

*dl-r*- (*p*-Hydroxyphenyl) - 4 - (4<sup>t</sup>-hydroxycyclohexyl)-hexane has been isolated from the alkali soluble material and its structure has been established. The remaining mixture of *cis*- and *trans*-

isomers appears to be resistant to the usual methods of separation. It contains small amounts of isomers belonging to the meso series.

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## Oxidation-Reduction Potentials of Thiol-Disulfide Systems. II. Dithiobiuret-3,5-Diimino-1,2,4-dithiazoline

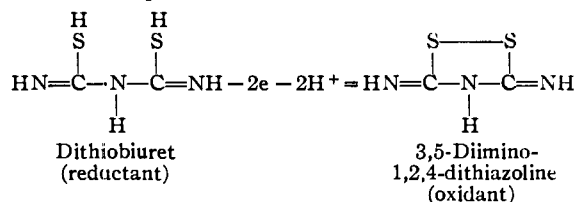
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The activation of certain enzymes by the formation or liberation of the thiol (—SH) groups of the enzyme molecule or their inactivation by alteration or blocking of these groups has been indicated in the recent literature.

Since the reduction of (—SS—) to two (—SH) is a possible mode of activation and the reverse oxidation a possible mode of inactivation, the oxidation-reduction potentials of these components of the enzyme molecule or the potentials of the reagents which react with them are of interest.

The measurement of the simpler systems involving (—SH) and (—SS—) has been undertaken as one approach to the study of the mechanism of such reactions. A further aim is to establish a graded series of reagents which might be useful for such studies.

The following simple type system is the first to be reported which involves the opening and closing of a ring structure as part of the oxidation-reduction equilibrium.



The potentials, within the *pH* range studied, are stable and follow the equations applicable to systems of the type: reductant - 2 H<sup>+</sup> = oxidant. The relatively low position of the system on the oxidation-reduction scale (*E*'<sub>0</sub> = +0.251 at *pH* 0 and *E*'<sub>0</sub> = +0.102 at *pH* 5) indicates that dithiobiuret is a moderately strong reducing agent and suggests its use for this purpose.

### Experimental

The oxidation-reduction potential apparatus and procedure generally used for such measurements were employed. The potentials of platinum wire electrodes immersed in the test solution were measured against a saturated potassium chloride calomel half-cell connected by a saturated potassium chloride-4% agar bridge. A Leeds and Northrup Type K potentiometer and a No. 2420-C galvanometer were used. Liquid junction potentials were considered negligible. The electrode, vessel and calomel cell were kept in a water-bath at 30° and the test solution was deoxygenated and stirred with cylinder nitrogen gas previously purified by passing over hot copper.

The recorded potentials have been converted into *E*<sub>h</sub> values (normal hydrogen electrode taken as zero). The potentials of the two electrodes usually were within 0.0001 volt and, after the first two minutes allowed for mixing and reaction, did not change more than 0.0002 in thirty minutes.

The materials and solutions were prepared from commercial analytical grade reagents.

Dithiobiuret<sup>1</sup> was recrystallized from hot 0.01 *N* hydrochloric acid. At this acidity the slight amount of oxidized product formed by air oxidation remains in solution and does not decompose significantly into sulfur and other undesired products. Analysis of the material showed: N, 30.5, 30.7; calculated, 31.1.

3,5-Diimino-1,2,4-dithiazoline, the oxidation product of dithiobiuret was isolated as the hydrochloride. Twenty-five grams of recrystallized dithiobiuret was suspended in 350 cc. of *N* hydrochloric acid. While cooling in an ice-bath, 21.5 cc. of 30% hydrogen peroxide was added dropwise and stirring was continued for about a half-hour or until, by microscopic examination, all of the needle-like crystals of dithiobiuret had disappeared and only the granular crystals of the oxidation product hydrochloride were present. These were filtered off by suction, washed with cold *N* hydrochloric acid, acidified ethanol, ethanol, and finally ether. The 5% water remaining after drying over calcium chloride was removed by heating at 110° for about two hours. Analyses showed: N, 24.4, 24.4; Cl, 21.1, 21.0; calculated N, 24.7; Cl, 20.9, indicating the monohydrochloride, C<sub>2</sub>H<sub>4</sub>N<sub>3</sub>S<sub>2</sub>Cl.

Ceric or thallic sulfate solutions in sulfuric acid were prepared from the corresponding oxides and standardized. Formamidine disulfide solutions in *N* hydrochloric acid were prepared from weighed amounts of the di-trichloroacetate.

The *pH* of the acid solutions or buffers were calculated from their corresponding quinhydrone electrode potentials. Their composition, within 1% accuracy, was as follows: *pH* 0.05—1 *N* HCl; *pH* 0.73—0.2 *N* HCl, 0.8 *M* KCl; *pH* 1.32—0.05 *N* HCl, 0.95 *M* KCl; *pH* 1.99—0.1 *N* HCl, 0.1 *M* glycine, 0.9 *M* KCl; *pH* 2.60—0.06 *N* HCl; 0.14 *M* glycine, 0.94 *M* KCl; *pH* 3.36—0.02 *N* HCl, 0.18 *M* glycine, 0.98 *M* KCl; *pH* 3.97—0.16 *N* HAc, 0.04 *M* KAc, 0.96 *M* KCl; *pH* 4.58—0.1 *N* HAc, 0.1 *M* KAc, 0.9 *M* KCl; *pH* 5.19—0.04 *N* HAc, 0.16 *M* KAc, 0.84 *M* KCl.

Quantitative solutions of the reductant or oxidant were made with deoxygenated solvents and protected by the passage of a stream of purified nitrogen. Moderate heating was usually necessary to facilitate solution of the reductant.

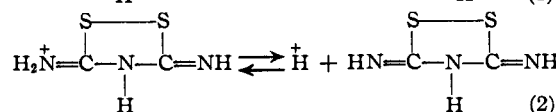
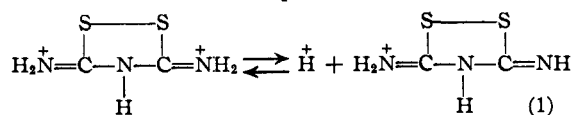
### Calculations

The mathematical equations applicable to the potentials of the system may be developed in the conventional manner<sup>2</sup> from the fundamental electrode equations.

(1) Obtained through the courtesy of the American Cyanamid Company, 30 Rockefeller Plaza, New York 20, N. Y.

(2) W. M. Clark and B. Cohen, U. S. Public Health Repts., **38**, 670 (1923); **40**, 1158 (1925).

The oxidant has two possible ionizations



the first being a strong acid of  $K = 10^{+1}$  or greater, assumed for convenience in developing the equations to be  $K_{a1} = 10^{+1}$ , and the second measured as  $pK_{a2} = 7.4$ , or  $K_{a2} = 4 \times 10^{-8}$ . The reductant does not ionize appreciably within or near the  $pH$  range studied.

The sum,  $S_0$ , of the concentrations of all of the species of the oxidant present may be expressed

$$S_0 = (\text{OxH}_2^+) + (\text{OxH}^+) + (\text{Ox}) \quad (3)$$

Since the reductant has essentially only one species, its concentration may be represented simply by  $S_r$ .

The potential of one of the equilibria participating may be calculated by

$$E_h = E_0 + \frac{RT}{nF} \log_e \frac{(\text{OxH}_2^+)}{S_r} \quad (4)$$

where  $E_h$  is the potential of the platinum oxidation-reduction electrode referred to the normal hydrogen electrode as zero;  $E_0$  is a constant characteristic of this system and based on the values of the specific ionization constants as designated and assumed above; and  $R$ ,  $T$ ,  $n$  and  $F$  have their usual significance. At  $30^\circ$ ,  $(RT/nF) \log_e ( )$  becomes  $0.03 \log_{10} ( )$ .

$$K_{a1} = \frac{(\text{H}^+)(\text{OxH}^+)}{(\text{OxH}_2^+)} \quad (5) \quad \text{and} \quad K_{a2} = \frac{(\text{H}^+)(\text{Ox})}{(\text{OxH}^+)} \quad (6)$$

so that the successive elimination of  $(\text{Ox})$  and  $(\text{OxH}^+)$  by a combination of (6) then (5) with (3) results in

$$(\text{OxH}_2^+) = \frac{S_0}{\frac{(\text{H}^+)^2 + K_{a1}(\text{H}^+) + (K_{a1}K_{a2})}{(\text{H}^+)^2}} \quad (7)$$

Then by substituting  $(\text{OxH}_2^+)$  in (7) into (2) and separating terms the final complete equation becomes

$$E_h = E_0 + 0.03 \log_{10} \frac{S_0}{S_r} + 0.03 \log_{10} \frac{(\text{H}^+)^2}{(\text{H}^+)^2 + (K_{a1}\text{H}^+) + (K_{a1}K_{a2})} \quad (8)$$

It should be noted that  $E_0$  is not the value of  $E'_0$  at  $(\text{H}^+) = 1$ , or  $pH = 0$ , as is the case with many systems but it is the  $E'_0$  of that portion of the  $E'_0 - pH$  curve where  $\text{H}^+$  is not involved in the equilibria, and the slope is zero. ( $E'_0$  has its customary definition as the  $E_h$  of an equi-molar

mixture of oxidant and reductant at some specified  $pH$ .) Graphically  $E_0$  may be considered as the intersection of the projected 0.00 slope portion of the  $E'_0 - pH$  with the  $pH = 0$  ordinate line. Since  $K_{a1} = 10^{+1}$  was assumed for convenience for illustrative purposes, no particular significance should be attached to the magnitude of the value so selected nor to the  $E_0$  so obtained.

The effect of  $pH$  or acidity upon the potentials of an equi-molar mixture of oxidant and reductant is shown in Fig. 1, where  $E'_0$  is plotted against  $pH$ . The curve follows the equation (8) and is a straight line from  $pH$  0 to 5, except for slight deviation at each end due to the effect of the ionizations.

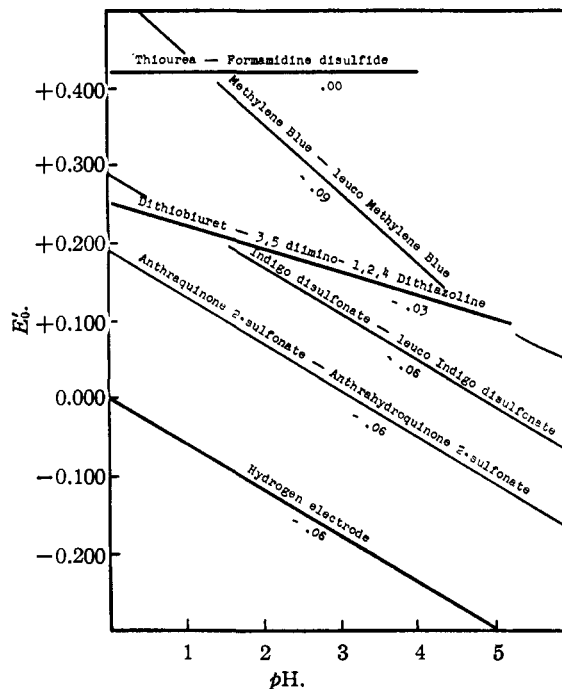


Fig. 1.—Relationship of the  $E'_0$  of common oxidation-reduction systems at various  $pH$  to the thiol-disulfide systems.

The potentials of mixtures of oxidant and reductant are shown in Tables I and II. The observed values agree with those calculated by the appropriate equation usually to within 0.0001 volt.

The calculated value of  $E_0$  for each  $pH$  is shown in Table II; the arithmetical average is 0.2820 with a maximum variation of  $\pm 0.0013$ . Using this average,  $E'_0$  at  $pH = 0$  may be calculated by equation (8) as  $+0.2510$  volt.

“Semiquinone” formation for this system is negligible. A “semiquinone” is a structure<sup>3</sup> intermediate between the fully oxidized and fully reduced form of a two-valent change reversible oxidation-reduction system which differs from each

(3) L. Michaelis, *Chem. Rev.*, **16**, 243 (1935); L. Michaelis and M. P. Shubert, *ibid.*, **22**, 437 (1938).

TABLE I

COMPARISON OF EXPERIMENTAL AND THEORETICAL POTENTIALS OF MIXTURES OF DITHIOBIURET AND 3,5-DIIMINO-1,2,4-DITHIAZOLINE AT 0.01 *N* (0.005 *M*) TOTAL CONCENTRATION IN 1 *N* HYDROCHLORIC ACID

% Total Reductant	equivalents Oxidant	$E_h$ Exptl.	$E_h - E'_0$	
			Calcd.	Theor.
90	10	0.2201	-0.0280	-0.0286
80	20	.2310	-.0171	-.0181
75	25	.2336	-.0145	-.0143
70	30	.2377	-.0104	-.0110
60	40	.2428	-.0053	-.0053
50	50	.2481	.0000	.0000
40	60	.2532	+.0051	+.0053
30	70	.2594	+.0113	+.0110
25	75	.2624	+.0143	+.0143
20	80	.2664	+.0183	+.0181
10	90	.2775	+.0294	+.0286

Based on the following assumptions, equation (6) may be modified to accommodate the quantities involved in this case. Since the amount of dissociation is small, the concentration of the oxidant hydrochloride, designated as (salt), is practically equal to (OxH). It follows from equation (2) that  $(Ox) = (H)$ . Therefore, equation (6) becomes

$$K_{a_2} = \frac{(K) \times (H)}{(\text{salt})} = \frac{(H)^2}{(\text{salt})} \quad (9)$$

$$-\log K_{a_2} = -\log (H)^2 + \log (\text{salt}) \quad (10)$$

$$pK_{a_2} = 2 pH + \log (\text{salt}) \quad (11)$$

The pH of a 0.1 *M* solution of oxidant hydrochloride was found to be 4.2 and that of a 0.01 *M* solution 4.7, when brom cresol green was employed with the usual indicator techniques.  $pK_{a_2}$  was calculated at 7.4 for both cases.

TABLE II

POTENTIALS OF MIXTURES OF DITHIOBIURET AND 3,5-DIIMINO-1,2,4-DITHIAZOLINE IN BUFFERS AT VARIOUS pH  
Total concentration of mixture, oxidant + reductant, = 0.01 *N* (0.005 *M*); ionic strength = 1.0

pH	$E_{1/2}$ ( $E'_0$ )	$E_{3/4}$ (75% oxid. 25% red.)	$E_{1/4} - E_{1/2}$ (calcd.)	$E_{1/4}$ (25% oxid. 75% red.)	$E_{1/4} - E_{1/2}$ (calcd.)	$E_0$ (calcd.)
(0.00)	(+0.2510)					(+0.2820)
.05	+.2481	+0.2624	+0.0143	+0.2336	-0.0145	+.2807
.73	.2296	.2439	.0143	.2155	-.0141	.2818
1.32	.2124	.2268	.0144	.1982	-.0142	.2820
1.99	.1916	.2061	.0145	.1774	-.0142	.2814
2.60	.1745	.1884	.0139	.1599	-.0146	.2825
3.36	.1525	.1667	.0142	.1382	-.0143	.2833
3.97	.1331	.1480	.0149	.1189	-.0142	.2822
4.58	.1144	.1290	.0146	.1003	-.0141	.2818
5.19	.0958	.1101	.0143	.0810	-.0148	.2815

of these forms by one equivalent. A structural formula with completely satisfied valences for each element cannot be written for semiquinones. Their presence may be best detected by the deviation of the slope of the potential curve from that of the theoretical curve when  $n = 2$ . The slope at each pH (see Table II) is estimated from the difference between the value at 75% or at 25% oxidized and that at 50% oxidized. The theoretical difference is respectively + or - 0.0143 v. and there is no significant deviation from this value.

The ionization constants of the components of the system could not be determined from the changes in slope of the  $E'_0$ -pH curve, since only one distinctive slope portion was measureable. The rate of decomposition of the oxidant at pH higher than 5.2 prevents reliable measurement of potentials for the region where both components are un-ionized and the uncertainty of the liquid junction potentials with concentrated acids rendered difficult the interpretation of results with such solutions.

The second acidic dissociation constant of 3,5 diimino-1,2,4-dithiazoline,  $pK_{a_2}$ , could be estimated from the measurements of the pH of aqueous solutions of the oxidant hydrochloride salt by employing the equation (11) below.

Dithioiuret does not ionize appreciably within the pH range of 0 to 5.

The effect of total concentration was negligible or within reasonable experimental error. For a total concentration of 0.01 *N* with ratio of oxidant to reductant concentration of one, in 1 *N* hydrochloric acid  $E_h = +0.2481$  volt; for 0.001 *N* concentration  $E_h = +0.2493$ . The potentials at 0.0001 *N* concentration were quite unstable (unpoised) while stirring the solution with nitrogen gas but attained the value of +0.251 within a few minutes after stirring was discontinued.

Titration of the reductant with ceric or with thallic sulfate in 1 *N* sulfuric acid solutions, or with formamidine disulfide in *N* hydrochloric acid, gave potentials which were within 0.001 volt of the theoretical values at all of a great number of measured points between 10 and 90% reduced or oxidized. Titrations of the oxidant with titanous sulfate, or of the reductant with vanadic (5+) sulfate or with molybdcyanide, in acid solutions were unsuccessful because of the slow rate of reaction with the reagents employed.

### Discussion

Because of the importance of the (-SH) and (-SS-) groups in biological materials, many at-

tempts have been made in the past twenty years to measure the oxidation-reduction potentials of the apparent equilibrium,  $2\text{RSH} - 2\text{H} = \text{RSSR}$ . The potentials were generally unstable and difficult to duplicate and did not follow with sufficient accuracy the equations believed to be applicable to the systems.

Recently some results for cysteine-cystine and other thiol-dithio systems were reported which were obtained under special conditions of titrating agents and catalysts.<sup>4</sup> These results, while indicating apparent agreement with the theoretical calculations, are not in accord with the many prior investigations cited in the same publication<sup>4</sup> with regard to the reliability and possible significance of the measurements of these systems; further study and confirmation seem desirable to definitely establish the potentials of these systems.

The potentials of the thiourea-formamidine disulfide system, measured under a variety of conditions of acidity and concentration,<sup>5</sup> agree with the calculated values well within the tolerances usually set for organic systems and demonstrate the reversibility of the thiol-dithio equilibrium.

The measurement of the oxidation of dithiobiuret to its oxidant, 3,5-diimino-1,2,4-dithiazoline, seemed a desirable step in the development of this subject because of the two thiol groups on the same molecule which offered the possibility either of the formation on oxidation of a ring structure with two sulfur atoms or of the formation of a chain compound with two or more sections connected by  $-\text{SS}-$  linkages. The conformity of the potentials to the simple equation for a two-valent step oxidant-reduction affected by changes in the ratio of oxidant to reductant but unaffected by changes in the total combined concentrations and also the symmetry of the curve shows that the disulfide formed is the single ring structure.

The relative position of the  $E'_0$ - $p\text{H}$  curve of dithiobiuret-3,5-diimino-1,2,4-dithiazoline system toward several other common reversible systems is shown in Fig. 1. When comparing potentials of a particular mixture of reductant and oxidant of a system of the type  $2\text{RSH} + 2\text{H} = \text{RSSR}$  (thiourea-formamidine disulfide) with any other, due

consideration must be taken of the total combined concentration of the components, as well as the ratio of the reductant to the oxidant, because the equation for such systems involves the square of the RSH concentration.<sup>4,5</sup>

At low  $p\text{H}$ , dithiobiuret is among the strongest of the common organic reducing agents. When the side products of the decomposition of the unionized oxidant ( $pK_{a_1} = 7.4$ ) are not objectionable, dithiobiuret may also be useful for reducing purposes above  $p\text{H}$  5.2.

Formamidine disulfide, the oxidant of thiourea, readily oxidizes dithiobiuret as predicted by the potentials of the two systems, the expected potentials being rapidly and accurately attained. This result demonstrates that systems of the  $(-\text{SH})$  and  $(-\text{SS}-)$  type can react with each other and suggests the extension of the present use of  $(-\text{SH})$  and  $(-\text{SS}-)$  compounds for the interaction with similar groups when these are part of the enzyme molecule for activations or inactivations by oxidative or reductive reactions.

The measurements are being extended to include the potentials of various thiol-disulfide systems in order to prepare a series of graded reagents of this type and to study the effect of substitutions.

#### Summary

The oxidation-reduction potentials of the thiol-disulfide system, dithiobiuret-3,5-diimino-1,2,4-dithiazoline have been measured in the  $p\text{H}$  region of 0.05 to 5.2 and found to follow the equations applying to systems involving a reversible two-equivalent change from reductant to oxidant. This result establishes the oxidation step as the removal of  $2\text{H}$  from the  $2-\text{SH}$  of dithiobiuret to form a five-membered ring structure with two adjacent S atoms, the first system of this type whose potentials have been measured.

The  $E'_0$  of the system at  $p\text{H}$  0 was estimated as  $E_h = +0.251$  volt and the second acidic ionization constant of the oxidant as  $pK_{a_2} = 7.4$ . The low  $E'_0$  suggests the use of dithiobiuret as a reducing agent.

Formamidine disulfide oxidizes dithiobiuret giving the theoretical potentials and showing that  $(-\text{SH})(-\text{SS}-)$  type systems can react rapidly and reversibly with each other.

(4) L. R. Rykjan and C. L. A. Schmidt, University of California publications in *Physiology*, **8**, 257 (1944).

(5) P. W. Preisler and L. Berger, *THIS JOURNAL*, **69**, 322 (1947).