Halwer, M., Nutting, G. C. & Brice, B. A. (1951). *J. Amer. chem. Soc.* **73**, 2786.
Heller, W. & Klevens, H. B. (1946). See Bull & Currie (1946).
Hoch-Ligeti, C. & Hoch, H. (1948). *Biochem. J.* **43**, 556.
Jacobsen, C. F. (1949). *C.R. Lab. Carlsberg, Sér. Chim.*, **26**, no. 18, 455.
Jacobsen, C. F. & Christensen, L. K. (1948). *Nature, Lond.*, **161**, 30.
Johnston, J. P. (1945). Thesis, Oxford.
Johnston, J. P. & Ogston, A. G. (1946). *Trans. Faraday Soc.* **42**, 789.
Kekwick, R. A., Lyttleton, J. W., Brewer, E. & Dreblow, E. S. (1951). *Biochem. J.* **49**, 253.
Li, C. H. (1946). *J. Amer. chem. Soc.* **68**, 2746.
Li, C. H. (1951). In *Amino acids and Proteins*. Springfield, Illinois: Thomas.
Longsworth, L. G. (1947). *J. phys. Chem.* **51**, 171.
Lundgren, H. P. & Ward, W. H. (1949). *Annu. Rev. Biochem.* **18**, 115 (p. 122).
McMeekin, T. L., Polis, B. D., DellaMonica, E. S. & Custer, J. H. (1948). *J. Amer. chem. Soc.* **70**, 881.
McMeekin, T. L. & Warner, R. C. (1942). *J. Amer. chem. Soc.* **70**, 881.
Palmer, A. M. (1934). *J. biol. Chem.* **104**, 359.
Pedersen, K. O. (1936). *Biochem. J.* **30**, 961.
Polis, B. D., Schmukler, H. W., Custer, J. H. & McMeekin, T. L. (1950). *J. Amer. chem. Soc.* **72**, 4965.
Riley, D. P. (1951). *Nature, Lond.*, **168**, 242.
Senti, F. R. & Warner, R. C. (1948). *J. Amer. chem. Soc.* **70**, 3318.
Smithies, O. (1953). *Biochem. J.* **55**, 57.
Smithies, O. (1954). *Biochem. J.* **58**, 31.
Sørensen, M. & Palmer, A. H. (1938). *C.R. Lab. Carlsberg, Sér. chim.*, **21**, 283.
Stein, W. H. & Moore, S. (1949). *J. biol. Chem.* **178**, 79.
Svensson, H. (1946). *Ark. Kemi Min. Geol.* **22A**, no. 10, 1.

Sulphydryl Groups in Haemoglobins

By V. M. INGRAM
Medical Research Council Unit for the Study of the Molecular Structure of Biological Systems, Cavendish Laboratory, University of Cambridge

(*Received* 23 *September* 1954)

Recently Green, Ingram & Perutz (1954) crystallized derivatives of horse haemoglobin in which two sulphydryl groups were combined either with *p*-mercuribenzoate groups or with silver ions. The crystals obtained from these mercaptide compounds proved to be isomorphous with free oxyhaemoglobin, but they showed significant changes in the X-ray diffraction pattern from which the phases of the diffracted rays could be deduced. Further development of this method required a more detailed knowledge of the number and the reactivity of the SH groups in various haemoglobins than was available in the literature; for this purpose the present study was undertaken.

The presence of sulphydryl groups in horse globin was first demonstrated by Anson & Mirsky (1931). The same authors later titrated horse haemoglobin with potassium ferricyanide and found no reactive SH groups at pH 6·8, whereas raising the pH to 9·5 led to the oxidation of 1·3 SH groups per four iron atoms. The maximum number of reactive groups was found in denatured horse globin in guanidine hydrochloride solution and amounted to two SH for four iron atoms (Mirsky & Anson, 1936). Greenstein (1939), using the porphyrindin titration, concluded that there are three sulphydryl groups per four iron atoms in horse globin. No further work on horse haemoglobin seems to have been reported. According to Hughes (1950) a molecule of human haemoglobin at pH 7·5 reacts with 2 molecules of methylmercury iodide. Ingbar & Kass (1951) titrated human haemoglobin amperometrically with silver nitrate in ammoniacal 0·85% sodium chloride. They found two SH groups per molecule which increased to 4–5 with the addition of 20% methanol. By a similar method Benesch (1950) found four thiol groups in dog haemoglobin.

The proteins studied in the present experiments included the adult haemoglobins of horse, man, ox and sheep. The number of available SH groups was determined by measuring the number of silver atoms per molecule bound as Ag-S-protein. For this the amperometric titration method with silver nitrate was used (Benesch & Benesch, 1948; Ingbar & Kass, 1951). Silver nitrate is added to the protein in weakly ammoniacal solution until unbound silver ammine ions are present; the end point is detected by the appearance of the diffusion current of these ions to a rotating platinum electrode. The binding of mercuric chloride by the haemoglobins was studied with a similar method (Kolthoff, Stricks & Morren, 1954).

The reactivity of the SH groups was investigated by allowing them to react with known molecular proportions of such mercury derivatives as mercuric chloride, sodium *p*-chloromercuribenzoate or

methylmercury nitrate or with *N*-ethylmaleimide (Friedmann, Marrian & Simon-Reuss, 1949; Marrian, 1949). Some or all of the sulphydryl groups would then be blocked to the reaction with silver nitrate; investigation of the stoicheiometry of these blocking reactions gave results which allowed certain tentative conclusions to be reached about the spatial dispositions and availabilities of the SH groups.

MATERIALS AND METHODS

Horse oxyhaemoglobin. Fresh horse blood was defibrinated by shaking with glass beads. The red cells were then sedimented by centrifuging at a low speed and washed 4 times, sedimenting each time from 4 vol. 0·9% NaCl. The packed cells were lysed by freezing and thawing and the cell debris was removed in a high-speed centrifuge. Addition of an equal volume of 4 M phosphate buffer (K_2HPO_4:NaH_2PO_4 = 2:1) caused most of the oxyhaemoglobin to crystallize. A solution of the moist filter cake in water (2 vol.) was obtained by the cautious addition of 2 N-NaOH, and from this the protein was crystallized by adding the above phosphate buffer. The preparation remained unchanged when stored as a moist cake of crystals in the refrigerator. Before use portions were dissolved in a small volume of water and dialysed for 1 or 2 days at 3° against water or 0·9% NaCl. Usually no denatured material was visible and additional SH groups were liberated only when dialysis lasted for longer than a week.

Human oxyhaemoglobin. The red cells from fresh human blood, defibrinated as above, were washed in a similar manner. Addition of an equal volume of water caused them to lyse; the NaCl concentration was raised to 1% and the cell ghosts were removed by centrifuging at 10000 rev./min. for 20 min. Before use samples of this solution were dialysed in the cold against 0·9% NaCl.

Ox and sheep oxyhaemoglobins. Fresh blood was treated as described for horse oxyhaemoglobin. In some cases clotting was prevented by the addition of sodium citrate, and the step of shaking the blood with glass beads could then be omitted. These haemoglobins were not recrystallized, but samples were dialysed in the usual way before use.

Reagents. The *p*-chloromercuribenzoic acid (PCMB) used had been prepared by Mr B. R. Slater in the Department of Biochemistry, Cambridge University; another sample was obtained from the Sigma Chemical Corporation Chicago, U.S.A. A solution of the acid in an excess of 0·1 N-NaOH was diluted to give an approx. 1 mM solution. To prepare methylmercury nitrate, the iodide was converted into a solution of the hydroxide by shaking an aqueous suspension with fresh silver oxide in the cold. The supernatant was neutralized with dil. HNO_3 and diluted to give a 1 mM solution. All other reagents and buffer solutions were made with A.R. chemicals and glass-distilled water was used throughout.

Amperometric titrations. The apparatus and technique were very similar to those used by Kolthoff & Harris (1946), Benesch & Benesch (1948), Ingbar & Kass (1951) and Kolthoff *et al.* (1954); the diffusion current to the rotating platinum electrode was measured with a Pye Scalamp galvanometer, cat. no. 7904/S. Unless otherwise stated, aqueous 0·05 M-NH_4NO_3 and the indicated concentration of NH_3 formed the background electrolyte for both the $AgNO_3$ and the $HgCl_2$ titrations. The volume of solution in the titration vessel was 30 ml. and this contained 1–5 μequiv. of sulphydryl groups. Initially $AgNO_3$ and $HgCl_2$ were used as 1 mM solutions, but it was soon found more convenient to add them from microburettes as 20 and 5·5 mM solutions, respectively. Of the two reagents, $HgCl_2$ gave very much sharper end points, indicating the higher affinity of this reagent for SH groups.

Where reagents were added to block SH groups, the reaction was allowed to proceed for a few min. before titration. It was found that $AgNO_3$ titration could be used to investigate these blocking reactions, since silver has a lower affinity for the SH groups in haemoglobin than the three mercury derivatives. Thus the number of SH groups which remain available after blocking, can be determined directly in this way. On the other hand, titration with $HgCl_2$ of the blocked protein does not give useful information, because PCMB and probably methylmercury, though not of course $HgCl_2$, are completely displaced from their sites by $HgCl_2$ during titration with this substance.

Denaturation experiments. Sodium dodecyl sulphate (SDS) was used exclusively, because earlier experiments using methanol and ethanol to denature the protein had given erratic titration values. The commercial product (L. Light and Co., Colnbrook, Bucks) was recrystallized twice from absolute ethanol and washed thoroughly with light petroleum. The indicated amount of SDS was added shortly before the titration, giving a clear solution which showed the colour change characteristic of denaturation. The reaction appeared to be complete almost immediately after addition of the reagent. In the presence of SDS the end point of the silver titration became less sharp, but reproducibility remained good. The Ag:SH ratio of 1:1 obtained in the titration of reduced glutathione in 0·10 M-NH_3 was not affected by the presence of SDS. No decrease in sharpness was observed in titrations with $HgCl_2$.

When the SH groups were partially or completely blocked before titration, such blocking usually preceded denaturation; occasionally this order was reversed with no difference in the results.

RESULTS

Horse haemoglobin. Both silver and mercury are firmly bound by horse oxyhaemoglobin. The titration of the native and the denatured protein with $AgNO_3$ and $HgCl_2$ gave results which are summarized in Table 1. They fall into two parts: titration results with the native haemoglobin in 0·03 M-NH_3 and with the denatured protein in 0·10 M-NH_3. These ammonia concentrations gave the most satisfactory plots for the $AgNO_3$ titration, as with higher concentrations the end points were less distinct and with lower ones the amount of silver used was sensitive to chloride ions and other interfering substances. The number of silver atoms bound per four iron atoms shown in Table 1 indicates the total number of SH groups in the protein or the number left available after previous reaction with mercury derivatives. The former is seen to be four in the native and six in the denatured protein.

The results show that it is possible to block the sulphydryl groups completely to $AgNO_3$ both in the native and the denatured proteins. In the native protein each equivalent of mercury compound can block two SH groups and similarly the titration with $HgCl_2$ reaches the end point after only two molecules have been bound by the four sulphydryl groups. After reaction with one equivalent of methylmercury nitrate the native protein showed two end points during $AgNO_3$ titration, the first at approximately two atoms of silver and a second when four silver atoms had been taken up. This can be explained by assuming that the methylmercury group has combined with the protein and has blocked two SH groups, leaving the other two freely available; these then account for the first end point. On raising the concentration of silver ions further these can displace the methylmercury group; two more atoms of silver are thereby used up giving a final end point to the titration of 4 Ag:4 Fe. The SH groups of horse haemoglobin which were left after reaction with PCMB or methylmercury were not titrated with $HgCl_2$, since this reagent will completely displace such blocking compounds; hence the results of the titrations would have been the same as for the untreated protein.

Table 1. *Sulphydryl groups of horse oxyhaemoglobin*

NH_3 concn. (M)	Blocking agent added before titration (Hg atoms per 4 Fe)	Titration results: $AgNO_3$ bound (Ag atoms per 4 Fe)	Titration results: $HgCl_2$ bound (Hg atoms per 4 Fe)
0·03	—	4·0	2·0*
0·03	1·0 $HgCl_2$	2·0	—
0·03	2·0 $HgCl_2$	0	—
0·03	1·0 PCMB	1·9	—
0·03	2·0 PCMB	0	—
0·03	1·0 CH_3HgNO_3	(1·9)†	—
After denaturation with sodium dodecylsulphate (200 molecules per 4 Fe)			
0·10	—	6·0	4·1
0·10	2·0 $HgCl_2$	0·1	2·1
0·10	4·0 PCMB	0	—
0·10	1·0 PCMB	4·0	—
0·10	1·0 PCMB‡	3·9	—

* In either 0·10 M-NH_3 or in 0·5 M-KCl–phosphate buffer pH 7·42 (Kolthoff *et al.* 1954).
† Titration shows two end points (see text).
‡ Added after denaturation.

The six SH groups in the denatured haemoglobin can be blocked progressively to titration with $AgNO_3$ by either PCMB or $HgCl_2$ as is shown in Fig. 1*a*. However, $HgCl_2$ appears to be the more efficient, since nearly all six SH groups are masked after the addition of only two molecules of $HgCl_2$, whereas four molecules of PCMB are required to produce the same effect.

It is interesting to note that on denaturation with SDS the number of SH groups detectable by $AgNO_3$

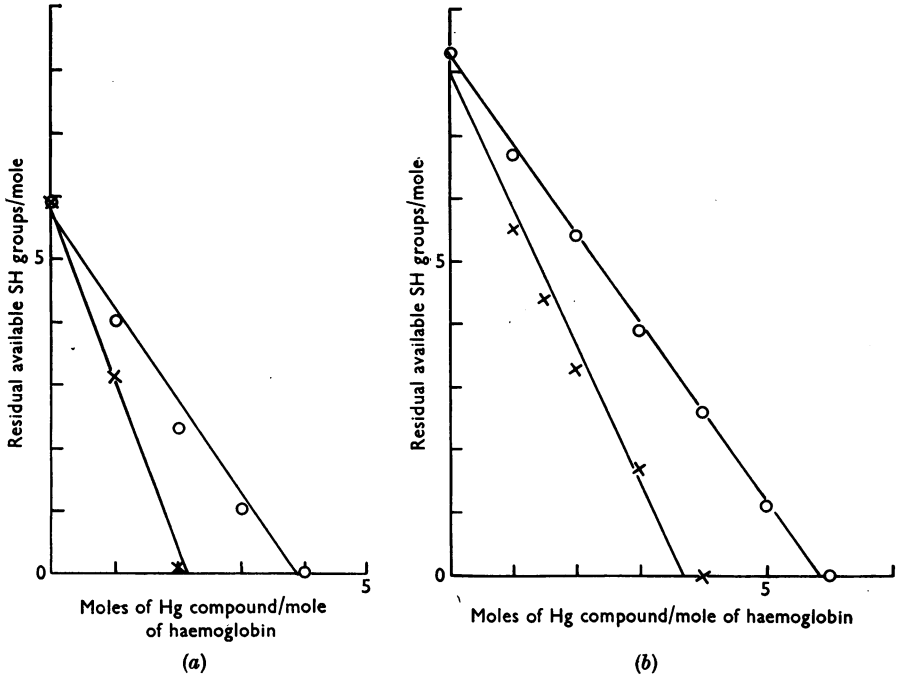

Fig. 1. Progressive blocking of SH groups of SDS-denatured haemoglobin with $HgCl_2$ and PCMB. *a*, Horse haemoglobin; *b*, human haemoglobin. ×—×, $HgCl_2$ as blocking agent; O—O, PCMB as blocking agent.

titration increases from four to six whereas the number of $HgCl_2$ equivalents goes up from two to four and not, as might have been expected, from two to three. The significance of these findings will be discussed in a later section.

The reaction of the sulphydryl groups of native horse oxyhaemoglobin with PCMB was also investigated by the method of Boyer & Segal (1954). Various amounts of PCMB were added to a 0·02 mM haemoglobin solution in 0·1 M phosphate buffer at pH 7·33 and allowed to react at room temperature for 90 min. The optical density at 250 mμ. increases as the result of combination of PCMB with SH groups. A plot of this increase against the molecular proportions of PCMB per haemoglobin molecule is shown in Fig. 2. The break in the plot near PCMB: Fe = 2:4 indicates that two molecules of the mercurial combine with each native haemoglobin molecule. This agrees with the results obtained by the $AgNO_3$ titration method (Table 1).

It may be concluded that native horse haemoglobin has four SH groups available to $AgNO_3$, but binds only two $HgCl_2$, two PCMB and, probably, two

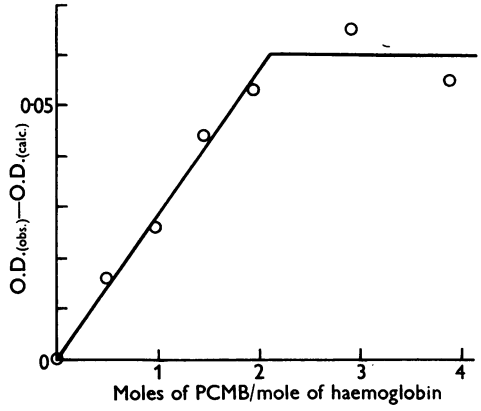

Fig. 2. Reaction between PCMB and native horse oxyhaemoglobin. O.D. = optical density.

Table 2. *Sulphydryl groups of ox oxyhaemoglobin*

NH_3 concn. (M)	Blocking agent added before titration (Hg atoms per 4 Fe)	Titration results: $AgNO_3$ bound (Ag atoms per 4 Fe)	Titration results: $HgCl_2$ bound (Hg atoms per 4 Fe)
0·03	—	4·0	2·0*
0·03	1·0 $HgCl_2$	1·9	—
0·03	2·0 $HgCl_2$	0	—
0·03	1·0 PCMB	2·0	—
0·03	1·0 CH_3HgNO_3	2·0	—
After denaturation with sodium dodecylsulphate (400 molecules per 4 Fe)			
0·10	—	3·8	2·2

* In either 0·10 M-NH_3 or in 0·5 M-KCl–phosphate buffer pH 7·42 (Kolthoff *et al.* 1954).

methylmercury nitrate molecules. After reaction with these proportions of the mercury compounds no SH groups can be detected by the silver titration. SDS-denatured horse haemoglobin has six SH groups, which can be completely blocked to combination with $AgNO_3$ by either two $HgCl_2$ or four PCMB. It will combine with four $HgCl_2$ during the titration with this reagent. The significance of these findings is discussed in a later section.

Ox haemoglobin. The titration results presented in Table 2 show the great similarity in the behaviour of the SH groups of the haemoglobin of ox and horse. They do differ, however, in their affinity to methylmercury, since silver does not displace this reagent from its combination with the sulphydryl groups of ox haemoglobin. Again it appears that one molecule of each of the three mercury compounds is capable of blocking two SH groups in the native protein.

A detailed study of the thiol groups of SDS denatured ox haemoglobin was not carried out, but it is clear that in contrast to horse haemoglobin no additional SH groups are liberated by SDS.

Human haemoglobin. Native human haemoglobin shows a strong similarity to the horse and ox proteins (Table 3). As stated below, the number of SH groups found by $AgNO_3$ titration varies with the concentration of ammonia and with the ionic strength. This made it difficult, at first, to get reproducible values.

Human haemoglobin, denatured with SDS, has eight SH groups and binds six $HgCl_2$ and not four as might have been expected (Table 3, Fig. 1*b*). This finding, as with horse haemoglobin, was surprising since in both cases the native protein binds twice as many silver atoms as mercury. All the eight groups

Table 3. *Sulphydryl groups of human oxyhaemoglobin*

All titrations in 0·10 M-NH_3.

Blocking agent added before titration (Hg atoms per 4 Fe)	Titration results: $AgNO_3$ bound (Ag atoms per 4 Fe)	Titration results: $HgCl_2$ bound (Hg atoms per 4 Fe)
—	3·9	2·2*
2·0 $HgCl_2$	0†	—
1·0 PCMB	1·9	—
2·0 PCMB	0	—
After denaturation with sodium dodecylsulphate (400 molecules per 4 Fe)		
—	8·3	6·0
4·0 $HgCl_2$	0	—
4·0 $HgCl_2$‡	0	—
6·0 PCMB	0	—

* Same value in 0·5 M-KCl–phosphate buffer pH 7·42 (Kolthoff *et al.* 1954).

† In 0·03 M-NH_3.

‡ Added after denaturation.

can be blocked in an understandable manner, as in horse, with $HgCl_2$ or PCMB. Again the former is a more efficient blocking agent, and two more molecules of PCMB are necessary to cover all the sulphydryl groups.

Sheep haemoglobin. Native sheep haemoglobin differs from the other three species by taking up twice as many equivalents of silver and mercury (Table 4). Here, as in human haemoglobin, it was necessary to control the ammonia concentration and the ionic strength of the medium to obtain reproducible results. Again the mercury derivatives block two SH groups each and the compound with methylmercury is stable.

Titration with $AgNO_3$ in the presence of SDS proved impossible as the end points were not reproducible. The mercuric chloride titration was unaffected and showed that no new SH groups were made available by SDS.

Effect of ammonia concentration and ionic strength. When the ammonia concentration of the titrating medium was increased from 0·03 to 0·25 M, it was found that the number of atoms of silver bound decreased in human and sheep haemoglobin (Table 5), but there was no definite effect on the horse and ox proteins. In all cases the sharpness of the end point was impaired. Titrations with $HgCl_2$ were not affected. The ammonia concentration chosen for most of the $AgNO_3$ titrations was either 0·03 M or 0·10 M. These are lower than those used by Benesch & Benesch (1948) or Ingbar & Kass (1951). Nevertheless they are high enough to prevent interference by chloride and bromide and also probably high enough to counteract binding by sites in the protein molecule other than the SH groups. Under these conditions no silver at all was bound by samples of ribonuclease, chymotrypsinogen, insulin or by the myoglobins of horse, sperm whale and elephant seal. Reduced glutathione gave the theoretical Ag:SH ratio of 1:1 in 0·03 M-NH_3. Of the proteins, ribonuclease and elephant-seal myoglobin were also titrated with $HgCl_2$, but again there was no uptake of the metal.

Table 4. *Sulphydryl groups in sheep oxyhaemoglobin*

Unless otherwise indicated all titrations were carried out in 0·03 M-NH_3.

Blocking agent added before titration (Hg atoms per 4 Fe)	Titration results: $AgNO_3$ bound (Ag atoms per 4 Fe)	Titration results: $HgCl_2$ bound (Hg atoms per 4 Fe)
—	8·0*	4·1†
2·2 $HgCl_2$	3·7	—
4·3 $HgCl_2$	0	—
2·1 PCMB	3·7	—
4·2 PCMB	0	—
2·0 CH_3HgNO_3	4·2	—
After denaturation with sodium dodecylsulphate (200 molecules per 4 Fe)		
—	—‡	3·9§

* Mean of seven close determinations,
† Mean of five close determinations.
‡ Titration unreliable.
§ In 0·10 M-NH_3.

Since the results obtained with native horse and ox haemoglobin are unaffected by increasing the ammonia concentration to 0·25 M, where binding is generally assumed to be specifically by SH groups, the results with the lower ammonia concentrations must also be valid. A number of blocking experiments have also been carried out in 0·25 M-NH_3 and the results were the same as with lower concentrations. If the concentration of ammonia is reduced below 0·03 M (0·10 M in the presence of SDS) then non-specific binding of silver does occur, variable but high values are obtained, and the protein soon precipitates if SDS is not present. Although the end point improves as the NH_3 concentration is lowered, there is a limit below which one must not go.

As there is an ammonia effect in human and sheep haemoglobins, one cannot be quite so certain of the results obtained with NH_3 concentrations below 0·25 M. However, the analogy with horse and ox is strong, and the figures reported are reproducible and are whole numbers. In human haemoglobin they also agree with the known symmetry of the molecule. The preferred assumption is that in 0·25 M-NH_3 some of the —S—Ag groups begin to dissociate as they also seem to do at higher ionic strengths.

In human and sheep haemoglobins it was observed that increasing the ionic strength by the addition of NaCl or NH_4NO_3 decreased markedly the number of SH groups found by the $AgNO_3$ titration, but it was not possible to obtain reproducible results. No such effect could be demonstrated for the $HgCl_2$ titration of these proteins, nor was it found with horse and ox haemoglobins.

Table 5. *Number of sulphydryl groups found by titration with* $AgNO_3$ *at different* NH_3 *concentrations*

Haemoglobin	NH_3 concentration (M): 0·03	0·10	0·25
Horse	4·1	4·0	4·2
Ox	4·0	3·7	3·9
Human	3·9	3·4	3·3
Sheep	7·9	7·6	6·6

Reaction of haemoglobins with N-ethylmaleimide. It has been shown (Friedmann *et al.* 1949; Marrian, 1949) that *N*-ethylmaleimide (NEMI) combines readily with the SH groups of such compounds as mercaptoacetic acid and glutathione. Tsao & Bailey (1953) have used this reagent to block stoicheiometrically all the sulphydryl groups of actin and myosin which had been denatured with

guanidine hydrochloride. These authors have also reported that NEMI reacted with only 40% of the SH groups in native myosin.

NEMI will react with the thiol groups of native horse and ox haemoglobin, but not stoicheiometrically. At a neutral pH and room temperature two moles of the reagent blocked only 1·5 out of the four SH groups present in horse haemoglobin. In the case of ox haemoglobin four moles of NEMI combined with two of the four available thiol groups (Fig. 3).

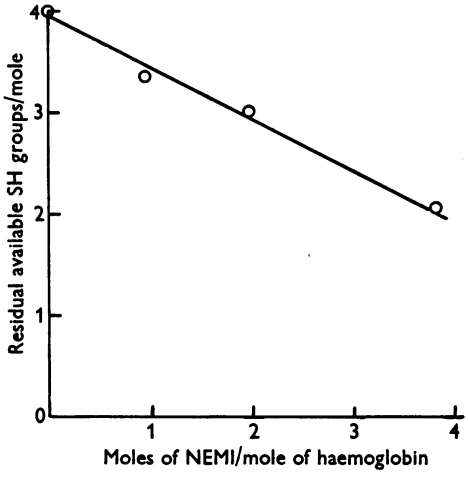

Fig. 3. Blocking of the SH groups of native ox haemoglobin with *N*-ethylmaleimide.

Reaction of $HgCl_2$ *with PCMB-blocked* SH *groups.* PCMB reacts with the SH groups of haemoglobins to form a compound from which it cannot be displaced by $AgNO_3$ in either neutral or ammoniacal solution. $HgCl_2$, on the other hand, competes successfully for the sulphydryl groups of glutathione and of horse haemoglobin denatured with SDS. In both cases the end point of the $HgCl_2$ titration is quite unaffected by any previous addition of PCMB or indeed of $AgNO_3$. For these two SH compounds the order of affinity of the three reagents is therefore $HgCl_2 > PCMB > AgNO_3$.

DISCUSSION

In the amperometric titration method for the estimation of SH groups in proteins, when silver nitrate is added it is bound by the test substance until an excess of silver is present; a diffusion current to a rotating platinum electrode is then observed and indicates the end point of the titration and therefore the number of silver atoms held by the protein.

The underlying assumption in the use of this method is that silver will be bound covalently to a protein in the presence of ammonia (0·25 M) only by the sulphydryl groups; the number of silver atoms bound is then equal to the number of available thiol groups. This assumption is based on experiments with simple sulphydryl compounds (Kolthoff & Harris, 1946; Benesch & Benesch, 1948). Whilst none of the other groups which are known to occur in proteins will bind silver under these conditions, the possibility of specific binding by some unknown non-thiol group cannot be excluded. In this connection, it is of interest that several non-thiol proteins have shown no uptake of silver under the conditions described here (see above). In the present paper the above assumption will be used. Similar considerations apply to the titration with mercuric chloride, with the added complication that this metal is capable of combining with two SH groups.

In their paper on the estimation of SH groups by amperometric $HgCl_2$ titration Kolthoff *et al.* (1954) state that the end point corresponds to a 1:1 ratio of $HgCl_2$ to SH. Of the test SH compounds used by them, glutathione, cysteine and serum mercaptalbumin, only the first was titrated during the present work and the ratio Hg:SH was found to be 1:1. On the other hand the results obtained by this titration method with the native haemoglobins (Tables 1–4) show clearly that one $HgCl_2$ group can effectively block at least two SH groups to combination with silver. Hughes (1950) was led to a similar conclusion by the finding that mercaptalbumin which has one SH group per molecule can form a compound with $HgCl_2$ of the type Hg—(S—protein)$_2$. Addition of more $HgCl_2$ led to dissociation and to the compound Cl—Hg—S—protein; presumably this last is the final product during the amperometric titration with $HgCl_2$ described by Kolthoff and accounts for his 1:1 ratio of Hg:SH.

It is not possible to decide on the basis of the results obtained whether in the haemoglobins the ratio of one $HgCl_2$ to two SH is due to the formation of a —S—Hg—S— bridge or whether it is a steric effect as in the case of PCMB. The former explanation is perhaps the more likely in view of the ability of $HgCl_2$ to block more SH groups in SDS-denatured human and horse haemoglobins than the even more bulky PCMB. Hitherto PCMB and methylmercury derivatives have been regarded as specific blocking agents for SH groups and as forming compounds with a 1:1 Hg:SH ratio. Whilst the specificity is unchallenged, it can no longer be assumed that the number of sulphydryl groups in a protein is always equal to the number of molecules of mercury derivative bound.

Previous X-ray crystallographic studies (Perutz, 1942; Perutz, Liquori & Eirich, 1951) have shown that the molecules of horse and human haemoglobin possess diad axes of symmetry; there is as yet no

evidence for or against such symmetry in the other haemoglobins. The finding in the present work that the SH groups of horse and human haemoglobin occur always in even numbers agrees well with such symmetry requirements. It may be that the ox and sheep proteins also have twofold symmetry, since the numbers of thiol groups in them are also multiples of two, but there is as yet no X-ray evidence to that effect. Fourier projections of horse haemoglobin with two PCMB per molecule (Bragg & Perutz, 1954) have shown the positions in projection of the mercury atoms and hence of at least two of the four SH groups in the native proteins (Figs. 4*a*, *b*). The silver atoms of horse haemoglobin-Ag_2 have been located similarly, and were found to be near to the mercury positions.

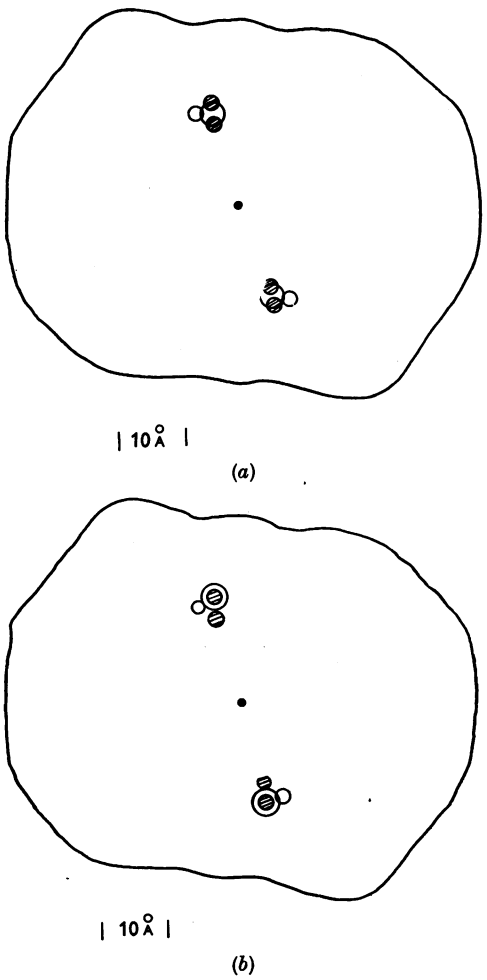

Fig. 4. Diagrammatic representation of the suggested positions of the SH groups in horse haemoglobin. ●, —SH group available in the native protein; o, —SH group available in the SDS-denatured protein; ○, —Hg atom of $HgCl_2$ or PCMB. *a*, Combination with 2 $HgCl_2$/molecule; *b*, combination with 2 PCMB/molecule.

The symmetry of the native molecule, together with the fact that on the Fourier projections the silver atoms were found in only two sites, although there are four SH groups, leads one to suppose that the four groups occur in two symmetrically situated pairs, one in each half molecule, and that the partners of each pair are near neighbours. An atom equivalent of mercury or silver distributed between the two groups of a pair might easily have appeared as a single peak in the Fourier projection at the resolution employed, particularly if the distance between them had been foreshortened by the process of projection. On the other hand, if the four groups were all widely apart, the appearance of two silver peaks in the Fourier projection could only be explained by assuming two of them to have a higher affinity for Ag^+ and PCMB. This is not supported by the result of the amperometric $AgNO_3$ titration, which showed unbroken uptake of silver to a total of four atom equivalents. In addition it would then be difficult to explain the finding that two equivalents of $HgCl_2$, PCMB and, probably, methylmercury are sufficient to block all four SH groups to reaction with silver. For these reasons arrangement of the SH groups of the native protein into pairs is preferred as an explanation of the experimental facts. Very recently a Fourier projection of crystalline horse haemoglobin-Ag_4 has been calculated (Ingram & Perutz, unpublished). This shows the four silver atoms as only two peaks, each slightly elongated and each high enough to account for two metal atoms close together. This finding is in agreement with the proposed pairing of the SH groups.

Native ox and human haemoglobins also appear to have four SH groups in two pairs, each of which has the sulphur atoms close together. Native sheep haemoglobin, on the other hand, has eight groups, apparently in four pairs. In human haemoglobin, as in horse, the symmetry found in X-ray studies demands that the two pairs be in equivalent positions.

On denaturation with SDS, another two SH groups appear in horse haemoglobin (Fig. 4*a*). In speculating about their positions in the molecule the following facts have to be taken into consideration. When two $HgCl_2$ are bound by a molecule of the protein nearly all the six SH groups are sterically blocked to silver ions, but not to further reaction with $HgCl_2$, which has a higher affinity. Therefore, a total of four $HgCl_2$ can be taken up after denaturation. When two molecules of PCMB combine with a molecule of protein, one PCMB to each group of three SH sites (Fig. 4*b*), only four out of the six sulphydryl groups are blocked to $AgNO_3$ and another two PCMB are required to complete the

process. These results suggest that each of the two additional SH groups uncovered on denaturation is probably near one of the pairs of 'native' groups; thus there would be two clusters each containing three SH groups. The configuration of SH groups within each cluster of three is probably not much altered by denaturation since blocking with mercuric chloride or PCMB is equally effective when the mercurial is added to the already denatured haemoglobin.

SDS-denatured human haemoglobin shows eight SH groups, two more than horse. Correspondingly two more molecules of the blocking reagents $HgCl_2$ and PCMB are required to make all the sulphydryl groups unavailable to silver ions. A simple explanation, though probably not the only one, is to assume that six of the groups occur, as in horse, in two clusters of three and that the remaining two groups are in a general position and its symmetrical equivalent. It seems that the last-mentioned groups are not near the others, since any blocking of them does not affect the other groups or vice versa; also they combine in a 1:1 ratio with the mercury reagents.

On the other hand, ox and sheep haemoglobins show no extra SH groups on treatment with SDS. As no extra $HgCl_2$ is taken up, the distance between the groups in a pair can hardly have been altered by the treatment.

The report of Hughes (1950) that native human haemoglobin combines with two molecules of methylmercury iodide agrees very well with its reactions with the closely related PCMB and methylmercury nitrate in the present work. Nevertheless, there are four SH groups in the native protein which react in the amperometric $AgNO_3$ titration; it is interesting to note that this is also the number found by Benesch (1950) in dog haemoglobin by the same $AgNO_3$ method. However, the two SH groups per molecule found by Ingbar & Kass (1951) do not agree with the four groups found during the experiments reported here. This discrepancy may be due to the higher ammonia concentration employed by them for the $AgNO_3$ titration, which would have had the effect of depressing the number of SH groups detected.

The amino acid composition of horse haemoglobin (Tristram, 1953) shows that each molecule contains six sulphur atoms derived from cysteine or cystine. Since all these appear as SH groups in the SDS-denatured protein, we can deduce that in horse haemoglobin the six polypeptide chains (Porter & Sanger, 1948) are not joined together by disulphide bridges. It is not likely that the two extra thiol groups of denatured horse haemoglobin are derived from the breaking of a disulphide group with SDS; this reagent does not affect the disulphide links of insulin, where experiments showed that there was no uptake of $AgNO_3$ even in the presence of the denaturant.

The rather inadequate analytical data available for ox haemoglobin (Haurowitz, 1954; Block & Bolling, 1947) indicate the presence in the molecule of 3·5 cysteine–cystine sulphur atoms. Together with the fact that four SH groups have been found in the native and the denatured protein this makes it seem likely that here also there are no disulphide bridges between peptide chains. The published analytical figures for sheep and human haemoglobin do not warrant any speculations on this subject.

SUMMARY

1. The numbers of available sulphydryl groups in the native and sodium dodecylsulphate denatured haemoglobins of horse, ox, man and sheep have been determined by amperometric titration with silver nitrate and with mercuric chloride.

2. Native horse, ox and human haemoglobins have four sulphydryl groups as two pairs of closely neighbouring groups. Native sheep haemoglobin shows eight sulphydryl groups as four such pairs.

3. Horse haemoglobin denatured with sodium dodecylsulphate has altogether six sulphydryl groups probably situated symmetrically with respect to a diad axis as two clusters of three. Since the number of cysteine–cystine sulphur atoms is six, there can be no disulphide linkages in this haemoglobin.

4. Denaturation of human haemoglobin with sodium dodecylsulphate reveals the presence of eight sulphydryl groups, six of which are probably arranged in two clusters of three as in horse haemoglobin, while the remaining two appear to be separate.

The author wishes to thank Dr M. F. Perutz, F.R.S., for his interest and encouragement during the course of this work. The gift of specially purified sodium dodecylsulphate by Dr B. A. Pethica, Cambridge, is gratefully acknowledged, as is a sample of methylmercury iodide from Dr F. R. N. Gurd, Boston.

REFERENCES

Anson, M. L. & Mirsky, A. E. (1931). *J. gen. Physiol.* **14**, 605.

Benesch, R. (1950). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 83.

Benesch, R. & Benesch, R. E. (1948). *Arch. Biochem.* **19**, 35.

Block, R. J. & Bolling, D. (1947). *The Amino Acid Composition of Proteins and Foods*, p. 176. Springfield, U.S.A.: Charles C. Thomas.

Boyer, P. D. & Segal, H. L. (1954). *The Mechanism of Enzyme Action*, p. 528. Baltimore: Johns Hopkins Press.

Bragg, W. L. & Perutz, M. F. (1954). *Proc. Roy. Soc.* A, **225**, 315.

Friedmann, E., Marrian, D. H. & Simon-Reuss, I. (1949). *Brit. J. Pharmacol.* **4**, 105.
Green, D. W., Ingram, V. M. & Perutz, M. F. (1954). *Proc. Roy. Soc.* A, **225**, 287.
Greenstein, J. P. (1939). *J. biol. Chem.* **128**, 233.
Haurowitz, F. (1954). *The Proteins*, vol. 2, Part A, p. 291. New York: Academic Press.
Hughes, W. L. (1950). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 82.
Ingbar, S. H. & Kass, E. H. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 74.
Kolthoff, I. M. & Harris, W. E. (1946). *Industr. Engng Chem. (Anal.)*, **18**, 161.
Kolthoff, I. M., Stricks, W. & Morren, L. (1954). *Analyt. Chem.* **26**, 366.
Marrian, D. H. (1949). *J. chem. Soc.* p. 1515.
Mirsky, A. E. & Anson, M. L. (1936). *J. gen. Physiol.* **19**, 439.
Perutz, M. F. (1942). *Nature, Lond.*, **149**, 491.
Perutz, M. F., Liquori, A. M. & Eirich, F. (1951). *Nature, Lond.*, **167**, 929.
Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
Tristram, G. R. (1953). *The Proteins*, vol. 1, Part A, p. 181. New York: Academic Press.
Tsao, T. C. & Bailey, K. (1953). *Biochim. biophys. Acta*, **11**, 102.

The Assimilation of Amino Acids by Bacteria

20. THE INCORPORATION OF LABELLED AMINO ACIDS BY DISRUPTED STAPHYLOCOCCAL CELLS

BY E. F. GALE AND JOAN P. FOLKES
Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge

(*Received* 2 *November* 1954)

In a previous paper (Gale & Folkes, 1953*a*) it was shown that washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) will incorporate [^{14}C]glutamic acid into the protein portion of the cells if these are incubated with glucose and the labelled amino acid either alone or as part of a complete mixture of amino acids. The sensitivity of the incorporation process differed according to the incubation conditions; when a complete mixture of amino acids was present the incorporation was highly sensitive to chloramphenicol and aureomycin but insensitive to penicillin, whereas when glutamic acid was the only amino acid present, the incorporation was much less sensitive to chloramphenicol but markedly affected by penicillin. Studies of the effects of varying the composition of the amino acid mixture and of adding inhibitors on the rate of incorporation of [^{14}C]glutamic acid or [^{14}C]phenylalanine compared with the effects on the rate of protein synthesis showed that the former rate was no measure of the latter (Gale & Folkes, 1953*a*, *b*). When glutamic acid is the only amino acid present in the incubation mixture, the incorporation process appeared to take place as the result of an exchange between glutamic acid residues in the protein and the free glutamic acid accumulated within the cells. If this were the case, it should be possible to demonstrate a removal of labelled residues from the cell protein (after incorporation) by exchange with non-labelled free glutamic acid; this could not be demonstrated with intact cells since it proved impossible to remove free labelled glutamic acid from the internal medium of the cells after incorporation experiments. In the present paper, a reversal of incorporation has been demonstrated by the use of disrupted cells which possess no internal medium.

Incorporation of labelled amino acids into a variety of tissue preparations has now been demonstrated by many workers (see reviews by Borsook, 1953; Gale, 1953) and in many cases it has been proved beyond reasonable doubt that the incorporated material has become part of the tissue protein, although protein synthesis as such has not been demonstrated during the experiments. Typical investigations of this point have included studies of the effect of prolonged and repeated washing of the protein precipitates with hot and cold trichloroacetic acid (TCA), solution and reprecipitation of the protein fraction, demonstration that the rate of release of labelled amino acid is the same as that of non-labelled amino acid during hydrolysis of the protein, and detection of the labelled residue in peptide fragments of the protein after mild hydrolysis. Incorporation requires a source of energy which can be provided by glucose for intact staphylococci (Gale & Folkes, 1953*a*) or by adenosine triphosphate (ATP) for disrupted cells (Gale & Folkes, 1953*c*) or microsome preparations (Zamecnik & Keller, 1954). Holloway & Ripley (1952) found that the incorporation of labelled leucine into rabbit reticulocytes could be correlated with their ribonucleic acid content and it is shown below that the incorporation of [^{14}C]glutamic acid