stant at finite ionic strength, to k_0, the rate constant at zero ionic strength, by the equation

$$\log k_\mu/k_0 = 2.06 \times 10^7 Z^2 d \quad (9)$$

where Z is the charge born by the dipolar activated complex, d is the distance of charge separation and μ is the ionic strength. Assuming Z is approximately $^1/_2$, d is of the order of magnitude of the C–N bond distance (~1.4 Å.), this reduces to

$$\log k_\mu/k_0 = 0.0722\mu \quad (10)$$

It is apparent from the equation that the effect will be relatively small in water at 90.0°. An inspection of our results obtained at varying ionic strengths (Table IV) reveals that because of experimental uncertainty it is not possible to make a clear-cut distinction between the ionic mechanism and the Werner mechanism from these data. The tendency for the rate in water alone to be somewhat lower than that in acid can be explained on two bases. First, if a small amount of reverse reaction were occurring even at the low per cent. conversions studied, added electrolyte would inhibit the reverse reaction and cause an apparent increase in the forward reaction. This phenomenon was most probably observed in the earlier work[2,4] done at relatively high per cent. conversions in water alone. A second explanation is that the ionic mechanism for the decomposition of urea is the correct one, and the rate in acid is faster because of the higher ionic strength of the acid solution. Fawsitt's observation[3] that the decomposition in water–alcohol mixtures is slower than in water alone can, perhaps, be interpreted as also favoring this point of view.

Pertinent thermodynamic data, an analysis of the entropy and energy of activation, and a discussion of the relevance of these findings to the mechanism of the urease-catalyzed hydrolysis of urea will be presented in a subsequent communication.

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[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE UNIVERSITY OF MINNESOTA]

Equilibrium Constants of Exchange Reactions of Cystine with Glutathione and with Thioglycolic Acid Both in the Oxidized and Reduced State

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Equilibrium constants have been determined of the following reactions: RSSR + GSSG(TSST) ⇄ 2RSSG(2RSST); RSSR + GSH(TSH) ⇄ RSSG(RSST) + RSH; RSSG(RSST) + GSH(TSH) ⇄ GSSG(TSST) + RSH and RSSR + 2GSH(2TSH) ⇄ GSSG(TSST) + 2RSH in which RSSR, GSSG and TSST denote the oxidized forms and RSH, GSH and TSH the reduced forms of cystine, glutathione and thioglycolic acid. The constants were found from solubility determinations of cystine in the various equilibrated reaction mixtures. The results were substantiated by some polarographic determinations. All the equilibrium constants were found of the order of one and unaffected by variations in the charge type of amino and carboxyl groups. From the equilibrium constants the oxidation potentials of the glutathione (GSH–GSSG) and thioglycolic acid (TSH–TSST) systems were calculated to be practically equal to that of the cystine–cysteine systems. The mixed disulfide RSST was found to be subject to a relatively rapid fission process in buffers of *p*H 5 to 7, while the other simple or mixed disulfides were stable under our experimental conditions.

Cysteine and thioglycolic acid have long been used as reducing agents for disulfide groups in proteins.[1] In recent years much importance is being attached to the sulfhydryl–disulfide interaction in the interpretation of phenomena observed in protein denaturation.[2–5]

A quantitative study is planned in this Laboratory of reactions involving low molecular weight thiols and disulfides on the one hand and native and denatured proteins on the other. In order to gain further insight into the characteristics of such reactions we have first studied interchange reactions between low molecular weight disulfides with formation of mixed disulfides and the reactions between disulfides and thiols. The equilibrium constant of the latter type of reaction allows the calculation of the oxidation potential of the particular systems. The results of these investigations are reported in this paper.

After Goddard and Michaelis[6] had shown that thioglycolic acid reacts with cystine with formation of cysteine Bersin and Steudel[7] made a quantitative study of the optical rotation of this system. They derived the equilibrium constant of the above reaction, assuming that dithiodiglycolic acid and cystine do not form a mixed disulfide. As shown in the present paper this assumption is entirely incorrect. Studies on reactions between simple and water-insoluble alkyl disulfides and mercaptans were reported by Gorin, *et al.*[8] In recent years mixed disulfides ($RSSR_1$) involving cystine and glutathione

(1) See *e.g.*, F. G. Hopkins, *Biochem. J.*, **19**, 787 (1925); A. E. Mirsky and M. L. Anson, *J. Gen. Physiol.*, **18**, 307 (1935); *ibid.*, **19**, 427 (1936); F. H. Haurowitz and M. Kennedy, *Proc. Fed. Am. Soc. Exp. Biol.*, **II**, 227 (1952).

(2) C. Huggins, D. F. Tapley and E. V. Jensen, *Nature*, **167**, 592 (1951).

(3) Sequence of papers by W. Kauzman, *et al.*, THIS JOURNAL, **75**, 5139 (1953).

(4) V. D. Hospelhorn, B. Cross and E. V. Jensen, *ibid.*, **76**, 2827 (1954).

(5) V. D. Hospelhorn and E. V. Jensen, *ibid.*, **76**, 2830 (1954).

(6) D. R. Goddard and L. Michaelis, *J. Biol. Chem.*, **106**, 605 (1934).

(7) T. Bersin and J. Steudel, *Ber.*, **71B**, 1015 (1938).

(8) G. Gorin, G. Dougherty and A. V. Tobolsky, THIS JOURNAL, **71**, 2551 (1949).

or their derivatives have been isolated by various authors.[9-12]

Denoting TSST, RSSR and GSSG as the oxidized forms of thioglycolic acid, cysteine and glutathione and TSH, RSH and GSH as their reduced forms, we have determined the equilibrium constant of the reaction

$$\text{RSSR} + 2\text{GSH(2TSH)} \rightleftarrows \text{GSSG(TSST)} + 2\text{RSH} \quad (1)$$

$$K_1 = \frac{[\text{GSSG}][\text{RSH}]^2}{[\text{RSSR}][\text{GSH}]^2}$$

Interpretation of the experimental data was complicated by the formation of mixed disulfides and equation 1 represents the over-all reaction of the following two steps

$$\text{RSSR} + \text{GSH(TSH)} \rightleftarrows \text{RSSG(RSST)} + \text{RSH} \quad (2)$$

$$K_2 = \frac{[\text{RSSG}][\text{RSH}]}{[\text{RSSR}][\text{GSH}]}$$

$$\text{RSSG(RSST)} + \text{GSH(TSH)} \rightleftarrows \text{GSSG(TSST)} + \text{RSH} \quad (3)$$

$$K_3 = \frac{[\text{GSSG}][\text{RSH}]}{[\text{RSSG}][\text{GSH}]}$$

$$K_1 = K_2 \times K_3.$$

In order to calculate the constants from the experimental data it was necessary to know the equilibrium constant of mixed disulfide formation

$$\text{RSSR} + \text{GSSG(TSST)} \rightleftarrows 2\text{RSSG(2RSST)} \quad (4)$$

$$K_4 = \frac{[\text{RSSG}]^2}{[\text{RSSR}][\text{GSSG}]}$$

In these studies use was made of the fact that cystine is only slightly soluble in the *p*H range between 5 and 7. The solubility of cystine is increased in the presence of freely soluble disulfides or thiol compounds. From the increase of the solubility of cystine the amount reacted with other constituents in the solution was found. The results were substantiated by a polarographic study which, although less accurate than the solubility method, also reveals some of the polarographic characteristics of RSSG.

Materials.—Cystine was a C.P. reagent grade product from Merck. Cysteine which was used in the form of its hydrochloride was a C.P. product from Pfanstiehl. Glutathione in the reduced state was also a product from Pfanstiehl; it is better than 90% pure as determined by titration with mercury.[13] Thioglycolic acid was an Eimer and Amend "pure" product which was purified by vacuum distillation and then found 99.8% pure by iodometric titration.[14] Stock solutions of the thiol compounds which were prepared in air-free water were 0.02 *M* in sulfhydryl. Oxidized glutathione and dithiodiglycolic acid were prepared by methods[14,15] described previously. The stock solutions of GSSG and TSST were 0.02 *M* and 0.01 *M* in disulfide, respectively. They were analyzed for disulfide by amperometric mercurimetric titration.[16] All the other chemicals used were commercial C.P. reagent grade products.

Experimental

Stability Measurement of Cystine in the Presence of Disulfide or Thiol Compounds.—Solubility measurements were carried out in narrow-mouth screw cap bottles of about 200-ml. capacity. Into the metal caps of the bottles was placed a self-sealing Buna-N gasket. Small holes in the cap provided access to the interior of the bottle through the gasket by means of a hypodermic needle which allowed the withdrawal of samples by means of a syringe. About 0.3 g. of solid cystine was introduced into the bottles and air-free phosphate or phosphate-citrate buffer containing a given concentration of glutathione (GSSG or GSH) or of thioglycolic acid (TSST or TSH) added. After complete removal of air the bottles were rotated at 25 ± 0.1° in a water thermostat. The time of attainment of equilibrium under the conditions of rotation depends on the *p*H, concentration of the disulfide or thiol compound in solution, and on the temperature. The interaction between cystine and disulfide was always carried out in the presence of a trace (usually 2×10^{-5} *M*) of cysteine which acts as a catalyst.[11] The mixtures investigated required from 70 hours to 70 days to attain equilibrium. The rotation was continued until the disulfide content of the solution had become constant.

After a given period of rotation the bottles were placed in vertical position in the thermostat until the excess of solid cystine had settled (about 12 hours). Appropriate volumes of the clear supernatant solution were then withdrawn and analyzed for disulfide by amperometric mercurimetric titration.[16] In separate blank experiments solutions of cysteine, glutathione and thioglycolic acid (both in the oxidized and reduced state) were kept in buffer solutions at *p*H 5 to 9 over periods of time required in the equilibrium experiments to attain equilibrium and analyzed for disulfide and sulfhydryl. The blank experiments indicated that none of the disulfide or thiol compounds undergoes decomposition during the time of equilibration of the reaction mixture.

Polarographic Procedure.—In a polarographic study of mixtures initially composed of GSSG and RSH or of RSSR and GSH 50 ml. of air-free mixtures of GSSG(RSSR) of initial concentrations of 10^{-3} to 2×10^{-3} *M* and RSH(GSH) (initially 2×10^{-3} to 10^{-2} *M*) in a borax buffer (0.05 *M* borax) was placed in bottles similar to those used in the solubility experiments. After various periods of time (up to 60 hours) samples were withdrawn from the bottles which were kept in a water thermostat at 25 ± 0.1°. The samples were introduced into the proper volume of an acetate buffer at *p*H 3 which was 1.75×10^{-4} *M* in thymol and polarographed under conditions suitable for the determination of GSSG and RSSR in mixtures.[17] The polarograms were taken with a Heyrovsky type Sargent polarograph Model XII. All potentials refer to the saturated calomel electrode. The characteristics of the capillary were $m = 2.02$ mg. sec.$^{-1}$ and $t = 3.83$ sec. at open circuit. The height of the mercury column was 80 cm.

The *p*H of the solutions was measured with a Beckman type H2 *p*H meter with a general purpose glass electrode 1190-80.

Results

Solubility Method. System RSSR + GSSG; Saturated with RSSR.—In order to ascertain that the increased solubility of cystine in the presence of glutathione is due to an exchange reaction, the solubility was also determined under comparable conditions in the presence of glycylglycine which does not contain disulfide or thiol. In a phosphate buffer of *p*H 6.95 containing 4×10^{-3} *M* glycylglycine the solubility of cystine at 25° was found to be 8.4×10^{-4} *M* as compared to a solubility of 7.8×10^{-4} *M* in the same buffer but in the absence of glycylglycine.

From the total disulfide content of mixtures obtained by shaking solid cystine in buffered solutions of oxidized glutathione and from the solubility of cystine in the buffer alone the equilibrium constant

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K_4 of reaction (4) was calculated as follows. Concentrations are given in mole per liter.

A is initial concn. of GSSG

s is solubility of RSSR in absence of GSSG (detd. in separate expt.)

T is total disulfide content at equilibrium

$$T = [\text{RSSG}] + [\text{RSSR}] + [\text{GSSG}]; \qquad T - s = [\text{RSSG}] + [\text{GSSG}]$$

$$A = [\text{GSSG}] + \frac{[\text{RSSG}]}{2}; \quad [\text{GSSG}] = 2A - T + s; \qquad [\text{RSSG}] = 2(T - s - A)$$

$$K_4 = \frac{[\text{RSSG}]^2}{[\text{RSSR}][\text{GSSG}]} = \frac{4(T - s - A)^2}{s(2A - T - s)}$$

The results are given in Table I.

TABLE I

EQUILIBRIUM CONSTANT K_4 OF THE RSSR–GSSG SYSTEM AT 25°

pH	Ionic strength (μ)	Initial concn., of GSSG, $M \times 10^3$	Total disulfide in[a] equilibrium mixture, $M \times 10^3$	K_4
5.90	0.08	1.0	2.34	3.7
5.90	.08	2.0	3.59	2.8
6.00	.4	4.0	6.16	2.6
6.95	.15	0.25	1.22	3.1
6.95	.15	0.50	1.62	3.7
6.98	.15	1.00	2.34	3.7
7.00	.15	2.00	3.51	2.2
7.02	.15	3.00	4.72	2.1
7.70	.19	0.50	1.93	3.0
7.70	.19	1.00	2.66	3.0
7.70	.19	2.00	3.94	2.4
			Av.	3.0

[a] Solubility of RSSR in phosphate buffer of pH 5.9 to 7.0 (μ = 0.08 to 0.15) is 7.8×10^{-4} M, in phosphate buffer of pH 7.7 (μ = 0.19) $s = 1.08 \times 10^{-3}$ M and in phosphate or phosphate–citrate buffer of pH 6 (μ = 0.40) $s = 9.0 \times 10^{-4}$ M. Time of equilibration: 70–80 hours at pH 7.7, 200–240 hours at pH 6.

System RSSR–GSH; Saturated with RSSR.—From the constant K_4 and from solubility data of cystine in reduced glutathione the constants K_2 and K_3 of equations 2 and 3 can now be calculated as follows. B is initial concentration of GSH. s is solubility of RSSR in absence of GSH. a and b are equilibrium concentrations of GSSG and RSSG, respectively. T_1 is total disulfide concentration in equilibrium mixture.

From equation 4

$$b = \sqrt{K_4 a s}$$

$$T_1 = [\text{RSSR}] + [\text{GSSG}] + [\text{RSSG}] = s + a + \sqrt{K_4 a s}$$

The equilibrium concentration (a) of oxidized glutathione can be calculated from the quadratic equation

$$a^2 - a(2T_1 - 2s + K_4 s) + (T_1 - s)^2 = 0 \quad (5)$$

and the concentration of RSSG is $b = T_1 - s - a$.

From equations 2 and 3 it is seen that the equilibrium concentrations of cysteine [RSH] and of reduced glutathione [GSH] are

$$[\text{RSH}] = 2[\text{GSSG}] + [\text{GSSR}] = 2a + b = T_1 - s + a$$

$$[\text{GSH}] = B - 2a - b = B - T_1 + s - a$$

The constants K_2, K_3 and the over-all constant K_1 can now be expressed as

$$K_2 = \frac{(T_1 - s)^2 - a^2}{(B - T_1 + s - a)s}, \qquad K_3 = \frac{a(a + T_1 - s)}{(T_1 - s - a)(B - T_1 + s - a)}$$

while $K_1 = K_2 K_3$.

An example of the calculation of the four constants follows.

Calculation of K_4.—0.3 g. of solid cystine was shaken at 25° with 50 ml. of a solution which originally was 2.5×10^{-4} M (A) in GSSG, 2×10^{-5} M in RSH (as catalyst), 0.027 M in NaH_2PO_4 and 0.04 M in Na_2HPO_4 (pH 6.95, μ = 0.15). Equilibrium was attained after about 70 hours; total disulfide concentration in equilibrium mixture: $T = 1.22 \times 10^{-3}$ M.

$$[\text{RSSR}] = s = 7.8 \times 10^{-4}\ M; \qquad [\text{RSSG}] = 2(T - A - s) = 3.8 \times 10^{-4}\ M$$

$$[\text{GSSG}] = A - (T - A - s) = 6 \times 10^{-5}\ M$$

$$K_4 = 3.1$$

Calculation of K_2, K_3 and K_1.—0.3 g. of solid cystine was shaken at 25° in the same buffer as above in the presence of 4×10^{-3} M (B) reduced glutathione (pH 6.90, μ = 0.15); sum of total disulfide (T_1) and sulfhydryl determined analytically: 1.078×10^{-2} M.

$$T_1 = 1.078 \times 10^{-2} - 4.0 \times 10^{-3} = 6.78 \times 10^{-3}\ M$$

$$[\text{RSSR}] = s = 7.8 \times 10^{-4}\ M$$

$$[\text{GSSG}] = a(\text{calcd. from eq. 5}) = 7.1 \times 10^{-4}\ M$$

$$[\text{RSSG}] = 1.29 \times 10^{-3}\ M, \quad [\text{RSH}] = 2.71 \times 10^{-3}\ M, \quad [\text{GSH}] = 1.29 \times 10^{-3}\ M$$

$$K_2 = 3.5, \ K_3 = 1.2, \ K_1 = 4.2$$

Experimental data and the constants K_2 and K_3 are given in Table II. From the average values of K_2 and K_3 the value of K_1 for the over-all reaction (1) is calculated to be 2.8 at 25°.

TABLE II

EQUILIBRIUM CONSTANTS K_2 AND K_3 FOR REACTIONS OF RSSR WITH GSH AT 25°

pH	μ	Initial concn. of GHS, $M \times 10^3$	Total disulfide in equilibrium mixture, $M \times 10^3$	K_2	K_3
5.65	0.08	4.00	2.75	3.2	1.1
5.77	.08	2.00	1.93	2.9	0.97
5.80	.08	1.00	1.41	2.0	0.71
5.70	.40	4.00	2.82	2.5	1.0
6.00	.40	8.00	4.13	2.8	1.1
6.02	.40	4.00	2.84	2.7	1.0
6.95	.15	1.00	1.43	2.5	1.1
6.93	.15	2.00	1.93	3.0	1.0
6.90	.15	4.00	2.78	3.5	1.2
6.75	.15	8.00	3.98	3.1	1.0
			Av.	2.8	1.0

The solubility of RSSR in GSSG solutions of pH 5.80 (phosphate buffer, μ = 0.08) was also measured at 50° and the average value of K_4 was found to be 6.1. From the K_4-values at 25 and 50° the heat of reaction of (ΔH_4) of reaction (4) was calculated to be +5420 cal. Considering the small value of ΔH_4 and the small changes in free energy in reactions (2) and (3) the ΔH values for these reactions are also expected to be small; therefore K_2 and K_3 were not determined at 50°.

Systems RSSR + TSST and RSSR + TSH.—Experiments on the reaction between cystine and dithiodiglycolic acid, carried out in the same way as described for oxidized glutathione, indicated

that media of pH 6 and 7 are not suitable for the determination of the equilibrium constant.

While mixtures of GSSG and RSSR do not give evidence of sulfhydryl formation under the conditions of our experiments the RSSR–TSST system undergoes decomposition to form sulfhydryl. From the anodic diffusion current measured polarographically in a reaction mixture obtained after shaking solid RSSR with 10^{-3} M TSST at pH 7.7 for 19 days it was concluded that the sulfhydryl concentration in the solution was 9.2×10^{-4} M. The same result was also obtained in the presence of 0.1 M Versene, indicating that traces of metals which might possibly be present as impurities in the mixture are not the cause of this decomposition. A 2×10^{-3} M TSST solution (phosphate buffer pH 7.7) in the absence of RSSR gave after 21 days an anodic current which corresponded to only 5.5×10^{-5} M sulfhydryl, while cystine did not yield any sulfhydryl under these conditions. The above facts indicate that the mixed disulfide RSST is considerably less stable than TSST and TSST is less stable than RSSR. Apparently the disulfide group in RSST undergoes hydrolytic fission with formation of a thiol compound. Symmetrical disulfides undergo such fission in much more alkaline media.[17] The rate of fission of RSST decreases markedly with decreasing pH. At pH 5 the extent of disulfide splitting is small enough to allow the determination of the equilibrium constant of the cystine–dithiodiglycolic acid system even though the time required for attainment of equilibrium is considerably larger at this low pH than at pH 7.7. The amount of sulfhydryl in the equilibrium mixture was determined by amperometric mercurimetric titration (in the absence of sulfite) and also polarographically. From the titration results and the anodic current the diffusion coefficient of the thiol compound was calculated to be 1.2×10^{-5} cm.^{2}/sec. which compares favorably with the value for thioglycolic acid (TSH^-) and cysteine ($RSH^{\pm}$) of 1.13×10^{-5} [14] and 1.06×10^{-5} [17] cm.^{2}/sec., respectively. The polarographic method does not indicate whether the anodic wave is due mainly to cysteine or to thioglycolic acid because of the equality of the diffusion coefficients.

At pH 5 the carboxyl groups of RSST can be considered to be ionized while the amino group is positively charged at this pH. Considering the proximity of the negative carboxyl group to the sulfur of the thioglycolic acid residue and that of the positive amino group to the sulfur of the cystine residue in the mixed disulfide it appears likely that hydrolytic fission of the mixed disulfide proceeds according to the equations

$$2RSST + 2H_2O \rightleftarrows 2TSH + 2RSOH \quad (6A)$$

$$2RSOH \rightleftarrows RSH + RSOOH \quad (6B)$$

$$2RSST + 2H_2O \rightleftarrows 2TSH + RSH + RSOOH \quad (6)$$

Our assumption that TSH is formed in reaction (6A) and not RSH has only a slight effect on the value of K_{4T} (Table III) under the experimental conditions. The assumption also finds some support in the fact that the thiol group in TSH is much less dissociated than that in RSH, the corresponding K-values being 2.8×10^{-11} and 2.2×10^{-9}. The sulfenic acid RSOH is extremely unstable and rapidly undergoes dismutation to form sulfinic acid RSOOH. The concentration of sulfhydryl formed in reaction mixtures was determined after various periods of time and found to attain constant values which are listed in Table III. For instance on shaking solid RSSR in a solution of 4×10^{-3} M TSST at pH 4.93 the sulfhydryl content was found to be 2×10^{-4} M after 30 days and remained unchanged on further shaking over a period of 80 days. The constancy of the sulfhydryl concentration in these solutions is indicative that reaction (6) is an equilibrium reaction. An attempt was made to calculate the over-all equilibrium constant

$$K_T = \frac{[TSH]^2[RSH][RSOOH]}{[RSST]^2} = \frac{\left(\frac{2\Sigma SH}{3}\right)^2 \times \left(\frac{\Sigma SH}{3}\right)^2}{[RSST]^2}$$

of this reaction from the total sulfhydryl (ΣSH) and disulfide (T) content of the equilibrium mixtures given in Table III. The values of K_T varied from 2×10^{-12}, 8×10^{-12} and 4×10^{-11} for mixtures which were originally 2, 4 and 8 $\times 10^{-3}$ M in TSST. Although the variations are too large to allow an exact estimate of the constant, it is evident that the equilibrium of reaction (6) lies far to the left.

TABLE III

EQUILIBRIUM CONSTANTS OF THE CYSTINE–THIOGLYCOLIC ACID SYSTEM AT 25°

The pH of the reaction mixtures was 5 ± 0.1 (μ = 0.3).

Time of equilibration, days	Initial concn. TSST, $M \times 10^3$ (A_T)	Total disulfide in[a] equilibrium mixture (T), $M \times 10^3$	Sulfhydryl formed during reaction (ΣSH), $M \times 10^3$	K_{4T}	K_{3T}	K_{2T}
70	2.0	3.87	0.11	5.4	...	...
50	4.0	6.33	.20	4.9	...	...
27	8.0	11.08	.37	4.9	...	...
	TSH, $M \times 10^3$					
20	2.0	2.02	Negligible	..	(2.8)[b]	(0.6)[b]
30	4.0	3.01	Negligible	..	4.3	0.8
40	8.0	4.35	Negligible	..	4.0	0.8
				Av.	5.1	4.1	0.8

[a] Solubility of cystine in buffer (0.049 M citric acid, 0.103 M Na_2HPO_4) $s = 0.83 \times 10^{-3}$ M. [b] The figures in brackets have not been considered in the calculation of the average values of the constants.

In the following calculations and table all constants referring to the cystine–thioglycolic acid systems are denoted by the index T.

From the disulfide and sulfhydryl content of mixtures obtained after shaking RSSR in TSST solutions the equilibrium constant K_{4T} was calculated as follows.

Denoting the solubility of RSSR as s, the total disulfide and sulfhydryl concentration of the equilibrium mixture as T and ΣSH, respectively, and the initial concentration of TSST as A_T

$$T - s = [TSST] + [RSST]$$

$$A_T = [TSST] + \frac{[RSST]}{2} + \frac{[TSH]}{2}$$

$$[RSST] = 2(T - s - A_T) + [TSH]$$

$$[TSST] = 2A_T + s - T - [TSH]$$

$$K_{4T} = \frac{[RSST]^2}{[TSST][RSST]} = \frac{(2(T - s - A_T) + [TSH])^2}{(2A_T + s - T - [TSH])s}$$

from equation 6

$$[TSH] = 2/3\Sigma SH$$

In the reactions between TSH and RSSR the extent of fission of RSST is extremely small. This fission is neglected in the calculation of the constants K_{2T} and K_{3T} which were found in the same way as described for the system RSSR + GSH. Experimental results and the constants are listed in Table III. The value of $K_{1T} = K_{2T} \times K_{3T}$ is calculated to be 3.3.

The over-all constant K_5 of the reaction between oxidized glutathione and thioglycolic acid

$$GSSG + 2TSH \rightleftarrows 2GSH + TSST \qquad (7)$$

is calculated from K_1 and K_{1T}.

$$K_5 = \frac{[TSST]\,[GSH]^2}{[GSSG]\,[TSH]^2} = \frac{K_{1T}}{K_1} = 1.7$$

It is seen that the values of all constants are of the same order of magnitude and do not deviate much from unity. This accounts for the fact that Bersin and Steudel[7] could find a value of K of one in the RSSR–TSH system even though they did not consider the appreciable formation of mixed disulfide.

Polarographic Measurements.—The formation of a mixed disulfide RSSG is clearly indicated by the difference in polarograms of unreacted and equilibrated mixtures of RSSR and GSSG. When dealing with an unreacted mixture of RSSR and GSSG the diffusion reduction current of GSSG can be measured satisfactorily in the presence of an appropriate amount of thymol[17] (see curve A in Fig. 1). The polarogram of an equilibrated mixture of RSSR and GSSG (curve C, Fig. 1) reveals that the RSSG wave overlaps with the RSSR wave and is more drawn out than the RSSR wave, indicating that the polarographic reduction of RSSG is even less reversible than that of RSSR. A comparison of curves B and C in Fig. 1 illustrates the catalytic effect of a trace of cysteine upon the rate of establishment of equilibrium. The height of the cystine wave measured at −1.0 volt in polarogram B corresponds to a cystine concentration in the reaction mixture of 8.6×10^{-4} M which is only slightly greater than the solubility (7.8×10^{-4} M) in a phosphate buffer of pH 7. Thus in 60 hours hardly any reaction between RSSR and GSSG had occurred. On the other hand, in the presence of 2×10^{-5} M cysteine equilibrium was established. The height of the wave at −1.0 volt corresponds to the sum of the diffusion currents of RSSR and RSSG. Expressed as cystine the wave height corresponds to a concentration of 1.6×10^{-3} M.

Mixtures of oxidized glutathione and cysteine and of cystine and reduced glutathione of various disulfide and thiol concentrations in a 0.05 M borax buffer of pH 9.2 were allowed to stand at 25° for various lengths of time (up to 60 hours) and then analyzed polarographically in an acetate buffer of pH 3 in the presence of thymol. The equilibrium concentration of GSSG was calculated from the diffusion current at −0.4 volt. From the known initial disulfide concentration (either as GSSG or RSSR) the sum of the concentrations of RSSR and RSSG was found. Using a value of K_4 of 3.0, [RSSR] and [RSSG] were calculated in the equilibrium

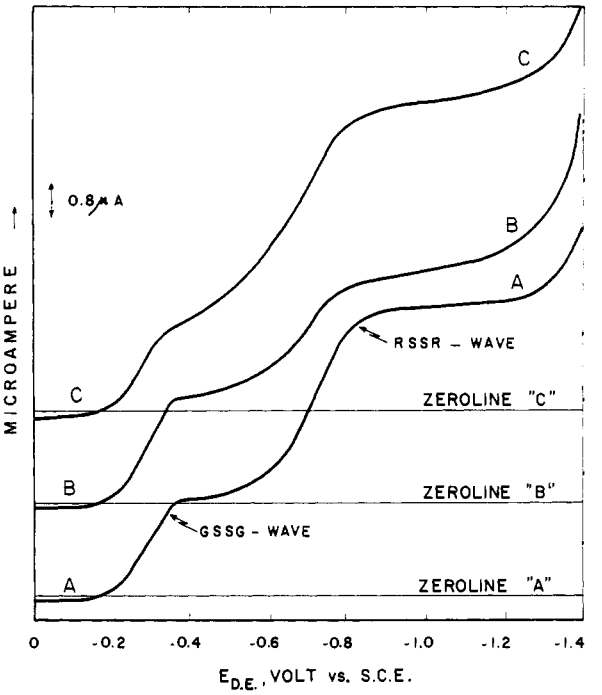

Fig. 1.—Current-voltage curves of: (A) 4.5×10^{-4} M GSSG and 7×10^{-4} M RSSR in 20 ml. acetate buffer pH 3, 1.5×10^{-4} M in thymol. Polarogram taken immediately after mixing. (B) 10 ml. solution of 10^{-3} M GSSG in phosphate buffer pH 7 after 60 hours shaking with solid RSSR. Polarogram taken in 20 ml. acetate buffer as in (A). (C) 10 ml. solution of 10^{-3} M GSSG and 2×10^{-5} M RSH in phosphate buffer pH 7 after 60 hours shaking with solid RSSR. Polarogram taken in 20 ml. acetate buffer as in (A).

mixture. Since equilibrium was attained at pH 9.2 the concentrations of the charge types of the thiol compounds had to be considered. From the total GSH and RSH concentrations and from the intrinsic dissociation constants[18] the concentrations of the species with the charged sulfhydryl group ($GS^{\pm}$ + $GS^{=}$ and $RS^{\pm}$ + RS^{-}) were calculated. Reactions involving charged sulfhydryl groups are considered in the discussion. From the concentrations in the equilibrium mixture the over-all constant K_1' of reaction (9) between RSSR and GS^- was calculated. Since the polarographic method is considerably less accurate than the solubility method, we report only the average value of K_1' of 11 ± 5 obtained in five experiments in which the initial concentrations varied widely. This is in fair agreement with the value of 11 (Table IV) derived from solubility measurements. From the diffusion current at −1.0 volt, from the known diffusion coefficient of cystine and from the concentrations of RSSR and RSSG in the equilibrium mixtures the diffusion coefficient of the mixed disulfide was calculated to be 5.9×10^{-6} cm.^{2} sec.$^{-1}$ which is of the same order of magnitude as those of GSSG (4.5×10^{-6})[15] and RSSR (5.3×10^{-6}).[15]

Discussion

In the calculations of the constants from solubility measurements the charge types of the acids have

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TABLE IV

COMPARISON OF THE EQUILIBRIUM CONSTANTS AND CHANGES IN FREE ENERGY AT 25° OF REACTIONS (4), (3), (2), (1), (7) (9A), (9C) AND (9) OF CYSTINE WITH GLUTATHIONE AND WITH THIOGLYCOLIC ACID

ΔF in cal. potential of reaction (1), E_1^0 in volt.

	K_4	K_3	K_2	K_1	K_5	K_3'	K_2'	K_1'	K_5'
Glutathione	3.0	1.0	2.8	2.8		2.0	5.6	11.2	
					1.7				2600
Thioglycolic acid	5.1	4.1	0.8	3.3		320	62	20000	

	ΔF_4	ΔF_3	ΔF_2	ΔF_1	ΔF_5	$\Delta F_3'$	$\Delta F_2'$	$\Delta F_1'$	$\Delta F_5'$	E_1^0
Glutathione	+650	0	+610	+610		+ 410	+1020	+1430		−0.013
					+310				+4660	
Thioglycolic acid	+960	+840	−130	+710		+3420	+2440	+5870		− .015

not been considered From the dissociation constants of the carboxyl groups in RSSR,[19] RSH,[19] GSSG,[20] GSH[21] and TSH[14] it is found that the carboxyl in these compounds occurs only in the charged form in the pH range of our experiments. From K_1[22] and K_2[22] of TSST it is calculated that about 76% is present as $^-$TSST$^-$ and most of the remainder as TSST$^-$ at the pH of our experiments (pH 5).

The dissociation of the amino group of cystine, oxidized glutathione and of the mercaptides varies in the pH range investigated. For instance with cystine at pH 7.7 66.5 and 33.4% are present as $^{\pm}$RSSR$^{\pm}$ and $^-$RSSR$^{\pm}$, respectively, while at pH 5.9 more than 99% is in the form of $^{\pm}$RSSR$^{\pm}$. The fact that the constants are the same (Tables I and II) between pH 7.7 and 5 indicates that the variation of the charge species of the disulfides between pH 7.7 and 5 does not affect the equilibria. The thiol group of the mercaptans is practically undissociated under all conditions of our solubility measurements and the predominant species of cysteine, reduced glutathione and thioglycolic acid are therefore RSH$^{\pm}$, GSH$^{\pm}$ and TSH$^-$, respectively.

The fact that the exchange reactions between two disulfides as well as between a disulfide and a mercaptan are considerably accelerated by an increase in pH suggests that the active species in these reactions is the mercaptide ion and not the undissociated sulfhydryl group. The interaction between two disulfides can thus be presented in a way similar to that suggested by Calvin.[23]

$$\text{RSSR} + \text{GS}^- \rightleftarrows \text{RS}^- + \text{RSSG} \qquad (8A)$$

$$\text{GSSG} + \text{RS}^- \rightleftarrows \text{GS}^- + \text{RSSG} \qquad (8B)$$

$$\text{RSSR} + \text{GSSG} \rightleftarrows 2\text{RSSG} \qquad (8)$$

This mechanism also explains that a trace of sulfhydryl strongly catalyzes the interaction between two disulfides which otherwise was found to be very sluggish under the conditions of our experiments.

The interaction between disulfide and mercaptide is presented by the following sequence of reactions: (the charge designations of amino and carboxyl groups are omitted)

$$\begin{array}{c} \text{GSH} \\ \uparrow\downarrow \\ \text{RSSR} + \text{GS}^- \rightleftarrows \text{RSSG} + \text{RS}^- \\ + \\ \text{H}^+ \end{array} \qquad (9A)$$

$$\text{RS}^- + \text{GSH} \rightleftarrows \text{RSH} + \text{GS}^- \qquad (9B)$$

$$\text{RSSG} + \text{GS}^- \rightleftarrows \text{RS}^- + \text{GSSG} \qquad (9C)$$

$$\text{RSSR} + 2\text{GS}^- \rightleftarrows \text{GSSG} + 2\text{RS}^- \qquad (9)$$

In the pH range of our solubility measurements (pH 5–7.7) the equilibrium constants K_2', K_3' and K_1' of reactions (9A), (9C) and (9) can be calculated in the following simplified way

$$K_2' = \frac{[\text{RSSG}][\text{RS}^-]}{[\text{RSSR}][\text{GS}^-]} = K_2 \frac{K_{\text{AR}}}{K_{\text{AG}}}$$

$$K_3' = \frac{[\text{GSSG}][\text{RS}^-]}{[\text{RSSG}][\text{GS}^-]} = K_3 \frac{K_{\text{AR}}}{K_{\text{AG}}}$$

$$K_1' = \frac{[\text{GSSG}][\text{RS}^-]^2}{[\text{RSSR}][\text{GS}^-]^2} = K_2' \times K$$

where K_{AR} (2.2×10^{-9})[18] and K_{AG} (1.1×10^{-9})[18] are the intrinsic dissociation constants K_{A} of the sulfhydryl group in RSH$^{\pm}$ and GSH$^{\pm}$ while K_2 and K_3 are the equilibrium constants of reactions (2) and (3). The corresponding constants for the RSSR–TS$^-$ system are calculated from the constant K_{AT} (2.8×10^{-11})[14] of TSH$^-$ and the constants $K_{2\text{T}}$ and $K_{3\text{T}}$.

A summary of the constants and of the changes in free energy of the equilibrium reactions involving cystine, glutathione and thioglycolic acid is given in Table IV. Also included in Table IV are the values of the e.m.f. E^0 of the cell corresponding to E^0 of reaction (1) for both GSH (−0.013 volt) and TSH (−0.015 volt). These values combined with the known oxidation potential of the cystine–cysteine system $^{\pm}$RSSR$^{\pm}$ + 2H$^+$ + 2e$^-$ ⇄ 2RSH$^{\pm}$, E^0 = +0.08 volt *vs.* N.H.E.[12] yield standard oxidation potentials of the GSSG–GSH and TSST–TSH

$$^{\pm}\text{GSSG}^{\pm} + 2\text{H}^+ + 2\text{e} \rightleftarrows 2\text{GSH}^{\pm} \qquad (10)$$

$$^-\text{TSST}^- + 2\text{H}^+ + 2\text{e} \rightleftarrows 2\text{TSH}^- \qquad (11)$$

systems of +0.093 and +0.095 volt *vs.* N.H.E., respectively. The value of E^0 for glutathione compares favorably with that (+0.068 volt *vs.* N.H.E.) reported by Ghosh and Ganguli.[24] No reliable E^0-value for the TSST–TSH system has yet been reported

It appears that the oxidation potentials of cys-

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(21) N. C. Li, O. Gawron and G. Bascuas, THIS JOURNAL, **76**, 225 (1954).

(22) B. Adell, *Z. physik. Chem.*, **A185**, 161 (1939).

(23) M. Calvin, Abstract, Symposium on Glutathine, Ridgefield Conn., Nov. 20–21, 1953.

(24) J. C. Ghosh and S. C. Ganguli, *Biochem. Z.*, **279**, 296 (1935).

teine, glutathione and thioglycolic acid are practically the same.

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[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE UNIVERSITY OF MINNESOTA]

The Polarographic Prewaves of Cystine (RSSR) and Dithiodiglycolic Acid (TSST) and the Oxidation Potentials of the Systems RSSR–RSH and TSST–TSH

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RECEIVED MAY 4, 1955

The two-step reduction wave of cystine has been reinterpreted. Evidence is given that the prewave is determined by the over-all reversible reaction RSSR + $2e^-$ + $2H^+$ ⇄ 2RSH where RSSR and RSH are cystine and cysteine. The prewave is found to be kinetic in nature. The electrode reaction is catalyzed by mercury which reacts with cystine to form mercury cysteinate, the latter being reduced rapidly to mercury and cysteine. The potential of the cystine–cysteine system at the dropping mercury electrode was found to correspond to the true oxidation potential. The same was found true for the thioglycolic–dithiodiglycolic acid couple but not for the glutathione system.

No satisfactory explanation has been offered for the two-step waves observed with cystine at the dropping mercury electrode in the *p*H range between 3 and 9.2.[2] The first and steeper part of the double wave, called the "prewave," has been attributed[2] to a mixed-potential effect. In the present paper it is shown that this interpretation is inadequate. Conclusive evidence is presented that the potential of the cystine–cysteine system measured at the dropping electrode after correction for the residual current corresponds to that of the couple

$$RSSR + 2H^+ + 2e^- \rightleftarrows 2RSH \qquad (1)$$

in which RSSR denotes cystine and RSH cysteine. At a platinum electrode this reaction is highly irreversible because of the slow rate of electroreduction of cystine. This reaction is catalyzed at the mercury surface which reacts rapidly with cystine according to

$$RSSR + Hg \rightleftarrows Hg(RS)_2 \qquad (2)$$

This reaction may occur in steps, *e.g.*, as

$$RSSR + Hg \rightleftarrows HgRS + RS\cdot \longrightarrow Hg(RS)_2 \qquad (2a)$$

but the experimental results do not allow further insight into the mechanism of this reaction. The mercuric cysteinate formed is rapidly reduced at the electrode

$$Hg(RS)_2 + 2e + 2H^+ \rightleftarrows Hg + 2RSH \qquad (3)$$

Thus equation 1 represents the over-all reaction which is the sum of equations 2 and 3. According to this interpretation the prewave of cystine should be kinetic in nature and the analysis of the wave should correspond to that of the reversible reaction (1). The effect of various factors upon the height of the prewave and the characteristics of the entire cystine wave are described in the experimental part.

The conclusion is arrived at that the potential of the cystine–cysteine system measured at the dropping mercury electrode corresponds to the oxidation potential of the system. In this connection the potentials of the thioglycolic–dithiodiglycolic acid and reduced and oxidized glutathione systems are briefly discussed.

(1) Tohoku University, Sendai, Japan.

(2) I. M. Kolthoff and C. Barnum, THIS JOURNAL, **63**, 520 (1941).

Materials.—Thioglycolic acid and dithiodiglycolic acid were prepared and purified by methods described previously.[3] All other chemicals were C.P. reagent grade products.

Experimental

Current–voltage curves were measured with the manual apparatus and circuit described by Lingane and Kolthoff[4] and automatically with a Heyrovsky type self-recording Sargent polarograph, Model XII. All potentials were measured against the saturated calomel electrode (S.C.E.). Oxygen was removed from the solution in the cell with 99.99% pure Linde nitrogen. The characteristics of the capillary were m = 2.53 mg. $sec.^{-1}$, t = 3.51 sec. (open circuit) at a height of the mercury column of 60 cm. The *p*H was measured with a Beckman Model H-2 *p*H meter.

Results

Characteristics of the Cystine Wave. Effect of *p*H.—The effect of *p*H on the current–voltage curves of 5×10^{-4} M cystine solutions is illustrated in Fig. 1. From the curves which were taken between *p*H 3 and 9 it is seen that the potentials at which the waves are shifted to more negative values with increasing *p*H, the shift for the prewave being approximately 60 millivolt per *p*H unit. The height of the prewave is little affected by *p*H and attains a maximum value at *p*H 8 while the height of the total wave (diffusion current) is constant over the *p*H range investigated.

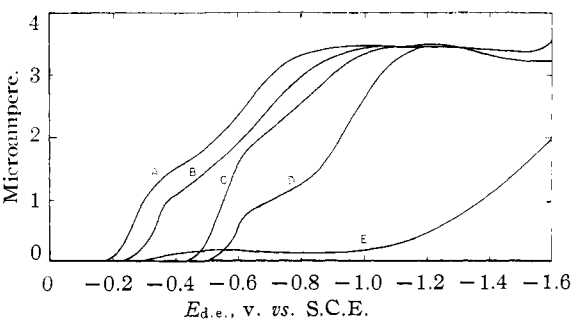

Fig. 1.—Current–voltage curves of 5×10^{-4} M RSSR at *p*H (A) 3.3, (B) 4.7, (C) 8.0, (D) 9.2 and of (E) 5×10^{-4} M TSST at *p*H 5.0.

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