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Further Studies on the Reaction of Disulphides with Silver Nitrate

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The kinetics of the reaction of silver nitrate with cystine and oxidized glutathione (GSSG) was the subject of previous studies (Cecil, 1950, 1951). It was found that GSSG reacted more rapidly than cystine but, since the reaction showed a number of complexities, comparisons were only semiquantitative. An attempt has been made to investigate these differences of reactivity by a quantitative comparison of eight disulphides under conditions where the complexities are reduced to a minimum. There is still much that is unexplained but, as this work is not being continued, the results obtained so far are reported here.

There has been some disagreement as to the stoichiometry of the reaction of disulphides with silver and with other metals. Lavine (1937), Challenger & Rawlings (1937) and Cecil (1950) found that the reaction stopped at the sulphinic acid stage represented by equation (1):

However, Vickery & Leavenworth (1930) and Preisler & Preisler (1930, 1932) found that some sulphonic acid was formed. We have confirmed there is a definite end point represented by equation (1), but if the reaction is continued for long periods then further changes may occur.

Cecil (1950) assumed, on the basis of earlier work (Lavine, 1937; Challenger & Rawlings, 1937), that

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the initial process is a hydrolysis of the disulphide bond to give thiol and sulphenic acid [reaction (2)]. The thiol is taken up by the excess of Ag^+ ion present (4), causing reaction (2) to proceed to the right. Simultaneously the unstable sulphenic acid dismutates to give sulphinic acid and more thiol (3).

An alternative hypothesis is that the initial reaction is a direct attack by Ag^+ ion on the disulphide bond:

followed by reactions (6), (3) and (4)

In both cases the over-all reaction is given by equation (1). The most direct evidence for reaction (2) is the work of Schiller & Otto (1876) and Fromm (1908), who boiled diphenyl and di-*p*-toluyl disulphides with ethanolic potassium hydroxide. The corresponding thiophenols and sulphinic acids were obtained, but no sulphenic acids. However, the fact that an attack by OH^- ions occurs under these conditions does not necessarily mean that it does so under the milder conditions in this work.

There are several criteria by which the two possible reaction mechanisms can be distinguished:

(i) The reaction kinetics will be different; this is dealt with in the next section. (ii) Equation (2) implies that there is an equilibrium concentration of thiol. It should therefore be possible by continued oxidation to convert all the disulphide into sulphenic acid. (iii) If the function of Ag^+ ion is solely to combine with preformed thiol [reactions (2) and (4)], then the reactivities of silver and other metals that form highly undissociated mercaptides should not differ greatly. A direct attack by the metal ion, however [reaction (5)], should result in considerable differences of reactivity.

THEORETICAL

Any kinetic treatment has to take into account the facts: (i) that there are two possible mechanisms for the splitting of the disulphide bond; (ii) that there are two successive reactions, namely the splitting of the disulphide bond and the subsequent dismutation of the sulphenic acid, either of which can be the limiting progress.

If the splitting of the disulphide bond is a hydrolysis as in equation (2), then the reaction will be first-order with respect to disulphide and zero-order with respect to Ag^+ ion. The expression for the over-all first-order reaction would then be

$$\frac{d[\text{R}\cdot\text{S}\cdot\text{S}\cdot\text{R}]}{dt} = k[\text{R}\cdot\text{S}\cdot\text{S}\cdot\text{R}].$$

If, on the other hand, there is a direct attack by Ag^+ ion on disulphide the reaction will be first-order with respect to both Ag^+ ion and disulphide, and the expression for the over-all second-order reaction will be

$$\frac{d[\text{R}\cdot\text{S}\cdot\text{S}\cdot\text{R}]}{dt} = k[\text{Ag}^+][\text{R}\cdot\text{S}\cdot\text{S}\cdot\text{R}].$$

The experimental results fitted the latter expression, which integrates to

$$k = \frac{2.303}{t(a-b)} \log \frac{b(a-x)}{a(b-\frac{2}{3}x)}, \quad (7)$$

where k is expressed in $\text{l. mole}^{-1} \text{ min.}^{-1}$, t is reaction time in minutes, a and b are the initial molar concentrations of AgNO_3 and $\text{R}\cdot\text{S}\cdot\text{S}\cdot\text{R}$ respectively, and x is the molar concentration of Ag^+ ion that has reacted, so that $(a-x)$ and $(b-\frac{2}{3}x)$ are the molar concentrations of AgNO_3 and $\text{R}\cdot\text{S}\cdot\text{S}\cdot\text{R}$ at time t . The factor $\frac{2}{3}$ arises from the stoichiometry of equation (1).

If the disulphide splitting [reaction (5)] is the limiting process, then disulphide concentrations calculated from the measured uptake of Ag^+ ion will be correct, and the values of k calculated from equation (7) should not vary with time. This happens with GSSG but with none of the other disulphides.

If the dismutation reaction (3) is the limiting process there will be an initial rapid reaction which slows down as the concentration of intermediate builds up, and the value of k calculated from equation 4 will change with time. However, since disulphide splitting must precede the dismutation reaction (5), this must be the limiting process in the early stages of the reaction. In these cases therefore the values of k obtained from equation 4 were extrapolated to zero time.

EXPERIMENTAL

Kinetic experiments. The reactions were carried out at 37° in buffered solution with AgNO_3 in excess over disulphide. The factors governing the choice of pH and buffer are discussed in the next section. The concentrations used were: disulphides, 0.1–0.38 mM; AgNO_3 , 0.5–3.0 mM; buffers, 20 mM. The reactions were stopped at the required time intervals by running 1 ml. samples into sufficient dil. HNO_3 to reduce the pH to 1.8–2. Excess of Ag^+ ion was then estimated by potentiometric titration with KBr (Cecil, 1950).

Analytical methods. Cecil (1950) used a back-titration procedure for the estimation of thiols; that is, a known excess of AgNO_3 was added and back-titrated with KBr. The method depended on the assumption that all the Ag^+ ion, except that bound as mercaptide, could be titrated by KBr at pH 2. Later work (Cecil & McPhee, 1955a) showed that this assumption was incorrect and that in most cases the silver mercaptides bind additional Ag^+ ion sufficiently strongly to interfere with KBr titration, resulting in high values for the thiol concentration. However, the results obtained with back titrations are reproducible so that correction factors can be applied. This has been done in evaluating the kinetic experiments.

In order to determine the correction factors, solutions of the different disulphides were allowed to react with Na_2SO_3 and the thiol formed was estimated both by the silver back-titration procedure and by potentiometric titration with HgCl_2 (Cecil, 1955). This latter method is more convenient than that of Cecil & McPhee (1955a) and Hg^{2+} ion is less liable than Ag^+ ion to form complexes with mercaptides. The Na_2SO_3 -disulphide reactions were carried out in evacuated Thunberg tubes. The reaction conditions for

Table 1. *Back-titration correction factors*

The thiols were produced by reaction of the corresponding disulphide with Na_2SO_3 and estimated both by the back-titration procedure and by potentiometric titration with HgCl_2 . The values derived by back titration have a positive error and must be multiplied by the factors.

Thiol	Factor
GSH	0.90
β -Mercaptopropionic acid	0.91
Homocysteine	0.84
Cysteine	0.85
N-Formylcysteine	0.85
N-Formylhomocysteine	0.77
β -Aminoethanethiol (cysteamine)	0.86
N-Acetylcysteine ester	0.98

GSSG, $\beta\beta'$ -dithiodipropionic acid [di-(2-carboxyethyl) disulphide] and diformylcystine were as described by Cecil & McPhee, 1955*a*. Of the other disulphides, cystine, homocystine, $\beta\beta'$ -diaminodiethyl disulphide (cystamine) and diacetylcystine ester were treated in the same way as GSSG, and diformylhomocystine was treated in the same way as diformylcystine. When reaction was complete each solution was acidified with dil. HNO_3 to pH 1.8–2 and samples were titrated by both methods. The correction factors are given in Table 1.

During the course of this work a comparison was made between the results obtained with the HgCl_2 titration and with the AgNO_3 titration of Cecil & McPhee (1955*a*), the disulphides listed in Table 4 being used. Agreement was within $\pm 1\%$, except for GSSG and homocystine where the results with AgNO_3 were 7 and 3% higher respectively than with HgCl_2 . Amperometric titration of cystine and GSSG with HgCl_2 by the method of Stricks, Kolthoff & Tanaka (1954) agrees with the potentiometric HgCl_2 titrations, and it is assumed that these are correct.

Preparation of materials

$\beta\beta'$ -Dithiodipropionic acid [di-(2-carboxyethyl) disulphide]. β -Mercaptopropionic acid (L. Light and Co. Ltd., Colnbrook, Bucks) was dissolved in 100 ml. of water and cooled to 0° . A trace of FeSO_4 was added, followed by 62 ml. of 0.3*N*. H_2O_2 (1 equiv.), also cooled to 0° . The mixture was allowed to come to room temperature and left overnight. The crystalline precipitate of disulphide was filtered and recrystallized from hot water. Yield 80%, m.p. $155\text{--}156^\circ$ (uncorr.). This procedure is more satisfactory than the oxidation with I_2 used by Biilmann (1905).

Other materials. GSSG, diformylcystine, homocystine and diacetylcystine ester were the samples used by Cecil & McPhee (1955*a*). Diformylhomocystine and $\beta\beta'$ -diaminodiethyl disulphide (cystamine) were the samples used by McPhee (1956).

RESULTS

Oxidation experiments

Solutions of homocystine and GSSG (about 0.1 mM) were buffered to pH 9.2 with $\text{Na}_2\text{B}_4\text{O}_7$ and a trace of FeSO_4 was added. Oxygen was passed through the solutions for 4 hr. at 37° . The initial and final concentrations of disulphide were measured by reaction with Na_2SO_3 , followed by titration with HgCl_2 as described above. There was no detectable difference.

Stoichiometry of the reaction

The evidence for this is based on the amount of silver that has reacted; no attempt was made to identify the reaction products in the dilute solutions used. If the reaction is continued for 5–20 times the normal time then there is a continued slow uptake of Ag^+ ion. This effect is more marked as the AgNO_3 concentration is increased. There was no evidence that this process reaches an end point. When the AgNO_3 concentration was high (more than tenfold molar excess over disulphide), Ag^+ ion uptakes of

more than 2/molecule of disulphide were observed, indicating that a proportion of sulphur–carbon bonds had been broken.

Binding of Ag^+ ion by disulphides

An equilibrium of the type

could affect the Ag^+ –disulphide reaction in two ways. The amount of Ag^+ ion bound could be an appreciable proportion of the total Ag^+ ion concentration and, secondly, if Ag^+ ion is bound in a complex with disulphide it would increase the probability, and hence the rate, of reaction.

Attempts were made to measure the possible extent of this equilibrium by adding small amounts of AgNO_3 to solutions of disulphide buffered in acetate and measuring the free Ag^+ ion concentration with a silver electrode. It was established that the amount of Ag^+ ion bound was small, but it was not possible to decide whether it was bound as a complex with intact disulphide or whether it had reacted to form mercaptide, even though the Ag^+ ion concentration was kept as low as possible.

Choice of reaction conditions

Buffers. The choice of buffers is limited to those which do not form precipitates with AgNO_3 , e.g. acetate, α -picoline and *s*-collidine. Table 2 shows some measurements made on the Ag^+ ion binding by these buffers at different pH values. Acetate binds relatively little Ag^+ ion, but both picoline and collidine bind more than 90%, under the conditions used. Acetate was therefore chosen as the buffer for this work, except for some experiments in urea solutions which were buffered with picoline.

pH. It was desirable to keep the pH above 5.4, as the rate of reaction falls off rapidly below this

Table 2. *Binding of Ag^+ ion by some buffer solutions*

The concentration of AgNO_3 was mM throughout, and that of sodium acetate, α -picoline and *s*-collidine was 20 mM. The pH was adjusted by the addition of HNO_3 . A silver electrode was immersed in the solutions together with a liquid junction and reference electrode. The measured potentials are expressed as molar concentrations of free Ag^+ ion. It is assumed that the activity of a mM solution of AgNO_3 in water is equal to its molar concentration.

Buffer	pH	Free Ag^+ ion concn. (μM)
Water	—	1000
Acetate	5.8	700
Acetate	4.6	680
α -Picoline	7.2	67
α -Picoline	6.2	100
<i>s</i> -Collidine	8.6	9
<i>s</i> -Collidine	7.7	25

value (Cecil, 1950). The upper limit of pH with acetate, which retains adequate buffering power, is 5.8. This pH was therefore used for experiments in which the reactivity of different disulphides was compared.

Experiments in acetate buffer at pH 5.8

GSSG will be considered first since it is the only disulphide studied which gives simple kinetics. The results are summarized in Table 3.

Constant values of k were obtained from equation (7) when the ratio $[\text{AgNO}_3]:[\text{GSSG}]$ was varied between 5.6 and 14.8, indicating that the reaction is then second-order.

There is no significance in the upper limit of the ratio $[\text{AgNO}_3]:[\text{GSSG}]$ apart from the experimental difficulty of measuring small differences in relatively large concentrations, and the fact that the tendency to side reactions increases with increasing AgNO_3 concentrations. When the ratio is reduced to 2.8 the rate constant falls by a factor of approximately 2. This result was entirely unexpected and the explanation has yet to be found. All the disulphides investigated showed the effect, although the ratio of AgNO_3 to disulphide concentration at which the change-over occurs varies with different compounds, and it is necessary to ensure that the excess of AgNO_3 is sufficient before comparisons are made.

The results with the other disulphides are given in Table 4. With all these compounds the rate con-

stants calculated from equation (7) for a single experiment decreased with time, suggesting that the dismutation reaction (3) is the limiting process. However, initial rate constants obtained by extrapolation to zero time did not vary over the concentration ranges indicated, so that the initial reaction must be first-order with respect to Ag^+ ion, and second-order over-all, indicating a direct attack of Ag^+ ion on disulphide. A typical extrapolation is illustrated in Fig. 1. The fact that extrapolation is necessary makes it difficult to estimate the accuracy of the rate constants so obtained. Ideally, readings should be taken very early in the experiments, but it is technically difficult to measure the small uptakes of Ag^+ ion involved with sufficient accuracy. It is likely that if the rate constants are in error the values should be higher than those quoted.

The values for the ratio $[\text{AgNO}_3]:[\text{R}\cdot\text{S}\cdot\text{S}\cdot\text{R}]$ are given; these were always sufficiently high to ensure that the reaction was second-order. It will be seen that whereas 2.7 was adequate for homocystine, 9.2 was required for dithiodipropionic acid. As with GSSG the upper limits represent only experimental limitations.

Experiments in the presence of urea

Cecil (1951) investigated the effect of added urea on the reaction of AgNO_3 with both cystine and GSSG. The idea was to test a hypothesis put forward earlier (Cecil, 1950) that a possible reason for the greater reactivity of GSSG was S-H-N bonding between the sulphur and the glutamyl- NH_3^+ . If this were so it was argued that the presence of a high concentration of urea (6–9M) should have the effect of breaking these bonds and making the GSSG behave more like cystine. It was found that although the initial rate of GSSG was not significantly affected by the presence of urea the

Table 3. Reaction of oxidized glutathione (GSSG) with silver nitrate at pH 5.8 and 37°

10 ⁴ × concn. (M)		Ratio [AgNO ₃]:[GSSG]	k (l. mole ⁻¹ min. ⁻¹)
AgNO ₃	GSSG		
30.5	2.06	14.8	29
17.6	1.57	11.2	29
20.4	2.06	9.9	26
9.6	1.71	5.6	28
9.6	3.48	2.8	17
5.1	1.82	2.8	15

Mean value (excluding the two results when $[\text{AgNO}_3]:[\text{GSSG}]=2.8)=28\pm 2$.

Table 4. Reaction of disulphides with silver nitrate at pH 5.8 and 37°

Substance	Ratio [AgNO ₃]:[R·S·S·R]	k (l. mole ⁻¹ min. ⁻¹)
Dithiodipropionic acid	9.2–16.0	80
Homocystine	2.7–4.9	2400
Cystine	5.3–10.1	9
Diformylcystine	5.0–10.0	9
Diformylhomocystine	5.3–10.0	10
Cystamine	5.1–10.2	3
Diacetylcystine ester	4.8–10.0	No reaction

Fig. 1. Reaction of diformylcystine with AgNO_3 in acetate at pH 5.8. Plot of second-order rate constant k against time: O, $\text{R}\cdot\text{S}\cdot\text{S}\cdot\text{R}=0.198$ mM; $\text{AgNO}_3=0.980$ mM; x, $\text{R}\cdot\text{S}\cdot\text{S}\cdot\text{R}=0.221$ mM; $\text{AgNO}_3=2.20$ mM.

reaction then showed the same complexity as cystine. The cystine reaction was not significantly affected by high concentrations of urea.

It was of interest to repeat these experiments with homocystine, in view of its high reactivity and the fact that interaction between the sulphur and the amino group is sterically possible. The homocystine concentration was 0.17 mM and that of AgNO_3 0.96 mM. The buffer used was α -picoline. Two solutions were run, one containing 6 M-urea and the other a control. Urea raises the pK of α -picoline and also binds Ag^+ ion slightly. To compensate for these effects HNO_3 was added to the solution containing urea to bring the pH to 6.7. The pAg was then 5.2. The pH and the pAg of the control solution were adjusted to these values by increasing the concentration of α -picoline and reducing that of HNO_3 . The reaction is complex in both cases and the initial rate constants are as follows: control solution 840 l. $\text{mole}^{-1} \text{min}^{-1}$; urea solution 530 l. $\text{mole}^{-1} \text{min}^{-1}$. This difference is probably significant, since the same compound is being compared under the two sets of conditions.

DISCUSSION

Mechanism of the initial reaction. There is the question of whether the initial splitting of the disulphide bond is a hydrolysis as in reaction (2) or a direct attack by the metal ion as in reaction (5). The evidence is as follows:

(i) The kinetic behaviour shows that the initial rate of reaction is proportional to the Ag^+ ion concentration as well as to that of disulphide. If reaction (1) occurred this would not be so. On the other hand, the anomalous behaviour at low Ag^+ ion concentrations suggests that the process is complex and makes this conclusion uncertain.

(ii) GSSG and homocystine are not converted into sulphinic acid by oxygen. It is possible, however, that the aerobic oxidation of thiols might be extremely slow at very low concentrations, and so this is not conclusive evidence that there is no hydrolysis.

(iii) The disulphide-silver nitrate reaction virtually stops in acid solution, yet Lavine (1937) found that cystine in sulphuric acid solution reacted rapidly with mercuric sulphate. Likewise diacetylcystine ester does not react with silver nitrate but reacts with phenylmercury hydroxide (Cecil, in preparation).

If the initial step is a hydrolysis then one would not expect such a marked difference between different reagents, but if the metal ion is the active agent such a difference is reasonable. The evidence thus favours the idea of a direct attack by Ag^+ ion on disulphide under the conditions used in this work. As mentioned earlier, however, Schiller & Otto

(1876) and Fromm (1908) have shown that hydrolysis can occur in alkaline solution.

Reactivities of different disulphides. The reactivity of the different disulphides may now be considered. Cystine has a net charge of approximately zero at pH 5.8, whereas cystamine has an over-all positive charge and dithiodipropionic acid an over-all negative charge. Since the reaction is with a positive ion, one would expect cystamine ($k=3$) to react more slowly than cystine ($k=9$) and dithiodipropionic acid more rapidly ($k=80$), as is the case. An analogous, but opposite, case is the reaction of the same compounds with SO_3^{2-} , where the respective second-order rate constants are cystamine 6700 (McPhee, 1956), cystine 1100, and dithiodipropionic 1.1 l. $\text{mole}^{-1} \text{min}^{-1}$ at 25° (Cecil & MCPhee, 1955b).

The behaviour of diformylcystine ($k=9$) and diformylhomocystine ($k=10$) cannot be explained on these grounds. The dissociation constants are likely to be similar to those of dithiodipropionic acid and, if charge effects were the main factor, one would expect the rate constants to be of the same order. As yet there is no explanation for this.

The complete failure of diacetylcystine ester to react is also unexplained. It reacts readily with SO_3^{2-} , though more slowly than the zwitterion form of cystine, the rate constants at 25° being 265 and 1100 l. $\text{mole}^{-1} \text{min}^{-1}$ respectively. One might expect a non-polar molecule to react less readily than a polar one, but the absence of any reaction is surprising.

The behaviour of homocystine is of particular interest because of its very high reactivity. Experiments with Fisher-Hirschfelder-Taylor models show that, as with GSSG, interaction between the sulphur and amino groups is sterically possible. One explanation for the reactivity is therefore that the amino groups are bonded by hydrogen to the sulphur as suggested by Cecil (1950) for GSSG. The Ag^+ ion will tend to form an ammine and so be held in proximity to the sulphur. If this were so then one would expect that, if urea were present in sufficient concentration to break hydrogen bonds by competitive action, the reactivity would be reduced. The rate constant in 6 M-urea is 530 as compared with 840 l. $\text{mole}^{-1} \text{min}^{-1}$ for the control. When the amino group is blocked by substitution the effect is much greater. The value of k for homocystine in acetate at pH 5.8 is 2400, whereas that for diformylhomocystine is 10 l. $\text{mole}^{-1} \text{min}^{-1}$. By contrast the rate constants for cystine and diformylcystine are both 9.

The evidence for S-H-N bonding was discussed by Cecil (1950). Since then Plant, Tarbell & Whiteman (1955) have examined the infrared spectra of compounds of the type $\text{R}_2\text{N}^+[\text{CH}_2]_n\text{SH}$ and found no evidence of hydrogen bonding, though the

evidence here suggests that it is likely to occur only when the amino group is unsubstituted. In view of the complexity of the reaction the idea of hydrogen bonding cannot be regarded as more than a working hypothesis, and the possibility of other forms of interaction must be considered.

The reactivity of GSSG could be explained by the fact that it has an over-all negative charge at pH 5.8, but the behaviour of diformylcystine and diformylhomocystine renders this argument uncertain. The other alternative is a mechanism similar to that suggested for homocystine. The effect of urea is somewhat ambiguous, in that it makes the reaction complex without affecting the initial rate. This is most likely to mean that the dismutation reaction (3) is being affected rather than the disulphide splitting.

Wright (1956) has investigated the X-ray structure of crystalline reduced glutathione (GSH) and has found no evidence for interaction between the amino and thiol groups in the solid state. On the other hand, Saunders (1933, 1934) has shown that there is chemical evidence for some interaction in solution. Following an earlier observation (Hopkins, 1929) that GSH does not give a crystalline derivative with naphthalenesulphonyl chloride, he showed the reaction to be

and that GSSG also fails to give a derivative. Cysteine, too, is oxidized to cystine but this gives the usual *NN'*-dinaphthalenesulphonyl derivative. However, if GSH is treated with chlorodinitrobenzene to give the *S*-dinitrophenyl compound, this reacts normally to give an *N*-naphthalenesulphonyl derivative.

The available evidence is thus conflicting and more will be needed to resolve this point.

SUMMARY

1. The kinetics of the reaction of silver nitrate with eight disulphides have been studied.

2. It has been found that, under given conditions, the reaction is first-order in respect of each reactant. Under these conditions the reactivity of the different disulphides may be compared.

3. Possible reasons for the observed differences in reactivity are discussed. It is suggested that the high reactivity of homocystine, and possibly of oxidized glutathione, is due to interaction between the amino groups and the sulphur atoms. Evidence for this is discussed.

4. The conditions for estimating the eight disulphides are given, a potentiometric titration with mercuric chloride being used. The results are compared with those obtained by other methods.

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