EXPLANATION OF PLATE 1

- A. Frog muscle prepared by injection and cut frozen ($\times 175$)
- B. Frog muscle prepared by injection and cut frozen. ($\times 175$)
- C. Longitudinal section of rat muscle prepared by injection, and cut after fixation. ($\times 280$)
- D. Frog muscle prepared by soaking and cut after fixation. ($\times 280$)

The Action of Urea on the Disulphide Groups of Oxidized Glutathione and Cystine

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In a previous paper (Cecil, 1950) it was suggested that the differences in reactivity between oxidized and reduced glutathione (G_2S_2 and GSH) on the one hand, and cystine and cysteine on the other, could be explained by hydrogen bonding between the sulphur and the glutamyl- NH_3^+ of the glutathione. If this explanation is correct then any substance which competes with the sulphur, by itself forming hydrogen bonds with the glutamyl- NH_3^+ , should tend to reduce this difference in reactivity. Crystalline urea forms intermolecular $N-H-O$ bonds (Wyckoff & Corey, 1934; Pauling, 1940) and it is likely that this also obtains to some extent in solution, particularly at high concentrations. It is therefore to be expected that urea will form hydrogen bonds with the glutamyl- NH_3^+ of G_2S_2 and so tend to make it behave like cystine.

It was shown by Cecil (1950) that the hydrolytic fission reaction of disulphides with an excess of silver nitrate can be used as a measure of comparative disulphide reactivity. The reaction, which may be represented by the following equations:

was followed by estimating the remaining free silver ion by potentiometric titration against potassium bromide.

Three different types of reaction kinetics were described and arbitrarily classified as types I, II and III. The type I reactions are unimolecular with respect to disulphide, (1) being the limiting process. Type II reactions are not unimolecular, suggesting that the dismutation (2) is the limiting process. Type III reactions occur at low pH and differ from I and II in that they do not go to completion. Under the conditions chosen for this work G_2S_2 gives a

type I reaction, and cystine a much slower type II reaction. This paper describes the effect of urea on these reactions.

EXPERIMENTAL

Reaction conditions

The apparatus and methods used were described in the previous paper (Cecil, 1950). The reactions were carried out in the dark at 37° in the range pH 7.0–7.6. α -Picoline, 0.02M, was used as the buffer, the acid component being either cystine or G_2S_2 . The concentrations of G_2S_2 and cystine were approximately 2×10^{-4} M, and that of the $AgNO_3$ 10^{-3} M. When high urea concentrations (4–9M) were used the reactions were carried out in 10 ml. Pyrex standard flasks. The urea was added as solid and the other reagents in solution. At lower urea concentrations Pyrex boiling tubes were used as previously.

Purification of reagents

Urea. This was twice recrystallized from hot ethanol. The ethanol used was first refluxed over Zn and NaOH, to remove chloride and peroxides, and redistilled. The halide content was always less than 1 part of Cl^- in 10^6 , measured as described by Cecil (1950).

α -Picoline. The sample used in the previous work gave no reaction with $AgNO_3$. In the presence of urea, however, a slow reaction took place involving an appreciable Ag uptake. This was shown to be due to an impurity in the picoline, which was removed by refluxing over Ag_2O for 2 hr. and redistilling. The fraction boiling at $128-130^\circ$ was used.

G_2S_2 and cystine. The samples described in the previous paper (Cecil, 1950) were used.

Stability of solutions

Dilute standard solutions are always liable to error because of adsorption on glass. In sunny weather particularly, $AgNO_3$ solutions were found to lose 3–4% of their Ag content during the day, which reappeared in solution overnight. The use of coloured bottles reduced, but did not eliminate, this effect. As a precaution the G_2S_2 and cystine solutions were

checked each day, and the AgNO_3 immediately before each experiment, using the methods described by Cecil (1950). Tests were also made with the Pyrex vessels used for the reactions. Under the conditions used, namely 37° in the dark, no adsorption was found.

Measurement of pH

pH measurements were made with a glass electrode calibrated with 0.05M-potassium acid phthalate, taken as pH 4.00 at 20° (MacInnes, Belcher & Shedlovsky, 1938), and 0.05M- $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ taken as pH 9.18 at 20° (Lauchlan, 1944). It was found that at the high urea concentrations the pH of the reaction mixtures rose to 7.6 as against 7.0 in the absence of urea. This effect was confirmed with a hydrogen electrode. Urea has no buffering power in this pH range and so this difference must have been due to the effect of urea on the dissociation constants of the ionizing groups present. Although the addition of acid would correct the pH readings it would not restore the original dissociation constants. Since there was therefore no way of making the reaction conditions identical in the presence and absence of urea, it was decided to maintain the same buffer concentrations throughout.

Previous work (Cecil, 1950) had shown that the hydrolytic fission of disulphides is favoured by increasing pH, and so it is possible that the effect of urea in slowing this reaction is greater than appears, but it cannot be less.

RESULTS AND DISCUSSION

The results are expressed graphically on the basis of the kinetic treatment given in the previous paper (Cecil, 1950). $\text{Log}(c-x)$ is plotted against time, where c =initial concentration of disulphide in equivalents, and x =reacted concentration in equivalents. Type I (unimolecular) reactions will therefore give linear, and types II and III curved plots.

G_2S_2 normally gives a type I reaction at pH 7. The effect of 1M-urea is only just detectable, but as the concentration is raised from 4 to 9M the reaction is progressively reduced in rate and becomes type II (Fig. 1). Previous work shows that, apart from interaction between the urea and G_2S_2 , this effect could be caused either by lowered pH, already discussed, or decreased silver ion concentration.

If urea binds silver ion sufficiently to interfere with the latter's reaction with GSH, then urea should also interfere with the silver ion reaction with bromide ion, since silver electrode potential measurements show that GSH has a higher affinity for silver ion than has bromide ion. Accordingly, silver nitrate was titrated with potassium bromide in the presence of 10M-urea at a pH of approximately 7.5. The titre, determined by the potentiometric method, was 3% low, which is little more than the error (1-2%) due to the use of the silver electrode at this pH. At low pH values (2-3) this error disappears completely. It is, therefore, considered unlikely that decreased silver ion concentrations are responsible for the effects observed.

Experiments were tried with G_2S_2 in 6M-urea in which the urea, G_2S_2 , and picoline were incubated at 37° for periods of up to 3 hr. before adding the silver nitrate. The effect was the same as when the silver nitrate was added simultaneously with the other reagents, showing that the action of urea on G_2S_2 is rapid. The reverse effect, namely the return of G_2S_2 to normal behaviour, is also rapid. Solid urea (0.6 g.) was added to 2 ml. of $9.6 \times 10^{-4}\text{M}$ - G_2S_2 solution, giving a urea concentration of approximately 4M, and this solution was incubated for 1 hr. at 37° . The total volume was then made up to 10 ml. by adding α -picoline and silver nitrate solutions and water, so that the final concentrations of all reagents was the same as in the previous experiment in 1M-urea. The same reaction rate was obtained.

Fig. 1. Comparative kinetics of the G_2S_2 - AgNO_3 reaction at pH 7.0-7.6 in the presence of varying concentrations of urea. A, $\blacktriangledown$, control reaction without urea; B, $\square$, reaction in 1M-urea; C, $\bullet$, reaction in 4M-urea; D, $+$, reaction in 6M-urea; E, $\circ$, reaction in 9M-urea.

The action of urea on the cystine-silver nitrate reaction was also investigated. Previous work (Cecil, 1950) had shown that cystine is normally more sensitive in this reaction to added reagents than is G_2S_2 . Thus acetate ion catalyses the cystine reaction quite markedly, but the G_2S_2 one very little. Barbitone slows the G_2S_2 reaction, but completely stops that of cystine. The impurity in the α -picoline, mentioned earlier, has no effect on the G_2S_2 reaction, but has considerable effect on that of cystine. This last effect was given as a correction in proof in the previous paper.

Fig. 2 shows the correct curve for the cystine-silver nitrate reaction in α -picoline at pH 7.0. Addition of 10^{-3}M -acetate swamps the effect of the impurity, the same increased rate being obtained whether the pure or impure picoline is used.

Unlike acetate and barbitone, urea has much less effect on cystine than G_2S_2 . The results for cystine

(Fig. 2) show a slight reduction in rate in the presence of 1M-urea, but no further reduction when the urea concentration is raised to 6M. In both cases the reaction remains type II. No effect was observed with 0.2M-urea.

Fig. 2. Comparative kinetics of the cystine- AgNO_3 reaction at pH 7.0-7.6 in the presence of varying concentrations of urea. A, ∇ , control reaction without urea; B, $\square$, reaction in 0.2M-urea; C, $\bullet$, reaction in 1M-urea; D, $+$, reaction in 6M-urea.

While these various results cannot be said to prove the hypothesis put forward earlier, they are in agreement with it. Thus, if the action of urea on G_2S_2 is to break $\text{N}-\text{H}-\text{S}$ bonds between the sulphur and the glutamyl- NH_3^+ by competitive action, one would expect G_2S_2 to behave increasingly like cystine as the urea concentration is raised. This does in fact happen. The reaction slows progressively and changes from type I to type II, though, even when the urea concentration is 9M, G_2S_2 reacts considerably more rapidly than cystine. The latter suggests that the glutamyl- NH_3^+ forms more stable bonds with the sulphur than with urea. This is in accord with the assumption that the sulphur bonds are

formed by chelation, whereas those with urea are intermolecular. It is thought possible that this effect of urea on glutathione may be related to its well known effect on the sulphur groups of certain proteins first reported by Ramsden (1940).

The effect on cystine is much smaller, and is maximal with 1M-urea. This effect differs from the inhibitory action of barbitone, or the catalytic action of GSH and G_2S_2 (Cecil, 1950). These three substances all form moderately stable complexes with cystine and in consequence they are effective at concentrations comparable with that of cystine, whereas urea shows no appreciable effect at 0.2M. A possible explanation is that cystine exhibits some intermolecular $\text{N}-\text{H}-\text{S}$ bonding in solution, insufficient to affect markedly its chemical reactivity, and which is easily interfered with by urea.

SUMMARY

1. The kinetics of the reaction between oxidized glutathione and silver nitrate at pH 7.0-7.6 were studied in the presence of varying concentrations of urea. Urea concentrations of 4M and above progressively reduced the reaction rate and changed the kinetics towards those characteristic of the cystine-silver nitrate reaction.

2. The rate of the cystine-silver nitrate reaction was slightly reduced by 1M-urea, but no further effect was produced by raising the urea concentration to 6M. The character of the kinetics was unchanged.

3. It is suggested that the urea acts by breaking $\text{N}-\text{H}-\text{S}$ bonds between the sulphur and the glutamyl- NH_3^+ of the oxidized glutathione, thus making it behave like cystine. The existence of this $\text{N}-\text{H}-\text{S}$ bond was discussed in a previous paper. The smaller effect of urea on cystine suggests that this substance normally exhibits some intermolecular $\text{N}-\text{H}-\text{S}$ bonding which hardly influences its chemical behaviour.

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