- Rapport, D. & Canzanelli, A. (1950). *Science*, **112**, 469.
 Roffo, A. & Correa, L. (1938). *Rev. sudamer. Endocr.* **20**, 548.
 Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
 Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
 Smith, I. (1958). *Chromatographic and Electrophoretic Techniques*. London: William Heinemann (Medical Books) Ltd.
 Spiegel-Adolf, M. (1935). *J. Amer. chem. Soc.* **57**, 1431.
 Tickner, A. (1959). *Rep. Brit. Emp. Cancer Campgn*, **36**, 594.
 Tickner, A. (1960). *Rep. Brit. Emp. Cancer Campgn*, **37**, 643.
 Tickner, A. (1961). *Rep. Brit. Emp. Cancer Campgn*, **38**, 764.
 Wheeldon, L. W. & Collins, F. D. (1958a). *Biochem. J.* **70**, 43.
 Wheeldon, L. W. & Collins, F. D. (1958b). *Biochem. J.* **70**, 46.

Biochem. J. (1963) **88**, 84

The Reaction of Protein Thiol Groups with some Disulphides

BY T. E. ISLES* AND P. C. JOCELYN†

Department of Clinical Chemistry, University of Edinburgh

(Received 10 December 1962)

Although the occurrence in Nature of mixed disulphides between a protein and non-protein moiety has often been proposed, only a few examples are known. The fractional protein SH content of the serum albumins is probably due partly to mixed disulphide formation with cystine (King, 1961), and there is evidence for mixed disulphide formation between glutathione residues and the protein sulphide residues of human haemoglobin (Huisman & Dozy, 1962) and of glutathione reductase (Mize, Thompson & Langdon, 1962). The protective effect of certain thiols and disulphides (e.g. cystamine) against radiation damage in animals may be due to their combination with sensitive enzyme SH groups to form mixed disulphides, thus protecting them from oxidation. Cystamine and its derivatives react readily with a number of proteins to give mixed disulphides by a thiol-disulphide exchange reaction (Eldjarn & Pihl, 1956):

If such mixed disulphides are normally present in biological systems, it is likely that they are formed analogously by reaction between protein SH groups and the physiologically occurring non-protein disulphides, cystine or oxidized glutathione.

The results of a study of the reaction of these two substances with protein SH groups are presented below.

* Present address: Department of Child Life and Health, University of Edinburgh.

† Present address: Department of Biochemistry, University of Edinburgh.

MATERIALS AND METHODS

Materials. Sodium *p*-chloromercuribenzoate (L. Light and Co. Ltd., Colnbrook, Bucks.) was purified and standardized by iodine titration (Boyer, 1954). The extinction at 247 mμ in 2% (w/v) metaphosphoric acid in 33 mm-phosphate buffer is linear with concentration. Bovine serum albumin (fraction V) and bovine γ-globulins (fraction II) were obtained from Armour Ltd., Eastbourne, and human serum albumin (fraction V) was from the Blood Transfusion Service, Royal Infirmary, Edinburgh. Bovine haemoglobin solutions were prepared from fresh bovine erythrocytes by haemolysis with water, prolonged dialysis against water at 4° and removal of stroma by centrifuging at 1000g for 30 min. Solutions were stored at -10°, thawed when required and standardized as oxyhaemoglobin in ammonia solution by measurement of the extinction at 576 mμ and comparison with standards (C. D. Keeler Ltd., London). Cystine was determined by the naphthoquinonesulphonate method (Sullivan & Hess, 1937).

Determination of protein thiol groups. The protein was dissolved in water and to the solution (1.0 ml. containing up to 0.1 μmole of protein) was added 0.2M-phosphate buffer, pH 7.4 (0.5 ml.), and either sodium *p*-chloromercuribenzoate (0.2 ml.; 0.16 μmole) or water (0.2 ml.). The volumes were adjusted to 2 ml., mixed and, after 10 min., 6% (w/v) metaphosphoric acid (1 ml.) was added. The precipitated proteins were sedimented by centrifuging and the extinction of the protein-buffer-*p*-chloromercuribenzoate supernatant was measured in a spectrophotometer (Unicam SP. 500) at 247 mμ. in a silica cell (1 cm. light-path) against that of the protein-buffer-water supernatant. The extinction found was subtracted from the extinction of a solution of the *p*-chloromercuribenzoate (0.2 ml.; 0.16 μmole) in 2% (w/v) metaphosphoric acid in 33 mm-phosphate buffer, pH 7.4, read against a buffer-metaphosphoric acid blank. From the decrease in ex-

tion (Fig. 1) the loss of *p*-chloromercuribenzoate was calculated and taken as equivalent to the thiol content of the precipitated protein (see the Results section).

RESULTS

The increase in extinction that occurs owing to mercaptide formation when *p*-chloromercuribenzoate is mixed with a thiol (Boyer, 1954) can be used to determine SH groups. A disadvantage in the use of this method for the determination of protein SH groups is that the protein itself absorbs

Fig. 1. Extinction due to *p*-chloromercuribenzoate in filtrates obtained after precipitating the proteins from a mixture of sodium *p*-chloromercuribenzoate (0.2 ml.; 0.16 μ mole) and various amounts of human serum albumin ($\circ$, $\bullet$), bovine serum albumin ($\triangle$, $\blacktriangle$), bovine γ -globulins ($\square$, $\blacksquare$) or bovine haemoglobin ($\times$). Black symbols refer to these proteins after previous iodination as described by Hughes & Straessle (1950). The *p*-chloromercuribenzoate filtrate was prepared as described for the determination of protein SH groups in the Materials and Methods section.

strongly in the same wavelength region. With bovine and human serum albumin, bovine γ -globulins and bovine haemoglobin precipitated in the presence of a known amount of *p*-chloromercuribenzoate, the extinction and thus the *p*-chloromercuribenzoate concentration of the remaining solution decreases linearly with increasing protein concentration up to 0.10 μ mole of protein/incubation mixture (Fig. 1). With bovine and human serum albumin and bovine γ -globulins this loss of *p*-chloromercuribenzoate is entirely due to its reaction with protein SH groups and consequent precipitation as the protein mercaptide because there is no loss of *p*-chloromercuribenzoate when the SH groups of these proteins have previously been oxidized with iodine. Treatment of bovine haemoglobin with iodine precipitates much of the protein and this test of specificity is therefore not applicable. Nevertheless, for bovine haemoglobin as for the other three proteins, values for the number of SH groups/protein molecule (Table 1) computed from this loss of *p*-chloromercuribenzoate during precipitation of a known amount of protein are in agreement with values obtained by other workers (see Cecil & McPhee, 1959). The method has therefore been used to study the reactions of protein SH groups as detailed below.

Reactivity of protein thiol groups. (a) Bovine haemoglobin. Incubation of a haemoglobin solution (12.5 μ moles/100 ml.) with oxygen or nitrogen for 5 hr. in 0.2 M-phosphate buffer, pH 7.4, did not significantly alter the protein SH content. There was also no significant difference when GSSG (up to 50 μ moles/mole of protein) was included and subsequently dialysed from the solution. In contrast, cystine (up to 0.6 μ mole/mole of protein) decreased the SH content of bovine haemoglobin from 2 to 1 groups/molecule (Table 2). Further loss of protein SH groups was not observed with higher concentrations of cystine.

(b) Bovine and human serum albumin. The SH content of bovine serum albumin was also unaffected by incubation with oxygen or nitrogen alone or in the presence of GSSG (up to 20 moles/mole of protein) and with or without added Cu^{2+}

Table 1. *Thiol group content of various proteins*

The SH groups were determined as described in the Materials and Methods section. The following values for the molecular weights of the proteins were used: bovine and human serum albumin, 66 000 (Edsall, 1954); bovine haemoglobin, 68 000 (Cecil & McPhee, 1959); bovine γ -globulins, 150 000 (Markus & Karush, 1957).

Protein	SH groups/molecule		No. of determinations
	Mean $\pm$ S.D.	Range	
Bovine serum albumin	0.66 $\pm$ 0.02	0.62–0.69	20
Human serum albumin	0.63 $\pm$ 0.02	0.60–0.65	7
Bovine γ -globulins	0.27 $\pm$ 0.01	0.26–0.28	5
Bovine haemoglobin	2.0 $\pm$ 0.1	1.8–2.2	13

ions ($40 \mu\text{g.}/\mu\text{mole}$ of protein). Cystine or homocystine, however, considerably decreased the SH content (Table 3).

Reaction of bovine serum albumin with cystine. This reaction was further studied as a model for thiol-disulphide interchange. The protein SH content diminishes linearly with increasing cystine concentration, and reaches zero at an initial cystine:protein SH ratio of 4.5 (Fig. 2). After incubation of bovine serum albumin with cystine, the proteins were precipitated and the residual

cystine was determined. One molecule of cystine was lost for the loss of 2 protein SH groups, and no formation of cysteine was detectable. However, if the SH groups of bovine serum albumin were first blocked by mercaptide formation with *p*-chloromercuribenzoate, and excess of *p*-chloromercuribenzoate removed by dialysis, no cystine was lost when subsequently incubated with the dialysed protein solution (Table 4). Measurements of the rate of loss of protein SH groups, with an initial cystine:protein SH ratio of 1:2, fit second-order kinetics (Fig. 3); the rate is affected by pH, there being no detectable reaction at or below pH 5.6 (Table 5). No difference in the rate at pH 7.4 was

Table 2. *Thiol group content of bovine haemoglobin after incubation with cystine*

Haemoglobin ($0.6 \mu\text{mole}$) was incubated at 37° in 0.2M -tris buffer, pH 7.4, for 24 hr. in a final vol. of 4 ml. The SH groups were determined as described in the Materials and Methods section.

Cystine added (moles/mole of haemoglobin)	SH content (groups/molecule of haemoglobin)
0.00	2.1
0.21	1.7
0.42	1.3
0.63	1.1
0.84	1.2
1.10	1.2
1.25	1.0

Table 3. *Effect of non-protein disulphides on the thiol content of bovine serum albumin*

Bovine serum albumin ($1 \mu\text{mole}$) was incubated for 20 hr. at 37° with the disulphide ($1 \mu\text{mole}$) in 0.2M -tris buffer, pH 7.4, in a final vol. of 8 ml. Protein SH groups were determined as described in the Materials and Methods section.

Disulphide	SH content (group/molecule of protein)	Percentage loss
None	0.62	0
GSSG	0.60	3
Cystine	0.22	63
Homocystine	0.30	52

Fig. 2. Thiol groups content of bovine serum albumin ($0.5 \mu\text{mole}$) after incubation at 37° for 20 hr. in 0.2M -tris buffer, pH 7.4, in a final vol. of 4 ml. with various amounts of cystine. Protein SH groups were determined as described in the Materials and Methods section.

Table 4. *Cystine concentrations after reaction with bovine serum albumin alone or pretreated with *p*-chloromercuribenzoate*

p-Chloromercuribenzoate-treated bovine serum albumin was prepared by mixing sodium *p*-chloromercuribenzoate (1 equiv.) with bovine serum albumin solution and, after 10 min., dialysing for 18 hr. against water. The protein solutions ($1 \mu\text{mole}$) were mixed with 0.2M -tris buffer, pH 7.4, with or without cystine (as indicated), in a final volume of 8 ml. Solutions were sampled before and after incubation at 37° for 20 hr. Protein SH groups and cystine were determined as described in the Materials and Methods section. Values are given as SH groups/molecule of cystine or protein.

Protein solution	Before incubation		After incubation		Loss	
	Protein SH	Cystine	Protein SH	Cystine	Protein SH	Cystine
Bovine serum albumin alone	0.64	—	0.64	—	0.0	—
Bovine serum albumin + cystine	0.66	0.49	0.12	0.24	0.54	0.25
<i>p</i> -Chloromercuribenzoate-treated bovine serum albumin + cystine	0.08	0.50	0.09	0.50	0.0	0.0

observed in the presence of catalytic amounts of selenium dioxide, ferrous sulphate, cobaltous sulphate, or cupric sulphate (up to 0.2 mole/mole of protein), or of thioglycollic acid (0.05 mole/mole of protein).

Regeneration of protein thiol groups. When bovine serum albumin, or bovine serum albumin that had previously been allowed to react with cystine, was treated with GSH or cyanide, there was a rise in the protein SH content up to a maximum value of 0.8–0.9 SH group/molecule of protein (Table 6). These values were decreased by 0.6–0.7 SH group/molecule after further interaction with cystine.

DISCUSSION

The SH groups of bovine haemoglobin react with excess of cystine, but only half of the SH is lost. Since the proteins possess two SH groups/molecule, one of them is evidently not able to form a mixed

disulphide. A difference between the two available SH groups in native human haemoglobin is also implied by the observation (Morell, Hoffman, Ayers & Taketa, 1962) that, on deoxygenation of oxyhaemoglobin, one SH group/molecule is no longer titratable by *N*-ethylmaleimide. In the present work, however, no change occurred in the SH content of bovine haemoglobin (measured with *p*-chloromercuribenzoate) as a result of incubation

Table 5. *Effect of pH on the reaction of bovine serum albumin with cystine*

Bovine serum albumin (0.5 μ mole) was incubated at 37° for 2 hr. with or without cystine (0.4 μ mole) in 0.2M-tris buffer, of indicated pH, in a final volume of 4 ml. Protein SH groups were determined as described in the Materials and Methods section.

pH	SH content (group/molecule of bovine serum albumin)	
	Bovine serum albumin alone	Bovine serum albumin + cystine
4.6	0.66	0.65
5.6	0.66	0.65
6.6	0.66	0.61
7.4	0.66	0.51
8.4	0.67	0.30

Fig. 3. Kinetics of the reaction of bovine serum albumin with cystine. The initial concentration of cystine was half that of the bovine serum albumin SH content (2.07 μ moles). The mixture was incubated at 37° in 0.2M-tris buffer, pH 7.4, in a final vol. of 24 ml., sampled at the times shown, and the protein SH groups were determined as described in the Materials and Methods section. The kinetic constant obtained from the slope (Glasstone, 1960) is 0.247 M⁻¹ sec.⁻¹.

Table 6. *Effect of reduced glutathione or cyanide on the thiol group content of bovine serum albumin before and after reaction with cystine*

Cystine-treated bovine serum albumin was prepared by allowing bovine serum albumin to react with cystine (1 equiv.) as described in Table 3 and subsequently dialysing the solution for 24 hr. against water. Untreated or cystine-treated bovine serum albumin (1 μ mole) was incubated anaerobically at 37° for 2 hr. alone or as indicated with GSH (10 μ moles) or sodium cyanide (2.5 mg./ml.) in a final vol. of 8 ml. The solutions were subsequently dialysed against water at 4°, and the protein SH groups were determined as described in the Materials and Methods section. Samples of the solutions, dialysed after incubation with GSH or cyanide, were again allowed to react with cystine (as described above), redialysed and their protein SH groups redetermined.

Protein	Addition to the incubation medium	SH content (group/molecule)	SH content after further incubation with cystine (group/molecule)
Bovine serum albumin alone	None	0.64	—
	GSH	0.91	0.22
	Cyanide	0.82	0.23
Bovine serum albumin + cystine	None	0.25	—
	GSH	0.90	—
	Cyanide	0.82	—

with oxygen or nitrogen. Human haemoglobin has been stated to form a mixed disulphide by reaction with GSSG, involving the loss of approx. 1.6 of its two available SH groups/molecule (Huisman & Dozy, 1962). Under similar conditions both SH groups of the bovine haemoglobin molecule appear to be unreactive towards GSSG.

Bovine and human serum albumins have SH contents corresponding to 0.6–0.7 SH group/molecule and this fractional titre has been attributed to the presence in the purified protein of mixed disulphides with cysteine or GSH (King, 1961). King has stated that the SH groups of human serum albumin react with both GSSG and cystine; in the present work, however, neither human nor bovine serum albumin showed any significant reaction with GSSG although they both lost their SH content in the presence of an excess of cystine.

The reaction of bovine serum albumin with cystine has been studied by parallel determinations of the fall in the protein SH content and the loss of cystine. Since no cystine is lost after masking the protein SH groups with *p*-chloromercuribenzoate, non-specific protein-binding of cystine does not occur. The stoichiometry of the reaction (two protein SH groups lost for the loss of one molecule of cystine), and the second-order kinetics found when the initial concentration of cystine is half that of the protein SH groups, suggest that in the reaction:

the cysteine produced is rapidly re-oxidized to cystine for re-utilization. This is in contrast with the finding (Klotz, Ayers, Ho, Horowitz & Heiney, 1958) that, after mixed disulphide formation between bovine serum albumin and a complex aromatic disulphide, the non-protein thiol first formed reacts further with a further protein disulphide group.

Treatment of bovine serum albumin with GSH or cyanide increases the protein SH titre almost to 1 SH group/molecule (an effect also observed with GSH by Hird, 1962) and has the same effect on the cystine-treated protein despite its much lower initial SH content. This greater increase in the SH content of the cystine-treated protein and its fall to the original value after re-exposure to cystine is strong evidence that GSH or cyanide both cleave

the mixed disulphide groups (with cyanide this implies that *S*-cyanocysteine is formed and the alternative cleavage giving the *S*-cyanoprotein does not occur). The smaller increase in the SH content of the native protein in the presence of GSH or cyanide is then probably attributable to the specific cleavage of pre-existing mixed disulphide groups.

SUMMARY

1. Protein SH groups have been determined by measuring the loss of *p*-chloromercuribenzoate from solution when the protein is precipitated in the presence of sodium *p*-chloromercuribenzoate.

2. Incubation with an excess of cystine decreases the SH group content of the serum albumins to zero and of bovine haemoglobin to 50 %. GSSG has no effect on the SH content of either protein.

3. The reaction of cystine with the SH groups of bovine serum albumin gives a loss of one molecule of cystine/two protein SH groups. The rate of this reaction follows second-order kinetics and is influenced by pH but not by heavy-metal ions.

4. GSH and cyanide regenerate the SH groups of bovine serum albumin lost in the reaction with cystine.

REFERENCES

- Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.
Cecil, R. & McPhee, J. R. (1959). *Advanc. Protein Chem.* **14**, 323.
Edsall, J. T. (1954). *J. Polym. Sci.* **12**, 253.
Eldjarn, L. & Pihl, A. (1956). *Proc. 4th int. Conf. Radiobiol., Camb.*, p. 249.
Glasstone, S. (1960). *Textbook of Physical Chemistry*, p. 1055. London: Macmillan and Co. Ltd.
Hird, F. J. R. (1962). *Biochem. J.* **85**, 320.
Hughes, W. L. & Straessle (1950). *J. Amer. chem. Soc.* **72**, 454.
Huisman, T. H. J. & Dozy, A. M. (1962). *J. Lab. clin. Med.* **60**, 302.
King, T. P. (1961). *J. biol. Chem.* **236**, pc5.
Klotz, I. M., Ayers, J., Ho, J. Y. C., Horowitz, M. G. & Heiney, R. E. (1958). *J. Amer. chem. Soc.* **80**, 2132.
Markus, G. & Karush, F. (1957). *J. Amer. chem. Soc.* **79**, 134.
Mize, C. E., Thompson, T. E. & Langdon, R. G. (1962). *J. biol. Chem.* **237**, 1596.
Morell, S. A., Hoffman, P., Ayers, V. E. & Taketa, F. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1057.
Sullivan, M. X. & Hess, W. C. (1937). *J. biol. Chem.* **117**, 425.