants must be considered. The same is true of cystine, except that the normal zwitterion is $\pm$RSSR$\pm$ and hence four ionization constants must be considered. After a few simplifing assumptions[6,47] equation (4) follows for the pH range 3 to 8.

4) $E_h^{RSSR} = E_o^{RSSR} + 0.06 \log (RSSR)^{\frac{1}{2}}/(RSH) - 0.06\ pH.$

If equilibrium could be established for the reaction

$$DDA + 2RSH \rightleftharpoons DTB + RSSR$$

$$K = \frac{(DTB)(RSSR)}{(DDA)(RSH)^2}$$

and

$$-RT \ln K = nF(E_o^{DDA} - E_o^{RSSR})$$

or

$$E_h^{RSSR} = 0.251 - 0.03 \log K + 0.03 \text{ pH}$$

Review of Literature

The first measurement of a mercaptan-disulfide potential was made by Dixon and Quastel[12] who investigated cysteine-cystine and glutathione-oxidized glutathione. They used both gold and platinum electrodes and carried out their measurements in a conventional manner. The potential was found to follow the following expression:

$$E_h = E_o + RT/F \ln (H) - RT/F \ln (RSH).$$

The cell is obviously not reversible to the disulfide.

Kendall and Nord[30] reported that under special conditions in the presence of indigo carmine, cysteine and glutathione would give stable potentials. These potentials were reversible only if a catalyst was present, such as hydrogen peroxide, sodium disulfide or molecular oxygen. It was believed that these catalysts aided cysteine to reduce indigo carmine and cystine to oxidize the reduced indigo dye. Dixon and Tunnicliffe[14] objected to their reasoning: they stated that cysteine reduces indigo-carmine

even in the absence of any catalyst. Kendall and Loewen[31] could not confirm this observation. Whatever the merits of this controversy, it was not shown that mixtures of thiol and disulfide establish potentials which are in accord with the appropriate thermodynamic equations.

In an attempt to account for his results Dixon[13] proposed that the potential of a thiol solution is determined by an equilibrium in which the rate of transference of hydrogen atoms is balanced by the diffusion of hydrogen gas from the electrode. Any nacent hydrogen thus formed is incapable of reacting with cysteine. Harrison and Quastel[26] felt that the presence of ferric or cupric ions should affect the acceptance of the nascent hydrogen by cystine. Since no such effect was observed, they felt they had disproved Dixon's mechanism.

Michaelis and Flexner[43] confirmed the electrode equation deduced by Dixon and Quastel and showed that all the proposed explanations were inadequate. Barron, Flexner and Michaelis[2] suggested that the cysteine potential at the mercury electrode is due to the following reaction,

$$(RS)_2Hg + 2H^+ + 2e^- \rightleftharpoons 2RSH + Hg$$

Under fixed experimental conditions, they said, the concentration of the mercuric cysteinate remains constant and is unaffected by the ratio of thiol to disulfide or the pH. They presented little quantitative evidence for this theory, but they did show that metallic mercury is attacked by

cysteine to form slightly soluble complexes.

Green[25] objected to the theories of Barron and Flexner and Michaelis. He and several others had obtained equations like that of Dixon and Quastel with different electrodes. If metal complexes were present in each case then all such complexes would have to be equivalent electromotively. Green rejected this possibility.

Lugg[40] assumed that a RS^- radical was anchored to the metal of an electrode. Starting with this postulate he proposed certain kinetic events to account for the observed facts but had too few basic data to make a convincing case.

William and Drissen[57] and Fischer[19] attempted to solve the problem by potentiometric titration of cysteine. They obtained different E_o values with different oxidizing agents. However, both these authors used erroneous calculations[47].

Ghosh and his coworkers[22,23,24] reported that it is possible to obtain a reversible thiol-disulfide system by the partial reduction of buffered solutions of disulfide at a mercury cathode. They reported the same E_o values for cysteine, thioglycolic acid and thiolactic acid; from this evidance they concluded that the force between the sulfur and the hydrogen atoms is independent of the rest of the molecule.

Green[25] was able to repeat the experimental results of Ghosh and his coworkers. Green believed, however, that a mercury-thiol complex was formed in the electrolysis, and

that this complex made the thiol-disulfide system electromotively active.

It was left to Freedman and Corwin[20] to prove Green's theories. They also repeated Ghosh's experiments and found similar results. Furthermore, they also found positive evidence for the existence of the Hg^{2+} ion in solution by the delicate dithizone method. Finally, they obtained an excellent titration curve (e.m.f. vs per cent oxidation) by titrating cysteine with $HgCl_2$.

Leyko[38] also confirmed Ghosh's results but found a significantly different E_0 (-0.021) for copper electrodes. He repeated Freedman and Corwin's experiments using a copper electrode but did not obtain a good titration curve, although he did find Cu^{++} by the dithizone method. Leyko concluded that complexes of cysteine with metals in concentrations of about 10^{-6} are responsible for the potentials observed.

Sometimes electrodes are not responsive to certain compounds unless a catalyst or a mediator is present. On this premise Ryklan and Schmidt[47] attempted to measure mercaptan-disulfide potentials in the presence of 1M HI and I_2. They published an impressive set of data, but did not attempt to resolve the role played by the I_2, I^- couple. They also failed to ascertain whether the mediator would operate at low concentrations. Several authors[10,20,45,46] have tried unsuccessfully to confirm Ryklan and Schmidt's results. Calvin[8] has expressed the opinion that the

potentials observed were those of the two couples (the mediator and the thiol). Caraway[10] found that the electrode responded definitely to the mediator and that the mediator system was not attaining equilibrium with whatever other systems may have been present.

A method free from the above objections would be to measure equilibrium constants and calculate relative potentials. By reacting thiols with oxidation-reduction indicators of known potential Fruton and Clarke[21] were able to obtain reproducible values. In no case did a potential disagree by more than 0.01 volt from one couple to another.

Hellerman[27] felt that the versatile reactivity of thiols makes the assumed reaction between dye and mercaptan questionable. On the other hand, Borsook, Ellis and Huffman[6] repeated the work of Fruton and Clarke and confirmed them in principle but not in detail. The potentials they found were about 50 mv. more negative than those reported by Fruton and Clarke. They felt that Fruton and Clarke's work suffered from numerous side reactions.

Eldjarn and Pihl[16] measured the equilibrium between cystine and glutathione and found the potential of the cysteine-cystine couple is 0.01 volt higher than that of the glutathione; only a relative value could be deduced in this case.

Borsook *et al.*[6] calculated the standard electrode potentials of thiol-disulfide couples from thermal mea-

surements. Their errors are large since their calculations involved differences of large numbers and assumptions concerning ionization constants. They estimate a possible error of 43 mv.

Tanaka, Kolthoff and Stricks[55] investigated the equilibrium,

$$2Fe^{++} + RSSR + 2H^{+} \rightleftharpoons 2Fe^{+++} + 2RSH$$

with cysteine-cystine. The potential of the couple can be calculated from the equilibrium constant and the value of E_0 for the ferrous-ferric ion couple. Leussing, Mislan and Goll[37] have questioned their work. They claim that Tanaka et al. made an erronous assumption concerning the spectrum of $FeOH(Cy)_2$ and that this invalidates the calculations of the equilibrium constant.

Kolthoff, Stricks and Kapoor[34] established equilibrium constants for other systems relative to cysteine-cystine by measuring the apparent solubility of cystine in the presence of other thiols and disulfides.

Polarographic studies have been also made[32,33,37]. The waves are complex and easily misinterpreted. Kolthoff, Stricks and Tanaka[35] showed the existance of a prewave caused by cysteine. The major wave was caused by mercuric cysteinate. But even with the prewave they found it difficult to demonstrate the loss or gain of one electron in the process.

Table V reviews the important potentials mentioned in the foregoing discussion.

TABLE V

OXIDATION-REDUCTION POTENTIALS OF

CYSTEINE AND GLUTATHIONE

Compound	E	E_h at pH=7	References
Cysteine	0.080	-0.33	Ghosh et al. (22)
	0.077		Green (25)
	0.082	-0.33	Ghosh et al. (23,24)
	0.19	-0.222	Fruton and Clarke (21)
	0.02	-0.39	Borsook et al. (6)
	0.27	-0.14	Ryklan and Schmidt (47)
	0.09		Leyko (38)
	0.08		Tanaka et al. (55)
	0.074		Kolthoff et al. (35)
Glutathione	0.068	-0.35	Ghosh et al. (23,24)
	0.45	0.04	Ryklan and Schmidt (47)
	0.19	-0.23	Fruton and Clarke (21)

Preisler and Bateman[45] and Preisler and Berger[46] apparently found two mercaptan-disulfide systems that give reversible cells; DTB-DDA and thiourea-formamidine disulfide, respectively. Their results were confirmed by Freedman and Corwin[20].

Electrometric Measurements

The chief objection to Ryklan and Schmidt's[47] measurements is that they varied the concentration of cystine over too small a range. With a small range of cystine concentration it would be difficult to detect any deviation from the thermodynamic relations. In this work it was decided to test the reversibility of cells in which the concentration of cysteine was kept constant and that of cystine varied. The potentials of these mixtures were measured in the presence

of 1M HI or 1M HCl, in the light or in darkness.

The measuring apparatus consisted of a Leeds and Northrup galvanometer model 2030-A and potentiometer, model K-2. A saturated calomel half-cell was coupled to the cysteine solution through a salt bridge and agar plugs. All solutions were degassed prior to measurements by using oxygen-free nitrogen. The temperature of the solutions was 30°C.

The DDA-DTB couple was first measured to confirm Preisler and Bateman's potentials. E_o values for the couple DDA-DTB are listed in Table VI below.

TABLE VI

OXIDATION-REDUCTION POTENTIALS
OF DDA-DTB

Ratio DDA/DTB	E_o
1	0.252
1	0.260
3:1	0.249
3:1	0.255
1:3	0.254
1:3	0.258

The potentials measured for cysteine-cystine were not reproducible. Erratic data resulted from all the measurements conducted in darkness or in the presence of HCl. The only results of any significance were obtained in 1M HI in the light. The values are recorded in Table VII. At a given cysteine concentration the E_h values remained constant as the cystine concentration was varied in the two cases by one-

hundred fold. This would serve to indicate that the potential is independent of the cystine concentration. E_h varied with cysteine concentration, but not systematically.

TABLE VII

APPARENT OXIDATION-REDUCTION POTENTIALS OF THE CYSTEINE-CYSTINE COUPLE

Cysteine Concentration Moles/liter	Cystine Concentration Moles/liter	E_h	E_o
0.001	0.000166	0.3155	0.2789
	0.0005	0.2956	0.2398
	0.0015	0.3115	0.2402
0.0125	0.001	0.3248	0.3175
	0.01	0.3264	0.2867
	0.1	0.3240	0.2596
0.01	0.001	0.3121	0.3229
	0.01	0.3111	0.2901
	0.1	0.3163	0.2497

Equilibrium Measurements

The couple DDA-DTB is unique. Both DDA and DTB have absorption spectra in the ultraviolet (Chapter II), and the oxidation potential of this couple is known[45]. Thus, if DDA is reacted with a thiol or DTB with a disulfide, the reaction can be readily followed. If equilibrium could be established and the concentration of all species were determined, the potential of the thiol-disulfide could be calculated (see equation 5).

The reaction was carried out at 30°C in oxygen-free water under an atmosphere of nitrogen as discussed in

Chapter II. The concentrations of DDA and DTB were calculated by the optical density equations (4) and (5) of Chapter III and subject to their limitations that only the symmetrical disulfide would be formed. Again, the results did not agree. A typical set of results obtained for the reaction of DDA with cysteine is illustrated in Figure 4. As can be seen, the extent of reaction rose initially, remained constant for some time and then decreased. The extent of divergence between the data at 246 and 282 mμ can also be seen.

Attempts were also made to attain equilibrium from the reaction of DTB with a disulfide, such as cysteine, and the time element is unfavorable since the reverse reaction should be slower by a factor of several thousand. DTB will decompose an prolonged standing in aqueous solution.

Attempts were made to improve the situation by increasing the concentrations of the reagents. This would increase the rate of reaction and possibly permit attainment of equilibrium before side reactions would occur to a large extent. Unfortunately, DTB is sparingly soluble. Solutions as concentrated as 10^{-3} M could be made up by first dissolving the DTB in 3 ml. of concentrated hydrochloric acid and 3 ml. water and gradually reducing the pH by titration with N/5 sodium hydroxide to pH $\sim$ 4.6. The exact pH was then stabilized by 0.05 M sodium acetate-acetic acid buffer.

It was hoped that the "plateau" region might correspond essentially to an equilibrium state, which was only slowly

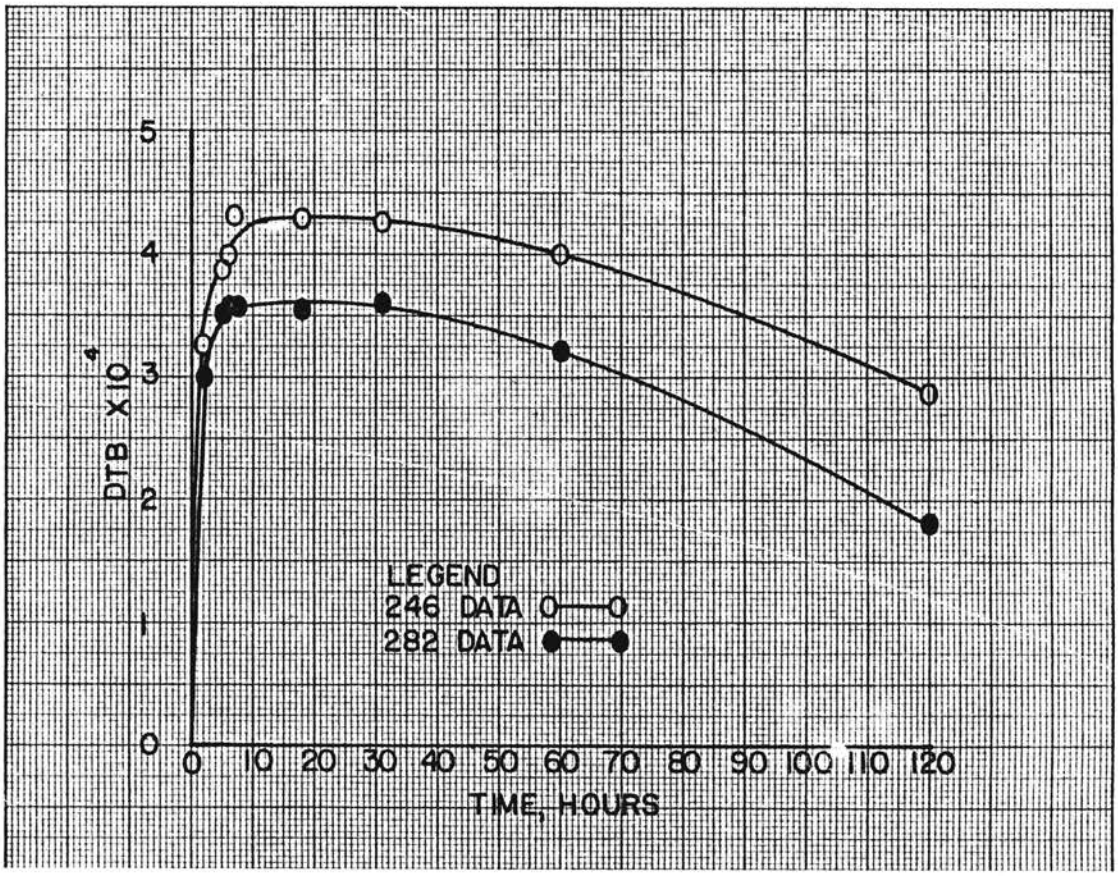

FIGURE 4

THE APPARENT FORMATION OF DTB AS A MEASURE OF THE EXTENT OF COMPLETION OF THE REACTION OF DDA WITH CYSTEINE

altered by some side reactions. For this reason mixtures were mixed that had approximately the composition corresponding to that in the plateau region; but it was observed that further reactions took place in these mixtures.

TABLE VIII

APPARENT REDOX POTENTIALS AND EQUILIBRIUM CONSTANTS OF CYSTEINE, MERCAPTOETHYLAMINE AND GLUTATHIONE

Compound	Forward		Reverse	
	$K \times 10^{-4}$	E_o	$K \times 10^{-4}$	E_o
Cysteine	16.63	0.2324	0.723*	0.2731*
	10.67	0.2382	1.551*	0.2633*
	13.47	0.2351		
	29.42*	0.2250*		
Mercapto-ethylamine	24.00*	0.2276*	1.004*	0.2689*
			1.964*	0.2602*
			0.618'	0.2663'
			0.656'	0.2655'
Glutathione	2.903*	0.2551*	1.115*	0.2676*
			1.964*	0.2602*

Note: * indicates solutions were premixed.

The equilibrium constants reported in Table VIII were all obtained at pH = 4.6, with the exception of the primed mercaptoethylamine values, which were obtained at pH = 4.3. The 246 mμ wavelength was used to calculate the data.

It appears that the mercaptan or DDA is being consumed in some other reaction which disturbs any mercaptan-disulfide equilibrium. The E_o values of the forward reaction differ for each mercaptan indicating that it is the mercaptan that is being consumed fastest in a side reaction. If DDA

were consumed then all E_o values should be similar. Such is the case in the reverse reaction where all values are similar. Here probably the common side reaction is the decomposition of DTB.

CHAPTER V

SUMMARY AND CONCLUSIONS

A kinetic study of the reactions between DDA and the mercaptans, cysteine, mercaptoethylamine and glutathione has been made. The reactions were followed spectrophotometrically at two wavelengths. The data calculated from these wavelengths did not agree indicating the formation of a third product. Despite this the data do conform to a simple kinetic relation, and it was thought worthwhile to calculate apparent rate constants using the data from the 246 mμ wavelength.

Different ratios of mercaptans to DDA were used with the mercaptans in large excess. Plotting the data in conformance to the first-order kinetic equations gave straight lines from which pseudo first-order specific rate constants could be calculated. These pseudo first-order rate constants differed for the various ratios of mercaptan to DDA by the reciprocal of the mercaptan concentration squared. The true kinetics are of third order. This can be interpreted in terms of the following mechanism;

$$2RSH + DDA \rightleftharpoons MTB$$

$$MTB + RSH \rightleftharpoons RSSR + DTB.$$

The rate constants also varied with the reciprocal of the (H^+) and it was shown that the ionization of DDA

essentially accounts for this pH dependence over the pH range (2.2 to 4.6) studied.

An attempt was made to measure the oxidation potential of cysteine by electrometric measurements. Experiments carried out in the presence of 1M HI showed that the cysteine-cystine couple did not give a reversible cell contrary to the claims of Ryklan and Schmidt[47].

The reaction of DDA with mercaptans were followed spectrophotometrically in an attempt to measure the equilibrium constant and evaluate the absolute potentials of the mercaptans. Although the reaction did appear to reach a stopping point, it is questionable whether this were a true equilibrium state. Side reactions appear to occur and they confuse the data.

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VITA

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