REFERENCES

- Aldridge, W. N. & Barnes, J. M. (1952). *Nature, Lond.*, **169**, 345.
- Augustinsson, K.-B. (1948). *Acta physiol. scand.* **15** (Suppl. 52), 182 pp.
- Augustinsson, K.-B. & Heimbürger, G. (1954). *Acta chem. scand.* **8**, 915.
- Babers, F. H. & Pratt, J. J. jun. (1950). *Physiol. Zool.* **23**, 58.
- Babers, F. H. & Pratt, J. J. jun. (1951). *Physiol. Zool.* **24**, 127.
- Bacq, Z. M. (1935). *C.R. Soc. Biol., Paris*, **120**, 247.
- Bacq, Z. M. (1946). *Biol. Rev.* **22**, 73.
- Beard, R. L. (1951). *J. econ. Ent.* **44**, 469.
- Bottger, G. T. & Yerington, A. P. (1952). *J. econ. Ent.* **45**, 131.
- Casida, J. E. (1954). *J. Physiol.* (Abstr.) (in the Press).
- Casida, J. E., Chapman, R. K., Stahmann, M. A. & Allen, T. C. (1954). *J. econ. Ent.* **47**, 64.
- Chadwick, L. E. & Hill, D. L. (1947). *J. Neurophysiol.* **10**, 235.
- Chamberlin, W. F. & Hoskins, W. M. (1951). *J. econ. Ent.* **44**, 177.
- Chefurka, W. (1953). *Agric. Inst. Rev.* **8**, 49.
- Cortegiani, E. & Serfaty, A. (1939). *C.R. Soc. Biol., Paris*, **131**, 1124.
- Duspiva, F. (1951). *Mitt. biol. Zent. Berl.* **70**, 91.
- Fernando, H. E., Roan, C. C. & Kearns, C. W. (1950). *Ann. ent. Soc. Amer.* **44**, 551.
- Gautrelet, J. (1938). *Bull. Acad. Méd., Paris*, **120**, 285.
- Hopf, H. S. (1952). *Ann. appl. Biol.* **39**, 193.
- Hopf, H. S. (1954). *Ann. appl. Biol.* **41**, 248.
- Hoyle, G. (1952). *Nature, Lond.*, **169**, 281.
- Hoyle, G. (1953). *J. exp. Biol.* **30**, 121.
- Johnson, G. A., Fletcher, J. H., Nolan, K. G. & Cassaday, J. T. (1952). *J. econ. Ent.* **45**, 279.
- Johnson, M. J. (1941). *J. biol. Chem.* **137**, 575.
- Karczmar, A. G. & Koppányi, T. (1953). *Arch. exp. Path. Pharmac.* **219**, 263.
- Krupp, H., Lendle, L. & Stapenhorst, K. (1952). *Arch. exp. Path. Pharmac.* **215**, 443.
- Lord, K. A. & Potter, C. (1950). *Nature, Lond.*, **166**, 893.
- Lord, K. A. & Potter, C. (1951). *Ann. appl. Biol.* **38**, 495.
- Lord, K. A. & Potter, C. (1953). *Nature, Lond.*, **172**, 679.
- Ludvik, G. F. & Decker, G. C. (1947). *J. econ. Ent.* **40**, 97.
- Ludvik, G. F. & Decker, G. C. (1951). *J. econ. Ent.* **44**, 405.
- Means, O. W. jun. (1942). *J. cell. comp. Physiol.* **20**, 319.
- Metcalf, R. L. & March, R. B. (1949). *J. econ. Ent.* **42**, 721.
- Metcalf, R. L. & March, R. B. (1950). *J. econ. Ent.* **43**, 670.
- Metcalf, R. L., March, R. B. & Sprott, M. (1953). Abstr. No. 65, National Meeting Ent. Soc. Amer., Dec. 8, 1953, Los Angeles, California.
- Mikalonis, S. J. & Brown, R. H. (1941). *J. cell. comp. Physiol.* **18**, 401.
- Mounter, L. A. (1954). *J. biol. Chem.* **209**, 813.
- Myers, D. K. (1951). *Arch. Biochem. Biophys.* **31**, 29.
- Nachmansohn, D. & Rothenberg, M. A. (1945). *J. biol. Chem.* **158**, 653.
- O'Brien, R. D. (1953). *Nature, Lond.*, **172**, 162.
- O'Brien, R. D. & Spencer, E. Y. (1953). *J. agric. Food Chem.* **1**, 946.
- Patton, R. L. (1953). Pp. 387-403 in *Insect Physiology*, K. D. Roeder, ed., John Wiley & Sons, Inc., N.Y.
- Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Richards, A. G. jun. (1943). *J. N.Y. ent. Soc.* **51**, 55.
- Richards, A. G. jun. & Cutkomp, L. K. (1945). *J. cell. comp. Physiol.* **26**, 57.
- Roan, C. C., Fernando, H. E. & Kearns, C. W. (1950). *J. econ. Ent.* **43**, 319.
- Roan, C. C. & Maeda, S. (1953). *J. econ. Ent.* **46**, 775.
- Rockstein, M. (1950). *J. cell. comp. Physiol.* **35**, 11.
- Roeder, K. D. (1948a). *Johns Hopk. Hosp. Bull.* **83**, 587.
- Roeder, K. D. (1948b). *J. cell. comp. Physiol.* **31**, 327.
- Stegwee, D. (1950). *Acta physiol. pharm. néerl.* **1**, 336.
- Stegwee, D. (1951). *Physiol. comp.* **2**, 241.
- Tahmisian, T. N. (1943). *J. exp. Zool.* **92**, 199.
- Tobias, J. M. (1948a). *J. cell. comp. Physiol.* **31**, 143.
- Tobias, J. M. (1948b). *Fed. Proc.* **7**, 340.
- Tobias, J. M., Kollros, J. J. & Savit, J. (1946). *J. cell. comp. Physiol.* **28**, 159.
- Truhaut, R. & Vincent, D. (1947). *Ann. pharm. franç.* **5**, 159.
- Truhaut, R. & Vincent, D. (1948). *C.R. Soc. Biol., Paris*, **227**, 738.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed. pp. 191. Minneapolis, Minn.: Burgess Publ. Co.
- Wilson, I. B. & Cohen, M. (1953). *Biochim. biophys. Acta*, **11**, 147.
- Yushima, T. & Chino, H. (1953). *Oyo-Kontyu*, **9**, 52.

A Kinetic Study of the Reactions on some Disulphides with Sodium Sulphite

BY R. CECIL AND J. R. MCPHEE†
Department of Biochemistry, University of Oxford

(Received 6 November 1954)

The reactivities of disulphide bonds in different compounds show considerable variation. For example, the rates of decomposition of simple cystine derivatives with alkali vary widely (Schöberl & Hornung, 1938) and oxidized glutathione (GSSG)

† Present address: C.S.I.R.O., Wool Textile Research Laboratories, Geelong, Australia.

reacts very much faster with silver nitrate than does cystine (Cecil, 1950).

This paper describes kinetic studies of the reactions of some disulphides with sodium sulphite. Clarke (1932) and Lugg (1932) showed that the reaction is $\text{RSSR} + \text{Na}_2\text{SO}_3 \rightleftharpoons \text{RSNa} + \text{RSSO}_3\text{Na}$. Sodium sulphite has since been used extensively for

the estimation of disulphides, a disadvantage being that the reaction is reversible at pH values above 7-7.5 (Footner & Smiles, 1925; Stricks & Kolthoff, 1951).

Schöberl & Ludwig (1937) and Kassel & Brand (1938) measured the approximate rates of splitting of certain disulphides with sodium sulphite, but the work was not sufficiently accurate for any attempts to be made at interpreting the kinetics. Schöberl & Krumei (1938) studied the effect of pH on the reaction with GSSG under their standard conditions. They observed a very rapid increase in thiol formed as the pH was raised from 3.5 to 5, the maximum amount being formed from pH 5 to 7. These results are in qualitative agreement with those presented here.

The reaction is useful for studying protein disulphide bonds, since it is specific and takes place under mild conditions. Thus, Fraenkel-Conrat (1942) found that only two-thirds of the total cystine of the intact lactogenic hormone reacted with sodium sulphite, and Elsworth & Phillips (1938) showed that about half the cystine of wool reacted at pH 5 at room temperature. Lindley (1948) extended the work on wool to other proteins, concluding that the disulphide bonds of many proteins can be divided into reactive and unreactive fractions. Some experiments with modified wools led Lindley & Phillips (1947) to propose that the reactive disulphide bonds are associated with carboxyl and acid amide groups. Since this work on proteins depended on results of thiol and disulphide estimations on hydrolysates, its quantitative significance is doubtful, although it is established that disulphide bonds differ considerably in reactivity towards sulphite.

Recently, it has been found that only two out of three disulphide bonds in insulin react with sodium borohydride (Edman & Diehl, 1952) and with sodium sulphite at pH 7 (Cecil, unpublished experiments).

The present work is concerned with the reactions of substances containing only one disulphide bond per molecule. With these simple compounds it has been possible to investigate the effects of different neighbouring groups on the disulphide bond.

THEORETICAL

Three complicating factors have to be considered in the description of disulphide-sodium sulphite reaction mixtures:

(i) The bisulphite-sulphite equilibrium between approximately pH 5 and 9; $\text{HSO}_3^- \rightleftharpoons \text{H}^+ + \text{SO}_3^{2-}$. The second dissociation constant of sulphurous acid is about 10^{-7} (Tartar & Garretson, 1941) and is represented as $K\phi$.

(ii) The reversibility of the reaction with disulphides above about pH 7.

(iii) Ionization of thiol and amino groups. In general, carboxyl ionizations can be ignored, since the reactions were studied above the pH range where these occur.

Competitive bimolecular reactions. It is possible that both HSO_3^- and SO_3^{2-} ions react with disulphide bonds so that, if the disulphide molecule contains no ionizing groups and the pH is between 5 and 9, the primary reactions (neglecting the reverse reaction for the moment) are:

If the reactions are bimolecular and k_1 and k_2 are independent of pH, then

$$\frac{d(\text{RSH})}{dt} = k_1[\text{RSSR}][\text{HSO}_3^-], \quad (1)$$

and
$$\frac{d(\text{RS}^-)}{dt} = k_2[\text{RSSR}][\text{SO}_3^{2-}]. \quad (2)$$

Let a be the initial concentration of RSSR, b the initial concentration of total sulphite, and x the concentration of total thiol formed at time t . Then at time t ,

$$[\text{RSSR}] = (a - x), \quad (3)$$

$$[\text{total sulphite}] = [\text{HSO}_3^-] + [\text{SO}_3^{2-}] = (b - x), \quad (4)$$

and
$$x = [\text{RSH}] + [\text{RS}^-]. \quad (5)$$

From these equations together with

$$K\phi = [\text{SO}_3^{2-}][\text{H}^+]/[\text{HSO}_3^-], \quad (6)$$

$$[\text{SO}_3^{2-}] = (b - x) \{K\phi/(K\phi + [\text{H}^+])\}, \quad (7)$$

and
$$[\text{HSO}_3^-] = (b - x) \{[\text{H}^+]/(K\phi + [\text{H}^+])\}. \quad (8)$$

Substituting (7) and (8) in (1) and (2) and adding:

$$\frac{d(\text{RSH})}{dt} + \frac{d(\text{RS}^-)}{dt} = \frac{dx}{dt} = k'(a - x)(b - x), \quad (9)$$

where
$$k' = k_1[\text{H}^+] + k_2K\phi/(K\phi + [\text{H}^+]), \quad (10)$$

i.e. an apparent rate constant, k' , can be found from the simple expression for a second-order reaction.

Two limiting cases can be considered:

(i) If $k_1[\text{H}^+] \ll k_2K\phi$ then from Eqn. 10

$$k' = k_2K\phi/(K\phi + [\text{H}^+]), \quad (11)$$

or $k_2 = k'\{(K\phi + [\text{H}^+])/K\phi\} \simeq k'$ when $K\phi \gg [\text{H}^+]$.

Eqn. 11 could only hold when $[\text{H}^+] > K\phi$ if k_1 were negligibly small.

(ii) When $k_1[\text{H}^+] \gg k_2K\phi$, from Eqn. 10

$$k' = k_1[\text{H}^+]/(K\phi + [\text{H}^+]), \quad (12)$$

$k_1 = k'\{(K\phi + [\text{H}^+])/[\text{H}^+]\} \simeq k'$ when $K\phi \ll [\text{H}^+]$.

This treatment can easily be extended to the system where one of the reactants ionizes as a dibasic acid in the pH range considered, e.g. cystine at high pH, when all the sulphite can be regarded as being in the form SO_3^{2-} .

Neglecting the reverse reaction, the system can be represented as:

If the forward reactions are bimolecular, the rate of formation of thiol is given by

$$\begin{aligned} \frac{dx}{dt} = & k_a \left[\begin{array}{c} \text{RSSR} \\ \diagup \quad \diagdown \\ \text{NH}_3^+ \quad \text{NH}_3^+ \end{array} \right] [\text{SO}_3^{2-}] \\ & + k_b \left[\begin{array}{c} \text{RSSR} \\ \diagup \quad \diagdown \\ \text{NH}_3^+ \quad \text{NH}_2 \end{array} \right] [\text{SO}_3^{2-}] + k_c \left[\begin{array}{c} \text{RSSR} \\ \diagup \quad \diagdown \\ \text{NH}_2 \quad \text{NH}_2 \end{array} \right] [\text{SO}_3^{2-}]. \end{aligned} \quad (13)$$

If a is the initial total disulphide concentration and b the initial total sulphite concentration, then at time t ,

$$\begin{aligned} & \left[\begin{array}{c} \text{RSSR} \\ \diagup \quad \diagdown \\ \text{NH}_3^+ \quad \text{NH}_3^+ \end{array} \right] + \left[\begin{array}{c} \text{RSSR} \\ \diagup \quad \diagdown \\ \text{NH}_3^+ \quad \text{NH}_2 \end{array} \right] \\ & + \left[\begin{array}{c} \text{RSSR} \\ \diagup \quad \diagdown \\ \text{NH}_2 \quad \text{NH}_2 \end{array} \right] = (a-x), \end{aligned} \quad (14)$$

$$\text{and} \quad [\text{SO}_3^{2-}] = (b-x). \quad (15)$$

The three forms of disulphide are related by the equations

$$K'_3 = [\text{H}^+] \left[\begin{array}{c} \text{RSSR} \\ \diagup \quad \diagdown \\ \text{NH}_3^+ \quad \text{NH}_2 \end{array} \right] / \left[\begin{array}{c} \text{RSSR} \\ \diagup \quad \diagdown \\ \text{NH}_3^+ \quad \text{NH}_3^+ \end{array} \right], \quad (16)$$

and

$$K'_4 = [\text{H}^+] \left[\begin{array}{c} \text{RSSR} \\ \diagup \quad \diagdown \\ \text{NH}_2 \quad \text{NH}_2 \end{array} \right] / \left[\begin{array}{c} \text{RSSR} \\ \diagup \quad \diagdown \\ \text{NH}_3^+ \quad \text{NH}_2 \end{array} \right], \quad (17)$$

where K'_3 and K'_4 are the dissociation constants of the amino groups.

Thus, it can be shown that

$$\frac{dx}{dt} = k''(a-x)(b-x), \quad (18)$$

$$\text{where } k'' = C(k_a[\text{H}^+]/K'_3 + k_b + k_c K'_4/[\text{H}^+]), \quad (19)$$

$$\text{and } C = K'_3[\text{H}^+]/([\text{H}^+]^2 + K'_3[\text{H}^+] + K'_3 K'_4).$$

Opposing reactions. Expressions for first- or second-order reactions opposed by second-order reactions are derived in standard text-books, e.g. Moelwyn-Hughes (1947). An important feature of reactions considered here is that the products ionize at pH values where the reactions are reversible.

If the reaction is

with neither reactants nor products containing ionizing groups, and no C or D present initially, then the net rate of formation of products is given by:

$$\frac{dx}{dt} = k_a \left[(a-x)(b-x) - \frac{x^2}{x_e^2} (a-x_e)(b-x_e) \right], \quad (20)$$

where a, b , are the initial concentrations of A and B respectively; x is the concentration of C and D at time t ; and x_e is the equilibrium concentration of products.

If the products contain ionizing groups and the pH is constant, then the amounts of C and D present at any time are not x but certain fractions of x , determined by the dissociation constants and the pH. The concentrations at time t are then

$$[A] = (a-x); \quad [B] = (b-x); \quad [C] = \delta x; \quad [D] = \epsilon x,$$

where δ, ϵ are constants. Therefore

$$\frac{dx}{dt} = k_a \left[(a-x)(b-x) - \frac{\delta\epsilon x^2}{\delta\epsilon x_e^2} (a-x_e)(b-x_e) \right]. \quad (21)$$

The product $\delta\epsilon$ cancels and Eqn. 21 is identical with Eqn. 20.

If one of the reactants contains dissociating groups, apparent rate constants for the forward reactions can be found from the integrated form of Eqn. 20 for particular pH values. The apparent constants can then be interpreted by the use of Eqns. 10 or 19.

Equilibrium constants. If the pH is above 9 and the disulphide contains no dissociating groups, the system at equilibrium is

The equilibrium constants are

$$K_1 = [\text{RS}^-]_e [\text{RSSO}_3^-]_e / [\text{RSSR}]_e [\text{SO}_3^{2-}]_e, \quad (22)$$

$$\text{and } 1/K_2 = K_{\text{SH}} = [\text{RS}^-]_e [\text{H}^+] / [\text{RSH}]_e, \quad (23)$$

where K_{SH} is the usual dissociation constant of a thiol group.

If K_{SH} and K_1 are unknown, both can be found from measurements at two pH values and the use of Eqns. 22 and 23 together with

$$\Sigma \text{RSH} = [\text{RS}^-]_e + [\text{RSH}]_e, \quad (24)$$

where ΣRSH is the total equilibrium concentration of products.

The equilibrium system is more complex when the disulphide contains ionizing groups, e.g. the cystine-sulphite equilibria which have been considered by Stricks & Kolthoff (1951). The system becomes simple when the pH is high enough for all the groups in products and reactants to be ionized so that K_1 can be determined directly from experimental measurements.

To calculate the equilibrium constants for the reactions involving the forms of the components containing ionizing groups, the dissociation constants of amino and thiol groups of cystine, cysteine and $\text{RSSO}_3^- \text{---} \text{NH}_3^+$ compounds must be known. Such calculations have not been made here since the uncertainty of the assumptions needed means that the figures obtained are of little value.

EXPERIMENTAL

Reagents. The materials used have been described previously (Cecil & McPhee, 1955). 0.1M- Na_2SO_3 solutions were made up daily and stabilized by the addition of ethylenediaminetetraacetic acid (EDTA) to a concentration of 2 mM, since without EDTA, small volumes of solutions could decompose to the extent of 20–30% during a day at room temperature. The concentration of EDTA was varied between 1 and 20 mM to see if reaction rates were affected, but no effect was observed.

Buffer solutions. Na_2SO_3 was used as its own buffer whenever possible, that is, when the sulphite was present in greater than tenfold molar excess over disulphide in the pH range 6.0–7.4. Other buffers used were acetate (pH 3.8–5.3), borate (pH 9.0–9.7) and carbonate (pH 9.8–11.3).

Kinetic experiments. These were done in boiling tubes kept in a thermostat at the required temperature. Na_2SO_3 was added to buffered disulphide solutions from pipettes calibrated for blowing, which took about 1 sec. to empty. The reactions were considered as starting when the pipettes were half empty, so that the zero-time error was less than 0.5 sec. The samples were withdrawn at measured time intervals and the reaction was stopped by blowing the samples from calibrated pipettes into titration vessels containing sufficient dil. HNO_3 to bring the pH to 1.8–2.2. The thiol concentrations were then found by potentiometric titration with 0.1M- AgNO_3 (Cecil & McPhee, 1955). Oxidation was negligible in the short times taken for these experiments.

The following experiments showed that acidification stopped the reactions. If a 20-fold excess of sulphite were added to a solution of any of the disulphides at pH 1.8–2.2 no thiol could be detected after standing for 2 hr. Also, if two samples from a reacting mixture were acidified simultaneously, there was no difference in titre between the one titrated immediately and the other 2 hr. later.

Equilibrium experiments. The time required to reach equilibrium was often considerable and to prevent errors from oxidation, the mixtures of disulphides and sulphite were kept in Thunberg tubes under nitrogen. The amounts of total thiol present at equilibrium were found by acidification of samples followed by titration with AgNO_3 . Several experiments were necessary for given conditions since calculated rate constants for the forward reactions between

disulphides and sulphite are sensitive to small errors in the equilibrium concentrations of thiol.

Ionic strength. The ionic strength of the reaction mixtures varied from 0.03 to 0.15. It was not practical to keep the ionic strength constant throughout because of the interference, at relatively high concentrations, of some of the buffer salts with the potentiometric titrations (see below). There is no evidence to suggest that the rate constants or equilibrium constants varied significantly with ionic strength in the range used.

Concentrations of reagents. The disulphide concentrations used were from 0.4 to 1.5 mM and the sulphite concentrations varied from approximately equimolar with disulphide to a 40-fold molar excess. The conditions were normally chosen so that samples could be taken for titration from times between 1 and 15 min.

Estimation of thiol concentrations at low pH. The titrations at pH 1.8–2.2 of *N*-acetylcysteine ester and β -mercapto-propionic acid presented no difficulties.

The mercaptides of both *N*-formylcysteine and cysteine complexed with Ag^+ in acid solution, giving titration errors of +4 and +17% respectively at pH 1.8–2.2 (Cecil & McPhee, 1955). Various means of eliminating the errors were tried. For example, by adding high concentrations of other cations known to have strong tendencies to form complexes (La^{3+} , Sr^{2+} , Ba^{2+}) and also by removing the sulphite at low pH before titrating at pH values where no complexing error is found. However, as the errors could not be avoided, they were accepted and correction factors applied to the titrations. The errors were found to be constant under the conditions used (Cecil & McPhee, 1955).

In the presence of borate and carbonate, GSH could be titrated without systematic error between pH 2.0 and 2.2. Positive errors of up to 10% were found below pH 2.0 while negative errors of 2–4% occurred above pH 2.2. There was no error in the range pH 1.8–2.2 if sulphite were present alone or with phosphate or acetate.

Measurement of pH. Glass electrodes were used, calibrated as recommended in British Standard 1647 (1950). The pH of reaction mixtures at 0° could not be found with glass electrodes since the electrical resistance is too high at this temperature, and hydrogen electrodes cannot be used in the presence of disulphides. The pH values of the buffer solutions used were therefore measured at 0° and 25° with a hydrogen electrode and the changes in pH with temperature recorded. The pH values of reaction mixtures at 0° could then be calculated from measurements made at 25° with glass electrodes. For the pH measurements, only the vessels containing the solutions to be measured were at 0° or 25°, the liquid junction and reference electrode being at room temperature throughout.

RESULTS

Order of reaction

Experiments designed to find the order of reaction with diacetylcysteine ester are described. The other compounds gave similar results.

(a) *With respect to disulphide.* Two experiments were done in which the initial concentration of diacetylcysteine ester in one was about half that in the other, the total sulphite concentration being approximately the same in both experiments, and

the sulphite in large excess. The pH was 6.4 and the reactions went quickly to completion under these conditions. The times taken for the same fraction of disulphide to react in each experiment were approximately the same, indicating a reaction first order with respect to disulphide.

If $\log_{10}(a-x)$ is plotted against time straight lines are not obtained (Fig. 1), but the initial slopes for the above two experiments (curves A and B) are approximately the same. This is consistent with the initial reaction being first order with respect to disulphide but suggests that the mechanism is complex, the rate constant for the reaction decreasing with time (see below).

Fig. 1. Reactions of diacetylcystine ester with sodium sulphite; plots of $\log_{10}(a-x)$ against time. a (mole l^{-1}) = initial disulphide concentration; x (mole l^{-1}) = total thiol concentration at time t ; b (mole l^{-1}) = initial total sulphite concentration.

Curve	pH	$10^4 a$	$10^4 b$
A	6.41	7.58	80.0
B	6.44	3.83	82.5
C	6.42	6.95	151.9

(For clarity, some of these graphs have been shifted along the ordinate. Only the scale is given, therefore, not the actual figures.) The lines have been drawn to show the initial rates. They are not lines of best fit.

(b) *With respect to sodium sulphite.* This was found from two experiments in which the disulphide concentration was nearly the same, but the total sulphite concentration differed by a factor of about 2 (Fig. 1, curves A and C). When the sulphite concentration was doubled, the initial slope of the graph of $\log_{10}(a-x)$ against time was approxi-

mately doubled, i.e. the initial pseudo-first-order rate constant was approximately doubled.

Thus, even though the reaction is complex, the initial reaction is first order with respect to each reactant.

(c) *Complex reaction mechanism.* The number of observations in any single experiment was too small to allow any sensitive test for linearity of regression of $\log_{10}(a-x)$ against time. Since the initial point in each experiment is the most accurate, the best-fitting straight lines through these points were computed for seven experiments with diacetylcystine ester. The correspondence between the data and these straight lines was tested by examining the distribution of the points about them. It was found that all the eleven observations between 0 and 40% completion of the reaction lay below the straight lines, and that there was significant regression of the deviations on percentage reaction. This implies that the data are better described by a curve passing through the initial value, and convex to the time axis, than by the best fitting straight line passing through the initial value.

The conclusions reached are that below pH 9 the reaction is complex, but the initial reaction is first order with respect to each reactant. Above pH 9 the reaction is reversible but there is no other complexity, since the experimental data fit the expression for reversible bimolecular reactions (Eqn. 20).

Reactions of diacetylcystine ester, diformylcystine and $\beta\beta'$ -dithiodipropionic acid

The pK values of the carboxyl groups of dithiodipropionic acid are 4.08 and 4.99 (Borsook, Ellis & Huffman, 1937) and those of diformylcystine would be expected to be close to these values. Since all the kinetic work with these compounds was done at pH > 6, it must refer (within the experimental limits) to the completely ionized forms. These disulphides can thus be grouped with diacetylcystine ester for presentation of results, since none contains groups whose state of ionization changes in the pH range used.

Diformylcystine involves an uncertainty due to the possibility of the formyl groups reducing the concentration of HSO_3^- or SO_3^{2-} ions by combining with one or both. Allowance can be made for this in the calculation of rate constants but this does not significantly alter the results (Table 2).

Reactions at pH > 9. Since the second pK of sulphurous acid is about 7 (Tartar & Garretson, 1941) all the sulphite can be considered to be in the form SO_3^{2-} at pH > 9. The reaction is then reversible and can be represented as

Table 1. *Reaction of diacetylcystine ester with sodium sulphite at 0° and pH > 9*

pH	<i>I</i>	Initial disulphide concentration (<i>a</i>) × 10 ⁴ (mole l. ⁻¹)	Initial total sulphite concentration (<i>b</i>) × 10 ⁴ (mole l. ⁻¹)	Time (min.)	Total thiol concn. (<i>x</i>) × 10 ⁴ (mole l. ⁻¹)	Rate constant for forward reaction <i>k_a</i> (l. mole ⁻¹ min. ⁻¹)	Apparent equilibrium constant <i>K_{app.}</i>
9.20	0.07	7.00	73.85	1.45	2.10	35.5	0.075
				2.67	3.10	37.0	
				3.92	3.50	35.2	
				5.15	3.83	38.9	
				<15	3.98 = <i>x_e</i> †		
9.31	0.11	6.57	135.7	1.20	2.90	39.6	0.070
				2.57	4.03	39.9	
				3.72	4.30	38.8	
				4.67	4.35	35.0	
				<15	4.43 = <i>x_e</i>		
9.43	0.08	7.00	73.08	1.20	1.93	39.3	0.065
				2.30	2.90	39.1	
				3.40	3.38	38.9	
				4.37	3.58	38.3	
				<15	3.80 = <i>x_e</i>		
9.61	0.09	7.00	73.08	1.72	2.23	33.8	0.060
				2.92	3.13	37.7	
				4.12	3.45	37.2	
				<15	3.70 = <i>x_e</i>		
10.15	0.07	7.77	79.62	0.92	1.78	37.5	0.050
				2.05	2.90	35.5	
				3.05	3.35	34.2	
				<15	3.85 = <i>x_e</i>		
Mean <i>k_a</i> = 37							

† *x_e* is the equilibrium concentration of total thiol.Table 2. *Reactions of diacetylcystine ester, dithiodipropionic acid and diformylcystine with sodium sulphite at pH > 9*

Substance	Temp. (°)	pH range of kinetic expts.	Rate constant for forward reaction <i>k_a</i> † (l. mole ⁻¹ min. ⁻¹)
Diacetylcystine ester	0	9.2–10.2	37 ± 2
	25	9.1–9.9	265 ± 9
Dithiodipropionic acid (ionized)	0	9.5–11.3	0.13 ± 0.005
	25	9.3–10.5	1.10 ± 0.07
Diformylcystine (ionized)	0	9.0–10.5	1.5 ± 0.1†
	25	9.1–10.5	9.0 ± 0.6†

† The figures given are the means and standard deviations of at least sixteen determinations of *k_a* from a minimum of four kinetic expts.

† These values were calculated assuming no SO₃²⁻ ions were bound by formyl groups. If each formyl group bound one SO₃²⁻ ion, the values would be 1.7 and 12 l. mole⁻¹ min.⁻¹ at 0° and 25° respectively.

The rate constant for the forward reaction, *k_a*, can be obtained from the integrated form of Eqn. 20 for opposing bimolecular reactions. A typical series of experiments with diacetylcystine ester is shown in Table 1.

Since the rate constant in carbonate at pH 10.15 is the same, within the experimental error, as that in

borate from pH 9.2 to 9.6, it is unlikely that either buffer is affecting the reaction. As a further check, small quantities of borate and carbonate were added to reaction mixtures at pH ≈ 7 without any effect, though at this pH different ionic species are involved. Similar experiments showed that neither acetate nor phosphate affects the reaction.

Table 2 summarizes the results for the three compounds at 0° and 25°. The rate constants for the reactions of the disulphide bonds with SO₃²⁻ ions (*k_a*) are in each case independent of pH and it is found that the negatively charged molecules (ionized dithiodipropionic acid and ionized diformylcystine) react much more slowly than the neutral diacetylcystine ester.

Reactions at pH < 9. Below pH 9, the equilibrium

must be considered and Eqns. 9 and 10 used to interpret the results. As shown earlier, the reaction is complex and only the initial reaction, which is bimolecular, is considered. Estimates of the initial bimolecular rate constants were obtained by calculating the apparent second-order rate constants during runs, plotting these against time and extrapolating the curves to zero time. Typical

results with diacetylcystine ester are shown in Fig. 2, and results with the three compounds at 25° are given in Table 3. It was expected that the apparent rate constants would become steady below pH 5 (just as k_a was constant above pH 9), giving a value for k_1 , the rate constant for reaction with HSO_3^- .

Fig. 2. Reactions of diacetylcystine ester with sodium sulphite; extrapolations for apparent initial rate constants, k' :

Curve	pH	$10^4 a$	$10^4 b$
A	6.88	7.67	82.1
B	6.41	7.58	80.0
C	6.42	6.95	151.9
D	6.44	3.83	82.5

a (mole l^{-1}) = initial disulphide concentration;
 b (mole l^{-1}) = initial total sulphite concentration. Curve A has been shifted 10 units down the ordinate.

However, k' continues to decrease with increasing $[\text{H}^+]$ and, in fact, is inversely proportional to $[\text{H}^+]$. That is, Eqn. 11 holds in this pH range, when $[\text{H}^+] \gg K\phi$, and not Eqn. 12; k_1 must therefore be negligibly small and Eqn. 11 can be used to describe the change in rate constant for the reaction between disulphides and SO_3^{2-} ions over the whole pH range.

The most recent determination of the pK of HSO_3^- is that of Tartar & Garretson (1941), who give $pK\phi$ at zero ionic strength as 7.20. Other literature values are 6.99 (Britton & Robinson, 1932) and 6.96 (Rumpf, 1933) at low ionic strengths. However, if 10^{-7} is used for $K\phi$ in Eqn. 11, the calculated value of k_2 (i.e., k_a) at 25° for diacetylcystine ester is about 370 $\text{l. mole}^{-1} \text{ min.}^{-1}$ compared with the experimental value of 265 $\text{l. mole}^{-1} \text{ min.}^{-1}$ (Table 2). If the experimental values of k_a from

Table 2 are used to calculate $pK\phi$ from Eqn. 11, the values shown in Table 3 are obtained; these are constant, the mean value being 6.8. This is rather lower than the accepted values, but since dissociation constants can vary considerably under different conditions, it is reasonable to assume that 6.8 is the value under the conditions of the present experiments. Similar series of experiments with the three compounds at 0° confirmed the results at 25°. The value for $pK\phi$ at 0° was the same, within the experimental error, as that at 25° showing that the heat of ionization of HSO_3^- is small. (The same conclusion was reached from measurements of the pH of sulphite solutions at the two temperatures.)

Table 3. Reactions of diacetylcystine ester, dithiodipropionic acid and diformylcystine with sodium sulphite at 25° and $pH < 9$

Substance	pH	Extrapolated value for apparent rate constant k' ($\text{l. mole}^{-1} \text{ min.}^{-1}$)	$pK\phi^\dagger$ calculated
Diacetylcystine ester	3.80	0.25	6.83
	4.33	0.9	6.82
	5.10	5	6.83
	6.10	46	6.78
	6.86	135	6.84
Dithiodipropionic acid (ionized)	6.71	0.5	6.80
	6.87	0.6	6.80
	7.16	0.75	6.83
	7.30	0.85	6.77
Diformylcystine (ionized)	6.22	2	6.77
	6.58	3.5	6.79
	6.68	4.5	6.70
	6.73	5	6.66

Mean $pK\phi = 6.8$

$\dagger$ These values were calculated from the apparent rate constants, using the values of k_a from Table 2 in Eqn. 11.

Equilibria at $pH > 9$. The method of calculation of equilibrium constants has been described earlier and some of the experimental data with diacetylcystine ester are given in Table 1. Apparent equilibrium constants, K_{app} , can be calculated using the experimental value for total thiol instead of $[\text{RS}^-]_0$, these of course varying with pH. It is assumed that $[\text{RSSO}_3^-]_0$ is equal to the equilibrium concentration of total thiol, x_0 , and that $[\text{RSSR}]_0$ and $[\text{SO}_3^{2-}]_0$ are the respective initial concentrations minus x_0 . At high pH when all the thiol is ionized, $K_{app} = K_1$.

From results at any two pH values, both K_1 and K_{SH} were calculated using Eqns. 22, 23 and 24. The values are given in Table 4.

There is a difference of about 0.4 unit in pK_{SH} between 0 and 25° for each of the thiols, *N*-acetylcysteine ester, *N*-formylcysteine and β -mercapto-propionic acid, corresponding to heats of ionization of +6000 cal. mole^{-1} for the thiol groups.

Table 4. *Equilibrium constants of the reactions of diacetylcystine ester, dithiodipropionic acid and diformylcystine with sodium sulphite and the pK values of the corresponding thiols*

Substance	Temp. (°)	I	$K_1 \times 10^{*}\dagger$	$pK_{SH}\dagger$
Diacetylcystine ester	0	0.07-0.11	4.97 ± 0.09	8.92 ± 0.04
	25	0.05-0.09	16.4 ± 0.4	8.53 ± 0.07
Dithiodipropionic acid (ionized)	0	0.13-0.15	0.110 ± 0.004	10.77 ± 0.02
	25	0.13-0.15	0.346 ± 0.005	10.35 ± 0.02
Diformylcystine (ionized)	0	0.09-0.11	0.65 ± 0.04	9.93 ± 0.04
	25	0.07-0.09	2.0 ± 0.1	9.50 ± 0.06

$\dagger K_1$ and pK_{SH} were calculated from Eqns. 22, 23 and 24. The figures given are each the means and standard deviations of at least six calculated values.

Table 5. *Dissociation constants of the amino groups of cystine and GSSG*

Temp. (°)	Cystine $\dagger$		Oxidized glutathione $\ddagger$	
	pK'_3	pK'_4	pK'_5	pK'_6
25	7.7	9.2	8.6	9.5
0	8.4	9.9	9.3	10.2

$\dagger$ From the data of Cannan & Knight (1927).

$\ddagger$ From the data of Li, Gawron & Bascuas (1954).

Other thermodynamic quantities can be calculated from the equilibrium data using the standard expressions (e.g. Glasstone, 1948). The standard changes in heat content (ΔH°), assuming them to be constant over the temperature range 0-25°, the standard free energy changes (ΔG°) at 25°, the standard entropy changes (ΔS°) at 25° for the reactions are given in Table 9.

Reactions of cystine and GSSG

The interpretation of these reactions is complicated by the presence of amino groups which ionize in the pH range used. The dissociation constants of

the amino groups of cystine (at 30°) and GSSG (at 25°) have been reported by Cannan & Knight (1927) and Li, Gawron & Bascuas (1954) respectively. Their figures were used to calculate constants at 0° and 25° (Table 5), assuming a heat of ionization for the $-\text{NH}_3^+$ groups of +11000 cal. (Cohn & Edsall, 1943).

Reactions at pH < 9. As with the other compounds, the reaction mechanism below pH 9 is complex, and the initial rate constants were estimated by extrapolating the second-order rate constants found during runs to zero time.

Taking the value of $K\phi$ to be that found in the experiments already discussed, i.e. 1.6×10^{-7} , k_2 values can be calculated from Eqn. 11 using the apparent rate constants, k' , found at various pH values. The results are summarized in Table 6. The k_2 values decrease as ionization of the amino groups begins. The nearly constant values found at low pH are the initial rate constants for the reactions of cystine (2+, 2-) and GSSG (2+, 4-) respectively. The mean values for these rate constants are: for cystine, 140 l. mole⁻¹ min.⁻¹ at 0° and 1100 l.

Table 6. *Reactions of cystine and GSSG with sodium sulphite at pH < 9*

Substance	Temp. (°)	pH	Extrapolated value for apparent rate constant k' (l. mole ⁻¹ min. ⁻¹)	Rate constants with SO_3^{2-} k_2 (l. mole ⁻¹ min. ⁻¹)
Cystine	0	5.01	2.4	150
	0	6.38	45	160
	0	6.45	45	150
	0	6.83	55	110
	0	7.06	60	90
	25	4.34	3.8	1120
	25	5.07	18.5	1100
	25	6.15	140	760
	25	6.90	400	720
GSSG	0	5.74	2	24
	0	6.09	4	24
	0	6.47	7.5	23
	0	6.65	9	21
	0	6.88	11	20
	25	4.85	2	180
	25	5.31	5.5	170
	25	6.70	60	140

mole⁻¹ min.⁻¹ at 25°; for GSSG, 22 l. mole⁻¹ min.⁻¹ at 0° and 160 l. mole⁻¹ min.⁻¹ at 25°.

Reactions at pH > 9. The apparent rate constants of the forward reactions between the disulphides and SO₃²⁻ ions were calculated from the integrated form of Eqn. 20. These apparent constants become the k'' values of a system involving one reactant ionizing as a dibasic acid as in Eqns. 18 and 19. A continuous drop in the apparent rate constants as the pH increased is shown by the experiments in Table 7. The k_a values are known from the results in Table 6 so that k_b and k_c can be calculated from Eqn. 19 by considering any two experiments together.

Table 7. *Reactions of cystine and GSSG with sodium sulphite at pH > 9*

Substance	Temp. (°)	pH	Apparent rate constant k'' (l. mole ⁻¹ min. ⁻¹)
Cystine	0	8.99	26
	0	9.20	18
	0	9.37	13
	0	9.79	3.2
	0	10.60	0.6
	25	9.04	64
	25	9.35	40
	25	9.55	8
	25	10.52	3
	25	10.52	3
GSSG	0	9.24	12
	0	9.42	11
	0	9.80	6
	0	10.58	2.3
	25	8.94	66
	25	9.10	57
	25	9.71	25
	25	10.22	15

With GSSG, the experiments in borate are consistent with those in carbonate, the values for k_b and k_c being shown in Table 10. With cystine, however, the reactions are slower in carbonate than in borate. It is now known whether this is due to catalysis by borate or inhibition by carbonate. A possible explanation is interaction between CO₂ and the cystine amino groups (Faurholt, 1925) but it is unlikely that this reaction will occur to any extent in this pH region.

If the experiments in borate are considered separately, then at 25° with $k_a = 1100$ l. mole⁻¹ min.⁻¹ it is found that $k_b = 50$ l. mole⁻¹ min.⁻¹ and $k_c = 16$ l. mole⁻¹ min.⁻¹. Similarly, at 0° with $k_a = 140$ l. mole⁻¹ min.⁻¹ it is found that $k_b = 5$ l. mole⁻¹ min.⁻¹ and $k_c = 1.5$ l. mole⁻¹ min.⁻¹. (These calculated values are sensitive to changes in the pK values used for the amino groups of cystine.)

When only the carbonate experiments are considered, the k_c values given in Table 10 are obtained.

These are considered as more suitable for discussion since they were obtained directly, although the possibility remains that they are lower than the true values.

Equilibria at pH > 9. Apparent equilibrium constants for the reactions with sulphite were calculated from the total equilibrium concentrations of all the components. These decrease with pH until, at the highest pH values used, K_{app} approximately equals the true equilibrium constants for the reactions with all the substances in their fully ionized forms. The results obtained with cystine and GSSG are given in Table 8.

Table 8. *Equilibria of cystine and GSSG with sodium sulphite at pH > 9*

Substance	Temp. (°)	pH	I	Apparent equilibrium constant $\times 10^2$ K_{app}
Cystine	0	8.97	0.07	18.0
	0	9.25	0.07	11.7
	0	10.02	0.13	4.3
	0	10.94	0.13	2.1
	25	9.24	0.13	13.8
	25	9.49	0.14	7.9
	25	10.14	0.13	2.2
	25	11.21	0.13	0.64
	25	11.21	0.13	0.64
	25	11.21	0.13	0.64
GSSG	0	9.33	0.10	5.5
	0	9.62	0.12	3.9
	0	10.24	0.10	2.3
	0	11.09	0.11	1.9
	25	8.94	0.08	14.3
	25	9.24	0.08	9.8
	25	9.86	0.10	5.8
	25	10.84	0.11	5.6

The standard thermodynamic quantities derived from the constants at the highest pH values of Table 8 are given in Table 9 and are close to the values for reactions with the three disulphides discussed earlier. The value of +8000 cal. for ΔH° between 0° and 25° for the cystine reaction given in Table 9 is close to that found by Stricks & Kolthoff (1951), viz. +7000 cal. between 12° and 37°. The actual equilibrium constants cannot be compared because of the difference in ionic strengths between the two series of measurements.

DISCUSSION

The mechanism of the complex reaction at pH < 9 is not known but the most likely complication is that an intermediate is formed which decomposes at a rate comparable with its rate of formation:

Another possibility is that, since the complex reaction occurs only when HSO₃⁻ ions are present, an equilibrium of the type

is set up, where Y does not decompose to thiol and RSSO_3^- . However, X and Y differ only by a proton, so that for the second hypothesis to be correct, the structure of Y would have to be such that it had no tendency to lose this proton.

Equilibrium constants have been used to calculate the pK value of the thiol groups of *N*-acetylcysteine ester, *N*-formylcysteine and β -mercapto-propionic acid (Table 4). Only the last of these has

responding to a heat of ionization of +6000 cal. An estimate of this heat of ionization does not appear to have been given in the literature, and various workers have assumed it to be negligible (e.g., Stricks & Kolthoff, 1951).

The kinetic data for the reactions of the various disulphides with SO_3^{2-} ions are summarized in Table 10. The rate constants for diacetylcysteine ester are much higher than those for the ionized

Table 9. *Thermodynamic quantities with sodium sulphite*

		$\text{RSSR} + \text{SO}_3^{2-} \rightleftharpoons \text{RS}^- + \text{RSSO}_3^-$	$\text{RSH} \rightleftharpoons \text{RS}^- + \text{H}^+$
ΔH° (cal. mole ⁻¹)	(1)	+7700	+5800
	(2)	+7400	+6300
	(3)	+7200	+6300
	(4)	+8000	—
	(5)	+7000	—
ΔG° (cal. mole ⁻¹)	(1)	+1100	+11 600
	(2)	+3300	+14 100
	(3)	+2300	+13 000
	(4)	+2000	—
	(5)	+2000	—
ΔS° (cal. mole ⁻¹ deg. ⁻¹)	(1)	+22	-20
	(2)	+14	-26
	(3)	+16	-23
	(4)	+14	—
	(5)	+17	—

(1) Diacetylcysteine ester.

(2) Dithiodipropionic acid (ionized).

(3) Diformylcysteine (ionized).

(4) Cystine

(5) GSSG } reactants and products in completely ionized forms.

Table 10. *Summary of kinetic data*

Substance	Rate constants with SO_3^{2-} k_2 (l. mole ⁻¹ min. ⁻¹)		Net charge on molecule	Activation energy E_a (cal.)	Frequency factor PZ_0 at 25° (l. mole ⁻¹ sec. ⁻¹)	Entropy of activation $\Delta S^\ddagger$ (cal. mole ⁻¹ deg. ⁻¹)
	25°	0°				
Diacetylcysteine ester	265	37	0	12 800	1×10^{10}	-14
Dithiodipropionic acid (ionized)	1.1	0.13	-2	13 500	9×10^7	-24
Diformylcysteine (ionized)	9	1.5	-2	11 500	4×10^7	-25
Cystine						
(1) Cy_2S_2 : 2+, 2-	1100	140	0	13 300	9×10^{10}	-10
(2) Cy_2S_2 : 1+, 2-	(5-70)	(0.5-5)	-1	—	—	—
(3) Cy_2S_2 : 0+, 2-	3†	0.5†	-2	11 600	2×10^7	-27
Oxidized glutathione						
(1) GSSG: 2+, 4-	160	22	-2	12 800	6×10^9	-15
(2) GSSG: 1+, 4-	(40-50)	(2-4)	-3	—	—	—
(3) GSSG: 0+, 4-	10	2	-4	10 400	4×10^8	-29

† Values for k_2 in carbonate buffers.

been reported previously, Borsook *et al.* (1937) giving a value of 10.47 at 25°, compared with the present value of 10.35 at the same temperature. This suggests that the other two thiol pK values can be relied upon to ± 0.1 unit.

The pK values of all three thiol groups change by approximately 0.4 unit between 0° and 25°, corre-

forms of dithiodipropionic acid and diformylcysteine, indicating that an overall negative charge on the disulphide molecule causes a low reaction rate with SO_3^{2-} ions. This effect is also strikingly illustrated by the large differences in rate constants between the various charged forms of cystine and GSSG.

The value of the rate constants for GSSG (2+, 4-) are close to those of the compounds with no net charge, whereas the values for GSSG (0+, 4-) are close to those of the compounds with net charges of -2. Thus, two of the COO⁻ groups of GSSG must have little effect on the reactivity of its disulphide bond, suggesting that in large molecules only those charged groups close to the disulphide bonds influence their reactivity.

The values for the activation energies (E_a), frequency factors (PZ_0) and entropies of activation (ΔS^*) given in Table 10 were calculated from the standard expressions (e.g. Frost & Pearson, 1953a). The ΔS^* values support the hypothesis that it is the charge in the environment of the disulphide bonds that influences the rates of reaction with SO₃²⁻ ions. Values for ΔS^* for reactions between ions and neutral molecules usually lie between 0 and -10 cal. deg.⁻¹ mole⁻¹, the figures for reactions between ions of like charge varying from approximately -20 to -40 cal. deg.⁻¹ mole⁻¹ (Frost & Pearson, 1953b). Here ΔS^* is found to be about -13 cal. deg.⁻¹ mole⁻¹ and -26 cal. deg.⁻¹ mole⁻¹ for the reactions of SO₃²⁻ with neutral and negatively charged molecules respectively.

The use of the sulphite reaction for the estimation of simple disulphides has been discussed in a previous paper (Cecil & McPhee, 1955). With molecules containing groups ionizing in the pH range 5-9, an optimum pH is found for the apparent rate constant for reaction with SO₃²⁻ ions, this optimum depending on the pK values of the various groups in the disulphide molecule. There is no way of predicting the optimum pH for reaction with the disulphide bonds of proteins. Values for wool are given in the literature as about pH 5 (Elsworth & Phillips, 1938), from measurements of the total amounts of thiol formed, and about pH 6 (Speakman, 1936), for the reaction of sulphite with stretched wool fibres. These determinations of the total amount of reaction, as distinct from rate constants, also depend on the reversibility of the reaction, which could be important above about pH 6 with proteins.

SUMMARY

1. The kinetics of the reactions of cystine, oxidized glutathione, diacetylcystine ester, di-formylcystine and $\beta\beta$ -dithiodipropionic acid with sodium sulphite have been studied.

2. The reaction above pH 9 is a simple reversible bimolecular reaction, but below pH 9 the mechanism is complex.

3. It was found that the disulphide bonds of these compounds react with SO₃²⁻ ions but not HSO₃⁻ ions.

4. The rates of reaction with SO₃²⁻ ions are dependent on the net charge in the vicinity of the

disulphide bonds. A net negative charge, due to COO⁻ groups, causes a large decrease in rate constant.

5. The pK values of the thiol groups of *N*-acetylcysteine ester, *N*-formylcysteine and β -mercapto-propionic acid have been calculated from equilibrium data.

6. The heats of ionization of the thiol groups of these three compounds have been found to be approximately +6000 cal.

We wish to thank Mr J. H. Fessler, Dr A. G. Ogston, and Dr R. B. Fisher for many most helpful discussions during the course of this work. One of us (J.R.M.) is indebted to the Commonwealth of Australia for C.S.I.R.O. traineeship. We are grateful to the Distillers Company (Biochemicals) Ltd. for a generous gift of glutathione (DCB 524).

REFERENCES

- Borsook, H., Ellis, E. L. & Huffman, H. M. (1937). *J. biol. Chem.* **117**, 281.
 British Standards Institution (1950). 1647 pH Scale.
 Britton, H. T. S. & Robinson, R. A. (1932). *Trans. Faraday Soc.* **28**, 531.
 Cannan, R. K. & Knight, B. C. J. G. (1927). *Biochem. J.* **21**, 1384.
 Cecil, R. (1950). *Biochem. J.* **47**, 572.
 Cecil, R. & McPhee, J. R. (1955). *Biochem. J.* **59**, 234.
 Clarke, H. T. (1932). *J. biol. Chem.* **97**, 235.
 Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino acids and Peptides*, p. 80. New York: Reinhold Publishing Corp.
 Edman, P. & Diehl, K. (1952). *2nd Int. Congr. Biochem., Paris* (Abstr.), p. 51.
 Elsworth, F. F. & Phillips, H. (1938). *Biochem. J.* **32**, 837.
 Faurholt, C. (1925). *J. Chim. phys.* **22**, 1.
 Footner, H. B. & Smiles, S. (1925). *J. chem. Soc.* p. 2887.
 Fraenkel-Conrat, H. (1942). *J. biol. Chem.* **142**, 119.
 Frost, A. A. & Pearson, R. G. (1953a). *Kinetics and Mechanism*, pp. 96-98. New York: John Wiley & Sons Inc.
 Frost, A. A. & Pearson, R. G. (1953b). *Kinetics and Mechanism*, p. 130. New York: John Wiley and Sons Inc.
 Glasstone, S. (1948). *Textbook of Physical Chemistry*, 2nd ed. p. 897. London: Macmillan and Co. Ltd.
 Kassel, B. & Brand, E. (1938). *J. biol. Chem.* **125**, 131.
 Li, N. C., Gawron, O. & Bascuas, G. (1954). *J. Amer. chem. Soc.* **76**, 225.
 Lindley, H. (1948). *Biochem. J.* **42**, 481.
 Lindley, H. & Phillips, H. (1947). *Biochem. J.* **41**, 34.
 Lugg, J. W. H. (1932). *Biochem. J.* **26**, 2144.
 Moelwyn-Hughes, E. A. (1947). *The Kinetics of Reactions in Solution*, 2nd ed. pp. 38-41. Oxford: Clarendon Press.
 Rumpf, P. (1933). *C.R. Acad. Sci., Paris*, **197**, 686.
 Schöberl, A. & Hornung, T. (1938). *Liebigs Ann.* **534**, 210.
 Schöberl, A. & Krumei, F. (1938). *Ber. dtsh. chem. Ges.* **71**, 2361.
 Schöberl, A. & Ludwig, E. (1937). *Ber. dtsh. chem. Ges.* **70**, 1422.
 Speakman, J. B. (1936). *J. Soc. Dy. Col.* **52**, 335.
 Stricks, W. & Kolthoff, I. M. (1951). *J. Amer. chem. Soc.* **73**, 4569.
 Tartar, M. V. & Garretson, H. H. (1941). *J. Amer. chem. Soc.* **63**, 808.