Lepage, G. A. & Umbreit, W. W. (1943). *J. biol. Chem.* **147**, 263.
Lepage, G. A. & Umbreit, W. W. (1945). In *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, by W. W. Umbreit, R. H. Burris & J. F. Stauffer. Minneapolis: Burgess Publishing Co.
Leva, E. & Rapoport, S. (1943). *J. biol. Chem.* **149**, 47.
Popják, G. & Muir, H. (1949). *Biochem. J.* **45**, xi.
Rapoport, S., Leva, E. & Guest, G. M. (1943). *J. biol. Chem.* **149**, 57.
Schmidt, G., Hershman, B. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 523.
Zilversmit, D. B., Entenman, C. & Chaikoff, I. L. (1948). *J. biol. Chem.* **176**, 193.
Zilversmit, D. B., Entenman, C. & Fishler, M. C. (1943). *J. gen. Physiol.* **26**, 325.

The Quantitative Reactions of Thiols and Disulphides with Silver Nitrate

By R. CECIL
Department of Biochemistry, University of Oxford

(*Received* 3 *November* 1949)

1. The Estimation of some Thiols and Disulphides

Most of the existing methods of estimating thiols depend on the quantitative oxidation of thiols to disulphide. Errors may result from the oxidation proceeding beyond this stage, or from other substances present being oxidized simultaneously. A further difficulty arises when the amount of oxidation is measured colorimetrically, as the readings have to be compared against that given by a standard solution of the thiol concerned. Since most thiols, with the exception of reduced glutathione (GSH), oxidize appreciably in the presence of air, their standard solutions must be checked simultaneously by another method if errors are to be avoided.

These remarks apply equally to the estimation of disulphides, since the available methods consist in reducing them in whole or in part to thiols and estimating the latter.

A method making use of the direct reaction of the thiol group with a heavy metal would have advantages over the oxidation methods. Kolthoff & Harris (1946) have devised an amperometric titration method for thiols with silver nitrate. Benesch & Benesch (1948) reduced the scale of the method and applied it to biologically important thiols. They also applied it to serum proteins, but in doing so they took no account of the reaction between silver ions and disulphide groups (see Part 2 of this paper), which may well have affected their results.

In the method to be described, which was developed independently, the same reaction, namely the formation of silver mercaptide, is used, but the amount of free silver ion present is measured potentiometrically using a silver electrode. The addition of an excess of silver nitrate and estimation of the remaining free silver ion by back titration with potassium bromide, using the silver electrode to determine the end point, was found to be more convenient than a direct titration with silver nitrate.

This method has also been adapted to the estimation of disulphides. These are first split by the action of sodium sulphite (Clarke, 1932), and the thiol formed is then estimated.

EXPERIMENTAL

Apparatus

Since absolute measurements of electrode potentials are not required in this type of work it is permissible to use a calomel reference electrode together with a salt bridge. So long as the diffusion processes at the liquid junctions cause only small variations in the e.m.f. of the cell, the measurements made can be interpreted with sufficient accuracy in terms of the concentration of the ion, or the activity of its salt, to which the electrode is reversible.

The arrangement used (Fig. 1) was designed to allow electrical contact to be made between the calomel electrode and the solution being titrated, without contaminating the latter with chloride. A good tap at *A* is essential, as any leakage results in chloride siphoning over into the titration vessel. Sufficient grease can be applied to the tap to prevent any leakage while still retaining adequate conductivity.

The Ag electrodes consisted of thin Pt wire which was lightly plated with Ag just before use. They gave the theoretical slope of 58 mV./pAg unit at 18° over the range pAg 3 to pAg 9–10, where $pAg = -\log [Ag^+]$. The glass electrodes were made according to MacInnes & Dole (1929) from Corning 015 electrode glass. All values of pH quoted were measured with them. Both the glass and the Ag-plated platinum electrodes were fused on to glass side arms which were conveniently mounted in lumps of plasticine on brass blocks.

Potentials were measured with a standard Cambridge pH meter, used throughout as a millivolt-meter. Readings with the glass electrodes were reproducible to 1 mV. and with the Ag to 0·5 mV. When a glass electrode was being used, a Weston cell was introduced into the circuit in such a way that the glass electrode was always negative to the calomel electrode and so could be connected to the grid of the electrometer valve.

The microburette used was of the type described by Heatley (1939), and was made from Hysil 'Veridia' tubing. Since the titration vessels could not be moved the burette was mounted on a counterpoised arm and raised and lowered by hand. The capacity was $1{\cdot}15 \pm 0{\cdot}01$ µl./cm. of length.

Reagents

Apart from those mentioned in this section, all the reagents used were commercial A.R. grade.

Reduced glutathione (GSH). Dr R. B. Fisher very kindly provided a sample of crystalline GSH which he had prepared from yeast by the method of Hopkins (1929) as modified by Pirie (1930).

Method

In one experiment GSH was titrated directly with 0·1 M-$AgNO_3$. The free Ag ion concentration was calculated from the equation

$$E = E^\circ + \frac{RT}{F} \ln [Ag^+].$$

E° was obtained from the Ag potential given by an 0·01 M-$AgNO_3$ solution. The amount of combined Ag was obtained by difference.

In all the other experiments an excess of $AgNO_3$ was added to the thiol, and the remaining free Ag estimated by titration against 0·1 M-KBr. The titrations were followed by

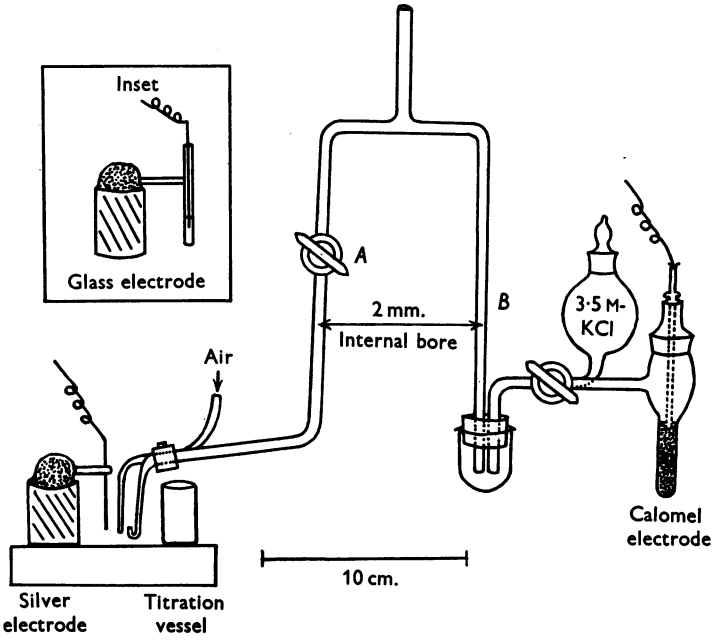

Fig. 1. Diagram of the apparatus used for the potentiometric Ag titrations. The liquid bridge is filled with 0·1 M-NH_4NO_3 from the curved tip to *B* and from there to the calomel electrode with 3·5 M-KCl. The T-tube at the top is used for filling and then sealed with a rubber tube and clip. The air tube, used for stirring, was connected to a small vibrator pump.

Oxidized glutathione (G_2S_2). This was prepared from the same sample of GSH by oxidation with H_2O_2 at 0° according to the directions given by Schöberl & Hornung (1938). Yield 82%.

Cystine. Commercial cystine was twice reprecipitated at pH 4·5 from acid solution.

Cystine is very insoluble near neutrality, but it was found possible to make stable supersaturated solutions of just below 10^{-3} M. Approximately 20 mg. of cystine are added to 100 ml. of water containing 0·5 ml. of 0·1 M-KOH. The suspension is shaken until the cystine has dissolved (about 1 hr.): it is then neutralized with 0·5 ml. of 0·1 M-HNO_3 and filtered to remove any nuclei that might start crystallization.

Nitric acid. Pure HNO_3 was freed from oxides of N by blowing a rapid stream of air through for 5 min., and then immediately diluted to 0·1 M.

Silver nitrate. A.R. $AgNO_3$ was used. Solutions of 0·01 M and below were found to be more stable if kept in Jena glass bottles which had been cleaned with ethanolic potash.

plotting the readings of the Ag electrode against the volume of KBr solution added. This gives a sigmoid curve, the mid-point of which corresponds to the state

$$a_{Ag^+} = a_{Br^-}.$$

This may be taken as the end point, since the activity coefficients of the two ions are unlikely to differ appreciably.

The solubility of AgBr is such that there will always be solid present as the end point is approached. In the presence of the solid

$$[Ag^+].[Br^-] = \text{solubility product},$$

and at the end point

$$[Ag^+] = [Br^-] = \sqrt{(\text{solubility product})}.$$

Hass & Jellinek (1932) give the solubility product as $6{\cdot}3 \times 10^{-13}$, so that at the end point, assuming the activity coefficients to be unity, the Ag ion concentration will be $7{\cdot}95 \times 10^{-7}$ g. ion/l. = pAg 6·1. The Ag electrode potential at pAg 3 = 380 mV., so that at pAg 6·1, $E_{Ag} = 200$ mV.

The midpoints of a large number of titration curves were determined graphically (by measuring the centre of the obviously straight part of the curve around the point of inflexion), and were found to lie within 197 ± 2 mV. under the conditions used. This suggests that the solubility product remains effectively constant under these conditions. Fig. 2 shows the titration curves of 0·1 ml. of 0·01 M-$AgNO_3$ (≡ 10 μl. of 0·1 M-KBr) in 1 ml. of water and in 1 ml. of 0·1 M-NH_4NO_3. Although the slopes of the two curves differ, the end-point potentials, determined graphically, are the same. The latter represents a much higher ionic strength than that normally used. The difference in end points (0·1 μl.) is due to a small amount of halide in the NH_4NO_3 solution.

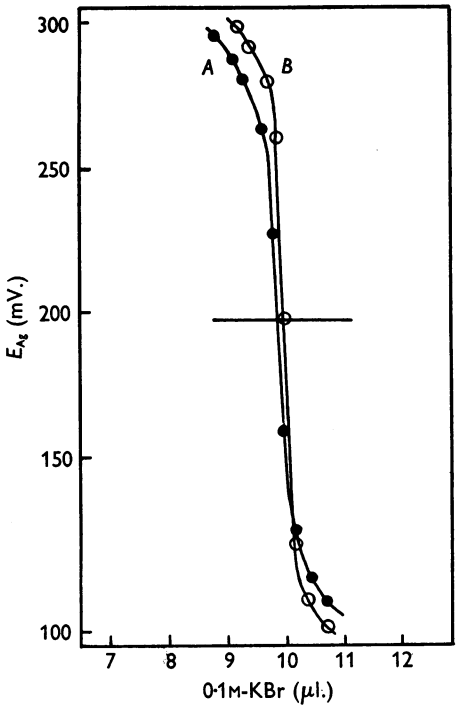

Fig. 2. Titration curve of 0·1 ml. of 0·01 M-$AgNO_3$ against 0·1 M-KBr. Curve *A* shows the $AgNO_3$ in 1 ml. of 0·1 M-NH_4NO_3 and curve *B* shows it in 1 ml. of water. The midpoint of both curves is indicated by the horizontal line.

Once the apparatus has been calibrated, therefore, it is necessary only to determine the amount of KBr needed to bring the Ag electrode potential to 197 mV. The end points obtained in this way are reproducible to 0·1 μl. of 0·1 M-solution, or 10^{-8} g.equiv.

In all these titrations proper mixing was essential, since very small volumes of relatively dense solution were being added. The air bubbler was arranged to switch on and off as required and was found to be a very efficient mixer. Sometimes trouble was experienced with frothing, in which case a small drop of caprylic alcohol could be added without otherwise affecting the titration.

Halide or silver content of reagents. This was determined by titrating a known amount of $AgNO_3$ mixed with a known amount of the reagent against standard bromide. When the reagent was the salt of a weak acid the pH was first reduced to 2–2·5 with 0·1 M-HNO_3. Any difference of titre from that of the $AgNO_3$ alone is caused by Ag (excess titre) or halide (short titre). The results are given in Table 1. Some of the buffers used in Part 2 are included.

Table 1. *Estimation of the halide or silver content of reagents by titration of a fixed quantity of silver nitrate against standard potassium bromide in the presence of a known amount of the reagent*

Substance	Vol. used (ml. 0·1 M soln.)	$AgNO_3$ added (μl. 0·1 M soln.)	KBr titre (μl. 0·1 M soln.)
NH_4NO_3	1·0	10	9·9
Sodium acetate	0·2	10	9·9
HNO_3	0·5	10	10·0
Sodium barbitone	0·2	10	9·8
Na_2SO_3	0·2	10	9·75
Picoline	0·2	10	10·0

RESULTS

Estimation of thiols

For most of this work GSH was used as it is sufficiently stable for standard solutions to be made up by weight.

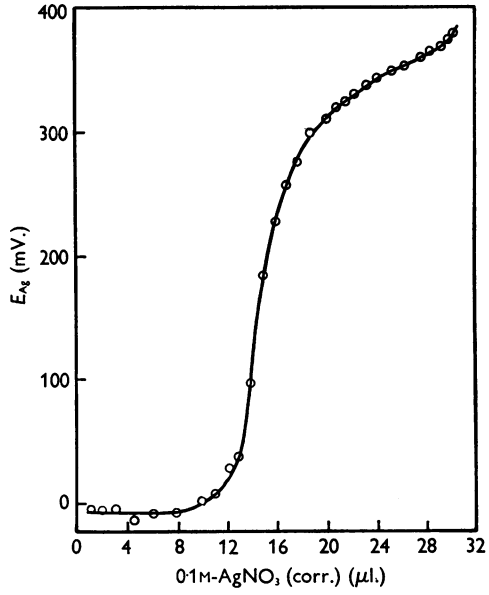

Fig. 3. Corrected titration curve of 2·0 ml. of $7·6 \times 10^{-4}$ M-GSH solution against 0·1 M-$AgNO_3$.

Fig. 3 shows the corrected curve, of combined silver against E_{Ag}, obtained when GSH (2·0 ml. of $7·6 \times 10^{-4}$ M) was titrated with silver nitrate. The first part of the curve was flat because solid GSAg was present. After one equivalent of silver had been added there was a jump in potential, followed by

further silver uptake while a second equivalent was being added. By this time all the GSAg had redissolved. Meanwhile the pH, initially 3·51, fell to 3·10 after the first equivalent had been added and then stayed constant.

In fact, the pH may be reduced before adding the silver nitrate without affecting the result.

Cysteine, prepared by the hydrolytic decomposition of cystine (see Part 2), was found not to form a soluble complex with excess silver nitrate, and the

Table 2. *Estimation of* GSH *by back titration against* 0·1 M-KBr *after the addition of a known excess of* $AgNO_3$

(The table shows the effect of variations in the pH of titration and of the amounts of GSH and $AgNO_3$ used. The GSH solution was made up by weight to $1·24 \times 10^{-3}$ M.)

Curve no.	GSH (ml.)	0·1 M-$AgNO_3$ (μl.)	0·1 M-KBr (μl.)	pH	M (calc.) of GSH $\times 10^3$
1	0·5	20	12·8	3·2	1·44
2	0·5	20	13·75	2·0	1·25
3	0·5	10	3·8	2·0	1·24
4	1·0	20	7·7	1·85	1·23
5	0·5	10	3·7	2·6	1·26
6	1·0	20	7·5	2·5	1·25
7	0·5	10	3·8	2·05	1·24
8	0·5	10	3·85	1·92	1·23

This kind of experiment showed the type of reaction that takes place between GSH and silver nitrate, but the curves obtained were too complex for precise end-point determination.

The difficulties were overcome by adding silver nitrate in excess and back-titrating with potassium bromide. This had the additional advantage that the silver potential at the end point was the same regardless of the thiol being estimated. Even so the estimation of GSH was complicated by the firmness with which the second equivalent of silver was held. Thus if the bromide titration was carried out at pH 3·2 the uptake of silver was greater than one equivalent. Reducing the pH to 2·5 or less with 0·1 M-nitric acid loosened the attachment of the second silver and gave an uptake of only one equivalent.

However, when this was done the curve was no longer symmetrical near the end point, and the uncertainty in end-point determination remained. This was resolved by carrying out a series of titrations, using a standard GSH solution, at varying pH and with varying ratios of GSH and silver nitrate. These are summarized in Table 2 and Fig. 4.

It appears that the following assumptions are justified: (*a*) that the slope of the curve below the inflexion point corresponds to that of silver nitrate titrated with potassium bromide in the presence of GSAg; (*b*) that the end point is the intersection of this part of the curve with the ordinate $E_{Ag} = 197$ mV., assuming this to be the correct end-point potential for the apparatus in use.

The end points given in Table 2 were obtained on these assumptions, and the correspondence of the GSH concentrations derived from them with the weighed concentration makes it unlikely that they are much in error. The curves in Fig. 4 also show that there is no reason to suppose that reducing the pH, at least to 1·85, liberates any of the mercaptide silver.

titration curve, with bromide, showed none of the complexity shown by GSH. The mercaptide pre-

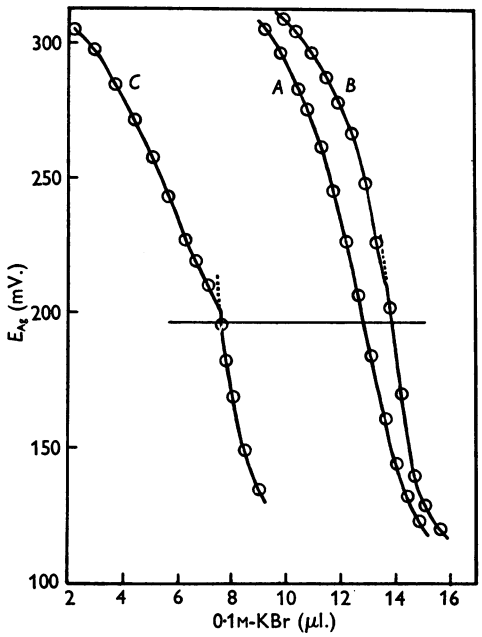

Fig. 4. Titration curves of GSH against 0·1 M-KBr after the addition of a known excess of $AgNO_3$ from the data given in Table 2. Titrations 3, 4, 7 and 8 give the same shaped curves as 2, and 5 the same shape as 6, so that only the curves relating to 1, 2 and 6 are shown. *A*, symmetrical curve at pH 3·2 giving the wrong end point. *B*, the same titration at pH 2·0 giving an asymmetrical curve but the correct end point. *C*, titration at pH 2·5 when the point of inflexion almost obscures the end point.

cipitate contained some loosely bound silver which was liberated during titration, suggesting that a rather unstable and insoluble complex is formed.

Cysteine and GSH were also estimated in the presence of their disulphide forms. Disulphides undergo hydrolytic decomposition in the presence of silver nitrate (see Part 2), but this reaction can be stopped by reducing the pH sufficiently, so that any silver uptake can be ascribed to the presence of thiol.

Samples of a G_2S_2 solution, 1·0 ml. of $9·8 \times 10^{-4}$ M, were acidified with 0·1 M-nitric acid, after which 0·1 ml. of 0·01 M-silver nitrate was added to each and then titrated with bromide. The results given in Table 3 show that, provided the pH is below 2, the error introduced by presence of G_2S_2 is small. An experiment with cystine at pH 2·0 showed no detectable decomposition. It is of course essential to acidify before adding the silver nitrate.

Table 3. *Titration of silver nitrate with* KBr *in the presence of* G_2S_2, 1·0 *ml. of* $9·8 \times 10^{-4}$ M *solution, at different hydrogen-ion concentrations*

(The proportion of the G_2S_2 reacting with the $AgNO_3$ before titration decreases with decreasing pH.)

pH	0·1 M-KBr (μl.)	Percentage of G_2S_2 split
3·05	9·5	3·4
2·4	9·7	2·0
1·9	9·9	0·7

Theory = 10.

The titration curves of silver nitrate in the presence of G_2S_2, from the experiments just quoted, showed none of the anomalies either in shape or slope, of those in the presence of GSAg. This suggests that G_2S_2 does not form a complex with silver nitrate.

An important difference between cysteine and glutathione silver mercaptides is that the former is photosensitive, whereas the latter is not appreciably so. Suspensions of cysteine mercaptide in silver nitrate solution go brown when exposed to normal daylight for any length of time. Since this coincides with a reduction in the amount of free silver ion present it is probable that the brown colour is due to the formation of colloidal silver. By contrast, a solution of GSAg in excess of silver nitrate gave no colour in daylight or after 45 min. of strong ultraviolet irradiation. Because of this effect, suspensions of the cystine mercaptide should not be left standing exposed to light before estimation.

Estimation of disulphides

Clarke (1932) showed that sodium sulphite reacts with disulphides according to the equation

$$RSSR + Na_2SO_3 \rightleftharpoons RSNa + RSSO_3Na.$$

Schöberl & Ludwig (1937) used a large excess of sulphite, 300 × the molarity of disulphide, in acetate buffer at pH 5·2, and estimated the thiol formed colorimetrically with Folin's reagent.

It was found that if the buffering were left to the sulphite, with a correspondingly higher pH, then very much less was needed to bring the reaction to completion. The thiol formed was estimated after acidification, as described in the previous section. Since sodium sulphite normally contains appreciable amounts of halide, this must be measured (see Table 1) and allowed for when calculating the amount of combined silver.

Oxidized glutathione. The reactions were carried out in the titration vessels at room temperature, 1·0 ml. of approx. 10^{-3} M-G_2S_2 solution being used for each estimation. Table 4 shows the effect of varying the sulphite concentration: 5–10 mol. of sulphite/mol. of G_2S_2 was shown to be sufficient. This gives a pH of 6·5–7. The reaction takes 10 min. to go to completion.

Table 4. *Estimation of* G_2S_2. *Determination of the amount of sodium sulphite required to react with all the* G_2S_2 *present*

(The GSH formed was subsequently estimated by the method described. The G_2S_2 solution was originally made up as $1·90 \times 10^{-3}$ M.)

Mol. Na_2SO_3/mol. G_2S_2	M (calc.) of $G_2S_2 \times 10^3$
3	1·74
4	1·92
5	1·88
10	1·90

Cystine. Since cysteine is rapidly oxidized by air the reactions were carried out at room temperature in 0·5 in. (12 mm.) test tubes into which a stream of oxygen-free nitrogen was bubbled through a fine-drawn capillary. Normally 1·0 ml. of approx. 10^{-3} M-cystine solution was used, together with the required amount of 0·1 M-sulphite: 1·0 ml. of the mixture was pipetted off at the end of the reaction, added to the silver nitrate, and acidified to pH 2–2·5.

Table 5. *Estimation of cystine. Determination of the amount of sodium sulphite required to react with all the cystine present*

(The cysteine formed was estimated by the method described. The cystine solution was made up by weight to $0·83 \times 10^{-3}$ M.)

Mol. Na_2SO_3/ mol. cystine	M (calc.) of cystine × 10^3
10	0·80
15	0·81
20	0·83
25	0·83

The reaction is complete in 20–25 min. and 30 min. was taken as standard. The effect of varying the sulphite concentration is shown in Table 5. There is a wide range of concentrations in which the reaction goes almost, but not quite, to completion; 25 mol. of sulphite/mol. of cystine was taken as standard. This

gives a pH of approximately 8·5. Cysteine silver mercaptide is soluble in sodium sulphite, but the solution is still photochemically active.

DISCUSSION

With silver nitrate, GSH undergoes the same type of reaction as it does with cuprous sulphate (Hopkins, 1929; Pirie, 1931). The fact that G_2S_2 does not show signs of complex formation with silver nitrate suggests that the second equivalent of silver in the GSAg complex is bound either to the sulphur or to the mercaptide silver.

The cysteine silver mercaptide precipitate also binds additional silver, though less firmly than GSAg, suggesting that it forms an insoluble and rather unstable complex. This is in accord with Vickery & Leavenworth's (1930) description of a compound $(C_3H_5NSO_2Ag)_2.Ag_2SO_4$ arising from the reaction of cystine with silver sulphate. Challenger & Rawlings (1937) found that ethanethiol reacts with mercuric chloride to give an insoluble complex of composition $C_2H_5SHgCl.HgCl_2$. These results suggest that most thiols will tend to give complexes with heavy metal salts, but leave unexplained the marked differences in solubility (Pirie, 1931) of these complexes.

The potentiometric titration method was worked out empirically, as the theoretical treatment appeared to be very complicated. The apparent constancy of the solubility product of silver bromide over the range of conditions used is rather surprising, but it agrees with the results of Ruka & Willard (1949). They found that the solubility of silver bromide in water, and in nitric acid solutions ranging from 0·03 to 1·0 M was the same within their experimental error.

It is hoped soon to adapt these methods of thiol and disulphide estimation to work on proteins. However, the reactivities of these groups in different proteins are likely to differ, and it will be necessary to reinvestigate the conditions of reaction for each protein.

2. The Hydrolytic Fission of Disulphides

It is well known that large differences exist in the reactivities of the sulphur groups of different thiols and disulphides. These differences have generally been demonstrated in the past under rather violent conditions. Thus Brand & Sandberg (1926) measured the amount of sulphur removed from thiols and disulphides by boiling in 0·1 N-sodium carbonate for 10 min. or by standing for many hours in a mixture of N-sodium hydroxide and lead acetate. Schöberl & Hornung (1938) heated the various sulphur compounds with N-sodium hydroxide at 100° for 2 hr. and analysed the breakdown products. It was thought that an attempt to relate such differences in sulphur reactivity in simple compounds to differences in their molecular structure might throw light on similar differences which exist in protein sulphur groups. In the experiments just quoted the substances concerned underwent considerable disintegration, bonds other than those involving sulphur being broken. For the study proposed a much milder reaction was required in which only the sulphur bonds were involved. Such a reaction was found in the hydrolytic fission of disulphides in the presence of silver nitrate. The nature of hydrolytic fission reactions was first demonstrated by Schiller & Otto (1876) and later by Fromm (1908). They boiled diphenyl and di-*p*-toluyl disulphides with ethanolic potash and showed that the reaction went according to the following equations (Ar = aryl radical).

$$2Ar.S.S.Ar + 2H_2O \rightleftharpoons 2Ar.SH + 2Ar.SOH,$$
$$2Ar.SOH \rightleftharpoons Ar.SH + Ar.SO_2H.$$

The same type of reaction takes place in the presence of heavy metal salts. The function of the metal is to remove the thiol from the system as an insoluble mercaptide. Thus Challenger & Rawlings (1937) have shown that when diethyl disulphide is treated with mercuric chloride 75 % of the sulphur is found in an insoluble precipitate of composition $C_2H_5SHgCl.HgCl_2$. 25 % remains in solution as the sulphinic acid (Blackburn & Challenger, 1938).

Lavine (1937) has shown that cystine reacts in the same way when it is treated with mercuric sulphate solution. Some of the cystine sulphinic acid was isolated, and this was used by Ryklan & Schmidt (1944) to demonstrate the reverse reaction, i.e. $RSO_2H + 3RSH \rightarrow 2RSSR + 2H_2O$. The oxidation-reduction potentials of mixtures of known amounts of cystine sulphinic acid, cysteine, and cystine were measured. The results agreed with those calculated on the assumption that all the sulphinic acid had reacted with cysteine to form cystine.

In a study of the dismutation products of cystine disulphoxide Lavine (1936) found that the sulphinic acid is the most stable of the intermediate oxidative products of cysteine and shows no tendency to dismute further, e.g. to cysteic acid.

Vickery & Leavenworth (1930) added a saturated silver sulphate solution to a solution of cystine in sulphuric acid and neutralized with sodium hydroxide. The precipitate $(C_3H_5NSO_2Ag)_2.Ag_2SO_4$, accounted for approximately 90 % of the original cystine. A small amount of cysteic acid was isolated from the filtrate. The reason for the reaction going beyond the sulphinic acid stage is not clear, though the local high pH's induced by the addition of 0·8 N-sodium hydroxide may have been partly responsible.

The substances chosen for comparative investigation in this work were cystine and G_2S_2. At concen-

trations of around 10^{-4} M and within the range pH 4·5–9 they reacted with silver nitrate at speeds suitable for kinetic studies. The evidence just quoted was considered sufficient to justify the assumption that the reactions would go according to the equations:

$$2RSSR + 2H_2O \rightleftharpoons 2RSH + 2RSOH, \quad (1)$$
$$2RSOH \rightleftharpoons RSH + RSO_2H, \quad (2)$$
$$3RSH + 3Ag^+ \longrightarrow 3RSAg + 3H^+. \quad (3)$$

This assumption was confirmed by the finding that whenever the reactions went to completion the amount of thiol formed agreed exactly with that calculated from these equations. The reactions were carried out in the presence of an excess of silver nitrate, so that estimation of the free silver ion remaining at any stage of the reaction (by the titration method described in Part 1) gave a measure of the amount of thiol that had been formed. It was found that all the buffers used, with one exception, played some part in the reactions. For this reason the experimental results are given under the main headings of the buffers with subheadings for the substrates.

The results obtained confirm that, over the range of conditions used, both the rates and the mechanism of the reactions of cystine and of G_2S_2 show considerable differences.

An attempt has been made to relate these differences to the molecular structures of the two substances.

KINETIC THEORY

A simple kinetic treatment was found useful in interpreting the results. The assumptions made were: (*a*) That (1) is the limiting reaction with the equilibrium on the side of the disulphide. Attempts to isolate the sulphenic acid have failed (Fromm, 1908; Lavine, 1937) supporting the assumption that (2) is rapid relative to (1). (*b*) That once RSH has reacted with silver ion to give RSAg (3), it takes no further part in the reaction. The reaction may be summarized as

$$2R_2S_2 + 2H_2O \xrightarrow{k} 3RSH + RSO_2H, \quad (4)$$

so that working in equivalents

$$\frac{4}{3}\frac{d(RSH)}{dt} = -\frac{d(R_2S_2)}{dt} = k(R_2S_2).$$

If c equiv./l. = initial concentration of R_2S_2 and x = reacted concentration of $R_2S_2 = \frac{4}{3}$ times silver uptake in equivalents, then

$$\frac{dx}{dt} = k(c-x),$$

and

$$\frac{1}{c-x}\frac{dx}{dt} = k;$$

integrating,

$$-\ln(c-x) = kt + \text{constant},$$

when $t = 0$, then constant $= -\ln c$, and so

$$\ln\frac{c}{c-x} = kt,$$

or

$$\log c - \log(c-x) = \frac{k}{2{\cdot}303}.t.$$

If the reaction is unimolecular in respect of disulphide, then a straight line should be obtained by plotting log $(c-x)$ against time: this was sometimes so and sometimes not. Attempts to explain the deviations on kinetic grounds were unsuccessful, but the comparative deviations from a linear plot proved to be a useful criterion.

EXPERIMENTAL

The reaction mixtures were incubated at 37° in tightly stoppered boiling tubes. Owing to the photosensitivity of cysteine silver mercaptide a light-proof water bath was used.

Samples (1·0 ml.) were pipetted off into titration vessels containing sufficient 0·1 M-HNO_3 to bring the pH to 2·0 in order to stop the reaction. The solutions containing cystine were exposed to light as little as possible during sampling and any that developed a brown colour through over-exposure were rejected. It was also necessary to stir the cystine solutions before sampling, by blowing through the pipette, as the cysteine mercaptide precipitate contained loosely bound silver which came off during titration. The free silver ion in the samples was determined by titration against 0·1 M-KBr as described in Part 1.

The choice of buffers was limited to those which do not precipitate silver ions or bind them strongly. This ruled out phosphate, borate and the glyoxaline derivatives. Those used were acetate, α-picoline and sodium barbitone. The buffer concentration was normally around 0·01 M. The halide content of all reagents used was measured as described in Part 1 and allowed for. This was particularly important for substances (such as buffers) which were present in relatively high concentrations.

The samples of cystine, GSH, and G_2S_2 were the same as those used in Part 1. The concentrations of the cystine and G_2S_2 solutions used were checked by the method described in Part 1.

RESULTS

Reactions in acetate buffer

Oxidized glutathione. The first two experiments with G_2S_2 in acetate buffer are given in detail (Table 6) to illustrate the method. The kinetic plots are shown in Fig. 5, curves *A* and *B*.

Solution *A* had a ratio of total silver added to mercaptide-bound silver at the end point of 3·0 and gave a linear, i.e. unimolecular plot. Solution *B* had a 'silver ratio' of 1·5 and the kinetic plot was not unimolecular although the reaction went to the end point. Accordingly in subsequent experiments the silver ratio was kept at 3·0 or higher.

As already pointed out two equivalents of silver are bound in the GSAg-silver nitrate complex, a fact which should be taken into account when calculating

Table 6. *Reaction of G_2S_2 with silver nitrate in acetate buffer*

(Solution *A*: 1 ml. G_2S_2, $2{\cdot}23 \times 10^{-3}$ M; 10 ml. $AgNO_3$, 10^{-3} M; 2 ml. sodium acetate, 10^{-1} M, pH 6·0. Solution *B*: 2 ml. G_2S_2, $2{\cdot}23 \times 10^{-3}$ M; 10 ml. $AgNO_3$, 10^{-3} M; 3 ml. sodium acetate, 10^{-1} M, pH 5·8. 1·0 ml. samples taken for titration. The titration figures are given in μl. 0·1 M-$AgNO_3$. The amount of G_2S_2/ml. in $A \equiv 1{\cdot}7$ μl. and in $B \equiv 3{\cdot}0$ μl. of 0·1 N-G_2S_2. Equation (4) shows that 1 mol. of G_2S_2 gives rise to 1·5 mol. of GSH, and so at the end point the uptake of silver should be 2·55 μl./ml. and 4·45 μl. 0·1 M-$AgNO_3$ for *A*, and *B*, respectively. The total Ag present initially per ml. was $A = 7{\cdot}7$ μl. and $B = 6{\cdot}7$ μl. of 0·1 M-$AgNO_3$; from this must be substracted 0·1 μl. to allow for the chloride content of the acetate.)

Time hr.	Time min.	Titre (μl. 0·1 M-$AgNO_3$)	Combined Ag (μl. 0·1 M)	c	x	$\log(c-x)$
			Solution *A*			
0	0	—	—	3·45	0	0·54
0	20	6·8	0·8	3·45	1·07	0·38
0	40	6·5	1·1	3·45	1·47	0·30
1	20	6·0	1·6	3·45	2·13	0·12
3	15	5·2	2·4	3·45	3·20	-0·70
4	15	5·0	2·6	3·45	—	—
10	0	5·0	2·6	—	—	—
			Solution *B*			
0	0	—	—	5·95	0	0·77
0	15	6·0	0·6	5·95	0·8	0·71
1	0	4·8	1·8	5·95	2·4	0·54
1	50	4·1	2·5	5·95	3·3	0·42
4	30	3·1	3·5	5·95	4·65	0·11
8	55	2·9	3·7	5·95	4·9	0·01
9	15	2·8	3·8	5·95	5·1	-0·07
20	30	2·7	3·9	5·95	—	—
48	0	2·2	4·4	—	—	—

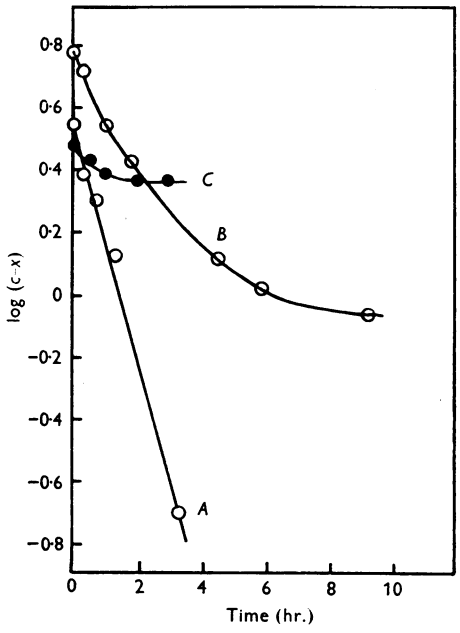

Fig. 5. Kinetic plots of the reaction of G_2S_2 with $AgNO_3$ in acetate buffer from the data given in Table 6. Ag ratio = Total initial Ag/Mercaptide-bound Ag at the end point. *A*, pH 6, Ag ratio = 3·0; *B*, pH 5·8, Ag ratio = 1·5; *C*, pH 4·9, Ag ratio = 3·0.

the silver ratio. However the cysteine mercaptide complex is less stable than the complex with GSAg and the stability of both complexes varies with the pH (see Part 1). In view of these complications the arbitrary method of basing the silver ratio on the mercaptide-bound silver only was used.

The next experiment, curve *C* in Fig. 5, shows the reaction of G_2S_2 in acetate at pH 4·5. Like '*B*' it gave a non-unimolecular plot, but differed in that it did not go to the end point in measurable time. These three types of reaction so far described are typical, and it is convenient to classify them.

Type I. Reaction unimolecular, end point reached.

Type II. Reaction not unimolecular, end point reached.

Type III. Reaction not unimolecular, end point not reached.

The reactions of G_2S_2 at pH 6 and 4·5 are profoundly different in character and it was desirable to find out at what pH the transition occurs. To this end four solutions were made up identical, except for their pH. One had a buffer of pure sodium acetate; the other three had, in addition, successive amounts of nitric acid. The silver uptake, expressed as the percentage of disulphide split, was plotted against pH, *A* after 2 hr., and *B* after 5 hr. (Fig. 6). This shows the transition to take place between pH 5 and 5·5.

Cystine. The results with cystine in acetate are shown by curves *A* and *B* in Fig. 7. '*A*', at pH 6·2, was a type II reaction since the end point was reached in 48 hr. '*B*', at pH 4·9, was a type III reaction since it practically stopped after 4 hr.

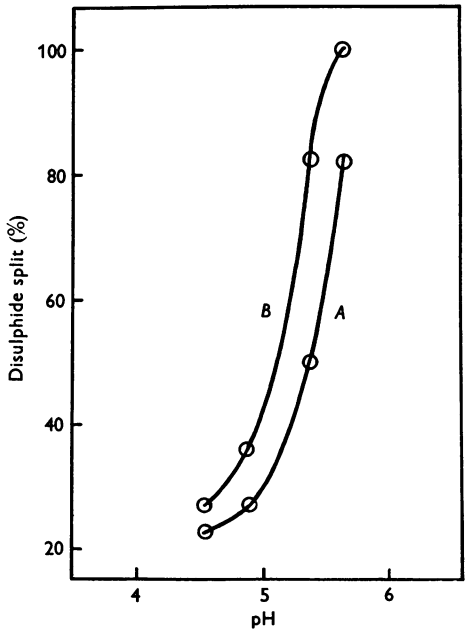

Fig. 6. Effect of pH on the G_2S_2-$AgNO_3$ reaction in acetate buffer. The reaction was carried out at four separate values of pH and measurements made after 2 hr. (curve *A*) and after 5 hr. (curve *B*).

There are reasons, which are mentioned in the Discussion, for thinking that the equilibrium concentration of cysteine in the presence of cystine is considerably lower than the corresponding concentration of GSH. In this and subsequent experiments with cystine the silver ratio was accordingly raised to 4·0 to reduce the possibility of (3) being the limiting reaction. This was as high as it could be raised without loss of accuracy in following the reactions.

Reactions in α-picoline buffer (a)

Cystine. α-Picoline was found to have a pK_a of approximately 6·1 by titration against nitric acid; it was used at a molar concentration 100 times that of the disulphide to give a pH of 6·9–7·0. At this pH cystine might have been expected to approach to a type I reaction. In fact it was nearer type III, (curve *C* in Fig. 7), though it did show differences from the reaction in acetate at pH 4·9 (curve *B* in Fig. 7). The reaction went further in picoline, 50 % of completion as against 22 %, and the curve does not show the marked change of slope that the reaction at low pH (of G_2S_2 as well as cystine) does.

It was found that small additions of acetate acted catalytically and restored the type II reaction, the rate being slightly greater than in acetate at pH 6·2. Curves *D*, *E* and *F* in Fig. 7 show the effect of adding acetate in 1, 5 and 25 times the molarity of the cystine. Increasing the amount beyond 5 mol. makes little, if any, difference to the rate of reaction.

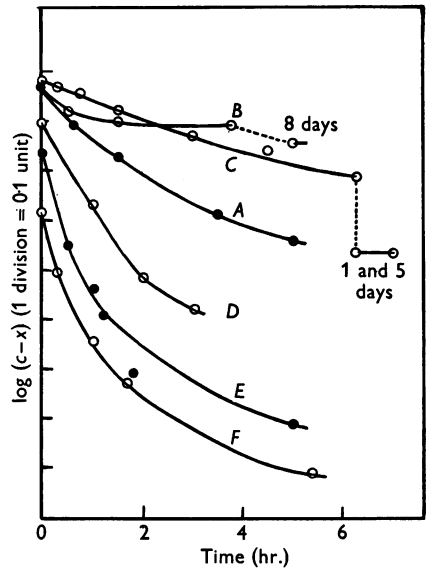

Fig. 7. Kinetic plots of the cystine-$AgNO_3$ reaction under various conditions. *A*, reaction in acetate buffer at pH 6·2; *B*, reaction in acetate buffer at pH 4·9; *C*, reaction in picoline buffer at pH 7·0. *D*, reaction in picoline buffer at pH 7 + acetate (1 mol./mol. cystine); *E*, reaction in picoline buffer at pH 7 + acetate (5 mol./mol. cystine); *F*, reaction in picoline buffer at pH 7 + acetate (25 mol./mol. cystine). For the sake of clarity in drawing this and subsequent graphs the curves were shifted along the ordinate when necessary, since only their slopes and shapes are to be compared. The figures relating to log $(c-x)$ are not given therefore, but the scale is.

Oxidized glutathione. The following experiments were tried with G_2S_2 in α-picoline: (1) G_2S_2 in picoline at pH 5·9; (2) G_2S_2 in picoline at pH 7; (3) G_2S_2 in picoline at pH 7 + acetate (25 times molarity of the G_2S_2); (4) G_2S_2 in picoline at pH 7 without silver nitrate.

Curves *A* and *B* in Fig. 8 show that the reaction in picoline at pH 5·9 was slightly slower than the reaction in acetate at pH 6. At pH 7 (Fig. 8, curve *C*) the reactions in the presence and absence of acetate proceeded identically. These results suggest that the G_2S_2 reaction is only very slightly affected by acetate catalysis. Exp. 4 was done to see whether any reaction takes place in the absence of silver. The solution was incubated at 37° for 2 hr. and then analysed for GSH (as described in Part 1). None was found.

Reactions in barbitone buffer

Cystine. An experiment was tried with cystine in this buffer at pH 8·9 in the hope that the higher pH might induce a unimolecular reaction. This time, however, the uptake of silver was entirely inhibited. Two explanations seemed possible: (1) the cystine remained intact, protected in some way by the barbitone; (2) the reaction took place, but the cysteine formed combined with the barbitone instead of with the silver.

To test (2) cysteine ester hydrochloride was incubated under nitrogen at 37° with an excess of barbitone and then analysed for thiol + chloride in the usual way. After 5 hr. incubation there was a 97 % recovery of cysteine ester hydrochloride. This small loss can be explained by a slight leakage of oxygen, and explanation (2) is therefore invalid.

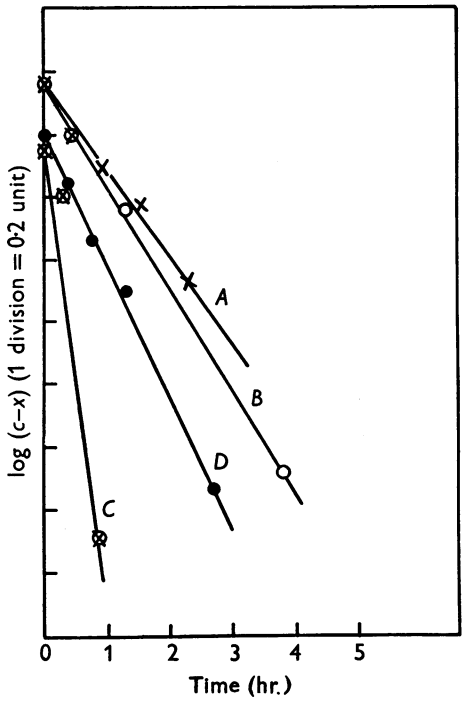

Fig. 8. Kinetic plots of the G_2S_2-$AgNO_3$ reaction under various conditions. *A*, reaction in picoline buffer at pH 5·9; *B*, reaction in acetate buffer at pH 6; *C*, reaction in picoline buffer at pH 7, and reaction in picoline buffer at pH 7 + acetate (25 mol./mol. G_2S_2). *D*, reaction in barbitone buffer at pH 9·1.

Oxidized glutathione gives a type I reaction in barbitone (Fig. 8, curve *D*), but it is considerably inhibited, the rate being less than that in picoline at pH 7.

Cystine + oxidized glutathione. An attempt was made to use this inhibitory effect of barbitone in analysing mixtures of cystine and G_2S_2. However, when a mixture of cystine and G_2S_2 was incubated with silver nitrate in barbitone buffer, the silver uptake showed that the cystine, as well as the G_2S_2, had reacted completely. It was then found that cysteine silver mercaptide is soluble in solutions of GSH and G_2S_2 and loses most of its photochemical activity once dissolved. This suggests that the cysteine mercaptide in these soluble complexes is behaving like GSAg. Thus the explanation for the unexpected absence of inhibition could be that cystine forms a reactive complex with G_2S_2 as well as an unreactive one with barbitone.

There are two further observations which may be included here. First, if acetate is added to the barbitone in place of G_2S_2 the inhibition remains. Secondly, if barbitone is added to a mixture of cysteine and sodium sulphite the reaction proceeds normally. These results are consistent with the fact that cysteine silver mercaptide, and therefore presumably cystine, does not form a complex with acetate but does with sodium sulphite.

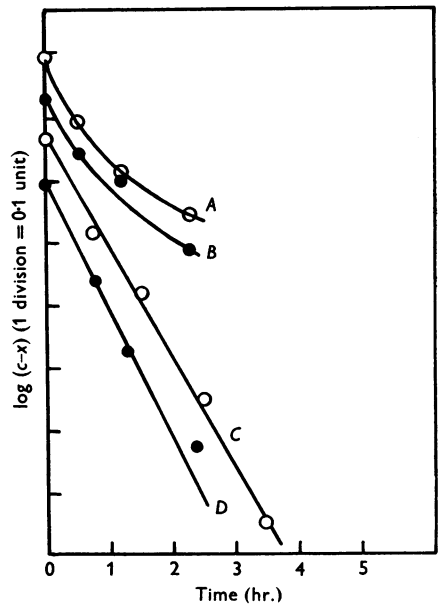

Fig. 9. Kinetic plots of the cystine-$AgNO_3$ reaction in picoline buffer at pH 7 when catalysed by GSH.

	Ag ratio	Mol. GSH/ mol. cystine
A	3·1	1·0
B	3·1	1·5
C	4·2	1·0
D	4·2	1·5

Reactions in α-picoline buffer (b)

Cystine. So as to investigate the effect of glutathione on cystine with a minimum of other complications, α-picoline was again used as the buffer with

GSH as the catalyst. Two solutions were made up containing, respectively, 1·0 and 1·5 mol. of GSH/mol. of cystine. The pH was 7·0 in each case. The reactions (Fig. 9, curves *A* and *B*) were still type II although the rate was the highest so far obtained with cystine. The silver ratio was 3·1 calculated on the assumption that the GSH was removing only one equivalent of silver from participation in the reaction. The experiment was repeated with a larger excess of silver, so that the silver ratio was 4·2, or 2·9 after allowing for the GSH removing two equivalents. This time (Fig. 9, curves *C* and *D*) type I reactions resulted, with the rates very similar for the two concentrations of GSH and comparable with the G_2S_2 reaction in acetate at pH 6. Thus, when in the presence of GSH, cystine can resemble G_2S_2.

DISCUSSION

The classification of the reaction kinetics into three types requires further explanation.

The reactions of G_2S_2 will be considered first as they are simpler than those of cystine. Three types of reaction have been found which have been arbitrarily classified as types I, II and III. In type I the initial splitting of the disulphide bond may be considered to be the limiting process as the kinetics show that the reaction is unimolecular in respect of disulphide. In the type II reactions this is not so, since the reactions become relatively slower as they proceed, and some other process must be the limiting one. This could be either the dismutation of sulphenic acid or the reaction of thiol with silver nitrate. With G_2S_2 it was shown to be the latter, since raising the silver nitrate concentration was sufficient to restore the type I reaction. The type III reactions are neither unimolecular, nor do they go to the end point. With G_2S_2 they occur at pH below 5. The fact that the end point is not reached suggests that an accumulation of the reaction products must occur and that equilibrium is reached after a time. This could be explained by assuming that, under the conditions used, it is RSO^- which dismutes and not RSOH. Thus at low pH when the unionized form predominates, the dismutation reaction becomes slow, so that the concentration of unionized sulphenic acid increases, and any thiol present will tend to react with it to give disulphide, rather than with silver ion. This argument presupposes the pK_a of glutathione sulphenic acid to lie in the region of 5–5·5. No figures are available, but such a value is thought not to be unreasonable.

The reactions of cystine are more complicated. Except in the presence of glutathione, it never gave a type I reaction. This means that in all other cases the limiting process was either dismutation or mercaptide formation. By analogy with the oxidation-reduction potentials and the pK_a's of the SH groups (Table 7), it is to be expected that the equilibrium concentration of cysteine in reaction (1) is considerably lower than that of GSH, and the increased silver nitrate concentration used for the cystine experiments may have been insufficient to allow for this. Even when catalysed by GSH the reaction of cystine was still more affected by variations of the silver-ion concentration than that of G_2S_2.

The type III reactions of cystine may be explained on the same basis as those of G_2S_2, though there is no sudden transition from type III to type I. In acetate buffer there is a transition from type III at pH 4·9 to type II at pH 6·2. By analogy with the pK_a's of the —SH groups (Table 7) the pK_a of cysteine sulphenic acid should be slightly higher than that of glutathione sulphenic acid. Thus one would expect the 'transition pH' of cystine to be slightly above that of G_2S_2, assuming that it is indeed related to the pK_a of the sulphenic acid. The behaviour of cystine in picoline at pH 7, when the reaction appears to be borderline between types II and III, can only be explained by assuming that, near the transition pH and in the absence of acetate, the dismutation of RSO^- is still extremely slow.

The mechanism suggested for the acetate catalysis is analogous to the reactions of sulphite and cyanide with disulphides, with the difference that the acetate compound must have a limited life.

$$RSSR + NaOAc \rightleftharpoons RSNa + [RSOAc].$$

The actual splitting of the —S—S— link is probably preceded by an electronic asymmetry, i.e.

$$RS^+\!\!-\!\!^-SR + H^+ + OH^- \rightleftharpoons RSH + RSOH,$$

and this could be initiated by collision with an acetate ion.

One still has to explain how it is that although acetate ion catalyses both the cystine and G_2S_2 reactions, yet type III reactions occur in acetate buffer at low pH. A possible explanation is provided by the reaction scheme below:

$$\begin{array}{ccccc} 2RSSR + 2H^+ + 2OAc^- & \longrightarrow & 2RSH + 2RSOAc & \xrightarrow{(b)} & RSO_2^- + RSH + 2OAc^- + 3H^+ \\ & & \downarrow (a)\ 2H_2O & & \uparrow (c) \\ & & 2HOAc + 2RSOH & \rightleftharpoons & 2RSO^- + 2H^+ \end{array}$$

In this it is assumed that (*c*) is a relatively slow reaction while (*b*) is relatively rapid: (*a*) is a strongly acid-catalysed reaction. Thus at low pH (*a*) is rapid so that (*b*) does not occur. Also at low pH, RSOH predominates over RSO^- so that altogether dismuta-

tion is very slow, and a type III reaction results. At higher pH (*a*) becomes slow so that (*b*) can occur rapidly.

The catalysis of the cystine reaction by GSH and G_2S_2 differs from acetate catalysis because it involves the formation of complexes. The behaviour of mixtures of cystine and GSH or G_2S_2 suggest that, in the complex, cystine takes on some of the properties of G_2S_2.

There is sufficient evidence available to suggest a possible reason for the difference in sulphur reactivity of cystine-cysteine and G_2S_2-GSH, respectively.

In Table 7 are given the reported values of the pK_a's of the thiol groups of cysteine and GSH, and the oxidation-reduction potentials of the two thiol-disulphide systems. Ryklan & Schmidt's (1944) values for the oxidation-reduction potentials are thought to be reliable because, in their experiments, in contrast to those of earlier workers (Dixon & Quastel, 1923; Michaelis & Flexner, 1928), true equilibrium between the thiol and disulphide forms was obtained.

Table 7. *Oxidation-reduction potentials and* pK_a*'s of the* SH *of certain thiols*

RSH	$E^{\circ\prime}$ (V.)*	pK_a
Cysteine	0·27	10·28†
GSH	0·45	9·62‡

* Ryklan & Schmidt (1944).
† Cannan & Knight (1927).
‡ Pirie & Pinhey (1929).

These figures, and the differences in photochemical activity, all point to the fact that the sulphur in G_2S_2-GSH is more electropositive than it is in cystine-cysteine. This can be accounted for by postulating some form of electrostatic bond in G_2S_2-GSH which has the effect of drawing electrons away from the sulphur. That which appears most likely is a hydrogen bond between the sulphur and the $—NH_3^+$ of the glutamyl radical, the hydrogen coming from the amino group so that the mercaptan hydrogen is left free to react. Experiments with Hirsch-Fischer models show that there is no obvious steric objection to this.

Because of the failure of hydrogen sulphide, the thioalcohols, and thiophenols to exhibit the high degree of molecular association shown by their oxygen analogues, it has often been assumed that sulphur has little tendency to form hydrogen bonds. However, Hopkins & Hunter (1942) have shown that a number of compounds, notably the thioamides, show molecular association in naphthalene solution, which they ascribe to S—H—N bonds. They found that this behaviour is only shown by compounds in which tautomeric exchange of the hydrogen between the nitrogen and sulphur is possible.

Obviously no tautomerism is possible in G_2S_2 or GSH. On the other hand, they both have a positively charged amino group which could form hydrogen bonds with the sulphur by chelation instead of by polymerization.

The idea is put forward as a working hypothesis because it does offer an explanation for a number of otherwise unexplained facts. For example the effect of GSH and G_2S_2 on cystine and cysteine may be explained by assuming that, in the complexes, some of the hydrogen bonding is transferred to cystine or cysteine.

It is possible, too, that the wide differences in sulphur reactivity in relatively simple compounds reported by Brand & Sandberg (1926) and Schöberl & Hornung (1938) may be explained by the existence of bonds of this nature.

More important are the well known differences in reactivity of the sulphur groups in proteins, in particular the 'appearance' of thiol groups on denaturation. In the native protein these groups could be hydrogen bonded to the extent that they will no longer react with nitroprusside and other thiol reagents. On denaturation these bonds are broken and the thiol groups then behave in a manner approximating to that of cysteine.

SUMMARY

Part 1

1. It was shown, by means of a potentiometric titration, that reduced glutathione (GSH) reacts with one equivalent of silver nitrate to form a silver mercaptide and with a second equivalent to form a soluble complex.

2. This reaction was used in the estimation of GSH. An excess of silver nitrate was added to the GSH, and the free silver ion remaining was estimated by back-titration with potassium bromide. This titration was also followed potentiometrically; the end points were reproducible to 10^{-8} g.equiv. The method can be used for other thiols.

3. It was shown that thiols may be estimated in the presence of disulphides by this method. The solution must first be acidified to pH 2·0 with dilute nitric acid in order to prevent the hydrolytic decomposition of the disulphides. The reaction between thiols and silver nitrate is not affected by this reduction of pH.

4. Cystine and oxidized glutathione (G_2S_2) were estimated by reaction with sodium sulphite, followed by estimation of the thiol formed by the method described. The conditions for the reaction with sodium sulphite were reinvestigated.

PART 2

5. The reaction kinetics of the hydrolytic decomposition of G_2S_2 and cystine in the presence of an excess of silver nitrate were studied under various conditions. The reactions were classified into three main types.

6. Acetate was shown to catalyse the reaction of cystine and, to a much lesser extent, that of G_2S_2. Similarly, barbitone inhibited the cystine reaction completely, but that of G_2S_2 only slightly.

7. In the presence of GSH and G_2S_2, cystine was shown to behave in a manner resembling G_2S_2.

8. Tentative explanations for the three different types of reaction kinetics are put forward.

9. A possible explanation for the greater reactivity of the sulphur in GSH and G_2S_2 as compared with that in cysteine in cystine is given. This involves a hydrogen bond between the sulphur in GSH and G_2S_2 and the $—NH_3^+$ group of the glutamyl radical.

My thanks are due to Dr A. G. Ogston for much helpful advice, and to Dr R. B. Fisher for the gift of glutathione.

Correction. Subsequent work has disclosed an impurity in the α-picoline used in Part 2 of this work. The cystine-silver nitrate reaction carried out with purified α-picoline at pH7·0 is Type II and not Type III. Curve *C* in Fig. 7 becomes intermediate between curves *A* and *D*. No other results are affected. Details will be given in a later publication.

REFERENCES

Benesch, R. & Benesch, R. E. (1948). *Arch. Biochem.* **19**, 35.
Blackburn, S. & Challenger, F. (1938). *J. chem. Soc.* p. 1872.
Brand, E. & Sandberg, M. (1926). *J. biol. Chem.* **70**, 381.
Cannan, R. K. & Knight, B. C. J. G. (1927). *Biochem. J.* **21**, 1384.
Challenger, F. & Rawlings, A. A. (1937). *J. chem. Soc.* p. 868.
Clarke, H. T. (1932). *J. biol. Chem.* **97**, 235.
Dixon, M. & Quastel, J. H. (1923). *J. chem. Soc.* p. 2943.
Fromm, E. (1908). *Ber. dtsch. chem. Ges.* **41**, 3403.
Hass, K. & Jellinek, K. (1932). *Z. phys. Chem.* **162**, 153.
Heatley, N. G. (1939). *Mikrochemie*, **26**, 147.
Hopkins, F. G. (1929). *J. biol. Chem.* **84**, 269.
Hopkins, G. & Hunter, L. (1942). *J. chem. Soc.* p. 638.
Kolthoff, I. M. & Harris, W. E. (1946). *Industr. Engng Chem.* (Anal. ed.). **18**, 161.
Lavine, T. F. (1936). *J. biol. Chem.* **113**, 583.
Lavine, T. F. (1937). *J. biol. Chem.* **117**, 309.
MacInnes, D. A. & Dole, M. (1929). *Industr. Engng Chem.* (Anal. ed.). **1**, 57.
Michaelis, L. & Flexner, L. B. (1928). *J. biol. Chem.* **79**, 689.
Pirie, N. W. (1930). *Biochem. J.* **24**, 51.
Pirie, N. W. (1931). *Biochem. J.* **25**, 614.
Pirie, N. W. & Pinhey, K. G. (1929). *J. biol. Chem.* **84**, 321.
Ruka, R. & Willard, J. E. (1949). *J. phys. colloid Chem.* **53**, 351.
Ryklan, L. R. & Schmidt, C. L. A. (1944). *Univ. Calif. Publ. Physiol.* **8**, 257.
Schiller, R. & Otto, R. (1876). *Ber. dtsch. chem. Ges.* **9**, 1637.
Schöberl, A. & Hornung, T. (1938). *Liebigs Ann.* **534**, 210.
Schöberl, A. & Ludwig, E. (1937). *Ber. dtsch. chem. Ges.* **70**, 1422.
Vickery, H. G. & Leavenworth, C. S. (1930). *J. biol. Chem.* **86**, 129.

Comparative Studies of 'Bile Salts'

1. PRELIMINARY SURVEY

BY G. A. D. HASLEWOOD AND VERYAN WOOTTON
Guy's Hospital Medical School, London, S.E. 1

(*Received* 3 *April* 1950)

Bile is apparently produced by all vertebrates, and the 'bile salts' might perhaps be described as physiologically the most important constituent. In the absence of complete data and a knowledge of the problems of digestion and absorption to be solved by each species, it is hardly possible at present to speculate profitably about the efficiency (for digestion and absorption) of different biles. It is, however, of some interest that the nature of the bile salts is by no means constant, but sometimes shows considerable differences as between species and species. Many of these differences are, chemically, considerable; they do not appear to be dependent on temporary variations of environment, but are apparently characteristic of, and invariable in, a species.

It seems, therefore, that we may have in the chemical nature of bile salts a character which will be of some value as a clue to species characterization, and which might even be an aid in the study of evolutionary history.

Any such possibility can only be explored after a wide comparative survey of the occurrence of different bile salts. Such a survey appears to have been begun by Japanese workers between about 1920 and 1941, largely as an attempt to find and identify intermediates in the assumed oxidation of cholesterol to the bile acids. A comprehensive collection of results does not appear in the literature: we have attempted to fill this gap in Tables 1–4. To avoid multiple references these tables refer to collections of data already made by Sobotka (1937) and