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The Estimation of Thiols and Disulphides by Potentiometric Titration with Silver Nitrate

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(Received 23 August 1954)

This paper describes further developments in the estimation of thiols by potentiometric titration with silver nitrate. A previous paper (Cecil, 1950) described a method in which the thiols were allowed to react with a known excess of silver nitrate followed by back-titration with potassium bromide. Although the results then obtained agreed well with the theoretical figures, it has now been shown that the agreement was due to compensation of errors.

It has been found that these errors can be avoided by titrating the thiols directly with the silver nitrate in buffered solutions. The silver mercaptides formed tend, in most cases, to form complexes with additional silver ion which can interfere with the determination of the thiol end point. However, the stability of these complexes varies greatly with pH, and a range of pH has been found for every compound in which the thiol group can be titrated satisfactorily.

As the pH is raised, so the concentration of silver ion in equilibrium with free thiol falls and becomes so low that reproducible potentials cannot be obtained with a silver electrode. This difficulty has been overcome by the development of the silver thiol electrode (Cecil, 1954) which is reversible to the thiol as well as to the silver ion.

As in the previous work, the disulphides are first allowed to react with sodium sulphite and the thiol formed estimated in the same way.

THEORETICAL

Reaction of thiols with silver nitrate. The reaction of thiols with silver nitrate is described by the equations

In the presence of solid silver mercaptide

where S is defined as the solubility product. The equilibrium equation for (2) is

$$K = \frac{[\text{RS}^-] \times [\text{H}^+]}{[\text{RSH}]}, \quad (4)$$

K being the dissociation constant of the thiol. This may be expressed in terms of total thiol concentration, c , where $c = [\text{RSH}] + [\text{RS}^-]$ so that (4) now becomes

$$[\text{RS}^-] = c \cdot K / (K + [\text{H}^+]), \quad (5)$$

and substituting in (3)

$$S = [\text{Ag}^+] c \cdot K / (K + [\text{H}^+]). \quad (6)$$

The end-point titre (c_{ep}) is reached when the total silver added equals the total thiol originally present:

$$[\text{Ag}^+]_{\text{ep}} + [\text{RSAg}] = c_{\text{ep}} + [\text{RSAg}],$$

or

$$[\text{Ag}^+]_{\text{ep}} = c_{\text{ep}}. \quad (7)$$

From (6) the silver ion concentration at the end point is

$$[\text{Ag}^+]_{\text{ep}} = \left(S \frac{K + [\text{H}^+]}{K} \right)^{\frac{1}{2}} = \sqrt{S'}, \quad (8)$$

where S' is the apparent solubility product under any one set of conditions.

This equation is of limited application at present because of the difficulties of defining the various quantities. K is known only for thiols that do not contain amino groups. Different ionic species of the same compound are likely to have different solubility products and these must be taken into account when using eqn. 6. Lastly, species other than thiol, for example sulphite and silver mercaptides, bind silver ion sufficiently to depress the end-point concentration below the predicted value. To this extent therefore the method is empirical, and sufficient of each titration curve must be plotted to determine the end-point potential.

If there is to be a simple relation between $[\text{Ag}^+]$ and c during a titration, then the term $K/(K + [\text{H}^+])$ must remain constant. Adequate buffering is therefore essential. Because of the low silver ion concentration prevailing in the presence of thiol, the usual buffer salts acetate, phosphate, borate, as well as nitric or sulphuric acid, may be used, provided their concentrations are kept low.

The titrations are followed potentiometrically with silver or silver thiol electrodes. At pH < 2.5 the silver ion concentrations are sufficiently high to be followed with a silver electrode. At higher pH this is not so, and a silver thiol electrode must be used. This consists of a silver electrode covered with a thin layer of silver mercaptide.

In the presence of thiol the reaction on the electrode surface is

and the potential is described by the equation

$$E_{\text{RS}^-} = E_{\text{RS}^-}^\circ - \frac{RT}{F} \ln [\text{RS}^-], \quad (10)$$

and substituting for $[\text{RS}^-]$ from eqn. 5

$$E_{\text{RS}^-} = E_{\text{RS}^-}^\circ - \frac{RT}{F} \ln c + \frac{RT}{F} \ln \frac{(K + [\text{H}^+])}{K}. \quad (11)$$

In the presence of silver ion the potential is described by the equation

$$E_{\text{Ag}^+} = E_{\text{Ag}^+}^\circ + \frac{RT}{F} \ln [\text{Ag}^+]. \quad (12)$$

In the presence of both thiol and silver ion, as the electrode can have but one equilibrium potential, this must be described equally by (11) and (12), i.e. $E_{\text{RS}^-} = E_{\text{Ag}^+} = E$.

It is convenient to use one standard potential only, so combining equations (6) and (12)

$$E = E_{\text{Ag}^+}^\circ - \frac{RT}{F} \ln c + \frac{RT}{F} \ln S + \frac{RT}{F} \ln \left(\frac{K + [\text{H}^+]}{[\text{H}^+]} \right), \quad (13)$$

and from eqns. 11 and 13

$$E_{\text{RS}^-}^\circ - E_{\text{Ag}^+}^\circ = \frac{RT}{F} \ln S. \quad (14)$$

The potentials obtained with a silver thiol electrode may be interpreted in terms of either thiol or silver ion concentrations, whichever is convenient. The titration curves obtained may be treated in the same way as those obtained with silver electrodes.

It was reported earlier (Cecil, 1954) that thiols containing free amino groups show anomalous electrode responses at high pH. This effect may be ignored in the present work because (a) the effect is small at concentrations below 10^{-4} M and so will not affect the titration curves near the end point, and (b) the effect is small at pH < 8 and the titrations described are normally done below this pH.

Reaction of disulphides with sodium sulphite

The reactions are

Although the precise conditions of reaction, i.e. time, temperature and SO_3^{2-} concentration, have to be determined for each compound, there are two points of general application which define the pH range. First, Stricks & Kolthoff (1951) have shown that reaction (15) is reversible, the extent of the back reaction depending on the amount of RS^- formed. Thus if the reaction is to go to completion the reaction pH must be sufficiently below the pK of the thiol. This effect is particularly important where the thiol formed must be titrated at a pH lower than that used for reaction.

Secondly, the present authors (Cecil & McPhee, 1954) have found from kinetic studies that the bisulphite ion does not react with any of these disulphides. The second pK of sulphurous acid is 7.2 (Tartar & Garretson, 1941) and so the rate of reaction will fall off rapidly with pH.

EXPERIMENTAL

Apparatus

The apparatus used for the titrations was the same as described previously (Cecil, 1950) with minor alterations, as follows:

(a) Stirring was effected by mounting the titration vessel on a rotating platform driven by an air-turbine directly beneath (Stock & Fill, 1950). The turbine was worked by suction from a filter pump. This method of stirring is efficient and, unlike the bubbling method previously used, does not affect the readings of the electrodes.

(b) The reference half-cell was Ag/AgCl instead of $\text{Hg}/\text{Hg}_2\text{Cl}_2$ —this simplified the apparatus and was equally satisfactory. The 3.5 M-KCl was saturated with solid AgCl and a silver wire immersed in it. The difference in potentials caused by this change was +35 mv.

Silver electrodes

(a) *Construction.* 28 s.w.g. platinum wire was sealed into a glass tube so that 5–10 mm. was exposed. The end of the wire was fused so as to eliminate sharp edges. In use the whole of the exposed part of the wire was immersed in the solution with the glass tube covering the wire at the air-water interface. 36 s.w.g. tinned copper wire was used for leads. Soldered joints on platinum are unsatisfactory and so a short piece of silver wire was fused to the platinum and the copper lead soldered to it. Mercury must not be used for making contact as it penetrates the glass seal and causes large errors in potential.

(b) *Gold plating.* Platinum is slowly attacked by thiols, even through a silver coating, and to prevent this a protective coating of gold was used. This too is attacked slowly but can be stripped off whenever the electrode is re-silvered. A potassium aurocyanide solution was used (Clarke, 1928) with a current density of 2–3 mA/cm.² for approximately 5 min. with stirring.

(c) *Silver plating.* A 'soft' deposit of silver was found to be most satisfactory. This was obtained by using a potassium argentocyanide solution with a high carbonate content, together with a high current density (Sanigar, 1929). The current density was 15–30 mA/cm.² and the composition of the plating solution was as follows: AgNO_3 , 3.5 g.; KCN, 4.0 g. (= 50% excess); K_2CO_3 , 10 g.; water, 100 g. The plating time is not critical, 10–60 sec. being satisfactory. After plating, the electrodes were washed thoroughly in distilled water.

Silver thiol electrodes

(a) *H_2S treatment.* Silver electrodes were dipped into aqueous H_2S (0.01 to 0.005 M) for 10–60 sec., to give a thin, even coating of Ag_2S . This should not produce any visible tarnishing of the electrode surface. If the sulphide layer is too thick, equilibrium will be slow and the potentials will drift when the stirring is started and stopped.

Solutions of H_2S oxidize rapidly. If degassed water is used the solutions keep much longer but the H_2S attacks the

silver too rapidly. It was found that the CO_2 normally present in water slows the action of H_2S on silver. The most satisfactory method is to dissolve the H_2S in degassed water but, before use, to mix this solution with water containing some dissolved CO_2 .

(b) *Equilibration with thiol.* This is an ion-exchange process between S^{2-} and the thiol concerned. It is rapid with RS^- and slow with RSH . Two concentrations of thiol were made up, approx. 10^{-3} and 10^{-4}M respectively, buffered in $\text{Na}_2\text{B}_4\text{O}_7$ at $\text{pH} \approx 9$. The electrodes were immersed in each solution alternately until reproducible potentials were obtained. Fresh solutions were used for each immersion: three immersions were usually sufficient. If the thiol was not available, a disulphide-sulphite mixture was used instead.

(c) *Cleaning of electrodes.* The silver layer was removed with conc. HNO_3 , and the gold layer with aqueous Cl_2 or by anodizing in HCl . This should leave the platinum bright and smooth. If not, it should be boiled in conc. HNO_3 for 10 min.

The preparation of these electrodes has been described in detail because they are the key to the whole method. The time required for plating and equilibration is not excessive and, once prepared, electrodes have been used for over a hundred titrations. Differences of potential between different electrodes do occur, and are reflected in differences in end-point potential. The titration end points, however, are not affected. The reason for these differences is still under investigation but they are generally due to attack of the gold or platinum by the thiol.

Reagents

β -Mercaptopropionic acid (L. Light and Co. Ltd., Poyle Trading Estate, Colnbrook, Bucks). A sample was dried over anhydrous MgSO_4 and distilled *in vacuo*. The capillary in the distillation flask was fed with dry, O_2 -free nitrogen. B.p. $58-60^\circ$ at approximately 1 mm. Hg.

L-Cysteine hydrochloride (Roche Products Ltd., Welwyn Garden City, Herts). The sample was kept in a desiccator over CaCl_2 .

L-Cysteine. Prepared from the hydrochloride by the method of du Vigneaud, Audrieth & Loring (1930).

DL-Homocysteine (L. Light and Co. Ltd.). A sample was sublimed at 180° at 0.02 mm. Hg.

Homocysteine thiolactone hydrochloride. Prepared from homocysteine by the method of Riegel & du Vigneaud (1936).

Reduced glutathione (GSH). A sample, DCB 524, was kindly supplied by the Distillers Company (Biochemicals) Ltd., Fleming Road, Speke, Liverpool 19.

$\beta\beta$ -Dithiodipropionic acid. Prepared from β -mercaptopropionic acid by oxidation with I_2 . After crystallization from water it had m.p. 156° (uncorr.). Biilmann (1905) gives $155-156^\circ$ (uncorr.).

L-Cystine (Roche Products Ltd.). Recrystallized once from hot dil. HCl .

Diformylcystine. Prepared from L-cystine by the method of Fruton & Clarke (1934). Potentiometric titration with NaOH gave an equiv. wt. of 146, theoretical = 148.

Diacetylcystine diethyl ester (diacetylcystine ester). Cystine ester dihydrochloride was prepared by the method of Abderhalden & Wybert (1916). The ester (0.2 g.) was dissolved in 2 ml. sat. aq. NaHCO_3 and extracted 3 times with

7 ml. portions of ethyl acetate. The combined extracts were dried over anhydrous Na_2SO_4 and filtered. A stream of ketene was passed through the solution at 20° . The ethyl acetate was evaporated *in vacuo* and a white crystalline product remained which melted sharply at 124° (corr.). Yield 60%, Hollander & du Vigneaud (1931) give m.p. $124-5^\circ$ (corr.).

DL-Homocystine (L. Light and Co. Ltd.). A sample was dissolved in hot dilute HCl and a little Norite added. The solution was filtered, and the homocystine precipitated by adjustment to pH 5. The precipitate was filtered and washed with water. The purification was repeated once.

Oxidized glutathione (GSSG). Prepared from GSH by the method of Schöberl & Hornung (1938).

Sodium sulphite. 0.1M solutions of reagent grade Na_2SO_3 containing $2 \times 10^{-3}\text{M}$ ethylenediaminetetraacetic acid (EDTA) were made up each week. The EDTA greatly reduced the rate of oxidation both of the Na_2SO_3 and of the thiols in contact with it. The halide content, measured as described by Cecil (1950), must not exceed 10^{-3} moles/mole of Na_2SO_3 .

Nitric acid. 0.1N solutions were prepared as described by Cecil (1950).

Silver nitrate. Reagent grade AgNO_3 was recrystallized once from water.

Buffer solutions. The following substances of reagent grade were used; KH_2PO_4 , K_2HPO_4 , $\text{Na}_2\text{B}_4\text{O}_7$, H_2SO_4 , sodium acetate, and acetic acid. Solutions of $\text{pH} > 5$ contained EDTA to the extent of one mole/50 moles of buffer. The following solution was used extensively and is referred to as phosphate A; KH_2PO_4 , 0.05M; K_2HPO_4 , 0.05M; EDTA, $2 \times 10^{-3}\text{M}$. Molarities quoted refer to the phosphate radical.

Distilled water. Glass-distilled water was used throughout.

METHOD

Solutions of thiols were made up in freshly degassed water and kept at 2° when not in use. The rate of oxidation was then 1–5% in 24 hr. for a 10^{-3}M solution, depending on the compound and its freedom from heavy metal impurities. Cystine and homocystine were dissolved in 0.01N- H_2SO_4 . The other disulphides were dissolved in water.

The reaction of disulphides with Na_2SO_3 was carried out either at room temperature or at $60 \pm 5^\circ$. At room temperature open test tubes or titration vessels could be used without appreciable oxidation occurring during the time needed for reaction. At 60° the rate of oxidation is greater and Thunberg tubes were used. These were flushed with O_2 -free nitrogen 3 times and finally evacuated. Since the Na_2SO_3 was present in excess it also served to control the pH, if necessary with the addition of a little HNO_3 .

Samples (1 or 2 ml.) of the thiol solutions were taken and the buffer was added. The total concentration of buffer should not normally exceed 0.05M or the electrodes may be slow in reaching equilibrium. Disulphide-sulphite mixtures were treated in the same way. 0.1N- AgNO_3 was used for titration and the volume added plotted against potential. The end points were taken as the mid-points of the titration curves, determined graphically, and were reproducible to $\pm 0.05 \mu\text{l.}$ of 0.1N- AgNO_3 .

pH was measured with a glass electrode calibrated as recommended in British Standard (1950) 1647.

Table 1. *Titration of thiols with AgNO₃*

β -Mercaptopropionic acid and GSH titrations were carried out with sufficient 0.1 N-HNO₃ or 0.1 N-H₂SO₄ added to bring pH to 1.8–2.2. Cysteine and homocysteine titrations were done at pH 6.5–7.0 using phosphate A, ca. 0.02 M.

Substance	Concn. range used (M)	pH range in which the thiol can be titrated without error	Titres expressed as % of the weighed concn.
β -mercaptopropionic acid	ca. 10^{-3}	1.5–4.5	98
GSH	5×10^{-5} to 6×10^{-3}	1.8–2.2	99.5
Cysteine	5×10^{-5} to 10^{-3}	2.5–7.5	99
Homocysteine	ca. 10^{-3}	3.0–9.0	97

Fig. 1. A: titration curve of 1 ml. of 8.6×10^{-4} M β -mercaptopropionic acid (in 0.02 N-HNO₃) with 0.1 N-AgNO₃ at pH 1.80. B: titration curve of 1 ml. of the same solution at pH 6.72 (0.02 M phosphate A). The apparent molarity is 1.16×10^{-3} , an error of +35%. The electrode potentials have been expressed in pAg units. 1 pAg unit = 58 mv at 18°.

RESULTS

Estimation of thiols

The range of pH in which the different thiols can be titrated with silver nitrate varies considerably. For routine purposes it is convenient to standardize conditions as far as possible. The compounds described here fall into two groups, namely those titrated in acid solution (pH 1.8–2.2) using a silver electrode, and those titrated in neutral solution at pH 6.5–7.0 using the appropriate silver thiol electrode. The buffers used were sulphuric acid or nitric acid in the acid range and phosphate A in the neutral range, all in a concentration of 0.01 to 0.02 M.

These results are summarized in Table 1; column 3 gives the full pH range over which each

Fig. 2. A: titration curve of 1.21×10^{-3} M GSH with AgNO₃ at pH 1.85. B: titration curve of 8.5×10^{-4} M cysteine hydrochloride at pH 6.75. C: titration curve of 9.2×10^{-4} M homocysteine at pH 6.75. Owing to the steepness of this curve the pAg scale has been halved. The electrode potentials have been expressed in pAg units. 1 pAg unit = 58 mv at 18°.

thiol can be titrated without error. The figures given in column 4 for the percentage recovery are each the mean of at least ten determinations. Repeated determinations on the same solutions agreed within $\pm 0.5\%$ of the mean.

β -Mercaptopropionic acid. This may be titrated without error between pH 1.5 and 4.5 (Fig. 1A). At higher pH the curves become flatter owing to silver complex formation, and more than one equivalent of silver is bound. At pH 6–7 the error is +35% (Fig. 1B).

GSH. GSAG complexes with silver ion over a wide range of pH and GSH can only be titrated satisfactorily between pH 1.8 and 2.2 (Fig. 2A). At pH > 2.5 the curves are asymmetrical and difficult

to interpret. At pH 1.7 and below, the curves become flatter and an additional 12% of silver is bound. Comparison of the present results with those obtained previously (Cecil, 1950) show that the bromide back-titration method gives results which are 4% high over the concentration range $5 \times 10^{-5} \text{ M}$ to $6 \times 10^{-3} \text{ M}$.

Cysteine and cysteine hydrochloride. Free cysteine may be titrated from pH 3.5 to 7.5. At pH > 7.5 the titration curves become very flat and the end points cannot be measured. At pH < 3.5 apparently normal curves are obtained, but the amount of silver bound increases as the pH falls, until at pH < 2.5 it reaches a constant amount 17% greater than the expected value. This factor of +17% was found to be constant over a wide range of concentration, namely $5 \times 10^{-5} \text{ M}$ to $1 \times 10^{-3} \text{ M}$. The same factor of +17% is found over the same range of concentration, if the bromide back-titration method (Cecil, 1950) is used.

Cysteine hydrochloride must be titrated at pH > 6 or the chloride interferes. The presence of 10^{-3} M cystine does not interfere. The upper halves of the cysteine (and cystine) titration curves are abnormally flat (Fig. 2B). The reason for this is not known but the steepness of the curves is such that it does not interfere with the end-point determination.

Homocysteine. This can be titrated without interference due to complex formation between pH 3.0 and 9.0. The curves are slightly asymmetrical but they are so steep that this is of no consequence (Fig. 2C). The silver homocysteine electrodes are rather slow in reaching equilibrium in phosphate A at pH 6.5 to 7.0. Equilibrium is reached more quickly in a sulphite buffer at the same pH and, provided the amount of any disulphide present is known, this buffer is preferable.

The behaviour in acid solution is curious. At pH 1.66–2.5 in nitric acid the titres of silver nitrate are 1–2% high and no precipitate of mercaptide is formed. If sulphuric acid is used, a precipitate is formed and the titre is 5% high at pH 1.92 and 17% high at pH 1.67.

In view of the possibility that this behaviour might be due to the formation of the thiolactone the

following experiments were tried. First, a sample of homocysteine thiolactone hydrochloride was titrated with silver nitrate at pH ≈ 2 and gave a titration for chloride only. The thiolactone itself did not react with the silver ion. Since the concentration of the free silver ion in a homocysteine titration is less than in a chloride titration, homocysteine can be estimated in the presence of its thiolactone without interference.

Secondly, two samples of homocysteine in nitric acid at pH ≈ 2 were titrated, one immediately, and one after standing for 1 hr. at room temperature. There was no reduction in the titre on standing, showing that the rate of formation of thiolactone under these conditions is negligible. Equally, if the behaviour of homocysteine in sulphuric acid were due to thiolactone formation, the titre would be expected to be low rather than high.

Estimation of disulphides

The conditions required for the sulphite–disulphide reaction fall into three groups. (a) Cystine, homocystine and diacetylcystine ester react rapidly at pH 6.5–7.0, and the thiols formed can be titrated at the same pH. These substances can therefore be titrated with silver nitrate immediately after adding the sodium sulphite. (b) Diformylcystine can also be titrated at the pH of reaction with sulphite, but the reaction is slow and must be allowed to proceed to completion before titration. (c) GSSG reacts rapidly with sulphite, while dithiodipropionic acid reacts slowly. Both, however, must be brought to pH 1.8–2.2 before titration, and the reaction must be complete before acidification. These results are summarized in Table 2.

N-Acetylcysteine ester and *N*-formylcysteine have not been studied as pure substances but only as sulphite–disulphide reaction mixtures. *N*-Acetylcysteine ester can be titrated with silver nitrate from pH 1.5 to 9.0 without any sign of silver complex formation. The titre falls off above pH 7.5 because of the reversibility of the reaction with sulphite. *N*-Formylcysteine may be titrated from pH 6.5 to 9.0, though the reverse reaction with sulphite becomes significant above pH 8.0. Below

Table 2. *Estimation of disulphides. Summary of results*

The temperature of reaction was 60° for diformylcystine and dithiodipropionic acid. The other compounds were allowed to react at room temperature.

Substance	Reaction conditions with Na_2SO_3			pH for titration	Titres expressed as % of the weighed concn.
	Moles of Na_2SO_3 /mole of RSSR	pH	Time allowed for reaction (min.)		
Cystine	15–25	6.5–7.0	0	6.5–7.0	100
Homocystine	15–25	6.5–7.0	0	6.5–7.0	99
Diacetylcystine ester	15–25	6.5–7.0	0	6.5–7.0	100
Diformylcystine	25–30	7.0–7.5	15	7.0–7.5	98
GSSG	15–25	6.5–7.0	30	1.8–2.2	98
Dithiodipropionic acid	50–60	7.5–8.0	30	1.8–2.2	98

pH 6.5, complexing with silver commences. The error is +15% at pH 4.5, falling to +4% at pH $\approx$ 2. *N*-Formyl cysteine is unusual in that it forms a silver mercaptide, which remains in solution under conditions which give a satisfactory titration.

Effect of chloride

If a mixture of two anions, both of which form insoluble silver salts, is titrated with silver nitrate, the jump in electrode potential corresponding to the end point of the less soluble anion will occur early, and its titre will be low. The size of the error depends on the difference in solubility products of the salts and the relative concentration of the two anions. A quantitative treatment is given by Kolthoff & Furman (1949).

In the estimation of GSSG and dithiodipropionic acid, mixtures of each disulphide with sodium sulphite are titrated with silver nitrate at pH $\approx$ 2. At this pH the apparent solubility products of the two silver mercaptides (*S'* of eqn. 8) are sufficiently close to that of silver chloride for small errors to arise if the sodium sulphite contains too much chloride as impurity. Thus, using approximately 10^{-3} M thiol solutions, GSH shows an error of -1% with 0.1 equivalent of chloride, and -4% with 0.5 equivalent. Similarly, β mercaptopropionic acid shows an error of -2% with 0.5 equivalent of chloride. These figures have been used in setting the limits for the chloride content of sodium sulphite given earlier.

The effect on the titrations at pH 6.5-7.0, using silver thiol electrodes, is less. Using approximately 10^{-3} M cysteine, there is no error with 3 equivalents of chloride, but with 5 equivalents the electrode becomes very sluggish and end points cannot be determined. This is probably because, under these conditions, the electrode potential is controlled by the thiol rather than the silver ion and, as the concentration of chloride is raised, it tends to displace the thiol from the surface of the electrode.

DISCUSSION

The range of pH in which the different silver mercaptides form stable complexes with silver nitrate is interesting, since it throws light on the groups involved. β -Mercaptopropionic acid complexes at pH > 4.5 but not below this pH. 1.02 equivalents of silver are bound at pH 4.6, and this rises to 1.35 at pH 6.7. The carboxyl *pK* is 4.32 (Borsock, Ellis & Huffman, 1937) and so it seems that the —COO^- form has a greater tendency to bind silver than the —COOH form. GSH and cysteine show an opposite effect. At pH < 1.7 GSH binds 1.12 equivalents of silver and at pH < 2.5 cysteine binds 1.17 equivalents. The carboxyl *pK* values of GSH are 2.12 and 3.53 (Pirie & Pinhey,

1929) and that of cysteine is 1.36 (Cannan & Knight, 1927). Although there is no precise correspondence, in these compounds the change from —COO^- to —COOH must be responsible for complex formation. Homocysteine shows the same tendency to complex in acid solution, but this tendency depends on the acid used. The reason for this is not known, but it is unlikely to be due to formation of the thiolactone, since this substance does not react appreciably with small amounts of silver ion at pH $\approx$ 2.

The carboxyl *pK* of *N*-formylcysteine does not appear to have been measured but is likely to be slightly below that of formylglycine, given as 3.4 by Zief & Edsall (1937). There is no complex formation between pH 6.5 and 9.0. At pH 4.7, however, the amount of silver bound rises to 1.15 equivalents and then falls to 1.04 equivalents at pH $\approx$ 2. It appears therefore that maximum complex formation occurs when the —COO^- and —COOH forms are both present, though once again there is no precise correspondence with the carboxyl *pK*. The behaviour of cysteine and GSH at high pH is complex and is still being investigated.

The kinetics of the sulphite-disulphide reaction have been reported briefly (Cecil & McPhee, 1954) and will be given in detail in a later paper.

It remains to compare the present results with those obtained previously by the bromide back-titration method. In the latter method a known excess of silver nitrate was added to the thiol solution at pH $\approx$ 2 and the excess measured by a potentiometric titration with potassium bromide. It depended on the basic assumption that, at this pH, any silver not bound by thiol was titratable by bromide. The agreement with the theoretical figures apparently indicated that this was so. One of the difficulties, however, in developing a method for the analysis of thiols is the lack of standards. Thiols are not easy substances to purify and they oxidize easily. In fact the GSH used in the previous work has since been shown to be slightly impure. Consequently, analysis by back titration, which gives an error of +4%, agreed well with the theoretical figures. The original sample of GSSG was made from this sample of GSH and so was subject to the same error.

Cystine was allowed to react with sulphite, acidified, and the cysteine formed estimated by back titration, apparently satisfactory results being obtained. It was not known then that the sulphite reaction is reversible at high pH and in fact it was carried out at approximately pH 8.5. Consequently, only 0.82-0.85 of an equivalent of cysteine was formed, but this fact was masked by the cysteine titration error of +17%. These errors have been shown to be constant over a wide range of concentration and so it is possible to allow for them. The back-titration method was used (Cecil, 1950) to

follow the reaction of GSSG and cystine with silver nitrate. Since GSSG and GSH were both subject to the same error the interpretation of this reaction is unaffected. The cystine concentrations on the other hand were correct, whereas the cysteine figures were 17% high. This reaction is therefore slower than it appeared and the difference in reactivity between the two substances greater. Details will not be given here, as work on these and other compounds is continuing.

SUMMARY

1. A method for the estimation of four thiols by potentiometric titration with silver nitrate is described. The titrations are carried out in buffered solutions, the pH used depending on the particular compound. In all cases one of two pH ranges, 1.8–2.2 or 6.5–7.0, can be used.

2. At pH < 2.5 the silver ion concentrations are sufficiently high for the titrations to be followed with a silver electrode.

3. At pH > 2.5 the silver ion concentrations are too low to be followed with a silver electrode and the appropriate silver thiol electrode must be used. The preparation of these electrodes is described and a simple thermodynamic treatment is given.

4. The method is applied to the estimation of six disulphides. These are first allowed to react with sodium sulphite to give one equivalent of the corresponding thiol. The factors governing the optimum pH for the reaction are discussed and the conditions for each compound are given.

5. The conditions under which the different silver mercaptides form stable complexes with additional silver are discussed.

6. This work disclosed errors in previous work on the estimation of thiols and disulphides. These are discussed and the corrections given.

Our thanks are due to Dr A. G. Ogston for many valuable discussions, to Dr E. M. Gal for help in purifying the thiols and to Miss S. Read for skilled technical assistance. We are grateful to the Distillers Company (Biochemicals) Ltd. for the generous gift of glutathione (DCB 524). One of us (J.R.M.) is indebted to the Commonwealth Government of Australia for a C.S.I.R.O. traineeship.

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Complex-Formation between Polypeptides and Metals

1. APPLICATION OF VARIOUS EXPERIMENTAL METHODS TO THE GLYCINE-COPPER SYSTEM

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(Received 21 July 1954)

In recent years complex-formation between amino acids and various metals has been extensively studied with the aid of potentiometric titration (Albert, 1950, 1952; Monk, 1951; Perkins, 1952). The theory and practical application of the method has been fully described by Albert (1950), and the results obtained are summarized in the form of stability constants which for a simple ligand L⁻

(such as an amino acid anion) and a divalent metal M are the equilibrium constants for the reactions

so that

$$K_1 = [ML^{+}]/[M^{2+}][L^{-}]$$

and

$$K_2 = [ML_2]/[ML^{+}][L^{-}].$$