4. From both fractions P3 and BL purified plasminogen fractions were obtained by batch adsorption on to diethylaminoethylcellulose resin followed by elution with lysine-containing buffers. The eluates obtained by the batch adsorption of fraction BL (fraction R) had a potency 190–315 times that of plasma, the preparation being soluble at neutral pH.

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The Reaction of Normal Adult Human Haemoglobin with Heavy-Metal Reagents

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All the mammalian haemoglobins which have so far been examined contain SH groups that form mercaptides immediately on the addition of heavy-metal reagents in the same way as simple thiols. Most, but not all, contain further SH groups that are unreactive in the native protein, but which react normally when the protein is denatured with acid or sodium dodecyl sulphate. It is not yet known why these groups are unreactive or what is their function.

The unreactive SH groups will react slowly with some heavy-metal reagents. This paper describes the reactions of mercuric chloride, phenylmercuric hydroxide, *p*-chloromercuribenzenesulphonate and silver nitrate with the SH groups of normal adult human haemoglobin in its various forms. Allison & Cecil (1958) have shown that this protein has a total of 6 SH groups/molecule, 2·2 of which are normally reactive and the remainder unreactive until the protein is denatured.

THEORETICAL

Abbreviations. HbO_2, Oxyhaemoglobin; HbCO, carboxyhaemoglobin; Met-Hb, methaemoglobin; Hb, reduced haemoglobin.

The slow reaction of haemoglobin with heavy-metal reagents was followed polarographically by observing the current due to the electroreduction of the heavy-metal reagent at the dropping-mercury electrode. In practice this is done by setting the applied potential to a value at the top of the polarographic wave and observing the fall in current with time. This section deals with the various factors that have to be taken into account when relating this change in current to the number of protein SH groups that have reacted to form mercaptide.

At any time during the reaction the heavy-metal ions will be distributed as follows: (*a*), free in solution; (*b*), combined as haemoglobin mercaptide

groups; (*c*), combined as complexes with haemoglobin mercaptide groups; (*d*), combined with other groups in the haemoglobin; the effect of sulphite in reducing the extent of this type of combination (see Table 1 and text below) suggests that it is weak compared with (*b*) and (*c*). [See also Benesch & Benesch (1948).] Because of this the decrease in polarographic current cannot be assumed to be directly proportional to the number of mercaptide groups formed. Amperometric titration of the reactive SH groups under the same conditions as those used for a slow reaction can provide information on the relative contributions of (*a*), (*b*), (*c*) and (*d*) to the observed current. A typical titration is shown in Fig. 1.

Residual current. This is the current observed before electroreduction of free heavy-metal reagent takes place. It is made up of the condenser current (i.e. that required to charge each succeeding mercury drop to the applied potential) together with that due to the electroreduction of any other species present (Meites, 1955). The condenser current will be affected by any substance which is attracted to the surface of the mercury drops, in particular the haemoglobin, which can be observed forming a coloured layer around each drop. The small change in residual current which is observed before the end point of a titration of the reactive SH groups (Fig. 1) is thought to represent a change in condenser current. Such a change is likely to be due to the different effects of haemoglobin and of haemoglobin combined with heavy-metal reagent as (*b*) or (*c*) on the capacity of the mercury drops. Since no polarographic waves can be observed before the end point of the titration, it is unlikely that electroreduction of heavy-metal reagent in the form (*b*) or (*c*) occurs to a significant extent.

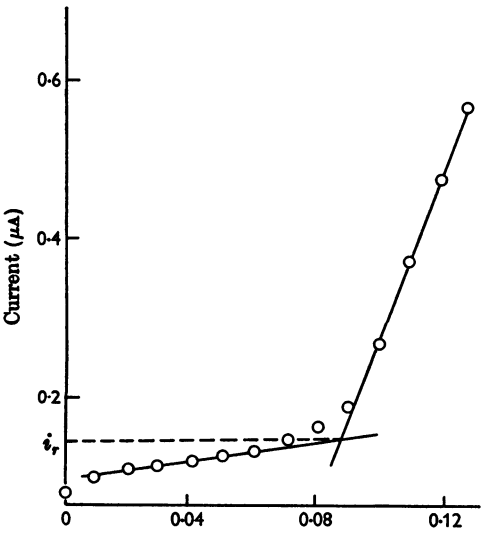

Fig. 1. Amperometric titration of HbCO with 10 mM-phenylmercuric hydroxide at −0·6 v; 3 ml. of 0·13 mM-HbCO–0·04 M-$NaNO_3$–0·04 M-$Na_2B_4O_7$ was used.

The problem is to decide what value of the residual current should be used when following the slow reaction of haemoglobin with an excess of heavy-metal reagent. The fact that free heavy-metal reagent is present prevents direct measurement of any change in residual current that may take place during the reaction. The procedure adopted in this work has been to carry out a titration of the reactive SH groups with the reagent under the conditions used in the slow reaction and assume that the residual current at the end points holds throughout the reaction. Provided any changes in residual current that take place during the slow reaction are small in relation to the changes in total current, the errors involved in this procedure will be small.

Diffusion current. After the end point of the titration of the reactive SH groups has been reached the change in current per increment of titrant increases, owing to the presence of free heavy metal in solution. If this experiment is repeated in the absence of protein the change in current per increment of titrant (i.e. the slope of the excess of reagent line) is greater. The difference is due to combination of the heavy-metal reagent with haemoglobin in the forms (*c*) and (*d*). As discussed above, it is unlikely that heavy-metal ions combined in the form (*b*) and (*c*) contribute to the diffusion current. It is not known, however, whether all the heavy-metal ions in the form of (*d*) can be reduced at the dropping-mercury electrode. The difference in the slopes of these two excess of reagent lines therefore cannot be used to calculate the amount of heavy-metal ions removed from solution in the forms (*c*) and (*d*). It can, however, be used to compare the relative amounts of (*c*) + (*d*) under different reaction conditions. As mercaptide groups are formed during the course of the reaction so the amount of heavy metal combined as (*c*) will increase. Since the concentration of free heavy-metal reagent will decrease during the reaction, so will the amount combined as (*d*).

Total current during the slow reaction. The diffusion current at any time, i_d, is represented as

$$i_d = D(m - Np)$$

where D is a coefficient relating diffusion current to the concentration of heavy-metal reagent in the form of (*a*) + (*d*) under a given set of conditions; m, the concentration of heavy-metal reagent at the start of the reaction in the form (*a*) + (*d*) [i.e. the

total concentration less that combined with the reactive SH groups in the form $(b)+(c)$]; p, the concentration of haemoglobin; N, the number of moles of heavy-metal reagent bound/mole of haemoglobin by the unreactive SH groups in the form of $(b)+(c)$ at time t (i.e. the total bound less that bound by the reactive SH groups). The total current is therefore:

$$i = i_r + i_d$$
$$= i_r + D(m - Np)$$

where i_r is the residual current at the end point of the titration of the reactive SH groups (as described under 'Residual current' above).

Therefore $$N = \frac{Dm + i_r - i}{Dp} \quad (1)$$

D may be determined in one of two ways: (i) If the current is extrapolated to zero time, then

$$D = \frac{i_0 - i_r}{m}$$

where i_0 is the current at zero time. (ii) If the initial reaction is rapid and the extrapolation cannot be made, a titration of the reactive SH groups is carried out under the same reaction conditions and D is taken as the slope of the excess of reagent line. (i) and (ii) differ in that the ratio of heavy-metal reagent to haemoglobin is high in (i) and low in (ii), but reasonable agreement was obtained between the two methods.

If kinetic studies are to be attempted it is necessary to know the concentration of free heavy-metal reagent and the number of unreactive SH groups remaining at any time. This is only possible when the amount of heavy-metal reagent bound as (c) and (d) is either known or insignificant.

EXPERIMENTAL

Materials

Haemoglobin. Normal human erythrocytes were kindly made available by the Biochemistry Department, Radcliffe Infirmary, Oxford. These were treated as described by Allison & Cecil (1958). The solutions of HbO_2 thus prepared were either kept as such or, when required, treated with CO to form HbCO. Both were estimated as HbCO with $10^{-3}\epsilon$ of 59·6 for 4Fe at 540 mμ.

Methaemoglobin. To a solution of HbO_2 (1 mole) in 0·02 M-Na_2HPO_4–0·02 M-NaH_2PO_4 at room temperature was added 8 moles of $K_3Fe(CN)_6$. The solution was dialysed against three changes of water. Concentrations were measured as methaemoglobin cyanide; $10^{-3}\epsilon$ at 540 mμ was taken as 46 per 4Fe.

Reduced haemoglobin. Nitrogen was bubbled through HbO_2 solution at 37° for 15–20 min. Frothing was prevented by a ring of 'Silicone' grease on the inside of the vessel.

p-*Chloromercuribenzenesulphonic acid.* This was obtained from the Sigma Chemical Co., St Louis 16, Mo., U.S.A., and used without purification. Concentrations of solutions (approximately 10 mM) were checked by amperometric titration with cysteine in 0·02 M-HCl. The method used for estimating phenylmercuric hydroxide [potentiometric titration with KBr (Allison & Cecil, 1958)] cannot be used since the halides are soluble in acid solution.

Other reagents. Those used were as described by Allison & Cecil (1958).

Apparatus and methods

Polarographic measurements. The dropping-mercury indicator electrode was used together with a saturated calomel reference electrode. The latter was connected to the cell with a 2% agar bridge containing either saturated KCl or M-NH_4NO_3. The polarograph was a Radiometer type PO4 recording instrument. This was used in the normal way for recording current–voltage curves. It was also used at fixed potential for recording amperometric titrations of haemoglobins with heavy-metal reagents. The polarograph was also used at a fixed potential for recording the slow reactions of haemoglobins with heavy-metal reagents, as described in the Theoretical section. In this case the voltage axis on the chart was used as the time scale. The capacity of the polarographic cell was 3·5 ml. It incorporated a water jacket through which water at any required temperature would be circulated. Oxygen was removed from all solutions before measurements were made by bubbling N_2 or, for HbCO solutions, CO.

Total-mercury analysis. A sample of the substance, either solid or in 0·2 ml. of water, was digested with 0·5 ml. of conc. HNO_3 at 100° for 1 hr. One drop of Br_2 was added (to convert all the mercury into Hg^{2+} ions) and the digestion continued for 15 min. Excess of Br_2 and N_2O_4 were removed by a stream of air, and 3 ml. of water was then added followed by 1 ml. of 0·2 M-Na_2SO_3 to remove traces of oxidizing material. The solution was made up to 10 ml. and analysed for Hg^{2+} ions by the dithizone method of Irving, Risdon & Andrew (1949).

Analyses for carbon and hydrogen. These were carried out by Alfred Bernhardt, Mikroanalytisches Laboratorium, Mülheim, Germany.

RESULTS

Reaction with phenylmercuric hydroxide

It was shown in the Theoretical section that the uptake of a heavy-metal reagent by haemoglobin can only be interpreted in terms of the number of SH groups that have reacted if the amount bound in the form (c) + (d) is small. One way of ensuring this is to add a high concentration of a low-molecular-weight substance that forms an electroreducible complex with the heavy-metal reagent [see discussion in Cecil & McPhee (1959)]. When the reactive SH groups of HbCO are titrated with phenylmercuric hydroxide at pH 9 and 37° in the presence and in the absence of sulphite the end points are 2·22 and 2·25 respectively. This suggests that the amount of phenylmercuric hydroxide bound by the reactive SH groups in the form (c) is small under these conditions. Accordingly the first experiments were carried out in the absence of a complex-forming agent.

Table 1. *Polarographic properties of phenylmercuric hydroxide*

Phenylmercuric hydroxide was at 37° in 0·04 M-$Na_2B_4O_7$–0·04 M-$NaNO_3$ in the presence and in the absence of HbCO, Na_2SO_3 and $Na_2S_2O_3$. The solutions were deoxygenated with CO. Currents were measured at −0·6 v.

Concn. of HbCO (mM)	Complex-form agent	Reactive SH titre	Diffusion current coefficient* D (μA/1 mM)		Half-wave potential of first wave of phenylmercuric hydroxide (v)
			Calculated from titration curve	Calculated from extrapolation of i to $t = 0$	
0	None	—	5·5	—	−0·14
0·065	None	2·25	3·4	3·2	−0·30
0	0·1 M-Na_2SO_3	—	4·8	—	−0·33
0·065	0·1 M-Na_2SO_3	2·22	4·0	4·8	−0·33
0	0·1 M-$Na_2S_2O_3$	—	4·8	—	−0·46
0·065	0·1 M-$Na_2S_2O_3$	1·95	4·0	4·8	−0·46

* See Theoretical section for explanation.

Reactions in the absence of a complex-forming agent. Carboxyhaemoglobin was used for most of the work but some comparative experiments were carried out with Hb and Met-Hb. The buffer used was 0·04 M-$Na_2B_4O_7$–0·04 M-$NaNO_3$. Chloride could not be used because, even at pH 9, it caused a slow disappearance of phenylmercuric hydroxide from solution. The basic polarographic data are given in Table 1. The presence of haemoglobin causes a fall in the value of D, which was partially restored by the addition of sulphite or thiosulphate. This indicates that there is appreciable combination of phenylmercuric hydroxide in the form (*c*) and (*d*) in the absence of a complex-forming agent.

Experiments were therefore carried out with various excesses of phenylmercuric hydroxide to discover the amount required for complete reaction of the SH groups, allowing for the fact that some will be bound as (*c*) + (*d*). The solutions were kept at 37° until the polarographic wave of phenylmercuric hydroxide had disappeared (6–8 hr.). Excess of phenylmercuric hydroxide was removed by dialysis against 0·02 M-$Na_2B_4O_7$–0·05 M-$Na_2S_2O_3$ for 24 hr. and subsequently against 0·02 M-$Na_2B_4O_7$. Sodium dodecyl sulphate (0·02 M) was added to the dialysed solution and the number of unreactive SH groups remaining uncombined determined by titration with phenylmercuric hydroxide. At least 10 equiv. of phenylmercuric hydroxide were required. Since it was shown that the reactive SH groups do not bind appreciably more than 1 equiv. of phenylmercuric hydroxide, this implies that each unreactive SH group must be binding nearly 2 equiv. under these conditions. As a further check total-mercury analyses were carried out on the dialysed reaction mixtures. The number of atoms of Hg/mole of HbCO corresponded with the total number of SH groups that were found to have reacted by the titration method. This shows that the dialysis against thiosulphate removed all the phenylmercuric hydroxide except that bound as (*b*) (i.e. as mercaptide).

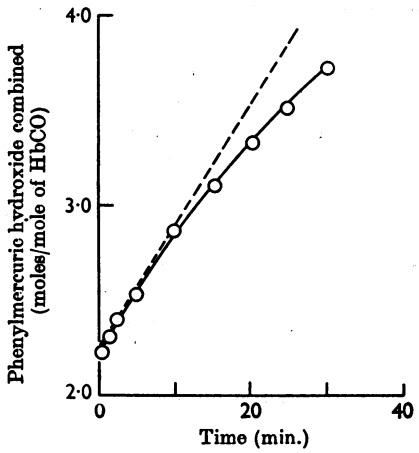

Fig. 2. Reaction of 0·47 mM-phenylmercuric hydroxide with 0·066 mM-HbCO in 0·04 M-$NaNO_3$–0·04 M-$Na_2B_4O_7$ at 37°. The rate curve was calculated from the polarographic diffusion current at −0·6 v by using equation (1). – – –, Initial slope (0·065 min.$^{-1}$) was determined graphically.

It is clear from these experiments that a considerable amount of phenylmercuric hydroxide was bound in the form of (*c*) + (*d*). This means that a kinetic treatment relating the uptake of phenylmercuric hydroxide to the number of SH groups reacting is not possible. Instead, the rate curve was calculated by using equation (1) and the initial slope used as a basis for comparison (see Fig. 2). A summary of results, including those with Hb and Met-Hb, is given in Table 2. These initial rates are dependent on the initial concentrations of the reactants. Comparisons can therefore only be made when the ratio of the phenylmercuric hydroxide and haemoglobin concentrations are the same in both experiments.

Table 2. *Initial rates of uptake of phenylmercuric hydroxide by haemoglobin*

Haemoglobin was approximately 0·1 mM in 0·04 M-$Na_2B_4O_7$ at 37° in the presence and in the absence of complex-forming agent. The solutions of HbCO were deoxygenated with CO and the others with N_2.

Haemoglobin	Phenylmercuric hydroxide (moles/mole of haemoglobin)	Concn. of $NaNO_3$ (M)	Complex-forming agent	Initial rate of uptake (min.$^{-1}$)
HbCO	6·2	0·9	None	0·15
HbCO	6·2	0·9	0·1 M-Na_2SO_3	0·065
HbCO	6·2	0·04	None	0·065
HbCO	6·2	0·04	0·1 M-Na_2SO_3	0·02
HbCO	6·2	0·04	0·1 M-$Na_2S_2O_3$	0·01
HbCO	8·0	0·04	None	0·17
Hb	8·0	0·04	None	0·27
Met-Hb	8·0	0·04	None	0·65

Before these complications were realized a second-order rate constant was calculated for HbCO in 0·04 M-$Na_2B_4O_7$–0·8 M-$NaNO_3$ on the basis that each SH group reacted with only one molecule of phenylmercuric hydroxide. A value of 0·4 l. mole^{-1} sec.$^{-1}$ at 37° was obtained. This value was constant throughout the reaction and so, on purely kinetic grounds, there was no reason to suspect that the reaction is complex.

Reactions in the presence of sodium sulphite. The reaction in the presence of sulphite was considerably slower than in its absence. Experiments were carried out, similar to those described above, in which the reaction was allowed to go to completion and the excess of phenylmercuric hydroxide was removed by dialysis against thiosulphate. When the HbCO was subsequently denatured with sodium dodecyl sulphate there was no release of SH groups but, instead, a liberation of free phenylmercuric hydroxide. A further complication was the production of a white precipitate during the reaction, which contained about 25% of the total mercury present. This substance was recrystallized from aqueous ethanol and shown to be diphenylmercury (Found: C, 40·5; H, 2·9; Hg, 55·2. Calc. for $C_{12}H_{10}Hg$: C, 40·7; H, 2·8; Hg, 56·5%), m.p. was 125° (uncorr.); Coates (1956) gives 125°. Diphenylmercury is precipitated slowly from a solution containing phenylmercuric hydroxide and an excess of sulphite or thiosulphate at pH 9, with a simultaneous appearance of Hg^{2+} ions. The reaction must therefore be of the type:

$$2PhHg^+ + 2X^- \rightleftharpoons (Ph)_2Hg + HgX_2,$$

where Ph is phenyl and X an anion which binds Hg^{2+} ions strongly. Such a reaction has been reported for cyanide and thiocyanate (Whitmore, 1919), but not for sulphite. The reaction with sulphite is slow and only causes significant error when long reaction periods are used. For short periods (1–2 hr.) phenylmercuric hydroxide–sulphite mixtures may be used provided suitable checks are made. The formation of Hg^{2+} ions in this way explains the liberation of phenylmercuric hydroxide that occurred when sodium dodecyl sulphate was added to HbCO that had reacted with phenylmercuric hydroxide in the presence of sulphite. Some of the SH groups will have formed $RSHg^+$ and others RSHgPh. Since the denatured HbCO forms $(RS)_2Hg$ with Hg^{2+} ions and since Hg^{2+} ions displace $PhHg^+$, the reaction:

$$RSHg^+ + RSHgPh \rightleftharpoons (RS)_2Hg + PhHg^+$$

will occur. The point of adding sulphite was to simplify the interpretation of the phenylmercuric hydroxide–HbCO reaction but this hope was not realized. The initial rates of the reaction with HbCO and Hb are given in Table 2.

Reaction in the presence of thiosulphate. The half-wave potential of phenylmercuric hydroxide in the presence of thiosulphate at pH 9 is −0·46 as compared with −0·33 v in the presence of sulphite and −0·14 v in borate alone. It would be expected therefore that, since the complex of phenylmercuric hydroxide with thiosulphate is stronger than that with sulphite, the reaction with HbCO in the presence of thiosulphate would be slower. The results given in Table 2 show that this is the case. Since diphenylmercury is also formed in the presence of thiosulphate, no further work was done with the reagent.

Precipitation of haemoglobin after reaction with phenylmercuric hydroxide. Haemoglobin is less stable after reaction with phenylmercuric hydroxide, and tends to precipitate. The extent of precipitation is greater with Hb than HbCO. It is reduced by sulphite and increased by exposure to air (Table 3).

Reaction with p*-chloromercuribenzenesulphonic acid*

The reactions with the sulphonic acid were investigated under conditions similar to those used for phenylmercuric hydroxide. The polarographic waves are somewhat different from those of phenyl-

mercuric hydroxide (Fig. 3). No evidence could be found for the formation of mercury bis(benzenesulphonate) in the presence of sulphite or thiosulphate.

Experiments designed to find the conditions necessary for complete reaction, similar to those described with phenylmercuric hydroxide, showed that only 4 out of the total of 6 SH groups reacted even after 48 hr. at 37°. It appears, therefore, that two of the 'unreactive' SH groups differ in their reactivity from the other two.

Table 3. *Haemoglobin precipitated after reaction with phenylmercuric hydroxide*

Measurements were made after reaction with phenylmercuric hydroxide for 16–19 hr. at 37°. The composition of the reaction mixture was approximately 0·2 mM-haemoglobin in 0·04 M-$NaNO_3$–0·04 M-$Na_2B_4O_7$ plus 10–12 equiv. of phenylmercuric hydroxide and complex-forming agent as shown. The solutions of HbCO were deoxygenated with CO, and Hb with N_2.

Haemoglobin	Complex-forming agent	Haemoglobin precipitated (%)
HbCO	None	10
HbCO	0·1 M-Na_2SO_3	5
Hb	None	71
Hb	0·1 M-Na_2SO_3	52
HbCO, but exposed to air	None	72

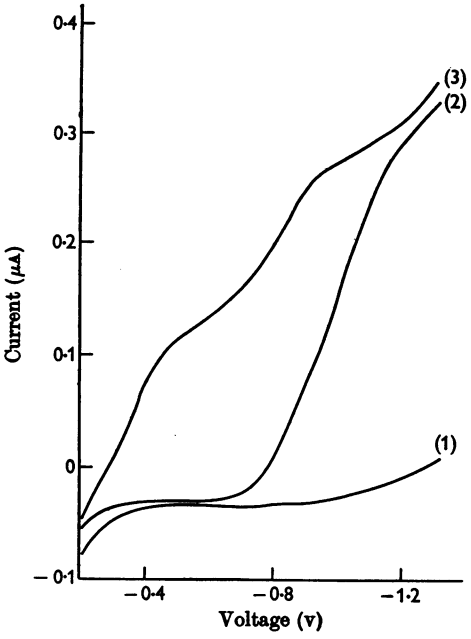

Fig. 3. Current–voltage curves of *p*-chloromercuribenzenesulphonic acid in the presence of HbCO with and without sodium dodecyl sulphate. (1), 0·13 mM-HbCO, 0·04 M-$NaNO_3$, 0·04 M-$Na_2B_4O_7$, 0·015 M-sodium dodecyl sulphate; (2), as (1) plus 0·4 mM-*p*-chloromercuribenzenesulphonic acid; (3), as (2) but without sodium dodecyl sulphate.

Reaction with silver nitrate

There is ample evidence that SH groups tend to bind more than one atom of Ag (see, for instance, Cecil & McPhee, 1959). Experiments with HbCO in the presence of sulphite showed that the diffusion current coefficient, D, was only 20 % of its value in the absence of HbCO. Titrations were also tried in the presence of thiosulphate but the end points were ill-defined. Accordingly no further work was done with $AgNO_3$.

Reaction with mercuric chloride

The reaction between haemoglobin and $HgCl_2$ was the last to be investigated because it was thought that the possibility of the bifunctional behaviour of Hg^{2+} ions might lead to ambiguities in the interpretation of results. Although the dimercaptide is formed with the denatured protein (Allison & Cecil, 1958), a ratio of one Hg atom to one S atom obtained throughout with the native protein.

The polarographic data for $HgCl_2$ are given in Table 4. The initial reaction of haemoglobin with $HgCl_2$ is rapid, making the extrapolation of the polarographic current to zero time unsatisfactory. The diffusion current coefficient was therefore calculated from the titration curve. Because of the rearrangement from the mono- to the di-mercaptide that is likely to occur on denaturation, titration in the presence of sodium dodecyl sulphate was not used to determine the number of residual SH groups. Instead, the dialysed protein was analysed for total mercury. Reaction of all the unreactive SH groups of HbCO took place with an eightfold molar excess of $HgCl_2$. In the presence of sulphite, only 4 out of the total of 6 SH groups reacted. In the presence of thiosulphate only the reactive groups reacted. The results, including experiments with Hb, are given in Table 5. The percentage of haemoglobin precipitated is also shown. In general this is less than occurs with phenyl mercuric hydroxide.

Fig. 4 shows the rates of uptake of $HgCl_2$ by HbCO under various conditions. Curve (*D*) shows the uptake in the absence of complexing agent, after correction for Hg^{2+} ions combined as (*c*) and (*d*). This was done by taking samples of the reaction mixture, adding thiosulphate, and measuring the concentration of the free mercury–thiosulphate complex polarographically. The difference between this and the total concentration gave the amount bound as mercaptide. This experiment showed that 4 moles of $HgCl_2$/mole of HbCO react

Table 4. *Polarographic data for mercuric chloride*

The polarographic properties of $HgCl_2$ at 37° in 0·04M-$Na_2B_4O_7$–0·04M-$NaNO_3$ in the presence and in the absence of HbCO, Na_2SO_3 and $Na_2S_2O_3$. The solutions were deoxygenated with CO. Currents were measured at −0·35v.

Concn. of HbCO (mM)	Complex-forming agent	Reactive SH titre	Diffusion current coefficient D* (calculated from titration curve) (μA/mM)
0	None	—	12·4
0	0·1M-KCl	—	12·0
0	0·9M-KCl	—	11·1
0·13	None	2·11	5·2
0	0·1M-Na_2SO_3	—	11·1
0·13	0·1M-Na_2SO_3	2·04	7·5
0	0·1M-$Na_2S_2O_3$	—	9·4
0·13	0·1M-$Na_2S_2O_3$	1·8	5·6

* See Theoretical section for explanation.

Table 5. *Reaction of mercuric chloride with haemoglobin*

Moles of $HgCl_2$ per mole of haemoglobin retained by the protein were measured after 15 hr. reaction at 37°, followed by dialysis against 0·05M-$Na_2S_2O_3$–0·05M-$Na_2B_4O_7$ for 24 hr. The composition of the reaction mixture was 0·14 mM-haemoglobin, 1·15 mM-$HgCl_2$, 0·04M-$NaNO_3$, 0·04M-$Na_2B_4O_7$ and complex-forming agent as shown. The solutions of HbCO were deoxygenated with CO, and Hb with N_2. After the reaction, denatured haemoglobin was removed by centrifuging and the supernatant dialysed. The amount of Hg^{2+} ions retained by the protein was estimated as total mercury.

Haemoglobin	Complex-forming agent	Haemoglobin precipitated (%)	Hg^{2+} ions after reaction and dialysis (g.atoms/mole of haemoglobin)
HbCO	None	1	6·1
HbCO	0·1M-Na_2SO_3	4	3·0
HbCO, 48 hr. reaction	0·1M-Na_2SO_3	10	3·7
HbCO	0·1M-$Na_2S_2O_3$	6	2·2
Hb	None	14	6·2
Hb	0·1M-Na_2SO_3	3	3·2

in 20 min., but that complete reaction takes 6 hr. Thus 2 of the unreactive SH groups may be assumed to react more rapidly than the other 2. Chloride was also tried as a complexing agent for $HgCl_2$. The amount of $HgCl_2$ reacting with HbCO in a given time fell as the concentration of chloride was increased (Fig. 5). This was presumably due to the excess of Hg^{2+} ions forming $HgCl_4^{2-}$ instead of being in the form of $(c)+(d)$.

DISCUSSION

The problem of the unreactive SH groups in proteins is one of long standing. The usual criterion for the existence of such groups is their release, i.e. reversion to normal activity, when the protein is denatured. Some of the explanations proposed, such as steric hindrance and the formation of labile chemical bonds, have been discussed in recent reviews (Boyer, 1959; Cecil & McPhee, 1959). These explanations do not, by themselves, account for the known facts. Another possibility is that the SH groups are involved in some form of 'non-polar' bonding. Although ideas on this type of bonding are still somewhat conflicting (Kauzmann, 1959; Klotz, 1960) the possibility must be given serious consideration.

Reactions of the heavy-metal reagents with haemoglobin were studied because they are the most specific reagents known for SH groups. Even so their specificity leaves much to be desired and this has given rise to difficulties in relating the uptake of heavy-metal reagent to the number of mercaptide groups formed. For this reason it has not been possible to use the data obtained for determining activation energies and other kinetic constants for these reactions. This work has not yielded any definite information about the state of the unreactive SH groups. However, the reactions of the heavy-metal reagents with these groups provide a sensitive means of detecting small differences in reactivity. Since valid rate constants could not be obtained the initial slopes of the rate curves were used for comparison. Although this procedure is not ideal the errors caused by combination of the heavy-metal reagent other than as

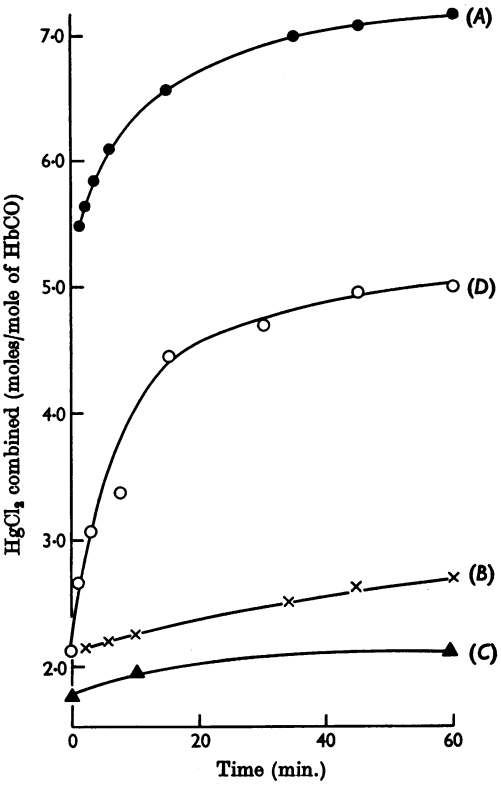

Fig. 4. Reaction of 1·0 mM-$HgCl_2$ with 0·12 mM-HbCO at 37° under various conditions. The rate curves were calculated from the polarographic diffusion current at −0·35 v by using equation (1). (*A*), In 0·04 M-$NaNO_3$–0·04 M-$Na_2B_4O_7$; (*B*), in 0·04 M-$NaNO_3$–0·04 M-$Na_2B_4O_7$–0·1 M-Na_2SO_3; (*C*), in 0·04 M-$NaNO_3$–0·04 M-$Na_2B_4O_7$–0·1 M-$Na_2S_2O_3$; (*D*), this shows the actual rate of mercaptide formation in 0·04 M-$NaNO_3$–0·04 M-$Na_2B_4O_7$. The method used is explained in the text.

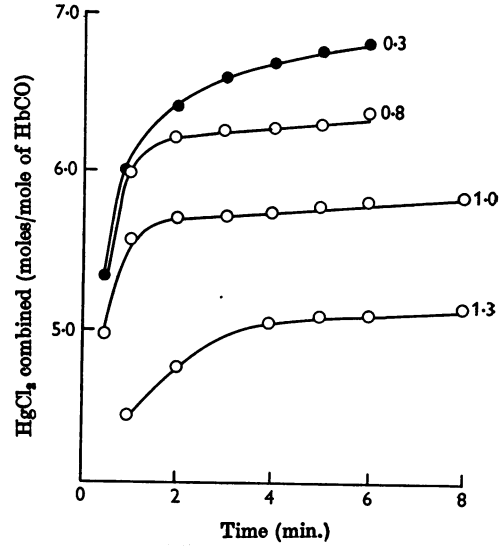

Fig. 5. Effect of KCl concentration on the reaction of 1·0 mM-$HgCl_2$ with 0·13 mM-HbCO in 0·04 M-$Na_2B_4O_7$–0·04 M-$NaNO_3$ at 37°. The rate curves were calculated from the polarographic diffusion current at −0·35 v by using equation (1). Figures on the curves refer to molarity of KCl.

mercaptide are likely to be least in the early stages of reaction. These initial slopes are dependent on the concentrations of the reactants and any comparisons must take this into account.

The study of the reaction of HbCO with mercuric chloride and *p*-chloromercuribenzenesulphonic acid has shown that 2 of the unreactive SH groups react more rapidly than the other 2. These reactions can be used to prepare haemoglobin derivatives with 2, 4 or 6 of the SH groups combined as mercaptide. Differences in the rates of reaction of phenylmercuric hydroxide with HbCO, Hb and Met-Hb show that the state of the iron atom has a considerable effect on the behaviour of the SH groups. These differences are discussed further in the next paper (Cecil & Snow, 1962).

The slow reaction of *p*-chloromercuribenzoate with several proteins has been studied with Boyer's (1954) spectrophotometric method. Thus Boyer (1954) quotes second-order rate constants for the reaction with β-lactoglobulin and ovalbumin at 28° as 7·5 and 20·9 l.mole^{-1} sec.$^{-1}$ respectively. Madsen & Cori (1956) quote 0·85 l.mole^{-1} sec.$^{-1}$ at 21° for the reaction with muscle phosphorylase. Boyer's method depends on the increase in absorption at 250 mμ that occurs when *p*-chloromercuribenzoate reacts to form mercaptide. The assumptions involved in interpreting the results of this method and the polarographic methods are different, and quantitative comparisons between the two must wait until more is known about both methods.

SUMMARY

1. Adult human haemoglobin has 2·2 reactive SH groups and 3·8 unreactive SH groups. The reactive SH groups can be titrated with heavy-metal reagents in the same way as simple thiols. The unreactive SH groups can only be titrated in this way after the protein has been denatured at pH < 4, or by an excess of sodium dodecyl sulphate. This paper describes the slow reaction of the unreactive SH groups with phenylmercuric hydroxide, mercuric chloride and *p*-chloromercuribenzenesulphonate.

2. The rates of reaction of the different forms of haemoglobin with these reagents are in the order methaemoglobin > reduced haemoglobin > carboxyhaemoglobin.

3. Phenylmercuric hydroxide and mercuric chloride react with all the unreactive SH groups but *p*-chloromercuribenzenesulphonate reacts with only two groups.

4. The mercaptides formed by the reactive SH groups with phenylmercuric hydroxide or mercuric chloride show little tendency to bind additional reagent, but those formed by the unreactive SH groups each bind up to one molecule of reagent. This additional reagent can be removed by dialysis against thiosulphate. Carboxyhaemoglobin with all 6 SH groups combined with phenylmercuric hydroxide or mercuric chloride can be prepared by this method.

5. The tendency of the unreactive SH groups to bind more than one molecule of heavy-metal reagent made it impracticable to apply a kinetic treatment to the reaction. Attempts to overcome this difficulty by carrying out the reactions in the presence of sulphite or thiosulphate were unsuccessful. Phenylmercuric hydroxide forms diphenylmercury in the presence of these reagents. Mercuric chloride reacts with only two unreactive SH groups in the presence of sulphite and with none in the presence of thiosulphate.

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The Function of the Unreactive Thiol Groups of Normal Adult Human Haemoglobin

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Adult human haemoglobin has a total of 6 SH groups. Of this 2·2 (the reactive groups) can be titrated with heavy-metal reagents in the same way as the simple thiols. The remainder (the unreactive groups) can only be titrated with heavy-metal reagents after the protein has been denatured with sodium dodecyl sulphate or acid (Allison & Cecil, 1958). The functions of the SH groups are still largely unknown. Riggs (1953) has shown that if thiol reagents (mercurials, formaldehyde or oxygen) are allowed to react with the reactive SH groups of haemoglobin, changes occur in its reaction with oxygen. Both the affinity for oxygen and the extent of haem–haem interaction are affected. It is not known whether the SH groups are directly concerned in these changes or whether the effects observed are a consequence of some other change in the molecule.

We have even less knowledge of the functions of the unreactive SH groups. The rate of reaction of these groups with phenylmercuric hydroxide and mercuric chloride is slow (Cecil & Snow, 1962), suggesting that they are probably involved in some type of bond, the chemical nature of which is unknown.

Some properties of the various forms of haemoglobin, namely reduced, oxy-, carboxy- and methaemoglobin as well as of globin, have been examined in the presence and absence of phenylmercuric hydroxide and mercuric chloride. From these studies it has been possible to make suggestions concerning the functions of the unreactive SH groups.

A short account of some of this work has already appeared (Cecil & Snow, 1961).

EXPERIMENTAL

Haemoglobin. Carboxy- (HbCO), oxy- (HbO_2), reduced (Hb) and met- (Met-Hb) haemoglobins were prepared as described by Cecil & Snow (1962).