configuration would lead to a correspondingly larger decrease in the stability of the naphthylazo compounds. The two dyes which contain an amino group in ortho position to the azo group again show a double band spectrum, probably as a result of the two possible paths of resonance, as described above. These compounds also show some phototropism (although their rate of reversal is also high), indicating that the hydrogen bond which is possible between the amino group and the azo nitrogen in these compounds is not as strong as that in dyes of Type III and V. The normal bathochromic effect of a methyl group is not discernible from the spectra of these two compounds.

The spectra of the four azo dyes derived from β-naphthol (Type V) are not affected by irradiation of this type, probably because of the existence of a strong hydrogen bond in these molecules, similar to that shown in Fig. 8. The strong displacement of the main absorption band toward longer wave lengths and the absence of a second band in the spectra of these compounds is consistent with this structure, since the formation of an additional chelate ring would be expected to cause a large bathochromic shift[24] and to provide a single preferred path for the resonance in the molecule.

Acknowledgment.—The authors express their appreciation to Miss Iva Halow who carried out a large part of the experimental work, and to the Bureau of Ships for financial support of the investigation.

(24) W. R. Brode and G. M. Wyman, THIS JOURNAL, **73**, 4267 (1951).

WASHINGTON 25, D. C.

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

Polarography of Glutathione

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The polarography of glutathione in the reduced (GSH) and oxidized form (GSSG) has been studied. Reduced glutathione gives two anodic waves at the dropping mercury electrode. The normal wave is well defined and corresponds to the formation of a mercurous compound (GSHg). The current-voltage curve in the *p*H region from 1 to 10.5 has been found to obey the equation for the reaction: $Hg + GSH \rightleftarrows GSHg + H^+ + e$. Normal diffusion currents have been observed with GSH over the entire *p*H range investigated. The diffusion coefficient of reduced glutathione is calculated to be 5.6×10^{-6} and 4.7×10^{-6} cm.^{2} sec.$^{-1}$ at *p*H 1 and 10.82, respectively, at an ionic strength 1 and at 25°. The characteristics of the second wave greatly depend upon the ionic strength of the medium. At an ionic strength of 1 it is fairly well defined and its height is of the same order of magnitude as that of the normal wave. It is suggested that the second wave corresponds to the formation of GSHg(II). Oxidized glutathione gives well defined reduction waves. Surface active substances like gelatin or thymol at low concentrations hardly affect the GSSG wave. Larger concentrations of thymol shift the waves to more negative potentials. This is accounted for by the electro-capillary behavior of thymol and GSSG. GSH is more capillary active at the dropping electrode than GSSG. The characteristics of the GSSG waves in the presence and absence of an excess of GSH are accounted for quantitatively by the sequence of reactions: (14) + (15) = (12) in which reaction (14) is the rate and potential determining step and equation (12) is the over-all reaction. The diffusion coefficient of oxidized glutathione at ionic strength 1 and *p*H 10.3 is calculated to be 4.5×10^{-6} cm.^{2} sec.$^{-1}$ at 25°.

A polarographic study of glutathione in the reduced (denoted as GSH) and oxidized form (denoted as GSSG) is described in this paper. The results are compared with those obtained with the amino acids cysteine and cystine.[1,2]

Catalytic polarographic waves obtained with glutathione have been described by Brdicka.[3] More recently Reiser,[4] Coulson, *et al.*,[5] and Tachi, *et al.*,[6] reported on diffusion-controlled current-voltage curves of glutathione.

Materials.—Glutathione in the reduced state was a Pfanstiehl product. The purity of this product was 99% as determined by titration with cupric copper.[7] Stock solutions 0.01 and 0.1 *M* in GSH were prepared in air-free water. Only freshly prepared stock solutions were used. A 10^{-2} *M* stock solution of oxidized glutathione (GSSG) was prepared by passing purified air through a 2×10^{-2} *M* GSH solution in an ammonia buffer (0.1 *M* in NH_4Cl, 0.1 *M* in NH_3) which contained a trace of copper (2×10^{-7} *M*) as a catalyst. The progress of the oxidation was followed polarographically. The air bubbling was continued until the GSH wave had disappeared. Toward the end of the reaction ammonia was driven out from the solution with air. The solution, which was stored in a refrigerator, was found to be stable for several months. Cysteine which was used in the form of its hydrochloride was a Pfanstiehl product. Cystine, C.P., was from Merck and Co., Inc. Stock solutions of cysteine and cystine were prepared in the same way as described in a previous paper.[8] All the other chemicals used were commercial C.P. reagent grade products.

Experimental Methods

Current-voltage curves were measured at 25.0 ± 0.1° with the manual apparatus and circuit described by Lingane and Kolthoff[9] and automatically with a Heyrovsky self-recording polarograph. All potentials were measured against the saturated calomel electrode (S.C.E.). Oxygen was removed from the solution in the cell with a stream of oxygen-free nitrogen which was purified by bubbling through vanadous sulfate.[10] During an experiment an atmosphere of nitrogen was maintained over the solution. Corrections were made for the residual current.

The characteristics of the capillary used were: m = 1.56 mg. sec.$^{-1}$, t = 4.82 sec. (open circuit); $m^{2/3}t^{1/6}$ = 1.748 mg.$^{2/3}$sec.$^{-1/2}$; h = 80 cm.

The *p*H was measured with a Beckman *p*H meter, Laboratory Model G. A glass electrode made of the usual 015 type electrode glass was used for solutions with *p*H below

(1) I. M. Kolthoff and C. Barnum, THIS JOURNAL, **62**, 3061 (1940).
(2) I. M. Kolthoff and C. Barnum, *ibid.*, **63**, 520 (1941).
(3) R. Brdicka, *Collection Czechoslov. Chem. Communs.*, **5**, 148 (1933).
(4) R. G. Reiser, *Univ. Pittsburg Bull.*, **40**, 220 (1944).
(5) D. M. Coulson, W. R. Crowell and S. L. Friess, *Anal. Chem.*, **22**, 525 (1950).
(6) I. Tachi and S. Koide, International Polarographic Congress in Prague I, p. 386 (1951).
(7) I. M. Kolthoff and W. Stricks, *Anal. Chem.*, **23**, 763 (1951).
(8) I. M. Kolthoff and W. Stricks, THIS JOURNAL, **72**, 1952 (1950).
(9) J. J. Lingane and I. M. Kolthoff, *ibid.*, **61**, 825 (1939).
(10) L. Meites and T. Meites, *Anal. Chem.*, **20**, 984 (1948).

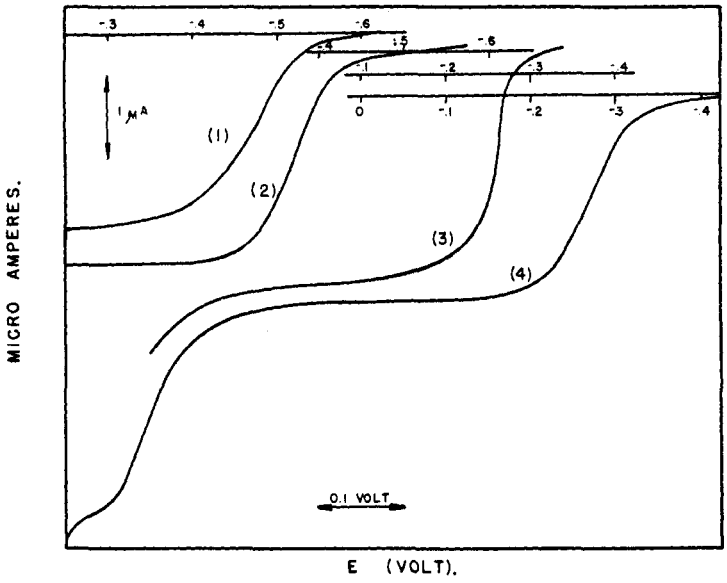

Fig. 1.—Polarograms of 10^{-3} M GSH in various buffers (μ 1): (1) phosphate, $NaNO_3$, pH 10.82; (2) borate, $NaNO_3$, pH 6.93; (3) acetate, $NaNO_3$, pH 5.13; (4) phosphate, $NaNO_3$, pH 5.22.

9.5 while measurements were made with the Beckman "General Purpose" glass electrode at pH above 9.5.

The ionic strength of the supporting electrolyte was adjusted by the addition of appropriate quantities of sodium nitrate or potassium chloride.

Results and Discussion

1. Reduced Glutathione (GSH).—Some 50 polarograms were taken with solutions of various concentrations in glutathione in buffers from pH 1 to 10.5. For the sake of brevity the results are not tabulated but in connection with the discussion essential data are presented in the form of graphs.

All GSH solutions gave a well-defined anodic wave and another relatively poorly defined second wave (see curve 4, Fig. 1) at potentials more positive than that where the normal diffusion current of GSH was observed. The shape of these waves was found to be affected by pH, nature of the buffer, and concentration of glutathione. In solutions with a pH considerably greater than 9 the normal wave becomes drawn out indicating an irreversible electrode process (compare curves 1 and 2 in Fig. 1). This was confirmed by analysis of the waves. A specific buffer effect can be seen from a comparison of the c–v curves obtained with an acetate and phosphate buffer of practically the same pH (5.13 and 5.22, respectively) and of the same ionic strength, as illustrated in curves 3 and 4 of Fig. 1. The analysis indicates that the electrode process is irreversible in acetate buffers. In ammonia buffers the half-wave potential is found to be more negative than that observed in phosphate or borate buffers of the same pH and ionic strength. In the latter two buffers as well as in dilute perchloric acid solutions reversible waves were obtained at glutathione concentrations of 10^{-3} M or less. At higher GSH concentrations the wave exhibits a flat maximum in borate, phosphate, ammonia or acetate buffers but has a normal appearance in dilute perchloric acid solutions.

Surface active substances like gelatin (0.005%) or thymol (saturated) have no effect on the shape of the anodic wave at various pH. Reduced glutathione markedly reduces the surface tension of mercury as is evident from the electrocapillary curves (drop time against applied potential) obtained in an ammonia buffer of pH 10.3 (μ = 1) and in 0.1 M perchloric acid (1 M in $NaNO_3$) (see Fig. 2), the effect being greater at higher pH. At pH 10.3 the electrocapillary maximum is shifted from −0.5 to about −0.65 v. (GSH is anion) while a shift from −0.6 to about −0.5 v. is found at pH 1.0 (GSH is cation). Figure 2 illustrates that the electrocapillary curves in the presence of GSH exhibit a discontinuity on both the positive and negative side of the half-wave potential (−0.502 and −0.028 v. at pH 10.3 and 1.0, respectively), indicating that the product of the anodic reaction (GSHg) as well as GSH are electrocapillary active. In fact, the electrocapillary curve obtained with a 5×10^{-4} M $(GS)_2Hg$ solution was found to be identical with that of 10^{-3} M GSH (see Fig. 2A).

It is shown below that the diffusion current of the GSH wave corresponds to a transfer of one electron per molecule of GSH. The following electrode reactions were considered.

$$GSH \rightleftarrows B + e \qquad (1)$$

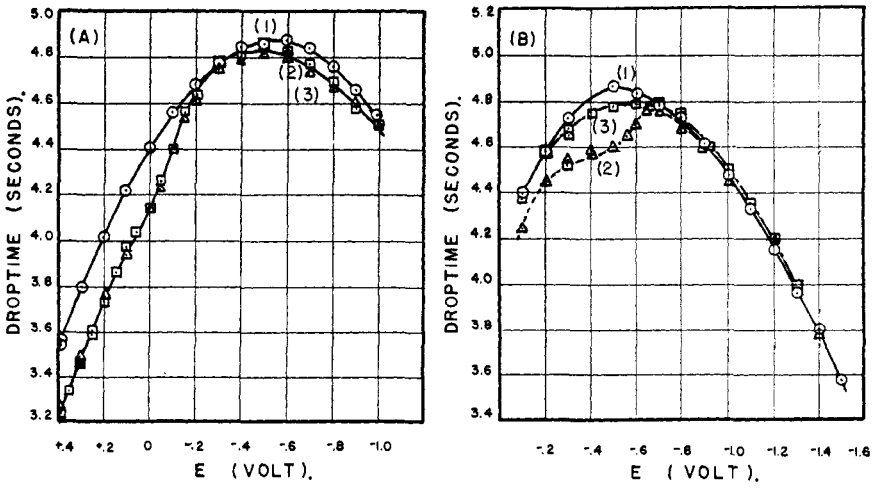

Fig. 2.—Effect of GSH, $(GS)_2Hg$ and GSSG on the electrocapillary curve: A, (1) ⊙ supporting electrolyte (0.1 M $HClO_4$, M $NaNO_3$, pH 1); (2) △ with 10^{-3} M GSH; (3) ⊡ with 5×10^{-4} M $(GS)_2Hg$. B, (1) ⊙ Supporting electrolyte (0.1 M NH_4Cl, M NH_3, 0.9 M KCl, pH 10.3); (2) △ with 10^{-3} M GSH; (3) ⊡ with 5×10^{-4} M GSSG.

$$2\,GSH \rightleftarrows B + 2e \qquad (2)$$

If the electro oxidation is reversible and occurs according to equation (1) the plot of log $(i_d - i)/i$ *versus* the potential should yield a straight line with a slope of 0.059. If equation (2) represents the (reversible) reaction a plot of log $(i_d - i)^2/i$ *vs.* E should be a straight line with a slope of 0.0295.

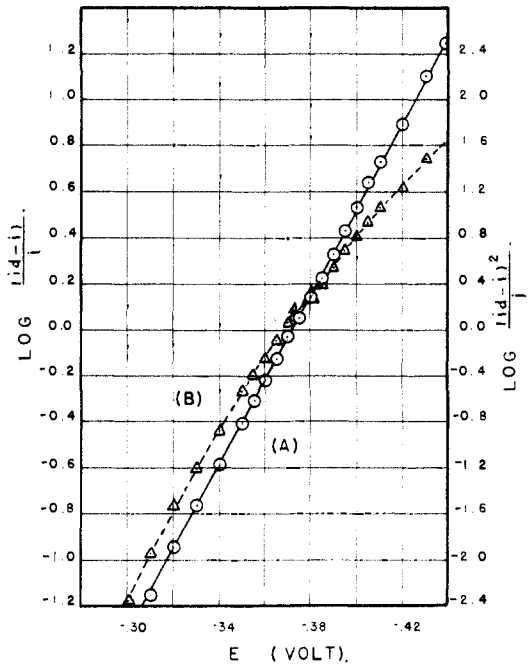

Fig. 3.—Analysis of wave of 10^{-3} M GSH at pH 6.93, μ 1: A, plot log $(i_d - i)/i$ *vs.* E; B, plot log $(i_d - i)^2/i$ *vs.* E.

An example of the plots is given in Fig. 3 corresponding to a solution 10^{-3} M in GSH in a borate buffer of pH 6.93. The plot of log $(i_d - i)/i$ *vs.* E gave a straight line with a slope of 0.055. This value is close enough to the theoretical slope of 0.059 to allow the conclusion that equation (1) represents the electrode reaction. Similar results were found upon analysis of waves obtained in 10^{-3} M GSH solutions over the pH range between 1 and 9.3. The equation of the wave is given by

$$E = E_{1/2} - 0.059 \log (i_d - i)/i \qquad (3)$$

This equation holds in perchloric acid solutions, and in buffers containing phosphate, borate and ammonia at concentrations of ammonia equal to or smaller than 0.1. The equation is not satisfied in acetate buffers and in buffer solutions with a pH greater than 9.5. Also in dilute perchloric acid solutions the equation does not hold when the GSH concentration is equal to or greater than 2×10^{-3} M. Equation (2) was not found to be satisfied in any of the solutions tested.

Kolthoff and Barnum[1] concluded that the anodic cysteine waves correspond to the formation of slightly dissociated mercurous cysteinate according to the equation

$$RSH + Hg \rightleftarrows RSHg + H^+ + e \qquad (4)$$

Apparently a similar reaction is involved in the one-electron anodic process of glutathione at the dropping electrode. If reaction (4) is reversible the mercury in GSHg should be reversibly reducible at the dropping mercury electrode.

Experiments with glutathione and mercurous nitrate indicated that GSHg is not stable and decomposes in solution with formation of mercury and $(GS)_2Hg$. A borate buffer (pH 6.94) which was 2.5×10^{-4} M in $(GS)_2Hg$ and 5×10^{-4} M in glutathione was polarographed. The composite wave obtained with this solution was analyzed and found to have the same characteristics as the anodic wave observed in a solution of 10^{-3} M GSH alone in the same buffer. Figure 4 gives the c–v curve of this wave and the plot log $(i - (i_d)_a/(i_d)_c - i)$ *vs.* E, where $(i_d)_a$ is the anodic and $(i_d)_c$ the cathodic diffusion current. i is taken positive for the cathodic and negative for the anodic current. The experiment was repeated in 0.1 M perchloric acid, the solution being 1 M, in sodium nitrate. The log plot has the same slope as at pH 6.9. The cathodic wave obtained in a borate buffer (pH 6.9) which was 5×10^{-4} M in mercuric glutathionate in the absence of GSH was also analyzed and found to have the same characteristics as the composite wave (plot log $i/(i_d - i)$ *vs.* E was a straight line of slope 0.051). An experiment carried out with a solution 5×10^{-4} M in $(GS)_2Hg$ in the presence of an excess of mercuric acetate (5×10^{-4} M) in a borate buffer (pH 6.9) did not give consistent results since the diffusion current of both the excess mercury and the $(GS)_2Hg$ were found to decrease on standing. A detailed study of the reactions of glutathione with mercury and other metals is planned.

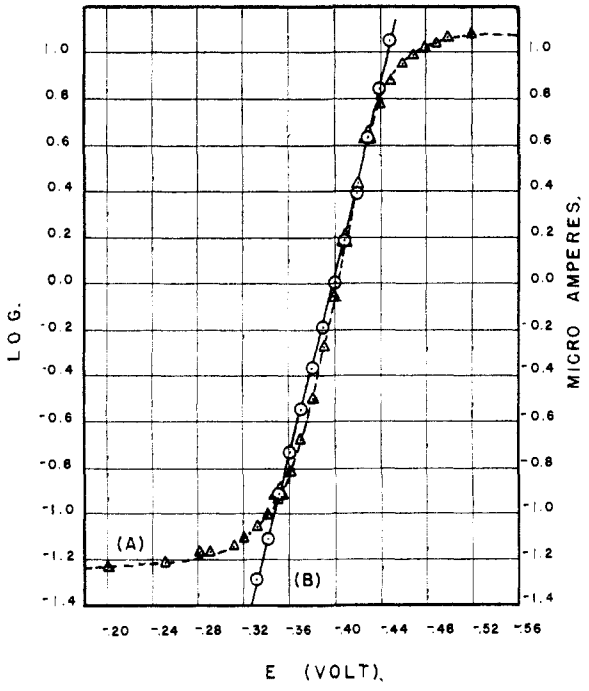

Fig. 4.—c–v curve A, and plot log $[i - (i_d)_a]/[(i_d)_c - i]$ *vs.* E. B, of a mixture of 5×10^{-4} M GSH, 2.5×10^{-4} M $(GS)_2Hg$ at pH 6.9, μ = 0.2.

The results of these studies allow the conclusion that GSH is not being oxidized to GSSG at the

dropping electrode but that it depolarizes the mercury with formation of (unstable) GSHg. The reduction wave of $(GS)_2Hg$ corresponds to the reduction of a univalent cation. Apparently the following reaction is very rapid

$$(GS)_2Hg + Hg \rightleftarrows 2\ GSHg \qquad (5)$$

the GSHg being the compound which is reduced. In this connection it may be mentioned that the reaction between mercuric chloride and mercury has been found to be very rapid at the dropping electrode.[11]

The equation of the anodic wave corresponding to reaction (4) is

$$E = E^\circ + RT/nF \ln [GSHg]^\circ [H^+]^\circ/[GSH]^\circ \qquad (6)$$

where E is the potential of the electrode, E° the standard potential of reaction (4) and the expressions in the brackets are concentrations (activity coefficients are neglected) of the reacting substances at the surface of the electrode. Considering that the sulfhydryl group in GSH is a weak acid with an ionization constant K it is easily derived from the previous discussion that E at 25° is given by

$$E = E^\circ + 0.059 \log k/k_1 + 0.059 \log \{[H^+] + K\} - 0.059 \log (i_d - i)/i \qquad (7)$$

where k and k_1 are constants which are proportional to the square roots of the diffusion coefficients of glutathione and the mercury glutathione compound (GSHg), respectively. In equation (7) $[H^+]$ is written instead of $[H^+]^\circ$, since we consider buffered solutions only.

The half-wave potential is expressed by

$$E_{1/2} = E' + 0.059 \log \{[H^+] + K\} \qquad (8)$$

where the constant E' is equal to $E^\circ + 0.059 \log k/k_1$ at 25°.

The half-wave potentials which were taken from the plots $\log (i_d - i)/i$ *versus* the potential are plotted *versus* *p*H in Fig. 5.

By extrapolation of the GSH-curve of Fig. 5 the value of E' which corresponds to the half-wave potential at "*p*H 0" is found to be +0.033 v. *vs.* S.C.E.

The experimental data agree well with equation (8). pK_4' (K of the sulfhydryl group) of glutathione is 9.12.[12] Thus K_4' becomes negligibly small as compared to the hydrogen ion concentration at a *p*H less than 8. In acid region the plot is a straight line with a slope of 0.058 as compared to the theoretical value of 0.59 at 25° (eq. 8). At a *p*H greater than 9.5 the hydrogen ion concentration becomes negligibly small as compared to K_4' and the half-wave potential becomes practically constant and independent of *p*H (see Fig. 5). Between *p*H 7.6 and 9.2 the line is curved as is theoretically to be expected. The data plotted on Fig. 5 also show the specific buffer effect. In acetate and ammonia buffers values of $E_{1/2}$ deviate from those on the drawn line.

With cysteine (RSH) in ammonia and phosphate buffers of various *p*H no effect of the kind and concentration of the buffer was found. From Fig. 5 it is seen that the plot for cysteine is similar to that for glutathione. The dissociation (titration) constant pK_3' of the sulfhydryl group of cysteine is 10.28[12] as compared to 9.12 of GSH. For this reason the $E_{1/2}$–*p*H plot for RSH is a curve between *p*H 9.3 and 10.5 and becomes a straight line at *p*H greater than 10.5 (Fig. 5). At such a high *p*H $E_{1/2}$ of cysteine is 0.1 v. more negative than that of GSH.

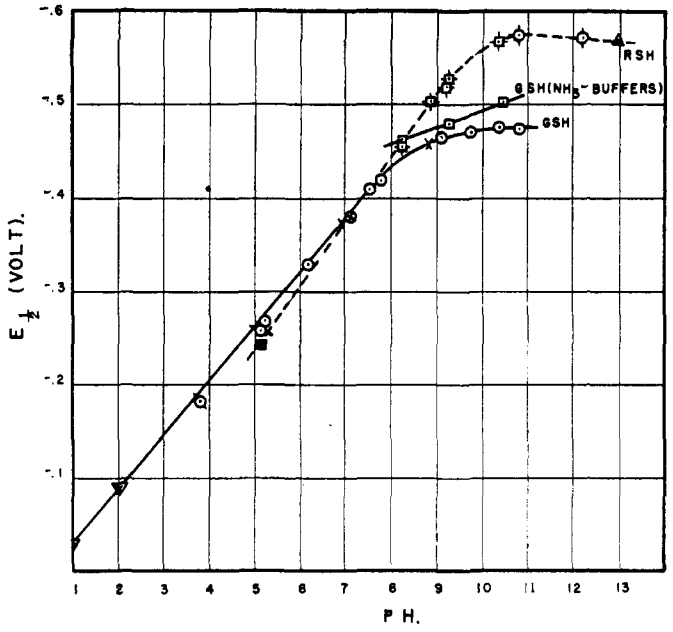

Fig. 5.—Half-wave potentials of GSH and cysteine (RSH) *vs.* *p*H: GSH, μ 1: △, $HClO_4$-$NaNO_3$; ⊙, acetate; ⊙, phosphate; ⊡, ammonia; ×, borate, μ 0.15 and 0.2: ⊗, borate. RSH, μ 1: ⊙, phosphate; ⊿, 0.1 *M* NaOH; ⊙, ammonia, μ 0.1 to 1.0; ⊠, 0.1 *M* NH_4NO_3.

The similarity of the plots $E_{1/2}$ *vs.* *p*H for glutathione and cysteine indicates that only a variation of the dissociation of the sulfhydryl group determines the characteristics of the anodic wave of each of the compounds while the electrode reaction is independent of the degree of dissociation of other groups in the molecule.

The diffusion current of reduced glutathione was found to be proportional to the GSH concentration in the concentration range between 5×10^{-4} to 2×10^{-3} *M* over a *p*H range between 1 and 10.8. This is made use of in the polarographic determination of glutathione. Cysteine does not give a true diffusion current in the *p*H range between 2 and 9. For a 10^{-3} *M* GSH solution the diffusion current is practically constant in the *p*H region from 1 to 9 and independent of the kind of buffer used. At *p*H greater than 9 the diffusion current decreases slightly.

Diffusion coefficients were calculated for glutathione at *p*H 1.0 and 10.82 from values of the diffusion current (2.42 and 2.26 μa, respectively, for 10^{-3} *M* solutions). Using the Ilkovic equation[13] for the diffusion current

(11) I. M. Kolthoff and C. S. Miller, THIS JOURNAL, **63**, 1405, 2732 (1941).

(12) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943.

(13) D. Ilkovic, *Collection Czechoslov. Chem. Communs.*, **6**, 498 (1934); *J. chim. phys.*, **35**, 129 (1938).

$$i_d = 605\, nD^{1/2}Cm^{2/3}t^{1/6} \quad (9)$$

and taking the proper values for t from Fig. 2 the values for D of reduced glutathione at ionic strength 1 and at 25° are found to be 5.6×10^{-6} and 4.7×10^{-6} cm.^{2} sec.$^{-1}$ at pH 1 and 10.82, respectively. In borate buffers of pH 7 and of ionic strength 0.2 and 1 the values for D are found to be 5.7×10^{-6} and 5.4×10^{-6} cm.^{2} sec.$^{-1}$, respectively. Kolthoff and Barnum[1] calculated a value for the diffusion coefficient of cysteine in 0.1 M perchloric acid of 7.0×10^{-6} cm.^{2} sec.$^{-1}$ at 25°. Considering the greater molecular weight of the tripeptide the smaller value of D of GSH as compared to that of cysteine is reasonable.

In polarogram IV of Fig. 1 a second anodic wave of GSH is seen which is almost of the same height as the first (normal) wave. It reaches a limiting value just before the anodic dissolution of mercury in the buffer used. Similar "abnormal" waves were found in perchloric acid solutions and in phosphate and borate buffers. In these media at an ionic strength of 0.15 this wave exhibited a flat maximum and changed only slightly with the GSH concentration. For example, in a borate buffer (pH 9.2, μ 0.15) the height of the wave varied from 0.59 to 0.75 μa when the GSH concentration was increased from 0.5 to 1.5×10^{-3} M. In the above media at an ionic strength of 1 the second wave was fairly well defined and did not exhibit a maximum. Its height was approximately the same as that of the first wave and roughly proportional to the GSH concentration. Thus in a phosphate buffer (pH 5.2) of ionic strength 1 the height of this wave was found to be 1.00, 2.31 and 3.45 μa at GSH concentrations of 5×10^{-4}, 10^{-3} and 1.5×10^{-3} M, respectively. At all pH and GSH concentrations investigated the wave was found to occur at a potential 0.55 v. more positive than the half-wave potential of the normal wave.

In amperometric titrations to be reported on in a subsequent paper it was found that under certain conditions GSH gives two end-points upon titration with mercuric mercury, the first one corresponding to $(GS)_2Hg$ and the second to GSHg(II). In GSHg(II) the mercury is bound to a carboxyl and mercaptide group. A possible interpretation of the second anodic wave is that it occurs by oxidation of GSHg(I) to GSHg(II). Equation (10) accounts for the normal wave and eq. (11) or (11a) for the second wave.

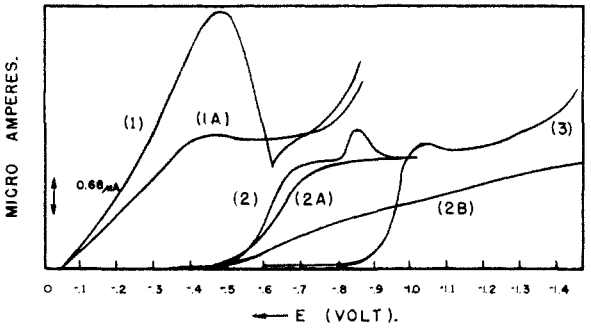

Fig. 6.—Reduction waves of 5×10^{-4} M oxidized glutathione in various buffers: (1) pH 1. 0.1 M $HClO_4$. M $NaNO_3$, (1A) same as (1) with 0.0025% gelatin; (2) pH 9.2, ammonia buffer, KCl, (2A) same as (2) with 0.005% gelatin, (2B) same as (2) with 0.0087% gelatin; (3) pH 7.0 borate buffer, $NaNO_3$, saturated with thymol.

$$HOOCGSH + Hg \longrightarrow HOOCGSHg(I) + H^+ + e^- \quad (10)$$

$$HOOCGSHg(I) \longrightarrow \underset{\underset{COO}{|}}{GSHg(II)} + H^+ + e^- \quad (11)$$

$$HOOCGSH + Hg \longrightarrow \underset{\underset{COO}{|}}{GSHg(II)} + 2H^+ + 2e^- \quad (11a)$$

2. Oxidized Glutathione (GSSG).—Some 100 polarograms were taken with GSSG in various buffers in a pH range between 1 and 10.3. The results are not tabulated but important data are presented in the form of graphs.

Polarograms of GSSG in strongly alkaline medium do not give consistent results since oxidized glutathione decomposes in these solutions with formation of GSH. In a 5×10^{-4} M GSSG solution which was 0.2 M in potassium hydroxide a marked decrease in height of the GSSG wave and an anodic GSH wave were observed on standing. Eight minutes after addition of alkali about 20% of the GSSG was decomposed by hydrolysis as determined polarographically. Cystine was found to be stable under these conditions. These findings are in agreement with Schöberl's[14] statement that cystine is more stable in alkaline medium than glutathione.

GSSG gives only one wave (compare GSH) over the entire pH range investigated. This wave is steeper and better defined than that of cystine.[2] In 5×10^{-4} M GSSG solutions a pronounced maximum occurs at pH 1 (see curve 1, Fig. 6) and in acetate buffer of pH 5. At pH 7 the maximum becomes considerably flatter. The height of the maximum decreases with increasing ionic strength and temperature. At a pH of 8 the maximum disappears, but reappears at a higher pH (9.2 to 10.3) at potentials when the diffusion current has been reached (see curve 2, Fig. 6).

Gelatin at concentrations of 0.00125 to 0.0025% suppresses the maximum and has hardly any effect on the half-wave potential and on the diffusion current. Increasing amounts of gelatin give rise to waves which are drawn out, the effect becoming more pronounced with decreasing pH and ionic strength. At a gelatin concentration of 0.0087% and at a pH of 9.2 a diffusion current region is not attained (see curve 2B, Fig. 6) even though the wave starts at the same potential as in the absence of gelatin. Thymol at concentrations lower than 10^{-4} M suppresses the maximum at pH 9 without affecting the half-wave potential markedly. Higher concentrations of thymol shift the wave to more negative potentials. Thus a 5×10^{-4} M GSSG solution at pH 7 which was 5×10^{-5} M in thymol gave a half-wave potential of −0.53 v. which upon saturation of the solution with thymol was shifted to −0.95 v. At this high thymol concentration the wave exhibits a rounded maximum (see curve 3, Fig. 6). It is interesting to note that thymol at concentrations of 10^{-4} to 1.5×10^{-4} M affects the location of the GSSG wave to a considerably smaller extent than that of the cystine wave.[2] This is

(14) A. Schöberl, *Ann.*, **507**, 111 (1933); **538**, 84 (1939).

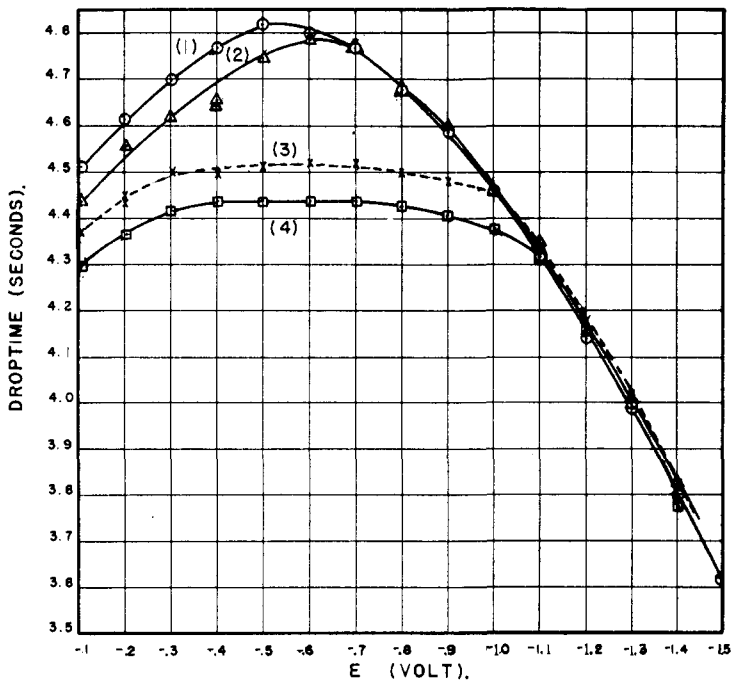

Fig. 7.—Effect of oxidized glutathione and of thymol on the electrocapillary curve in a borate solution (pH 7, μ 1) at 25°: (1) borate solution (2) with 5×10^{-4} M GSSG; (3) with 5×10^{-4} M GSSG, saturated with thymol; (4) borate solution, saturated with thymol.

being made the basis of an analysis of mixtures of cystine and oxidized glutathione.

From electrocapillary curves given in Fig. 7 it is seen that GSSG is capillary active at the mercury-solution interface. At pH 7 it displaces the electrocapillary maximum to more negative potentials. Saturation of the GSSG-free buffer with thymol gives a curve with a flat drawn-out top without distinct maximum. At −1.1 volts curves 1 and 4 coincide. At this potential thymol is desorbed. In a solution saturated with thymol the GSSG wave starts approximately at the potential where thymol is being desorbed (−1.0 v. see curve 3, Fig. 7). Comparison of Figs. 2 and 7 reveals that GSH is much more capillary active at the mercury-solution interface than is GSSG.

The over-all reduction of GSSG at the dropping mercury electrode may be represented by an equation similar to that of cystine[2]

$$\text{GSSG} + 2e + 2H^+ \longrightarrow 2\ \text{GSH} \qquad (12)$$

If this electrode reaction were reversible the potential at any point of the reduction wave at 25° should be expressed by

$$E = E' + 0.059 \log \{[H^+] + K\} + 0.0295 \log (i_d - i)/i^2 \qquad (13)$$

where the constant E' is equal to $E^\circ - 0.0295 \log k/k_1^2$. E° is the standard potential of reaction (12) and K is the titration constant (K_4') of the sulfhydryl group in GSH. Thus when E is plotted *versus* $\log (i_d - i)/i^2$ a straight line with a slope of 0.0295 should be obtained. An example of a plot of $\log (i_d - i)/i^2$ *vs.* E in an ammonia buffer is given in Fig. 8. This plot is a straight line with slope 0.058 instead of the expected value of 0.0295. The same result was found in solutions which were 10^{-3} to 5×10^{-4} M in GSSG at pH 10.3 to 5.1 in the presence of 0.0025% gelatin and at ionic strength 1. At lower ionic strength (0.35 to 0.1) straight lines of slope 0.055 to 0.060 were obtained only in the absence of a maximum suppressor. In the presence of 0.0025% gelatin or 7.5×10^{-5} to 10^{-4} M thymol GSSG solutions of ionic strength 0.1 to 0.35 and pH 8.2 to 9.1 gave waves with plots of $\log (i_d - i)/i^2$ *vs.* E consisting of two intersecting straight lines with slopes 0.055 (at potentials more positive than the half-wave potential) and 0.086 (at potentials more negative than the half-wave potential), respectively. GSSG solutions at pH markedly lower than 5.1 at ionic strength 1 and in the presence of 0.0025% gelatin gave drawn-out waves.

The slope of the plot of $\log (i_d - i)/i^2$ *vs.* potential was found to be unaffected by the height of the mercury column (30 to 100 cm.) and by an increase in temperature from 25 to 50°.

Ryklan and Schmidt[15] found that iodide ion catalyzes the establishment of the equilibrium between the oxidized and reduced forms of glutathione. A solution which was 5×10^{-4} M in GSSG and 0.2 M in potassium iodide at pH 8.9 (borax buffer, μ 0.35) in the presence of 10^{-4} M thymol

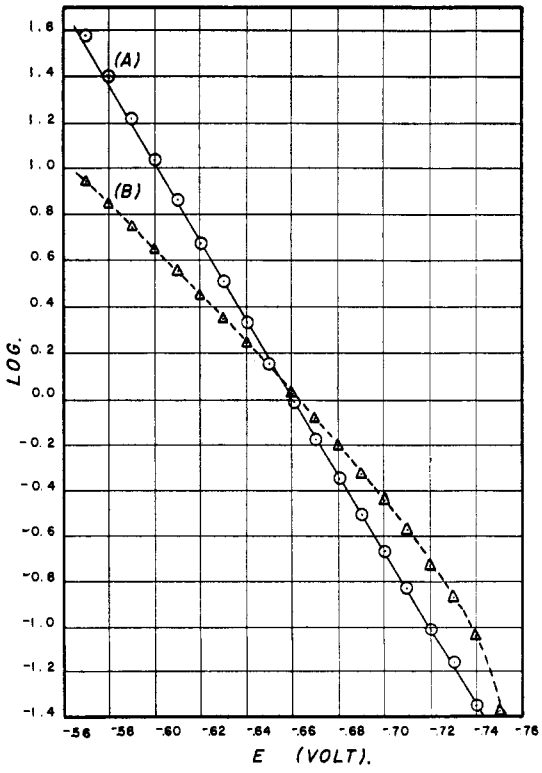

Fig. 8.—Plots of (A), $\log (i_d - i)/i^2$ and (B), $\log (i_d - i)/i$ *versus* potential of 5×10^{-4} M GSSG in M NH_3, 0.1 M NH_4Cl, 0.9 M KCl, 0.0025% gelatin; pH 10.34.

(15) L. R. Ryklan and L. A. Schmidt, *Univ. Calif. Publ. Physiol.*, **8**, 257 (1949).

gave a drawn-out wave with a half-wave potential of −0.664 v. which was 32 millivolts more negative than that of a solution containing 0.2 M KCl instead of 0.2 M KI. Apparently the mechanism of the reduction of GSSG is very complicated in the presence of iodide.[16]

Half-wave potentials of GSSG are plotted as functions of pH and log $i_d/2$, respectively, in Fig. 9. At pH equal to and lower than 8 the plot $E_{1/2}$ *vs.* pH is a straight line of slope 0.060 and is curved at higher pH as required by equation (13). In

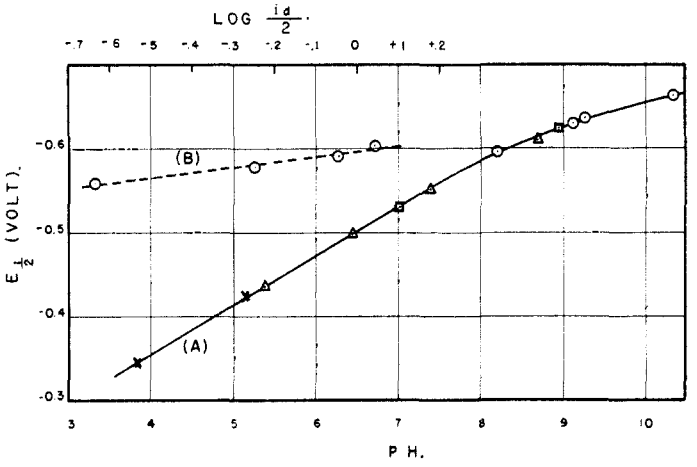

Fig. 9.—Half-wave potential of oxidized glutathione *vs.* pH and *vs.* log $i_d/2$ at 25°: A, $E_{1/2}$ *vs.* pH (5 × 10^{-4} M GSSG); B, $E_{1/2}$ *vs.* log $i_d/2$ at pH 8.3, ×, acetate: ○, ammonia; △, phosphate; □, borate.

contrast to reduced glutathione no specific buffer effects are observed with GSSG.

The variation of $E_{1/2}$ with the height of the mercury column which might be appreciable if adsorption phenomena affect the characteristics of the wave[17] was found to be negligibly small in a 5 × 10^{-4} M GSSG solution at pH 8.36 (ammonia buffer, μ 1). Thus at mercury heights of 30, 80, and 100 cm. the half-wave potentials were found to be −0.589, −0.597 and −0.597 v., respectively.

According to equation (13) the plot $E_{1/2}$ *vs.* log $i_d/2$ would be a straight line with a slope of 0.0295. At GSSG concentrations varying from 10^{-3} to 10^{-4} M at constant pH a straight line with slope 0.060 was found. An example of a plot of $E_{1/2}$ *vs.* log $i_d/2$ at pH 8.3 is given in Fig. 9.

From the above it appears that the slopes of the plots log $(i_d - i)/i^2$ *vs.* E as well as those of $E_{1/2}$ *vs.* log $i_d/2$ are twice as great as required by equation (13). Our results indicate that the potential determining step involves a reversible reaction with a one-electron transfer. The following mechanism accounts for the observed effects.

$$\text{GSSG} + e + H^+ \rightleftarrows GS^{\cdot} + \text{GSH} \quad (14)$$

$$GS^{\cdot} + e + H^+ \longrightarrow \text{GSH} \quad (15)$$

$$\text{GSSG} + 2e + 2H^+ \rightleftarrows 2\,\text{GSH} \quad (12)$$

It is reasonable to assume that reaction (14) which involves the cleavage of the disulfide bond is the rate-determining step of the electrode reaction. On this basis the equation of the wave at 25° is given by

$$E = E_0 + 0.059 \log [\text{GSSG}]^\circ [H^+]^\circ / [Gs^{\cdot}]^\circ [\text{GSH}]^\circ \quad (14A)$$

where E_0 is the standard potential of reaction (14). In the usual way[18] it can be derived that [GSSG]° is proportional to $(i_d - i)$ while [GS·]° and [GSH]° are proportional to i. Thus it is found that

$$E = E' + 0.059 \log \{[H^+] + K\} + 0.059 \log (i_d - i)/i^2 \quad (16)$$

where E' is a constant.

The half-wave potential at 25° is determined by

$$E_{1/2} = E' + 0.059 \log \{[H^+] + K\} - 0.059 \log i_d/2 \quad (17)$$

E' which corresponds to the half-wave potential at "pH 0" is derived from curve A of Fig. 9 to be equal to 0.119–0.059 log $i_d/2$. For a GSSG concentration of 5 × 10^{-4} M (i_d 2.26 μa.) E' is found to be +0.116 v. *vs.* S.C.E.

Equations (16) and (17) are in agreement with the experimental results. From equation (14) it is evident that GSH should affect the characteristics of the GSSG-reduction wave. If the concentration of the reduced glutathione is made sufficiently large such that the concentration of GSH at the interface of the drop can be assumed to be the same as that in the bulk of the solution equation (16) becomes

$$E = E' + 0.059 \log \{[H^+] + K\} + 0.059 \log (i_d - i)/i - 0.059 \log C_{GSH} \quad (18)$$

where C_{GSH} is the molar concentration of reduced glutathione added. The half-wave potential in the presence of an excess of GSH should be given by

$$E_{1/2} = E' + 0.059 \log \{[H^+] + K\} - 0.059 \log C_{GSH} \quad (19)$$

In the presence of sufficient GSH the plot log $(i_d - i)/i$ *vs.* E should be a straight line with slope of 0.059 and the half-wave potential should change by 0.059 v. to a more negative value as the GSH concentration is increased 10-fold. Also, the half-wave potential must be independent of the GSSG concentration in the presence of excess of GSH.

Experimentally these postulates were found to be correct. Solutions of 5 × 10^{-4} M GSSG in an ammonia buffer at pH 10.34 have been polarographed in the absence and presence of 1.6 × 10^{-2} M GSH. GSH at this high concentration suppresses the maximum of the GSSG wave and therefore gelatin was not added. The two plots of log $(i_d - i)/i^2$ and log $(i_d - i)/i$ *vs.* E, respectively, are given in Figs. 8 and 10. A comparison of these figures indicates clearly that the plot of log $(i_d - i)/i$ *vs.* E is a curve in the absence of GSH but a straight line with slope of 0.060 in the presence of GSH. On the other hand the plot of log $(i_d - i)/i^2$ *vs.* E is a straight line with slope of 0.058 in the absence of GSH but curved in the presence of GSH. A set of data obtained with a 5 × 10^{-4} M GSSG solution of pH 10.35 and in the presence of GSH at concentrations varying from 10^{-3} to 4

(16) T. F. Lavine, *J. Biol. Chem.*, **113**, 583 (1936); G. Toennies, *ibid.*, **122**, 27 (1937–1938).

(17) J. Kuta, *Collection Czechoslov. Chem. Communs.*, **16**, 1 (1951).

(18) I. M. Kolthoff and J. J. Lingane, "Polarography," Interscience Publishers, Inc., New York, N. Y., 1946.

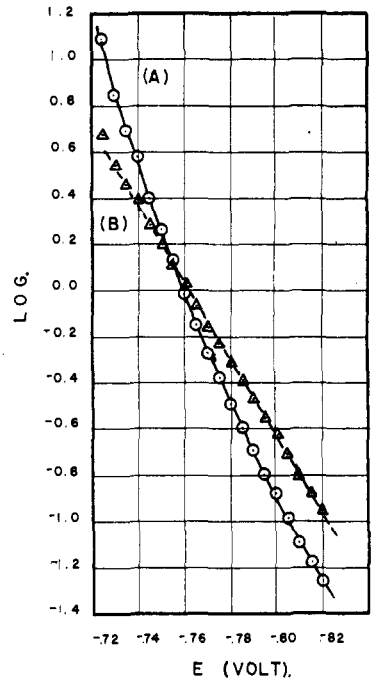

Fig. 10.—Plots of (A), log $(i_d - i)/i^2$ and (B), log $(i_d - i)/i$ *versus* potential of 5×10^{-4} *M* GSSG in 1.6×10^{-2} *M* GSH, *M* NH_3, 0.1 *M* NH_4Cl, 0.9 *M* KCl, *p*H 10.35.

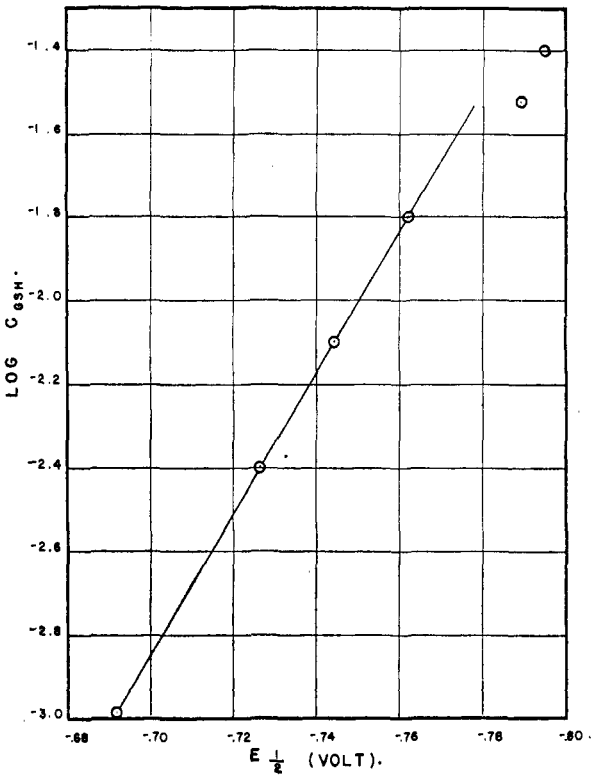

Fig. 11.—5×10^{-4} *M* GSSG in the presence of an excess of GSH at *p*H 10.3 (ammonia buffer); plot of $E_{1/2}$ *vs.* log C_{GSH}.

$\times 10^{-2}$ *M* are presented graphically in Fig. 11 which gives a plot of log C_{GSH} *vs.* $E_{1/2}$. It is seen that up to a GSH concentration of 1.6×10^{-2} *M* the plot is a straight line with slope of 0.059. At higher concentrations of GSH (3×10^{-2} *M* and higher) the half-wave potential of GSSG does not change any more with the GSH concentration but assumes a constant value of −0.79 v. This may be accounted for by the fact that the strongly capillary active GSH at higher concentrations displaces the GSSG wave to more negative potentials comparable to the effect of thymol. At a potential of −0.79 v. the GSH is desorbed (see Fig. 2). Experiments which were carried out with varying GSSG concentrations (2 to 7.3×10^{-4} *M*) but at the same concentration of GSH (1.6×10^{-2} *M*) confirm that the half-wave potential (−0.763 v. at *p*H 10.35) of GSSG is independent of the GSSG concentration in the presence of an excess of GSH as required by equation (19). A comparison of experiments with 5×10^{-4} *M* GSSG at *p*H 8.2 and 10.34, respectively, in the absence of GSH with the corresponding experiments in the presence of 1.6×10^{-2} *M* GSH shows that the shift of the half-wave potential of GSSG with *p*H (0.065 v. in the absence and 0.063 v. in the presence of GSH) is practically the same in the absence and presence of reduced glutathione. $E_{1/2}$ and i_d of reduced glutathione at a concentration of 10^{-3} *M* is not affected by the presence of 5×10^{-4} *M* GSSG in an ammonia buffer at *p*H 10.3 and ionic strength 1.

From experiments with a 5×10^{-4} *M* GSSG solution at *p*H 7 which were carried out at varying temperatures it is interesting to note that an increase in temperature from 25 to 50° causes a shift of $E_{1/2}$ by 31 millivolts to a more positive potential. Apparently the ease of breaking the disulfide bond of GSSG increases with increasing temperature.

The diffusion current of GSSG hardly changes with *p*H. The presence of reduced glutathione at concentrations of 10^{-3} to 4×10^{-2} *M* was found to have no effect on the diffusion current of GSSG. The diffusion currents measured with solutions 10^{-4} to 10^{-3} *M* in GSSG were found to be proportional to the concentration. Experiments carried out in an ammonia buffer at *p*H 8.36 and at a GSSG concentration of 5×10^{-4} *M* with heights (h) of the mercury column varying from 30 to 100 cm. gave $i_d/\sqrt{h}$-values of 0.24 to 0.25 after correction for back pressure. The wave can thus be considered to be diffusion controlled. Gelatin at concentrations not larger than 0.0025%, as well as thymol (7×10^{-5} *M*) have practically no suppressing effect on the diffusion current. The increase of the diffusion current with temperature is about 1.5% per degree as measured between 25 and 50°, a normal value for a diffusion controlled limiting current.[18] Using the Ilkovic equation the diffusion coefficient of oxidized glutathione at ionic strength 1 and at *p*H 10.3 is calculated to be 4.5×10^{-6} cm.^{2} sec.$^{-1}$, as compared to a diffusion coefficient of cystine of 5.3×10^{-6} cm.^{2} sec.$^{-1}$ in 0.1 *N* hydrochloric acid at 25°.[2]

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