Disulphide Interchange Reactions

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In studies designed to determine the distribution of the disulphide bridges, insulin was hydrolysed with cold concentrated hydrochloric acid and many more cystine peptides were obtained than could be accounted for by a unique structure for insulin. This result led to the suspicion that an interchange reaction took place at the disulphide bonds, and this suspicion was confirmed by experiments with model disulphides (Sanger, 1953). This paper reports the results of some further studies on the disulphide interchange reaction in acid, neutral and slightly alkaline solutions, which were made with the aim of finding how, and under what conditions, the interchange occurred, and particularly how it might be prevented during studies on the arrangement of the disulphide bonds of proteins.

In acid solution the reaction has been studied using a model system consisting of L-cystine and *NN'*-bis-2:4-dinitrophenyl-L-cystine (bisDNP-cystine). The coloured product of the reaction, unlike bisDNP-cystine, is not extracted from acid solution by ether, so that the progress of the reaction can readily be followed spectrophotometrically. It was assumed to be *N*-2:4-dinitrophenylcystine (monoDNP-cystine), and this has now been confirmed by isolation of the crystalline compound. The above model system could not be used for quantitative studies in neutral solutions because of the insolubility of cystine, which was therefore replaced by the more soluble cystylbisglycine or by oxidized glutathione (GSSG). A preliminary report of this work has been published (Ryle & Sanger, 1954).

EXPERIMENTAL

Materials

BisDNP-L-cystine was prepared by the method of Porter & Sanger (1948) and L-cystylbisglycine by the method of Bailey (1950). The insulin used was batch 9011G obtained from Boots Pure Drug Co., Nottingham.

Product of reaction between cystine and bisDNP-cystine. L-Cystine (100 mg.) and bisDNP-cystine (500 mg.) were dissolved in a mixture of 10 ml. 12N-HCl and 20 ml. acetic acid and allowed to stand at room temp. for 3–4 days. From part of the resulting mixture a small quantity of the product was obtained in crystalline form in the following way. The solution was diluted with 2 vol. of water, extracted with ether to remove unchanged bisDNP-cystine and concentrated to a small volume by evaporation *in vacuo* at 40°. A column of talc (Hopkins and Williams Purified B.P.C., previously washed with N-HCl) 3·5 cm. high and 4·5 cm. in diameter was prepared and washed with 0·1N acetic acid under applied pressure 5–10 cm. Hg to hasten the flow. The yellow solution was poured on to the top of the column and washed through with 0·1N acetic acid. Although the yellow material did not stick fast to the talc, the emerging liquid was found to be free of chloride before it reached the bottom of the column. At this stage the eluting liquid was changed to ethanol–N acetic acid (80:20, by vol.) and the yellow solution was collected as it came off the column. This solution was extracted with ether to remove ethanol and any bisDNP-cystine not already removed, and after concentration by evaporation *in vacuo* at 40° yellow crystals appeared on cooling. A second crop of crystals was obtained by further concentration of the mother liquor. The crystals were quickly washed with 0·2N acetic acid and dried in a vacuum desiccator. Under the microscope they appeared as bunches of fine yellow needles, m.p. 179° (uncorr.).

The preparation and characterization of monoDNP-L-cystine (m.p. 187°) has now been described by Bettelheim (1955).

The crystalline material was chromatographed on paper together with a sample of monoDNP-cystine kindly given us by Dr V. du Vigneaud. The unknown material moved as a single yellow spot which turned brown on treatment with ninhydrin. In descending runs (Whatman no. 1 paper) R_F values were: 0·64 in phenol–0·3% aq. NH_3 (Sanger & Tuppy, 1951); 0·84 in butanol–acetic acid–water (Partridge, 1948); 0·07 in *tert.*-amyl alcohol–phthalate buffer (pH 6) (Blackburn & Lowther, 1951). Both the authentic and the unknown material moved at the same speed in each case.

A small amount of the material was oxidized with 0·1 ml. performic acid solution (30% (w/v) H_2O_2–formic acid, 1:9 by vol.) at room temp. for 15 min. The formic acid was removed by repeated drying *in vacuo* and the residue chromatographed in the same three systems as before. In each case one yellow spot which did not react with ninhydrin and one ninhydrin-reactive spot appeared. These spots ran at the same speeds as DNP-cysteic acid (Sanger & Thompson, 1953) and cysteic acid respectively (see Table 1). The product is thus confirmed to be monoDNP-cystine.

The light absorption of the material in N-HCl and in 1% (w/v) $NaHCO_3$ was measured in the Beckman spectrophotometer. Beer's law was obeyed at the concentrations studied (less than 50 μM). The absorption is close to that of ϵ-DNP-lysine (Sanger, 1949). Absorption curves for acid and alkaline solutions are shown in Fig. 1.

The course of the interchange reaction in acid solution

The model system of cystine and bisDNP-cystine was used. In the experiments in HCl solution the reactants were dissolved separately in 12N-HCl and on mixing were

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Table 1. *R_F values of oxidation products of monoDNP-cystine*

Solvent system	Authentic DNP-cysteic acid	Yellow spot	Authentic cysteic acid	Ninhydrin-reactive spot
Phenol–0·3% aq. NH_3 (Sanger & Tuppy, 1951)	0·50	0·50	0·08	0·08
Butanol–acetic acid–water (Partridge, 1948)	0·73	0·74	0·16	0·15
tert.-Amyl alcohol–phthalate buffer (pH 6) (Blackburn & Lowther, 1951)	0·02	0·02	0·00	0·00

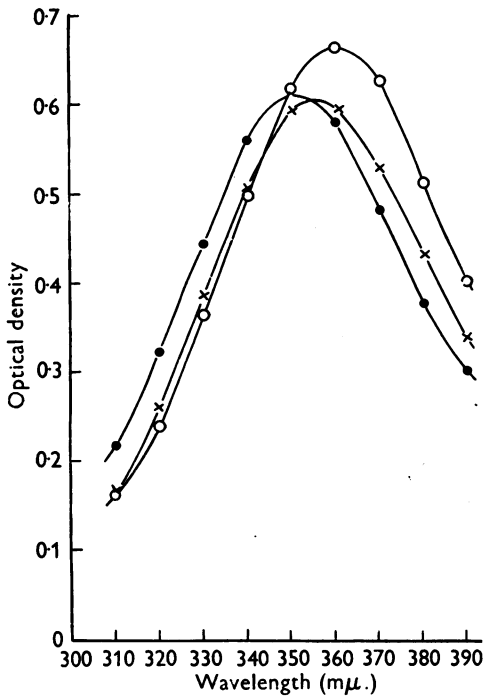


Fig. 1. Absorption curves of DNP derivatives of cystine: ●—●, 4·0 μM monoDNP-cystine in N-HCl; ○—○, 4·0 μM monoDNP-cystine in 1% $NaHCO_3$; ×—×, 2·0 μM bisDNP-cystine in 1% $NaHCO_3$.

diluted with HCl whose strength was such that the final mixture would be of the desired molarity. In this way the same stock solutions of reactants were used in all the experiments at different HCl concentrations. The incubations were carried out in a water bath at 35°. To follow the course of the reaction samples (2 ml.) were taken from time to time, pipetted into 4 ml. of water and shaken with 2 ml. ether. The ether was sucked off and the extraction repeated with a further 2 ml. ether. Trial experiments showed that this procedure was sufficient to remove the bisDNP-cystine from the aqueous phase. A sample from this phase was then diluted to a suitable volume and the absorption at 350 mμ. of the resulting solution was read in the Beckman spectrophotometer against water. The amount of monoDNP-cystine formed in each ml. of reaction solution was calculated from the absorption.

The course of the reaction in neutral and slightly alkaline solutions

The low solubility of cystine in neutral solution prevented its use in these experiments, so cystylbisglycine was used instead. The reactions were carried out in buffer solutions in a water bath at 35°, and were followed by the same means as in the experiments in acid solution except that the samples were pipetted into 2 vol. of N-HCl instead of into water.

The experiments in the absence of oxygen were carried out in Thunberg tubes in which the solutions of the two reactants were evacuated before being mixed. The tubes were re-evacuated after each sample had been taken.

RESULTS

Disulphide interchange in acid solution

Effect of acid. Fig. 2 shows the course of the reaction in various concentrations of HCl at 35°. It is very rapid in conc. HCl but falls off markedly as

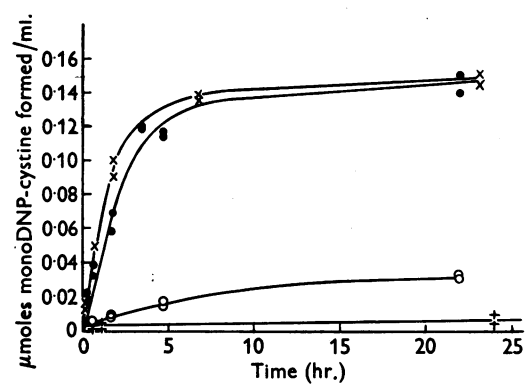


Fig. 2. Reaction of 10^{-3}M cystine and 10^{-4}M bisDNP-cystine in HCl at 35°: ×—×, 10N-HCl; ●—●, 9N-HCl; ○—○, 7N-HCl; +—+, 5N-HCl.

the concentration of acid falls below 9N. It was impossible to study the reaction at this temperature in solutions more dilute than 5N because of the insolubility of bisDNP-cystine. The results of some experiments carried out at 100° with more dilute acid are given in Table 2. Here excess cystine was used and the results are expressed as percentage of

the maximum interchange reaction theoretically possible if all the bisDNP-cystine were converted into monoDNP-cystine. In conc. acid the reaction is much more rapid in HCl than in corresponding strengths of H_2SO_4, though in dilute acids the reverse is the case. In 0·01 N acids or in water the reaction was more rapid than in 0·1 N acid but there was some production of H_2S, suggesting that other reactions were taking place. The reaction is at a minimum in 2 N-H_2SO_4 or in approx. 0·5 N-HCl.

Table 2. *Interchange reaction in various concentrations of* HCl *and* H_2SO_4 *at* 100°

3×10^{-4} M bisDNP-cystine refluxed 2 hr. in acid with 10^{-2} M cystine. Figures are % theoretically possible interchange reaction.

Normality of acid	% interchange in HCl	% interchange in H_2SO_4
0·1	4·6	18
0·5	—	3·3
1·0	4·7	2·8
2·0	—	1·5
3·0	—	2·3
5·0	26	4·7

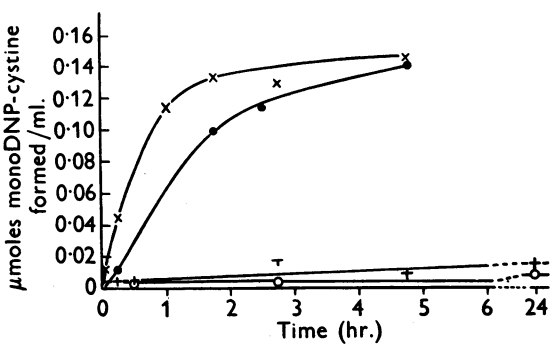


Fig. 3. Effect of cysteine on interchange reaction in acid solution: 10^{-3} M cystine, 10^{-4} M bisDNP-cystine, 10 N-HCl, 35°. Cysteine hydrochloride was added to give the following molar SH/SS ratios: ×—×, 0; ●—●, 10^{-3}; +—+, 10^{-2}; ○—○, 10^{-1}.

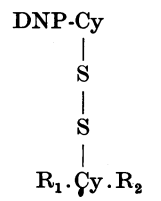
In an experiment at 35° with 10^{-3} M cystine and 10^{-4} M bisDNP-cystine in 10 N-H_2SO_4 containing 50 % (v/v) acetic acid in order to bring the DNP compound into solution the rate of disulphide interchange was found to be low, 10 % of the bisDNP-cystine having reacted after 24 hr. and 45 % after 5 days.

Effect of added thiol. Huggins, Tapley & Jensen (1951) described the gelation of various proteins in conc. urea solutions and produced evidence indicating that it was brought about by the rearrangement of some of the disulphide bonds of the protein, the rearrangement occurring by reaction between disulphide groups and thiols:

$$R^1SSR^2 + R^3SH \rightleftharpoons R^1SSR^3 + R^2SH.$$

It seemed possible that the disulphide interchange reaction studied here might occur by the same means. If this were so, the addition of thiols should accelerate the interchange reaction. Fig. 3 shows the effect on the reaction in 10 N-HCl of adding varying quantities of cysteine hydrochloride. It is apparent that, rather than accelerating the reaction, cysteine markedly inhibits it.

Conditions for protein hydrolysis. The above results suggest that the interchange reaction is slower in high concentrations of H_2SO_4 than in corresponding concentrations of HCl and that it is markedly inhibited by thiols. In order to determine how much interchange occurred during various conditions for the hydrolysis of insulin, samples of insulin (10 mg./ml.) were allowed to hydrolyse in the presence of bisDNP-cystine (0·02 M), and the amount of coloured material remaining in the aqueous solutions after ether extraction was taken as a measure of the extent of the interchange reaction. This colour is due to a mixture of peptides of the type



The results are given in Table 3 as the number of disulphide bonds of insulin that have reacted, assuming that when one such bond has reacted two DNP groups appear in the aqueous phase. It can be seen from Table 3 that no appreciable interchange occurs in 10 days when the hydrolysis is carried out at 37° with 10 N-H_2SO_4 in the presence of thiol, and there is only a very little after hydrolysis in the same solution at 100° for 2 hr. These two sets of conditions were used for the hydrolyses of insulin to determine the arrangement of the disulphide bonds (Ryle, Sanger, Smith & Kitai, 1955).

Similar experiments with cystine and bisDNP-cystine yielded similar results. The interchange reaction at 35° between 10^{-3} M cystine and 10^{-4} M bisDNP-cystine in 10 N-H_2SO_4 in 50 % acetic acid was completely inhibited by $1{\cdot}1 \times 10^{-5}$ M thioglycollic acid, no reaction being detectable after 5 days. At 100° $1{\cdot}1 \times 10^{-4}$ M thioglycollic acid provided complete inhibition of interchange over 1 hr., while in the same time in the presence of $1{\cdot}1 \times 10^{-5}$ M thioglycollic acid 15 % interchange occurred.

Table 3. *Interchange reaction occurring during the hydrolysis of insulin*

0·02 M bisDNP-cystine; 10 mg. insulin/ml.

Acid	Temp. (°)	Additions	Time	Approx. no. —S.S— bonds reacting/ molecule insulin (mol.wt. 6000)
12 N-HCl*	37	—	1 hr.	0·3
			2 hr.	0·65
			5 hr.	1·2
5·7 N-HCl in 50% (v/v) acetic acid*	37	10^{-3} M cysteine	1 day	0·35
			4 days	1·0
			10 days	1·35
10 N-H_2SO_4 in 50% (v/v) acetic acid	37	2×10^{-3} M cysteine	5 days	0
			10 days	0·01
10 N-H_2SO_4 in 30% (v/v) acetic acid	100	—	30 min.	0·35
			90 min.	0·7
10 N-H_2SO_4 in 30% (v/v) acetic acid	100	5×10^{-3} M thioglycollic acid	35 min.	0·05
			120 min.	0·15

* 2×10^{-3} M-bisDNP-cystine; 1 mg. insulin/ml.

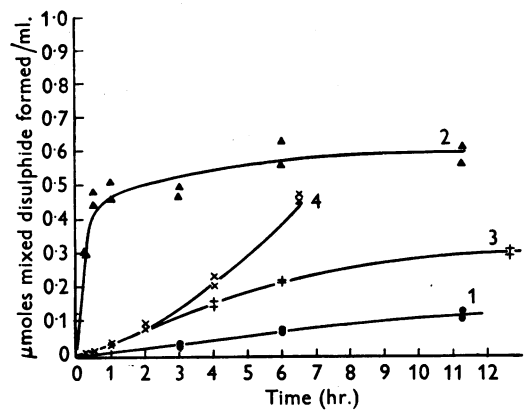


Fig. 4. Reaction of 10^{-3} M cystylbisglycine and 10^{-3} M bisDNP-cystine in 0·017 M-Na_2HPO_4–KH_2PO_4 buffer (pH 7·2), 35°. Effect of thiol and oxygen. ●—●, 1, no EDTA, not evacuated; ▲—▲, 2, as 1, with addition of 10^{-4} M GSH; +—+, 3, with 0·01 M EDTA, not evacuated; ×—×, 4, with 0·01 M EDTA, evacuated.

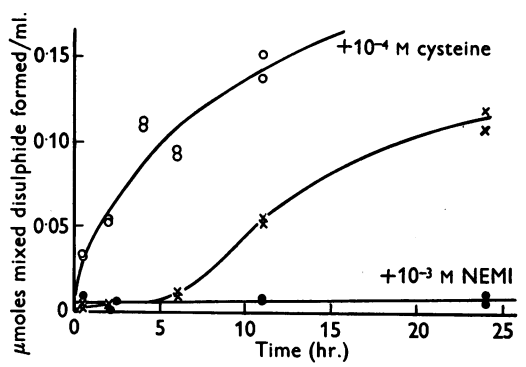


Fig. 5. Reaction of 10^{-3} M cystylbisglycine and 10^{-4} M bisDNP-cystine in 0·017 M-Na_2HPO_4–KH_2PO_4 buffer (pH 7·2), 35°, in evacuated tubes with 0·01 M EDTA.

Disulphide interchange in neutral and slightly alkaline solutions

Some preliminary experiments indicated that in neutral solution the disulphide interchange occurring between bisDNP-cystine and cystylbisglycine was catalysed by the addition of thiols, and since thiols are oxidized by oxygen in neutral solution in the presence of traces of heavy metal it seemed possible that the reaction might proceed faster if oxygen were removed, or oxidation prevented, by the addition of some chelating agent such as ethylenediaminetetraacetate (Versene).

In Fig. 4 are shown the results of some experiments which indicate that this is the case. The reaction in the presence of added thiol is rapid at the start but falls off sharply when oxygen is present and no ethylenediaminetetraacetate (abbr. EDTA) is added, presumably when the thiol has been oxidized. In the absence of added thiol the reaction is most rapid when oxygen is excluded and EDTA is added. In the presence of oxygen it is rather faster when EDTA is present than when none is added, so that it appears that the chelating agent has some, but not a complete, protective effect.

Fig. 5 shows the course of the reaction between 10^{-3} M cystylbisglycine and 10^{-4} M bisDNP-cystine (one-tenth of the concentration of the DNP compound used before). Catalysis by added thiol is again observed, and almost complete inhibition by *N*-ethylmaleimide (NEMI).

Fig. 6 shows the course of the reaction between GSSG and bisDNP-cystine. The rate is greater at the higher pH, and the reaction is almost completely

inhibited by the addition of *p*-chloromercuribenzoate (*p*CMB).

A curious feature of the interchange reaction in neutral solution in the absence of oxygen was the lag phase of somewhat variable duration occurring at the start of the reaction. In Fig. 7 are shown the results of four experiments which are identical, except that in two tubes freshly prepared solutions of the reactants were used, while in the other two solutions which had stood under vacuum at 37° for 16 hr. were used. In the experiments with these 'aged' solutions there is no lag phase.

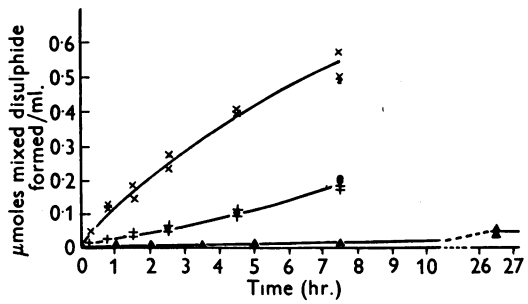


Fig. 6. Reaction of $0{\cdot}8 \times 10^{-3}$M GSSG with $0{\cdot}8 \times 10^{-3}$M bisDNP-cystine in 0·017M-Na_2HPO_4–KH_2PO_4 buffer at 35° in evacuated tubes. ×—×, pH 8·0 with 0·01M EDTA; ●—●, pH 7·2 with 0·01M EDTA; +—+, pH 7·2, without EDTA; ▲—▲, pH 7·2, with $0{\cdot}8 \times 10^{-4}$M *p*-chloromercuribenzoate.

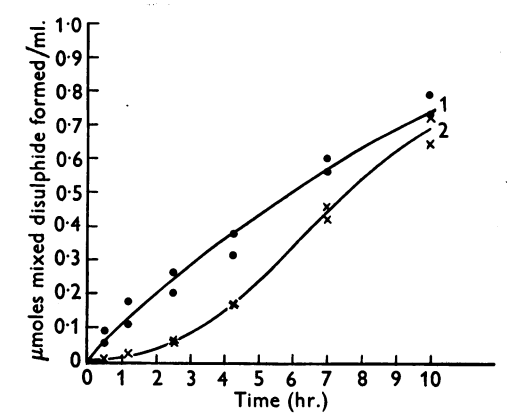


Fig. 7. Reaction of 10^{-3}M cystylbisglycine and 10^{-3}M bisDNP-cystine in 0·017M-Na_2HPO_4–KH_2PO_4 buffer (pH 7·2), 0·01M EDTA, 35°; ●—●, 'aged' solutions of disulphides; ×—×, fresh solutions of disulphides.

DISCUSSION

The most striking feature of the results reported above is the difference in the response of the disulphide interchange in acid and neutral solutions to the addition of thiols. The finding that thiols inhibit the reaction in acid solution and catalyse it in neutral solution must indicate that the mechanism under the two conditions is different. This conclusion accords well with the finding that the rate passes through a minimum in dilute acid and is greater in strongly acid or neutral solutions (Table 2).

The inhibition of the interchange reaction in neutral solution by NEMI which combines rapidly and quantitatively with thiols (Friedmann, Marriann & Simon-Reuss, 1949), by *p*CMB, which similarly removes thiols from solution (Hellerman, Chinard & Deitz, 1943), and by oxygen, and its catalysis by added thiol indicates that it is here carried by thiol groups in the same manner as the gelation reaction of Huggins *et al.* (1951). A catalytic amount of thiol must be produced from the disulphides, presumably by hydrolytic fission (Cecil, 1950), so that the interchange reaction would proceed as follows:

$$R^1SSR^1 + OH^- \rightleftharpoons R^1S^- + R^1SOH$$
$$R^2SSR^2 + R^1S^- \rightleftharpoons R^1SSR^2 + R^2S^-$$
$$R^2S^- + R^1SSR^1 \rightleftharpoons R^1SSR^2 + R^1S^- \text{ etc.}$$

At higher pH values the concentration of RS^- produced by the initial reaction with OH^- would be greater, so that the overall interchange reaction would be found to proceed faster, as observed in Fig. 6.

If it is assumed that the hydrolytic attack on the disulphides is slow, this mechanism may explain the lag phase observed in experiments in which fresh solutions of the disulphides were employed, as opposed to those in which 'aged' solutions were used (Fig. 7): in the experiments with fresh solution thiols are slowly formed from the disulphides during the lag phase, while in the experiments with 'aged' solutions the thiols will have been formed before the start of the experiment.

The inhibition of the interchange by thiol-binding reagents is most striking and, as described in the succeeding paper, such reagents have been employed in preventing interchange in neutral solutions during studies on protein disulphides.

It may not be out of place to speculate on the role which disulphide interchange reactions play in living systems. In the experiments described above a quite rapid interchange took place under neutral conditions in the presence of 10^{-4}M glutathione (3 mg./100 ml.) or cysteine. In the blood the concentration of glutathione found by Benedict & Gottschall (1933) is about 40 mg./100 ml. corresponding to about 80 mg./100 ml. for the red blood corpuscles in which all the glutathione is found. Bartlett & Stevenson (1954) give a lower figure, about 23 mg./100 ml., for whole blood and about 170 and 15 mg./100 g. wet weight for the liver and muscle, respectively, of growing rats. If it is

assumed that the glutathione is distributed uniformly through these two last tissues its concentration ranges from 0·5 to 5·0 mM—rather higher than that used in the experiments described above.

The conditions in the body are therefore such as could promote a rapid disulphide interchange, so that any small disulphides would be expected to react in this way. Whether such interchange can occur within and between protein molecules *in vivo* will depend on the rigidity of the protein structure and on the extent to which the disulphide bonds are accessible to glutathione. While little is known of the latter factor, the observations of Huggins *et al.* (1951) on the urea-induced gelation of albumins (a reaction which is essentially a disulphide interchange reaction) indicate that with these proteins interchange can only occur when the hydrogen bonding of the protein has been shattered by the high concentration of urea. If, as seems possible, other proteins behave in the same way, their disulphide bonds should be stable under physiological conditions, so that the interchange reaction may not be of importance.

Most of the small sulphur-containing molecules, such as glutathione and coenzyme A, are found in the reduced form, so are unlikely to play any except a catalytic part in disulphide interchange. The fact that these thiols are found side by side with proteins containing disulphide bonds is probably another reflexion of the stability conferred on the protein disulphides by the configuration of the molecule, maintained by other bonds within and between the polypeptide chains. Whilst it seems that in general proteins are unlikely to take part in disulphide interchange reactions under physiological conditions, it still remains possible that there are special cases in which such reactions are of importance.

No conclusions can be drawn here about the mechanism of the interchange reaction in acid solutions, but the most striking features here are the inhibition by thiols and the great speed of the reaction in 10N-HCl compared with that in 10N-H_2SO_4 or 5N-HCl. It should be pointed out that the activity of hydrochloric acid rises very rapidly with increasing concentration, so that that of the 10N acid is much greater than that of sulphuric acid of the same strength.

SUMMARY

1. A disulphide interchange reaction of the type

$$R^1SSR^1 + R^2SSR^2 \rightleftharpoons 2R^1SSR^2$$

has been studied in acid, neutral and slightly alkaline solutions.

2. The reaction is inhibited in acid solution by thiols.

3. The reaction in neutral and alkaline solution is catalysed by thiols and inhibited by thiol-binding reagents.

4. A mechanism for the reaction in neutral and alkaline solutions is suggested.

5. Suitable conditions have been determined for the hydrolysis of proteins without disulphide interchange occurring.

We wish to thank Dr V. du Vigneaud for a sample of monoDNP-cystine and Dr J. L. Bailey for help and advice in preparing cystylbisglycine. A.P.R. is indebted to the Medical Research Council for a scholarship for training in research methods.

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