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MERCAPTANS AND DISULFIDES:

SOME PHYSICS, CHEMISTRY, AND SPECULATION

Melvin Calvin

January 14, 1954

Berkeley, California

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ABSTRACT

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A general review and discussion of the chemistry of the lower and intermediate oxidation levels of sulfur. Evidence is presented for a cyclic (thiazoline) component in the structure of glutathione.

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** The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

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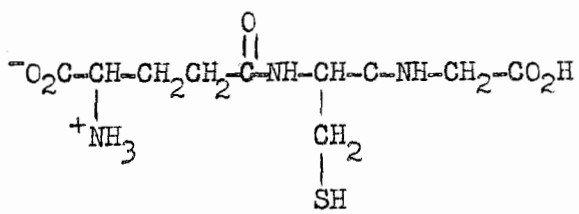
Introduction

The subject of this conference is given as "Glutathione." This is a very comprehensive subject. However, it does have some limitations, and the primary part of that subject to which this paper is addressed is the chemistry of the SH and SS systems such as might conceivably be involved in glutathione chemistry.

The generally accepted formula for glutathione is γ-glutamylcysteinylglycine, which is really a diamide, having the structure:



γ-glutamyl cysteinyl glycine

One of the major points of interest in the chemistry of glutathione has been the chemistry of the mercaptan group. More recently, there has been an additional interest developed in the possibility of glutathione being a common intermediate in the synthesis of all peptide links, particularly in

view of the existence of enzyme systems which transfer this γ-glutamyl residue to a whole variety of other amino acids. However, this is not the part of the subject to which I will be addressed. We will limit ourselves, then, to a discussion of sulfur chemistry.

In order to do this properly, one should have a more general view of the nature of sulfur chemistry, both inorganic and organic. For this purpose, I have prepared a table (Table 1) showing the various oxidation levels of sulfur, ranging from -2 to +6. Both inorganic sulfur compounds and some of their organic analogues are listed. They are arranged in two rows; the molecules with one atom of sulfur in them and the molecules containing two atoms of sulfur. There exist, of course, others with higher numbers, but for the moment we will not be concerned with them. Included also are a number of oxidation potentials in which we might be interested, connecting various inorganic species. It may be possible to use those inorganic redox values to estimate what the corresponding organic compound would have, since only three of these latter are listed and of the three only the mercaptan-disulfide ($-2 \leftrightarrow -1$) system has had any direct measurements attempted upon it. There will be discussed a bit more later. It is perhaps worth pointing out that for those compounds containing more than one sulfur atom, the oxidation number listed is the average one taken over all the sulfur atoms in the molecule. If they are separated in a non-redox process (such as hydrolysis) there may be a marked dismutation of oxidation number and reduction potential. This is, of course, especially true of molecules in an odd average oxidation number, i.e.,

$$\begin{array}{ccccccc} R\text{-}S\text{-}S\text{-}R & + & H_2O & \longrightarrow & R\text{-}SH & + & R\text{-}S\text{-}OH \\ -1 & & & & -2 & & 0 \end{array}$$

$$\begin{array}{ccccccc} R\text{-}\underset{\underset{O}{\|}}{S}\text{-}S\text{-}R & + & H_2O & \longrightarrow & R\text{-}\underset{\underset{O}{\|}}{S}\text{-}OH & + & RSH \\ +1 & & & & +4 & & -2 \end{array}$$

Such a process of average oxidation, usually with lower potential requirement followed by an internal rearrangement of redox potential may provide a route in biological systems for electron transfer through otherwise prohibitive (direct) potential barriers.

Now the particular interest we have in the present discussion is in the organic compounds containing sulfur corresponding to the two levels -2 and-1; that is, a mercaptan or a thioether, and a dialkyl disulfide, or to complete the analogy an alkyl hydrogen persulfide.

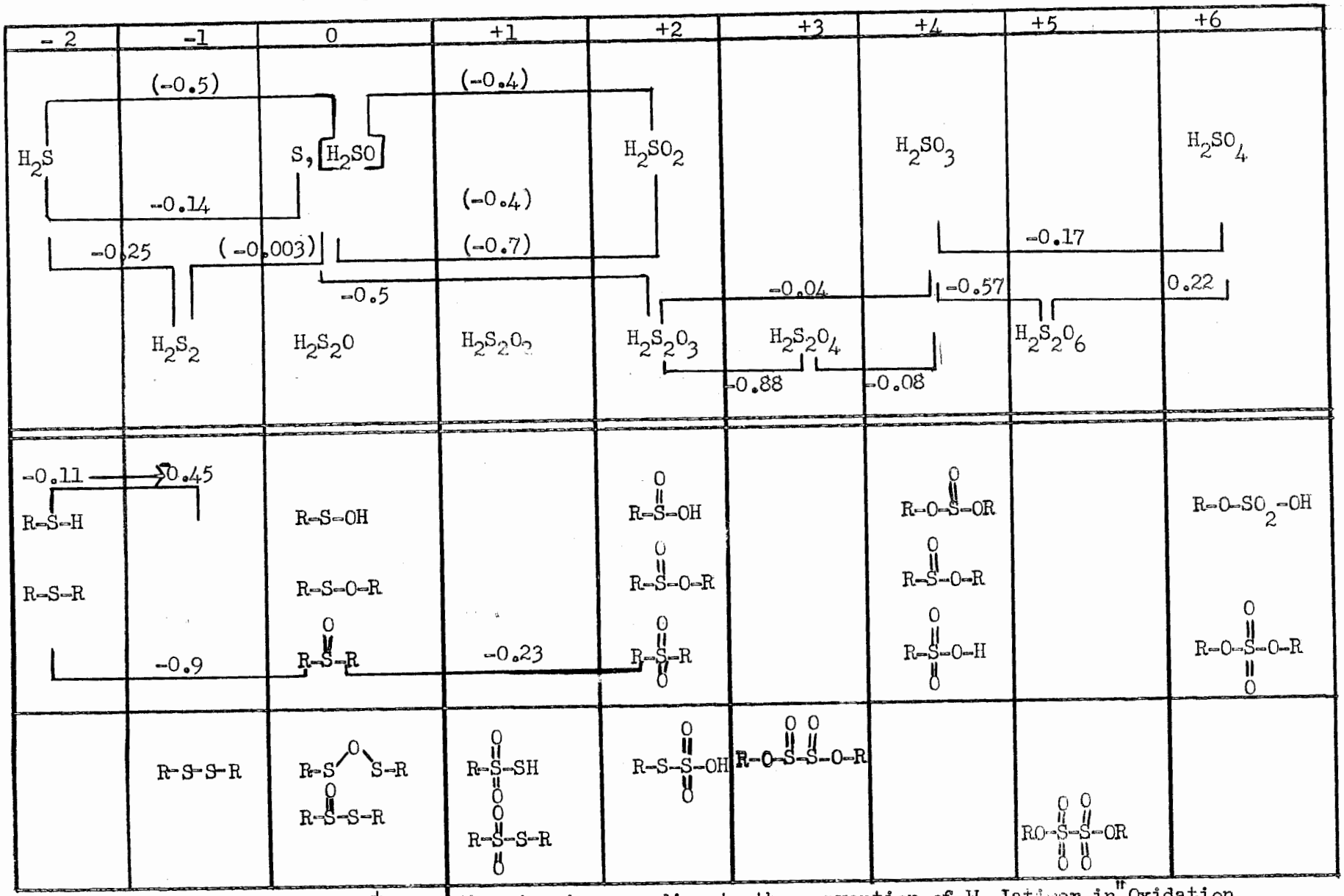
R-SH	R-S-S-H
or	or
R-S-R	R-S-S-R
-2	-1

Of these, by far the most important for glutathione chemistry, so far, is the alkyl mercaptan-dialkyl disulfide system.

Table 1*

Some Oxidation States of Sulfur Compounds and Their Organic Analogues

Only Compounds with One or Two Sulfur Atoms in the Molecule are Listed.



* Potentials are for 1 N H^+, and the sign is according to the convention of W. Latimer in "Oxidation Potentials," Prentice-Hall, Inc., New York, New York (1938).

Physics

The first thing I thought we would discuss is the physical evidence about the structure of these two groups. What do we know about such things as the thermodynamics of mercaptans and the disulfides? What do we know of the geometrical arrangement of the bonds around these groups? The distances are known some from spectroscopic data and some from crystal or gas diffraction data. They are given in Table 2. The bond energy can be computed. Before we go into the bond energy, let's say a little about the angles and geometry of the sulfides. The bond angle around a divalent sulfur is somewhat over 90° as far as it has been determined. The bonds in divalent sulfur might be considered as essentially p-bonds; the s-orbital playing relatively little part, and in this sense it is very similar to the bonding in oxygen compounds. For that reason, then, two bonds for the divalent sulfur atoms are roughly at 90° (it varies with the particular substituent being in general somewhat greater). The geometry of the disulfide-containing molecule is very interesting, the reason being that in this case one has two such sulfur atoms, presumably in both cases bonded by ordinary p electron pairs (to a first approximation). The s pair is spherically distributed about the nucleus, and the p pairs are on the 90° axes. The result is that in a disulfide the distribution leads to a very interesting geometry. An attempt to show this is made in Figure 1. The spatial configurations (dimethylsulfide), for example, are about as shown. First the S-S-C bond angle (γ) is about 107°, but the important and interesting thing is that the dihedral angle ( ) between the two S-C bonds is very nearly 90°. There is a restriction to the rotation about that S-S link amounting (at its maximum) to at least 10 Kcals., and probably nearer 20 Kcals., (from heat capacity measurements) which is a very high restriction for what appears to be a single bond between two atoms. The reason for this restriction is not simple. It probably has to do with

Table 2

Some Bond Energies (E) and Distances (d)

	E (Kcals.)	d (Å)
O-H	110	.957
S-H	82	1.345
C-O	74	1.44
C-S	52	1.81
H-H	104	.749
S-S	50	2.04
C-C	65	1.55
C=O	150	

For more complete lists, see M. L. Huggins, J. Am. Chem. Soc., 75, 4123 (1952); K. S. Pitzer, "Quantum Chemistry," Prentice-Hall, New York, New York (1953).

the tendency for the unshired p electrons on the two sulfur atoms to overlap when the dihedral angle is 0° or 180°. This overlap leads to a coulombic repulsion between non-bonding electron pairs and thus results in a rather large potential barrier at the coplanar position, the minimum then being roughly 90°. It may be assumed to vary as the cosine of the dihedral angle. Such a potential barrier, between 10-20 Kcals. at its high point, can have important consequences in any structure in which a disulfide is involved with groups which themselves have structural or steric requirements. This will, of course, be extremely important in any protein-peptide structure which involves the disulfide form of glutathione, cysteine or other mercaptan. So much, then, for the geometry of the SH and SS bonds.

The thermodynamics of the simple one-sulfur-atom compounds is well on its way to being worked out, and one can, with some degree of confidence, assign bond energies. Actually, the assignment of a bond energy from thermodynamic data is a more or less arbitrary thing, since the thermodynamics simply gives the energy of the whole molecule and not its individual parts, and the breaking down of that energy into bond energies involves the generation of a self-consistent system. It would be possible to have different systems give different bond energy assignments with the same total energy involved. A fiarly well accepted value now for most systems with SH lies between 82 and 87 Kcals. For CS, it is about 52-55 Kcals. Now, the difficult assignment is the SS assignment. Useful thermodynamic data are not yet available for ordinary dialkyl disulfides. The petroleum chemists, in whose hands most of this is, haven't quite got around to molecules of this size yet, with this carbon-sulfur ratio. They have done heat capacity measurements on these but they haven't done com-

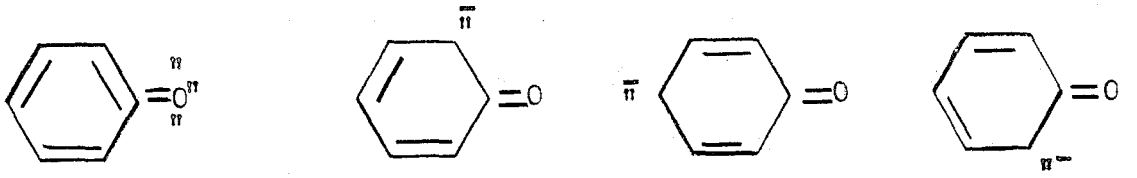
bustions yet. This information is expected within the next year or two, and presumably when such data become available it will be possible to assign a value for the SS bond in this self-consistent system without much ambiguity. But at present, that isn't possible. The only molecules that we have to work with are the inorganic ones, for which there is a heat of combustion value. From H_2S_2 and from S_8, which is a ring compound involving eight S-S bonds, one can calculate what an SS bond would be. I have done it for both cases, and it turns out to be roughly 49 Kcals. In order to obtain a value from H_2S_2 it is necessary to assume that the SH bonds in H_2S_2 are the same as in H_2S. Remember that these values are derived from thermodynamic quantities and not from kinetic quantities. They do not necessarily mean that, if one had a dialkyl disulfide and in some molecular kinetic act pulled the two sulfur atoms apart to form two sulfur free radicals, the energy required to do this would be 50 cals.; it may be so, but it is not necessarily so. This is simply a way of distributing the total heat of formation of the molecule.

General Chemistry - Acidity

With this kind of assignment of bond energy we can go on to have a look at some of the chemical reactions in which both of these molecules (mercaptans and disulfides) would be involved. The rather obvious one of the redox system between some mercaptans and disulfides I'm going to reserve for a later part of the discussion. I thought perhaps we would first look at the acidity of the mercaptans and then at some of the ordinary addition reactions which mercaptans can undergo.

The acidity of mercaptans is a subject all by itself, and I have prepared a table of acidities which upon close inspection may be rather surprising. H_2S has a pK (pK_1) of 7. When you replace one H by an ordinary alkyl group we have to estimate the pK value, because there are not as far as I know any simple alkyl mercaptans which have been titrated in water or salt solutions. Usually they are titrated in alcohol solutions. I estimate this at around 10. However, there are water-soluble mercaptans, for example, mercaptoethanol; we have just titrated some and it came out with a pK of 9.5. We have titrated mercaptoethylamine, and here there are two pK's, (i.e., the titration curve shows two buffer regions), one of 8.6 and the other 10.8. The question of assignment arises and there is not much ambiguity about it. The assignment is 8.6 for the mercaptan and 10.8 for the ammonium ion. In this case the mercaptan is strengthened even beyond the mercaptoethanol by the presence of the positive charge in the β-position. An ordinary alkylamine would be around 10; this is 10.8 because when it does dissociate the proton leaves a compound which already has a negative charge on it. Another interesting mercaptan which we titrated just the other day is the thioglycolic ester. This is almost as strong an acid as thiophenol, which was titrated in 50% alcohol, where it is 7.8. If it were done in water it probably would be lower than that; because the value in 90% alcohol is considerably higher, I believe around 9. So the phenol might be expected to be about 6.5 ~ 7.0 in water. At first, it was a little surprising that the thiophenol was as weak as it is — we thought it would be a lot stronger when compared to the acid strengthening effect of phenyl substitution for one of the hydrogen atoms of water. When you stop to think about it, the

reason that phenol is such a strong acid is because of the possibility of resonance in the phenolate ion:



These resonance forms of phenolate ion involving a double bond to the oxygen atom lead to the enhanced stability of the $^{-}O-\emptyset$ relative to the $^{-}O-H$, thus making the ΔF of ionization of phenol more negative than that of H_2O[1] ($pK_{H_2O} \cong 16$; $pK_{phenol} \cong 10$).

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(1) For further discussion see G.E.K. Branch and M. Calvin, "The Theory of Organic Chemistry," Prentice-Hall, Inc., New York, New York (1941).

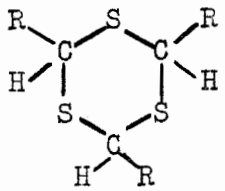
This type of resonance is of little help in increasing the acidity of thiophenol. One of the first principles of divalent sulfur chemistry is that such sulfur has very little tendency to form a double bond, and if this can be avoided it is done. So that the resonance effect which one uses to explain the enhanced strength of phenol over water or methanol is not important in strengthening thiophenol over H_2S. H_2S is 7, and thiophenol is hardly a stronger acid than H_2S. There is a remarkable lack of effect of the phenyl group on the acid strength of the SH group. This same resistance of S in the -2 oxidation level, to the formation of a double bond, is one way of viewing and understanding some of the properties of thioesters. Thus, the charge separation resonance forms play a significant role in determining the properties of ordinary esters

reducing the α-H activating influence of the ester carbonyl, although not to the extent that it is reduced in acids or acid anions. The resistance to such a form in thioesters

induced a behavior even more closely resembling that of ketones and their very active α-H atoms, with respect to their acidity[2] and their ability

(2) F. Lyman, Federation Proc., 12, 683 (1953).

to participate in aldol-type reactions. In fact the carbonyl itself in thioesters shows some of the addition reactions of true carbonyl compounds. (See addition reactions). If a deliberate attempt is made to produce a doubly-bonded sulfur as in thioaldehydes or ketones, polymerization generally takes place leading to singly-bonded sulfur atoms thus:



In the case of thiobenzophenone the monomer is known, and it is a pale blue compound extremely susceptible to oxidation. The 2- and 3-thione γ-phenyl propionic acids[3] were both formulated as thiolcinnammic acids thus:

Table 3

Acid Dissociation Constants of Some Mercaptans

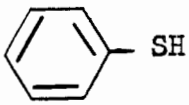
Compound	$-\log K_a$ (pK_a)	Reference
H - SH	7.0	Kubli. Helv. Chim. Acta, 29, 1962 (1946)
R - SH	~10	Estimate
$HO-CH_2CH_2-SR$	9.5	.15 N NaCl, 25°C. } Our own data *
CH_2-SH	8.6	" " " } Our own data *
$CH_2\overset{+}{N}H_3$	10.75	} Our own data *
$CH_3O-\overset{O}{C}-CH_2-SH$	7.8	" " " } Our own data *
	7.8	In 50% alcohol. (Schwarzenbach and Egli, Helv. Chim. Acta, 17, 1176 (1934).
$^-O_2C-CH_2-CH_2\underline{SH}$	8.3	Cohn and Edsall.** (Invert the assignment of $\overset{+}{N}H_3$ and SH)
$^+NH_3$	10.8	
(cysteine)		
$H-NCH_2-CO_2^-$		
$O=C-CH-CH_2-\underline{SH}$	8.7	Cohn & Edsall. Invert assignment.
NH		
C=O		
$(CH_2)_2$		
$CH-\overset{+}{N}H_3$	9.1	
(glutathione)		

Table 3 (Cont.)

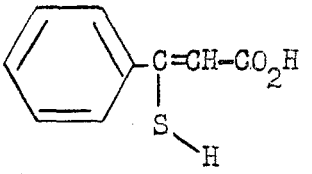
Acid Dissociation Constants of Some Mercaptans

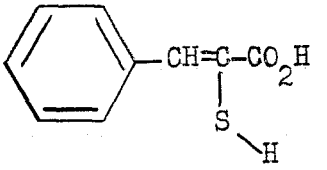
Compound	$-\log K_a$ (pK_a)	Reference
CH_2-SH	7.3	Cohn & Edsall. Invert assignment.
CH-$\overset{+}{N}H_3$	9.3	
C=O		
HN		
O_2C-CH-CH_2-SH	10.8	
(cysteinylcysteine)		

(*) We would like to thank Dr. J. Nielands for running these titrations on a Beckman titrimeter. The mercaptoethanol was purified by E. Schallenberg, who also prepared the methyl thioglycolate.

(**) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Company, New York, New York (1943), p. 84.

(3) E. Fischer and W. Brieger, Ber. d. Deutsch. Chem. Geo., 47, 2469 (1914).





For cysteine, besides the carboxylic acid which is not listed, there is the mercaptan and the α-amino group for which the two pK values which we have available to assign are 8.3 and 10.8. In all of the tables that I have seen, the assignment is 8.3 for the amino group and 10.8 for the mercaptan group. I think this must be wrong, and it should be the other way around. In most other cases, the α-ammonium group has pK values lying between 9 and 11. This assignment has important implications for protein systems because at physiological pH's of around 7.3 and with this kind of a pK value for a mercaptan, one would get an appreciable amount of ionized sulfur, which might be of some biological importance. As a matter of fact, I suspect that this view may provide the mechanism for the recently described exchange reaction between disulfides[4,5]. (See later section on S-S).

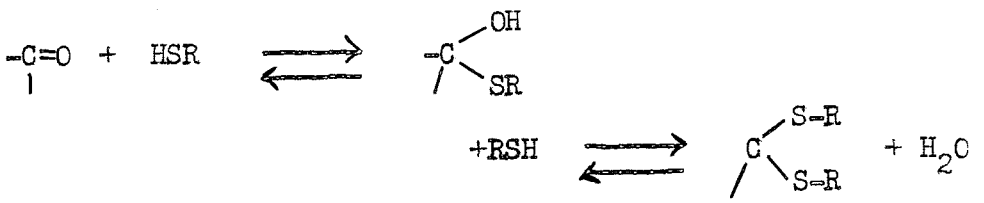
(4) F. Sanger, Nature, 171, 1025 (1953).

(5) J. A. Barltrop, P. M. Hayes and M. Calvin, in press.

General Chemistry - Addition Reactions

The addition reaction of mercaptans most familiar to organic chemists is the addition reaction of mercaptans with ordinary carbonyl

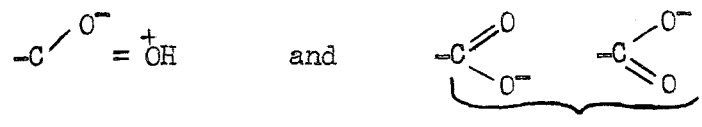
functions;



(either aldehyde or ketone carbonyl) to give semimercaptals, or semimercaptols. This, of course, could go on with another mercaptan as shown above to form the mercaptol from the semimercaptol. Unfortunately, I haven't been able to find any quantitative data regarding the equilibrium constants of such reactions. One thing we can say about them is that the equilibrium is very much farther to the right than it is for the corresponding oxygen compound (formation of acetals or ketals). In fact, it appears that if one were to use equimolar amounts of reactants, the equilibrium would be practically all over on the side of the fully formed mercaptal or mercaptol. One can make some estimates of where it might be with respect to the corresponding acetals and ketals by using our bond energy assignments that we made in Table 2, and calculating what the difference is between the bond energies involved in these various equilibria. Here, for example, in going from carbonyl to semimercaptol, we break an SH bond, which debits about 82 Kcals., and it costs a C=O which is $\sim$150 Kcals. This total cost is 232 Kcals. The receipts on the semimercaptal side are due to an hydroxyl group which in this system is $\sim$110 Kcals., the C-O which is $\sim$74 and the C-S which is $\sim$52 Kcals., making the return about 236 Kcals. The ΔE is -4 Kcals. -- very nearly the equilibrium that one might expect. If one carries this on to the complete mercaptal one finds that the ΔE for the next step

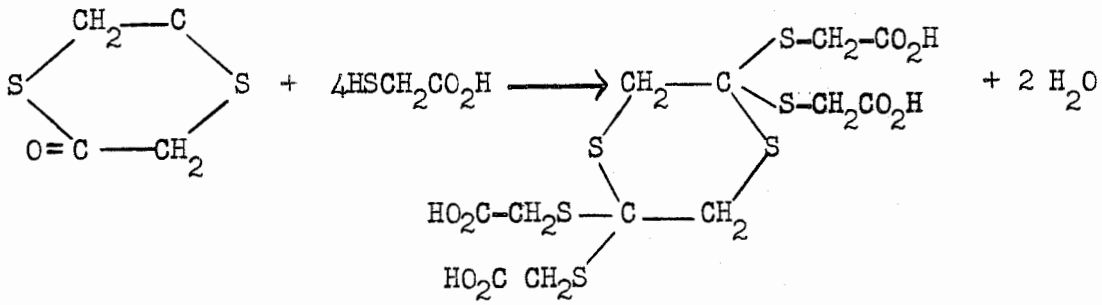
is ~-6 Kcals. Combining the mercaptans and carbonyl to thioketals or thioacetals, the ΔE for the whole reaction is ~-10 Kcals. This is not the case with acetal formation. As a matter of fact, the difference in these lies in the difference between two C-O bonds and the C=O, giving a $\Delta E \simeq +2$ Kcals. for both semi- and full ketal formation. I don't think one can use these values to calculate the equilibrium constants (K). I doubt still more whether it would have any great value for quantitative estimates of K in aqueous systems, since partial molar heats and entropies of solution have not been considered; nor even the entropy changes in the gaseous systems, which would probably favor the full ketals or acetals because of the loose molecule of water formed.

All this calculation can be taken for is to indicate the order of magnitude of the change that occurs when an SH would add across a carbonyl. Furthermore, any factors which would make the carbonyl markedly different from an ordinary aldehyde or ketone would have to be considered. Thus the formal carbonyl of an acid or acid anion is so strongly stabilized (~20 Kcals.) by resonance forms of the type



as to make addition reactions even by mercaptans, unfavored. The situation is not so unfavorable in esters, and with thioesters it appears that the carbonyl can be made to submit to addition at least by mercaptans, the following reaction having been observed:[6]

(6) A. Schoberl, Angew. Chem., 64, 82 (1952).



The situation with amides seems also to be on the verge of feasibility largely by virtue of the possibility for the elimination of H_2O with the formation of a C=N in primary and secondary amides. This will be discussed more fully later. Lastly, the reaction with CO_2 and its analogue CS_2 should be mentioned. While the addition of mercaptan across C=S in CS_2 in slightly alkaline media seems instantaneous and complete[5] according to the reaction

$$RSH + \overset{S}{\overset{\|}{C}}{=}S + OH^- \longrightarrow R{-}S{-}\overset{S}{\overset{\|}{C}}{-}S^- + H_2O$$

to form an alkyl trithiocarbonate, the corresponding reaction with CO_2 seems not to have been studied much, if at all. It might be expected that the equilibrium would be rapidly established and have an appreciable constant as written.

$$RSH + \overset{O}{\overset{\|}{C}}{=}O + OH^- \underset{H^+}{\overset{(OH^-)}{\rightleftharpoons}} R\,S\,\overset{O}{\overset{\|}{C}}\,O + H_2O$$

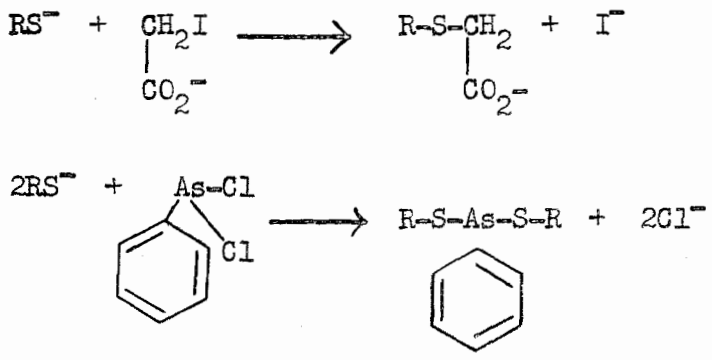
Presumably it would be reversed by acid to decompose the S-alkyl monothiocarbonate with the liberation of CO_2. Quantitative studies of this reaction would be of extremely great interest particularly in connection with biological systems involving the incorporation of CO_2. So much,

then, for the addition to carbonyl and its analogues which is really perhaps the most important single type of addition reaction which the mercaptans undergo in connection with the biological systems.

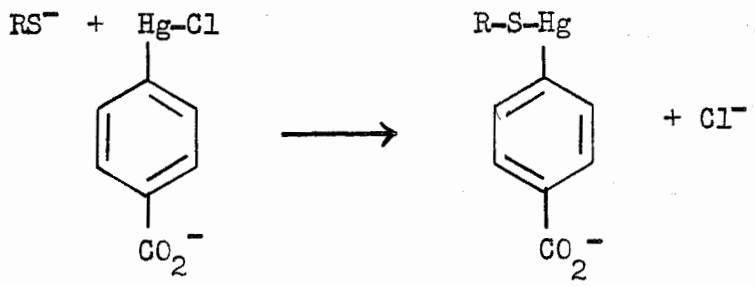
Mercaptans can also add to other types of unsaturation — simple C=C unsaturation. It is a common procedure now to use mercaptans and thiol acids to add to olefins, and it is a common method of introducing a sulfur into the compounds containing the olefinic group. However, in general, those reactions are much slower and require more vigorous catalytic conditions than do the ordinary carbonyl additions. There is more that could be described on the addition reactions, but I think that there are too many other items of interest to us, which will prohibit our spending any more time on them.

General Chemistry - Mercaptides and Displacement Reactions

While the mechanism of many of the addition reactions of mercaptans, particularly with carbonyl, might well be forumlated through mercaptide anion, there are a number of important reactions which are normally considered as directly involving mercaptide anions. These are particularly the formation of the very stable heavy metal mercaptides of Hg^{++}, Ag^{+}, and Cu^{++}, and the displacement reactions involving active halogen, as in the following:



(or Lewisite)



These and other similar reactions have been commonly used to test enzymatic systems for the presence in them of catalytically-important -SH groups. They owe their importance to the great stability of the products formed. They will not be further discussed here,[7] except to

(7) See E. S. G. Barron, "Advances in Enzymology, Vol. XI," 201-266, (1951); W. Stericks and I. N. Kalthoff, J. Am. Chem. Soc., 75, 5673 (1953).

point out that the specificity of many of them is far from complete, i.e., $I-CH_2-CO_2^-$ will quaternize (alkylate) amines and the heavy metals will form quite stable complexes with many amines, particularly when a chelate structure is possible.

General Chemistry - Oxidation

Finally, we should mention the notorious reducing ability of mercaptans, or conversely their susceptibility to oxidation. The first oxidation product, i.e., the one formed by the removal of one electron per sulfur atom, is the disulfide. This oxidation is usually accomplished by molecular iodine, although catalytic (Cu^{++}, Co^{++}) auto-

oxidation by O_2, as well as oxidation by peroxides may also be used.

$$2RSH + I_2 \rightleftharpoons R\text{-}S\text{-}S\text{-}R + 2H^+ + 2I^-$$

With the latter, however, there is the possibility and even the tendency for the oxidation to go further, for example,

$$R\text{-}S\text{-}S\text{-}R + \underline{t}\text{-}Bu\text{-}O\text{-}O\text{-}H \longrightarrow R\text{-}S\text{-}\underset{\underset{O}{\|}}{S}\text{-}R + \underline{t}\text{-}BuOH$$

While the redox system,

$$2RSH \rightleftharpoons R\text{-}S\text{-}S\text{-}R + 2H^+ + 2e^-$$

in all probability plays an important biocatalytic role,[8,9] there seems

(8) P. Massini and M. Calvin, Experientia, 8, 445 (1952).

(9) I. C. Gunsalus. In McCollum-Pratt symposium on "The Mechanism of Enzyme Action," (June 1953). Johns Hopkins University Press (1953).

little doubt of the structural importance of the disulfide link itself in such a protein[10] as insulin and in such a peptide as oxytocin.[11] It

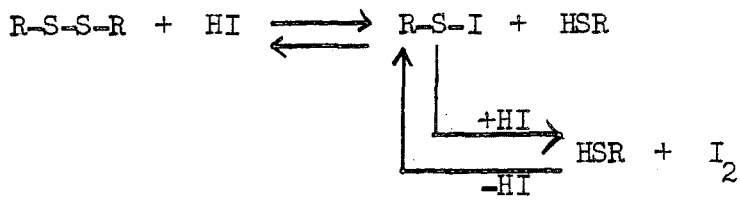
(10) F. Sanger and H. Tuppy, Biochem. J., 49, 481 (1951).

(11) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, J. Am. Chem. Soc., 75, 4879 (1953).

therefore behooves us to examine some of the reactions of the disulfide link itself, while reserving the quantitative aspect of the redox potential for a later section.

General Chemistry - Disulfides

Although great interest attaches to oxidative reactions on disulfides such as those producing mono- and dioxides and further oxidations and hydrolyses leading to sulfinic and sulfonic acids, it seems a bit beyond the scope of the present discussion to consider them. We will, therefore, be limited to reductive or dismutative splitting of the disulfide bond. In general, catalytic methods have not been used since the catalyst is either poisoned by the sulfur or the desulfurization takes place, i.e., fission of the C-S bond and its replacement by C-H. A common and widely useful method is reduction with Zn and HCl. The ease with which this takes place varies with the nature of the disulfide but generally an hour or two on the steam bath would be considered a vigorous condition. Some can be reduced in the cold with pH's as high as 2 or 3. It is interesting that BH_4^- seems to have considerable difficulty in reducing disulfides if it can do so at all. On the other hand, it can quite readily reduce sulfur from higher oxidation levels down to the mercaptan level. Apparently the disulfide link is susceptible to attack only by free radical type agents ($Zn\text{-}H^+$ or Cl_2) or by basic electron-pair containing species which might lead to reduction of one or both sulfur atoms. Such a one would be the reverse of the I_2 oxidation reaction, thus,



and there is evidence that it is indeed possible to find suitable disulfides for which at equilibrium there would be appreciable amounts of both oxidized and reduced sulfur present, namely, t-butyl mercaptan.[5] A more commonly known reaction of this type, however, and one in which the intermediate products seem to be known is that of disulfides with HCN. A recent reference[12] describes the reaction for some derivatives of cystamine

(12) A. Schoberl and M. Kawohl, Angew. Chem., 64, 274 (1952).

$$(RNHCH_2CH_2S)_2 + HCN \rightleftharpoons RNHCH_2CH_2SH + RNHCH_2CH_2SCN$$

The isothiocyanate is presumably known by its preparation from an isothiocyanate salt and the corresponding alkyl halide.

Work in our own laboratories on simple dialkyl disulfides has indicated a reluctance of these compounds to undergo this reaction and, in fact, the reverse reaction has been used to prepare aryl disulfides.[13]

(13) M. Nakazaki, J. Inst. Polytech. Osaka City Univ., 4, No. 1, Series C, Chemistry, 100 (1953).

Furthermore, there is some indication that when such compounds do undergo reaction with excess HCN both sulfur atoms of the disulfide may appear as mercaptan.[14] This would be completely unexpected in view of the fact that

(14) Private communication from T. Wieland, University of Frankfurt aum Main, Germany.

HCN is a better reducing agent than HI.[15] In the case of cystine a follow

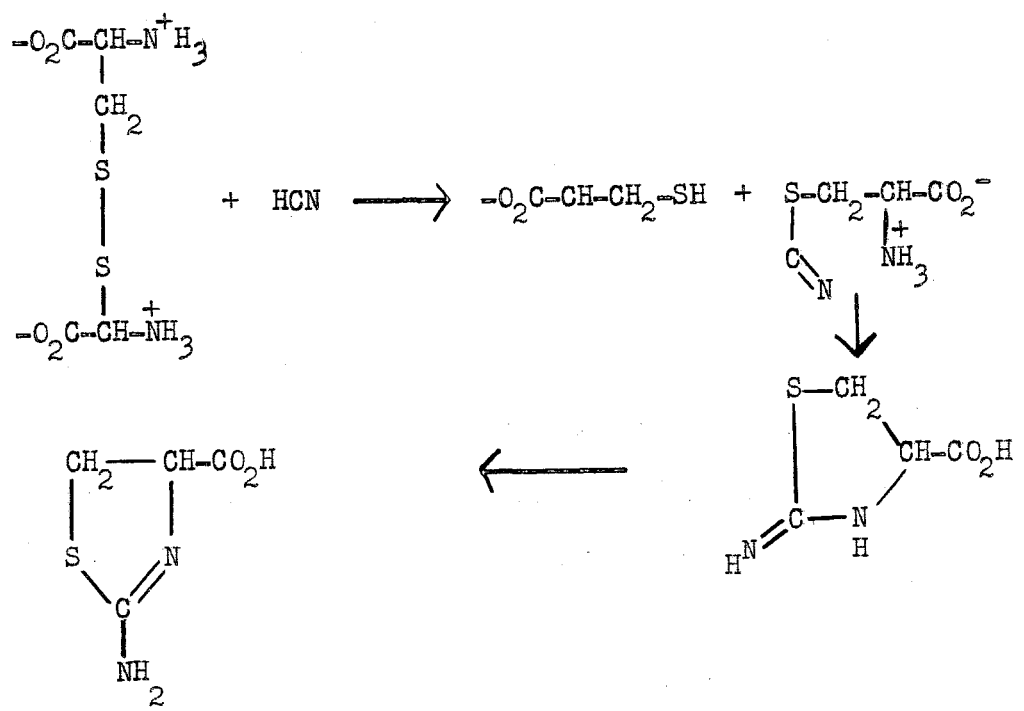
(15) $HCN \rightleftharpoons 1/2(CN)_2 + H^+ + e^-$ $E^o = -0.37$ (1N H^+)

$2I^- \rightleftharpoons I_2 + 2e^-$ $E^o = -0.54$ (1 N H^+)

reaction makes the splitting a very easy reaction to carry to completion.[16,17]

(16) J. L. Wood and S. L. Cooley, Federation Proc., 12, Abstract 967 (1953).

(17) H. Behringer and P. Zillikens. Ann., 574, 140 (1951).



2-amino-thiazoline-4-carboxylic acid

Perhaps one of the commonest methods of reducing disulfides which might be present in biological systems (proteins, cytoplasm and even living cells) is by introducing a relatively large amount of some mercaptan. This is usually cysteine or glutathione and more recently β-mercaptoethylammonium chloride. The mechanism of this reduction is that of an anionic exchange or displacement reaction of mercaptide with disulfide. Thus if R-S-S-R is the disulfide to be reduced and R'SH is the mercaptan added in excess, the following sequence of reactions would produce RSH and R-S-SR' from them.

$$(1)\qquad \begin{array}{c} R \\ | \\ R\text{-}S\text{-}S \end{array} + \begin{array}{c} R'SH \\ \uparrow\downarrow \\ {}^{-}SR' \\ + \\ H^{+} \end{array} \rightleftharpoons \begin{array}{c} R \\ | \\ S\text{-}S\text{-}R' \end{array} + RS^{-}$$

$$(2)\qquad RS^{-} + HSR' \rightleftharpoons RSH + R'S^{-}$$

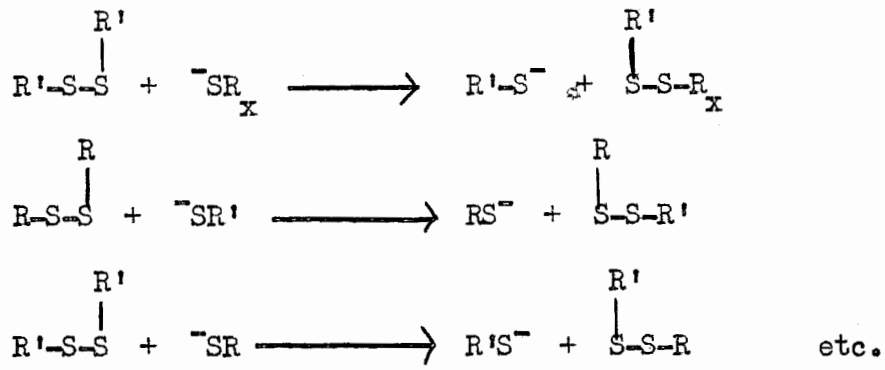
Reaction (2) is a rapid reversible proton exchange, the position of the equilibrium depending only on the relative pK's and concentrations. Since R'SH is in excess, it will be far to the right. This, in turn, will pull (1) far over to the right. Finally, since $[R'S] \ggg [RS^{-}]$ we have

$$(3)\qquad \begin{array}{c} R^{-} \\ | \\ R\text{-}S\text{-}S \end{array} + {}^{-}SR' \longrightarrow RS^{-} + \begin{array}{c} R' \\ | \\ S\text{-}S\text{-}R' \end{array}$$

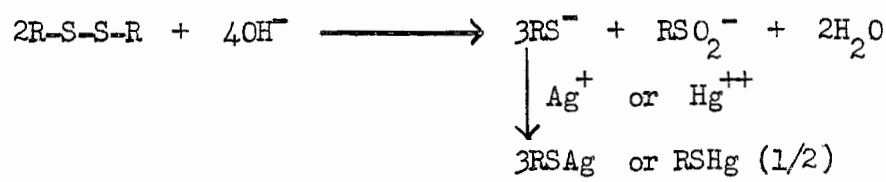
followed by removal of RS^{-} by reaction (2).

This same anionic exchange reaction is almost certainly responsible for the disulfide interchange mentioned earlier. A trace of mercaptan as catalyst would be all that is required and this might be produced by trace

reduction of hydrolysis (see later). Thus, accepting for the moment the presence of a trace of some mercaptan R_xSH and starting with the two disulfides R-S-S-R and R'-S-S-R', the following sequence would result in the apparent exchange of alkyl residues.



There is one other reaction which may be of this type and which should be mentioned. This is a dismutation reaction which takes place in alkali and in the presence of heavy metal ions such as Hg^{++} or Ag^{+}. The stoichiometry seems to be established[18,19,20] as



(18) S. Smiles and J. Stewart, J. Chem. Soc., 119, 1792 (1921).

(19) R. Cecil, Biochem. J., 47, 572 (1950).

(20) W. Sticks and I. M. Kolthoff, Anal. Chem., 25, 1050 (1953). ox-Glutathione only, even in the presence of cysteine.

The removal of the mercaptan as insoluble mercaptide is at least partially responsible for the quantitative character of the reaction which has been used as a method of analysis for oxidized glutathione (see (3) above).

It has been formulated as the sum of two reactions 1) an anionic attack on the disulfide by OH^- followed by 2) a dismutation of the unstable sulfenate and a removal of the mercaptide by heavy metal ion, i.e., for glutathione

$$2\ GSSG + 2OH^- \longrightarrow 2GS^- + 2\ GSOH$$

$$2\ GSOH \longrightarrow C\overset{O}{\overset{\|}{S}}SG + 2OH^- \longrightarrow GSO_2^- + GS^-$$

$$\overset{+}{H_2O} \qquad\qquad \overset{+}{H_2O}$$

$$3\ GS^- + 3/2\ Hg^{++} \longrightarrow 3/2\ GSHg(1/2)$$

The suggestion has been made that the entire system is a reversible one up to the point of Hg mercaptide formation. The equilibrium would be very far on the left for the first reaction, being drawn over by the two succeeding ones. The first reaction is at least formally a hydrolysis followed by a dismutation of the extremely unstable and as yet totally unknown aliphatic sulfenic acid or sulfenate ion. It is perhaps of interest to note that from Table 1 it is possible to estimate that the dialkyl sulfoxide, which is isomeric with an alkyl sulfenyl ester, is unstable to the dismutation reaction by about 30 Kcals.

$$2R_2SO \longrightarrow R_2S + R_2SO_2 \qquad \Delta F \simeq -30\ \text{Kcals.}$$

Oxidation Potential

We can at last turn to the question of the quantitative examination of the mercaptan-disulfide redox system. For about thirty years chemists and biochemists (Prof. H. T. Clarke among the earliest) have been trying to

determine the oxidation potential of this system.

$$2R\text{-}S\text{-}H \longrightarrow R\text{-}S\text{-}S\text{-}R + 2H^{+} + 2e^{-}$$

There have been a variety of determinations. In all cases, the mercaptan is a pretty good reducing agent, but the actual values of these potentials have been subject to variation and change. This is primarily due to the extreme difficulty of finding an electrode system which would respond to this mercaptan-disulfide system in a reversible way. The sulfur clearly forms very stable mercaptides, that is, salts, with heavy metals, and one might expect that even with platinum electrodes that one would find a surface coating of mercaptide which would interfere with the reversibility of the electrode. However, there is one set of measurements, make about ten years ago on the University of California (Berkeley) Campus, by Ryklan and Schmidt[21] (the late C. L. A. Schmidt of the Biochemistry Department) in which they determined the potentials for a series of mercaptans of different kinds. It looks as though they had a reversible electrode system responding not to the mercaptan-disulfide system but to another chemical redox system mixed with it, namely, the iodine-iodide system, which was believed to be in equilibrium with the mercaptan-disulfide system. The electro-active material was presumably the iodine-iodide, and not the mercaptan. It is well known that the iodine-iodide system, in acid solutions particularly, is a very easily reversible system and establishes a stable reversible potential at the platinum electrode. If one could assume

(21) L. R. Ryklan and C. L. A. Schmidt, University of California Publications in Physiology, 8, No. 17, p. 257-276 (1944).

that the sulfur is completely electro-inactive, that is, not affecting the electrode, and if the electrode potential is determined entirely via the iodine-iodide system which is in equilibrium with it and the mercaptan-disulfide system, then one could calculate what the mercaptan-disulfide potentials are. And this is what Ryklan and Schmidt did. They did a number of titrations with I_2 in 1 N HI on a series of eight mercaptans of different kinds, and arrived at a set of redox potentials which is of some interest. Their results are given in Table 4.

Without going any further into the absolute significance of the redox potentials, let us have a quick look at the relative values of the potentials. They are listed in the order of decreasing reducing ability of the reduced form, with the aromatics listed separately because of the different solvent; that is, the thiophenol is the best reducing mercaptan of the whole series of mercaptans -- its potential is -11.

The main determination was in 1 N HI, and the pH dependence was determined in a region from 1 N to a region of pH 3, and is .06, so this on the face of it looks pretty good, but there may be some reason for a systematic error in it. The work has been criticized on the grounds of irreproducibility, at least in the higher pH range (>4).[22] However,

(22) L. D. Freedman and A. H. Corwin, J. Biol. Chem., 181, 601 (1949).

it is probable that the 1 N HI titrations may have significance, nevertheless.

In any case, the thing that is most interesting here, which caught my eye and which was very important for us, in another connection, was the fact that glutathione is a relatively poor reducing agent, or saying it

Table 4

Reduction Potentials* for the Reaction

$$RSH \rightleftharpoons 1/2\ (R\text{-}S\text{-}S\text{-}R) + H^+ + e^-$$

	E^o (vs. N.H.E.) (pH = 0)	$E^{o\prime}$ (vs. N.H.E.) (pH = 7)	E_o' (vs. N.H.E.) (pH = 7)
Hydrogen	0.0	+0.42	-0.42
DPHN	—	+0.28	-0.28
Thioglycolic acid	-0.27	+0.15	-0.15
Gysteine	-0.27	+0.15	-0.15
Thiolhistidine	-0.32	+0.10	-0.10
Mercaptoethanol	-0.35	+0.07	-0.07
Ergothionine	-0.36	+0.06	-0.06
Glutathione	-0.45	-0.03	+0.03
Ascorbic acid	—	-0.07	+0.07
In 70% Ethanol			
Thiophenol	-0.11	+0.31	-0.31
o-Thiocresol	-0.30	+0.12	-0.12

* The mercaptan data are that of Ryklan and Schmidt[21]. The other values were taken from the table of Anderson and Plaut in "Respiratory Enzymes," edited by H. A. Iardy.

E^o and $E^{o\prime}$ are the potentials with sign according to the convention used by Iatimer in "Oxidation Potentials," Prentice-Hall, Inc., New York, New York.

E_o' is the potential with the sign convention (opposite of E^o) used by Anderson and Plaut, and more commonly used among biochemists.

in other words, to reduce oxidized glutathione is easy. It is one of the easiest disulfides to reduce. This accounts, at least in my mind, for the fact that enzymatically one can reduce glutathione with TPN,[23] whose po-

(23) See the communication of B. Vennesland in this volume.

tential we know to be about .28. In other words, reduced triphosphopyridine nucleotide has a larger reduction potential, i.e., is a better reducing agent, than is glutathione. And that would fit here. Many of the other values for glutathione are in the wrong direction. It is also of interest to note that dehydroascorbic acid, whose oxidation potential is fairly well established, can oxidize glutathione. This presumably places the reduction potenital of glutathione between that of the pyridine-nucleotide and that of ascorbic acid. Since it has so far not been possible to find conditions which could demonstrate any reduction of TPN by GSH the potential is probably nearer that of ascorbic acid, as given in Table 4.

Application to the Structure of Glutathione

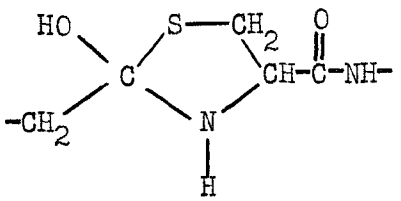
The interesting thing, then, is why this difference between cysteine and glutathione. The difference is quite large — about 7 Kcals. — or that order of magnitude, and in trying to devise an explanation for this it occurred to us that possibly glutathione was not the simple structure which we had on the board a moment ago with a free cysteine mercaptan group, but actually involves some kind of stabilization of the mercaptan; a process which does not occur in the other thiol compounds. An examination of the model of glutathione shows that this is indeed the case.

There is a very reasonable possibility of accounting for the enhanced stability of the mercaptan form of glutathione, namely, an interaction between the carbonyl oxygen of the γ-glutamyl residue and the SH-group of the cysteine residue. The first thing that one might suggest is a hydrogen-bonding which might stabilize the γ-glutamyl system. We have evidence for this possibility from an examination of the infra-red spectrum of benzyl mercaptan (in CCl_4) and in the presence of N-dimethylacetamide. The S-H band at 2565 cm.$^{-1}$ is very much broadened and shifted to 2525 cm.$^{-1}$ in the presence of the amide. This would correspond to the existence of a hydrogen bond between the mercaptan and the amide, since exactly similar shifts have been observed due to S-H interaction with pyridine or cyclohexylamine.[24] If there were as much as 8 cals. of

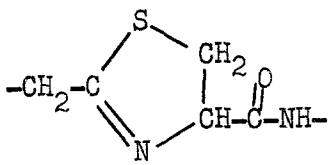
(24) W. Gordy and S. C. Stanford, J. Am. Chem. Soc., 62, 497 (1940).

extra stability in this interaction, that could account for the difference between cysteine and glutathione, because the potential is in the direction of stabilizing the mercaptans.

Now, in view of the thermodynamics which favors the addition of mercaptan over carbonyl, one might suspect that in addition to this hydrogen-bonding the next stage of the reaction might occur, and the mercaptan add across the amide carbonyl, at least to some extent in a dynamic equilibrium, to give a hydroxythiazolidine derivative of the character:



This looks as though it would be an unstable form but if it were in a dynamic equilibrium, one could go a step further and suggest that it is stabilized by the elimination of the water molecule to form a thiazoline, thus:



Construction of the models shows that all of these things are possible. In fact, not only possible, but likely. The next three figures show a sequence of photographs of the models. Figure 2 shows the hydrogen-bonding possible in the model system; the arrow points to the contact between the SH-hydrogen and the γ-glutamyl carbonyl. Figure 3 shows the photograph of the hydroxythiazolidine. Figure 4 shows, finally, the thiazoline formed by the elimination of water. The arrow indicates the nitrogen atom and the γ-glutamyl carbon atom between which the double bond has been formed.

The demonstration that this ring is actually formed in the glutathione has been made possible for us by virtue of the fact that thiazoline of this structure has a very interesting spectrum and we can find this spectrum in the solution of glutathione, under proper conditions. Figure 5 shows the ultraviolet spectrum of a group of compounds including 2-methyl thiazoline.[25] Curve 2 is the 2-methyl thiazoline (λ_{max} = 2610 Å;

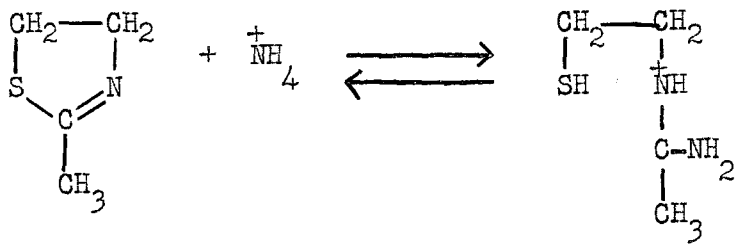
(25) Prepared in our laboratory by J. R. Quayle, according to S. Gabriel and C. v. Kirsch, Ber., 29, 2609 (1896).

ε_{max} = 5100) and curve 1 is the glutathione, showing the presence of a band with λ_{max} = 2685 Å (ε_{max} = 2000) approximately the same place that one finds it in thiazoline. Curve 3 is cystine and curve 6 is cysteine, curve 5, leucylglycine -- these are controls, none of them shows the clear band at 2600-2700 Å. While cystine shows clear evidence of the disulfide peak at 2500 Å even in 12 N HCl oxidized glutathione does not show it unless the acidity is much reduced (1 N). I think we have thus established the possibility of glutathione forming a thiazoline ring under the proper conditions.

The problem still remained of accounting for the ring chain equilibrium in glutathione. At this time a publication was discovered which not only seemed to provide the answer but also contained in it some of the elements of the ideas here presented. It turned out that Linderstrom-Lang and Jacobsen[26] had made a rather thorough study of methyl thiazoline,

(26) K. Linderstrom-Lang and C. F. Jacobsen, Comptes Rendes d. Lab. Carlsberg, Serie Chimique, Vol. 23, No. 20 (1950); J. Biol. Chem., 137, 443 (1941).

the very compound for which we have the absorption spectrum here, and found, very interestingly, that it exists in a relatively rapidly reversible equilibrium with ammonium ions in the reaction:



It forms an amidinium ion. This is a rapid reversible equilibrium, and now it seems clear why this rapid and reversible formation of thiazoline occurred in glutathione and only in glutathione. In glutathione the ammonium ion is always present, in the α-amino group of the glutamic residue. It can be brought right over the γ-glutamyl carbon atom to provide the mechanism for the ring opening and closing reaction.

I think, therefore, that in all our concerns with the reactions of glutathione and the possibility of its reversible formation of disulfide, and, indeed, the existence of cysteine in any peptide, we should consider the possibility of this thiazoline formation. It is more important in glutathione than it is in ordinary peptides, since in most ordinary peptides the circumstance of an available amino group to provide the mechanism of the opening and closing of the thiazoline ring might or might not be present -- but it is always present in glutathione. The last figure, Figure 6, presents a collection of the various structural forms in which glutathione may exist and which will manifest themselves to different extents, depending upon the conditions of the observation.

Figure Captions

Figure 1. The third non-bonding orbitals on each sulfur atom (P_z) are indicated only to show the orientation of the unshared electron pair density in each one, assuming no S or d hybridization.

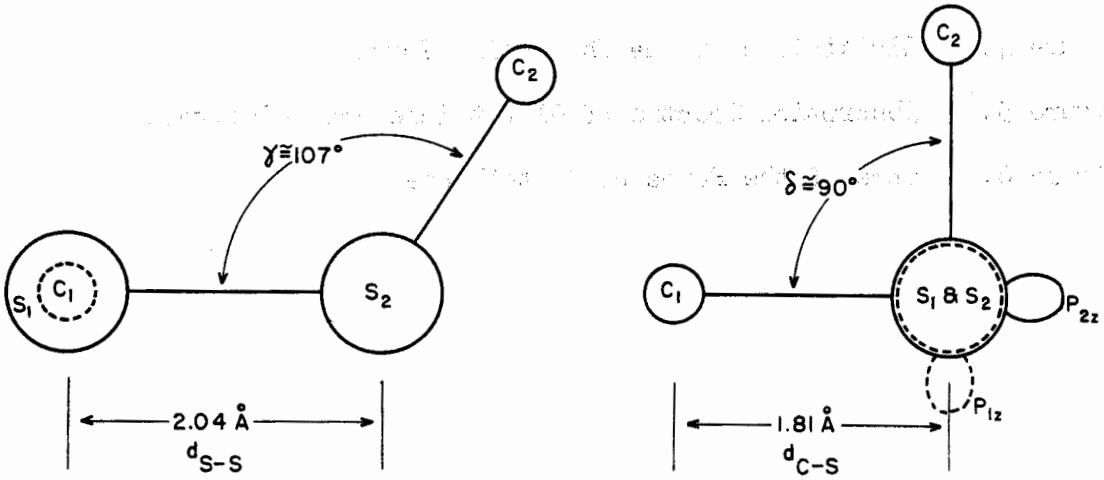
Figure 2. Glutathione Showing Possible H-Bond between S-H and γ-Glutamyl Carbonyl.

Figure 3. Glutathione in the Hydroxythiazolidine Form.

Figure 4. Glutathione in the Thiazoline Form.

Figure 5. Absorption Spectra of Glutathione and Relatives.

Figure 6. Some of the Forms of Glutathione.



GEOMETRY OF THE C-S-S-C SYSTEM

Fig. 1

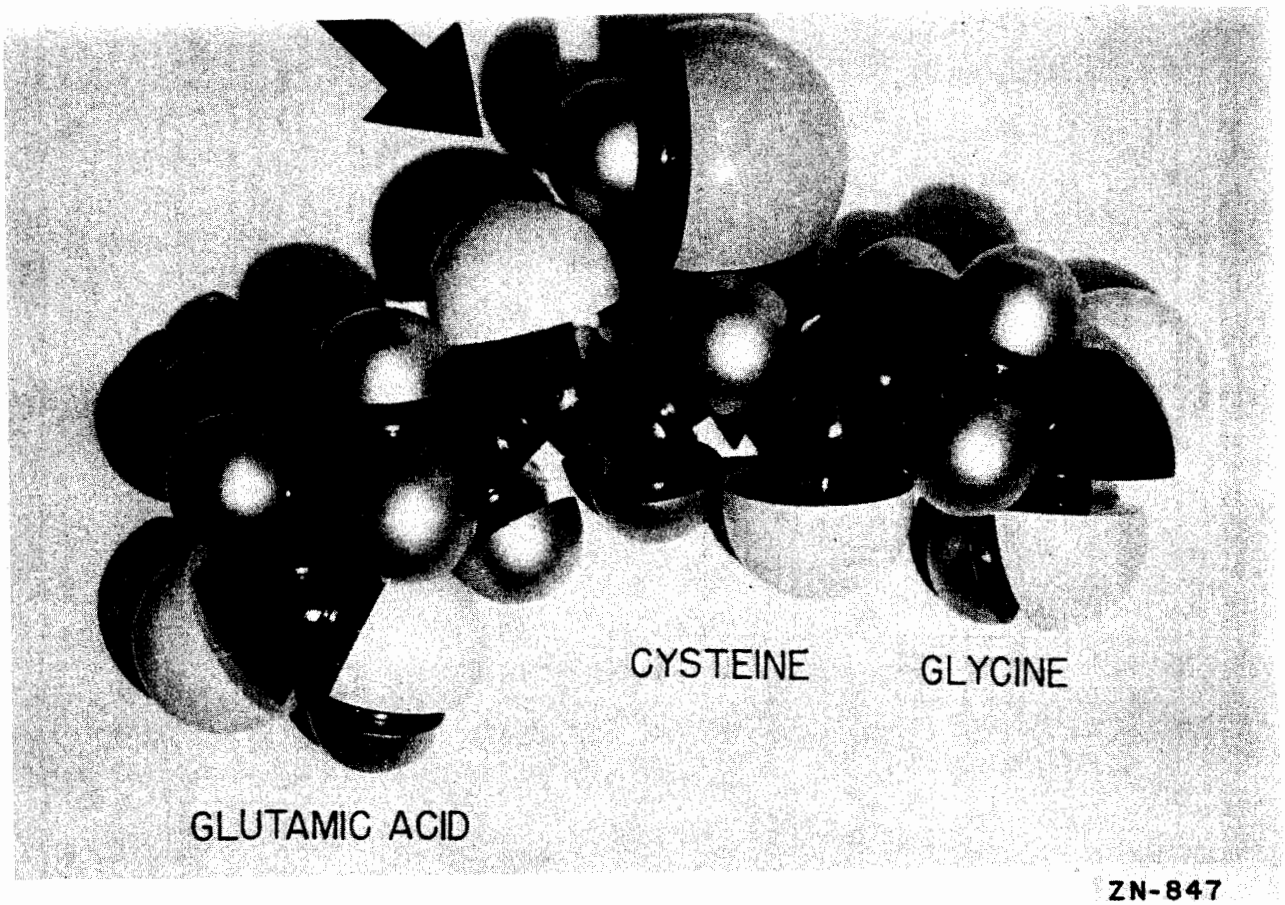
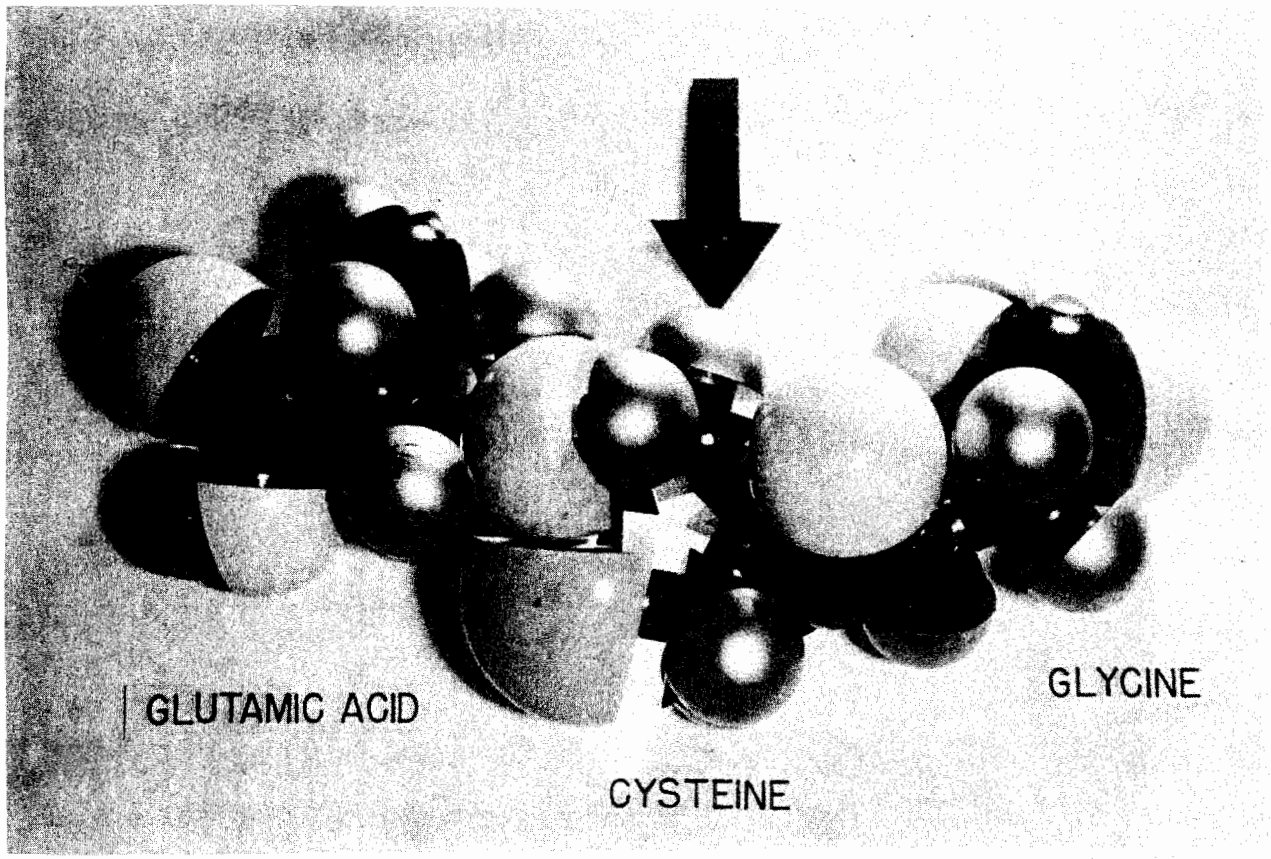


Fig. 2



ZN-846

Fig. 3

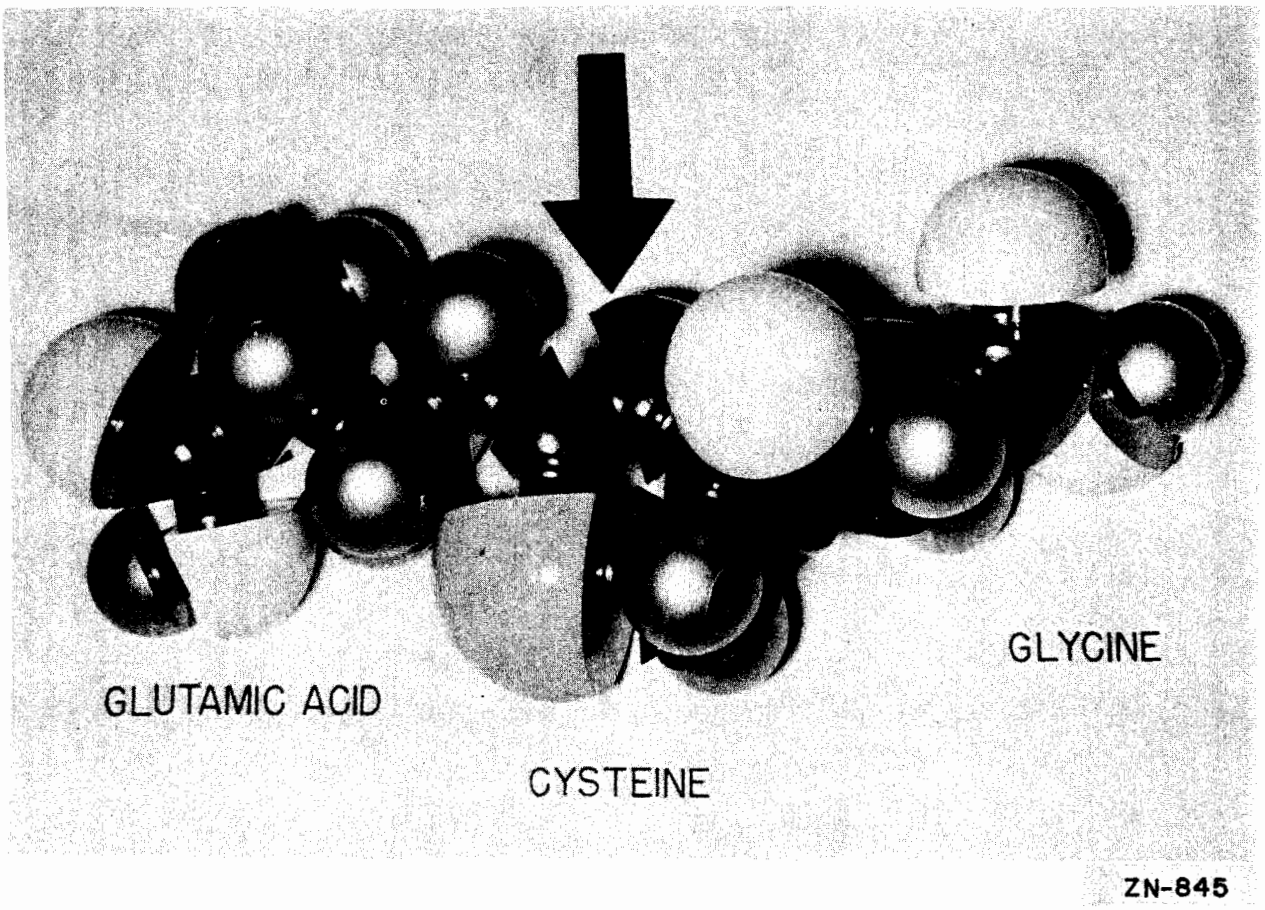


Fig. 4

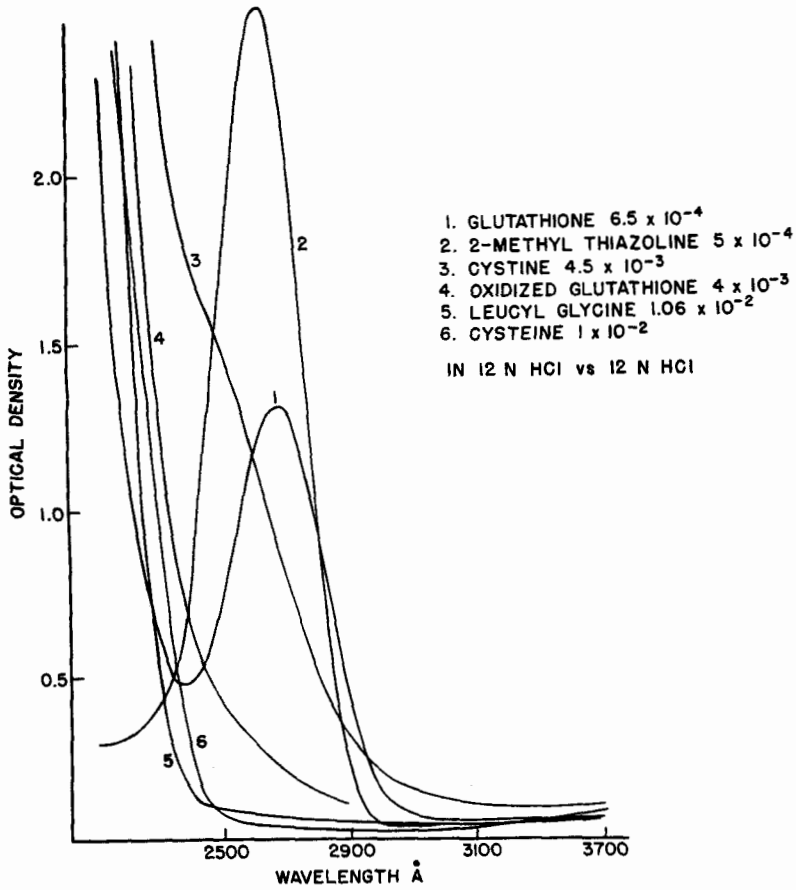
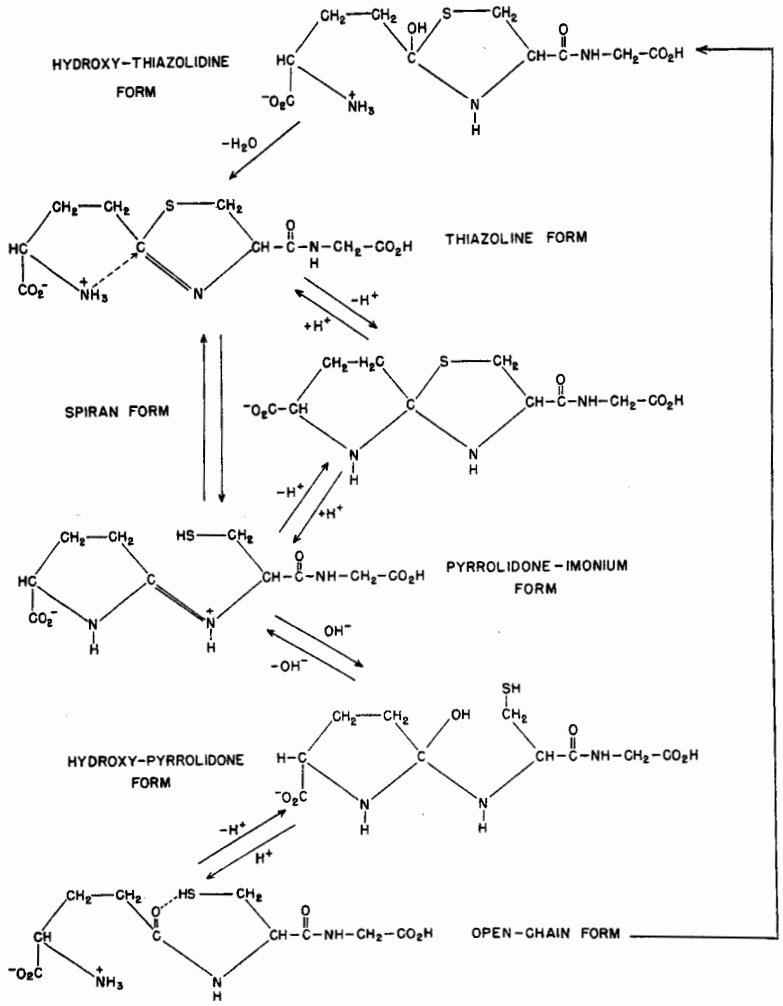

OPTICAL DENSITY
2.0
1.5
1.0
0.5
2500
2900
3100
3700
WAVELENGTH Å
1
2
3
4
5
6
1. GLUTATHIONE 6.5 x 10⁻⁴
2. 2-METHYL THIAZOLINE 5 x 10⁻⁴
3. CYSTINE 4.5 x 10⁻³
4. OXIDIZED GLUTATHIONE 4 x 10⁻³
5. LEUCYL GLYCINE 1.06 x 10⁻²
6. CYSTEINE 1 x 10⁻²
IN 12 N HCl vs 12 N HCl

Fig. 5



SOME OF THE FORMS OF GLUTATHIONE

Fig. 6