β -Glucuronidase finds its major application at the present time in the hydrolysis of urinary steroid glucuronides, and where rats are freely available the female preputial gland would appear to be a useful source of the enzyme in a concentrated form. It has an activity, in phenolphthalein units/g. of fresh tissue, 100 times greater than rat liver and 20 times greater than the visceral hump of the limpet (*Patella vulgata*). The product obtained at stage 3 above is probably sufficiently pure for most purposes.

SUMMARY

1. β -Glucuronidase from the female-rat preputial gland has been purified 25-fold to give a final activity of 455 000 phenolphthalein units/mg. of protein on assay in the presence of albumin.

2. In agreement with Bernfeld *et al.* (1954), the specific activity fell progressively on dilution, and this effect was abolished by adding albumin.

3. The purified enzyme also hydrolysed β -galacturonides.

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The Thiol Groups of Normal Adult Human Haemoglobin

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The analysis of the thiol groups in normal adult human haemoglobin has been the subject of a number of recent papers (Ingram, 1955; Benesch, Lardy & Benesch, 1955; Hommes, Santema-Drinkwaard & Huisman, 1956; Murayama, 1956). These workers all used the method of amperometric titration with silver nitrate (Kolthoff & Harris, 1946; Benesch & Benesch, 1948). They found that, in general, there were four reactive thiol groups/molecule of native protein and eight in the denatured protein. On the other hand, Ingbar & Kass (1951), using the same method but with sodium chloride in the supporting electrolyte, found two thiol groups/molecule of native protein. The same result was obtained by Hughes (1950) with methylmercuric iodide.

The reason for the discrepancy between the different findings is suggested by some of Ingram's (1955) results. As well as titration with silver

nitrate, he carried out amperometric titrations with mercuric chloride and a rotating platinum indicator electrode (Kolthoff, Stricks & Morren, 1954). Kolthoff and his colleagues found that, under the conditions specified, the endpoint of the mercuric chloride titration occurs when the ratio of mercury to thiol is 1:1. With simple thiols such as cysteine and glutathione this corresponds to the compound $(RS)_2Hg_2$, where one mercury atom is firmly bound as the dimercaptide $(RS)_2Hg$ and the other is less firmly bound, probably by carboxyl. With proteins steric considerations may make it difficult for two thiols to react with one mercury atom and a compound of the type $Prot \cdot S \cdot HgCl$ is formed. Thus Edelbach, Katchalsky, Maybury, Hughes & Edsall (1953) showed that mercaptalbumin ($Alb \cdot SH$) reacts rapidly with mercuric chloride:

A slow reaction follows with excess of protein:

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but the dimercaptide reacts rapidly with mercuric chloride:

Hence in the presence of an excess of mercuric chloride the product will be $\text{Alb} \cdot \text{S} \cdot \text{Hg} \cdot \text{Cl}$. Thus with simple thiols and with proteins the equivalence point of this titration will occur when the mercury to thiol ratio is 1:1, although for different reasons.

However, Ingram (1955) interpreted the results of his titrations of human haemoglobin with mercuric chloride on the basis of a ratio of mercury to thiol of 1:2, because only in this way could he account for the results obtained by silver titration. For instance, he found that native human haemoglobin binds 3.9 silver atoms and 2.2 mercury atoms/molecule. An alternative explanation is that the mercury results should be interpreted on the 1:1 basis and that the results with silver are high. Cecil & McPhee (1955) found that the silver mercaptides of simple thiols bind additional silver ions strongly and that large errors (30–40%) may result if the conditions of titration of each thiol are not checked with the pure substance.

Two lines of evidence from Ingram's paper support this interpretation: (i) the number of atoms of silver bound/molecule of haemoglobin fell as the concentration of ammonia in the supporting electrolyte increased. This suggests that the concentration was insufficient to prevent binding of additional silver ions by the haemoglobin silver mercaptide; (ii) when *p*-carboxyphenylmercuric chloride was added to haemoglobin before titration with silver nitrate, the addition of one molecule of the mercurial reduces by two the number of silver atoms bound. Since a mercurial of this type reacts with only one equivalent of thiol (Benesch & Benesch, 1952) it is extremely unlikely that it can block two. If, on the other hand, each thiol group is binding more than one silver atom the observation is explained.

EXPERIMENTAL

Preparation of materials

Haemoglobin. Specimens were obtained from three normal adult subjects, two male and one female. Blood was collected into 0.2M-sodium citrate, adjusted to pH 7.4. The erythrocytes were washed three times in a large volume of 0.15M-NaCl. After the final centrifuging an equal volume of water was added to the packed cells and the mixture frozen twice to lyse the cells. The haemolysate was dialysed at 4° against three changes of water and then centrifuged to remove stroma. Carbon monoxide was bubbled through the clear supernatant. Solutions of carboxyhaemoglobin (HbCO) were stored over CO in order to prevent the formation of methaemoglobin.

Concentrations were calculated from the absorption at 538 mμ. The millimolar extinction coefficient used (per four Fe atoms) was 59.6. This value was based on total-iron analyses by the method of Paul (1948).

Phenylmercuric hydroxide (PhHgOH). This was made from basic phenylmercuric nitrate (British Drug Houses Ltd.) by the method of Schramm (1947). Solutions were made up in water to approx. 10 mM. Concentrations were checked by potentiometric titration against KBr in HNO₃ at pH 2, with a mercury electrode as indicator. (For a description of the apparatus see Cecil, 1955.) The titres obtained were 96–98% of the weighed concentrations in different preparations. Schramm titrated his preparation with HClO₄ in the presence of NaCl. On a pure sample of PhHgOH this gives the same result as the KBr titration. The latter has the advantage, however, that it measures the concentration of PhHg⁺ ion rather than OH[−] ion and so can be used equally well for basic phenylmercuric nitrate, which is available commercially. If desired, this reagent can be used in place of PhHgOH.

Mercuric chloride. Reagent-grade HgCl₂ was recrystallized once from water.

Sodium dodecyl sulphate (SDS). A sample (L. Light and Co. Ltd., Colnbrook, Bucks) was recrystallized once from ethanol.

Nitrogen. Oxygen-free N₂ (British Oxygen Co. Ltd.) was passed through a gas-washing bottle containing vanadous sulphate (Meites, 1948).

Carbon monoxide. This was obtained from Imperial Chemical Industries Ltd., Gaskell Marsh Works, Widnes.

Apparatus

For titrations and current-voltage curves closed vessels were used and a stream of N₂ was passed through them to remove O₂ from the solutions. The vessels held 4–6 ml., and 0.2–1 μmole of HbCO was dissolved in this volume of solution. A dropping mercury electrode was used, the characteristics of the capillary being: $m = 1.95 \text{ mg. sec.}^{-1}$, $t = 3.2 \text{ sec.}$ (open circuit), $h = 57 \text{ cm.}$ The reference electrode was connected by a saturated KCl-agar bridge, and all potentials are expressed relative to the saturated calomel electrode. Titrations and current-voltage measurements were carried out at room temperature. A Tinsley Recording Polarograph type 12 was used to record current-voltage curves, and a manual circuit of the type described by Kolthoff & Lingane (1952) was used for titrations.

Frothing. In the early experiments caprylic alcohol (octan-2-ol) was used to stop frothing of HbCO when N₂ was bubbled through the solutions. It is effective but, being surface-active, influences some of the reactions at the dropping mercury electrode. Some examples of this are given. An alternative method of preventing frothing is to smear a ring of silicone high-vacuum grease round the top half-inch of the titration vessel. This procedure appears not to affect the electrode reactions and was used unless otherwise stated.

Analytical methods

Titrations with mercuric chloride. The technique was essentially that described by Stricks, Kolthoff & Tanaka (1954) for the estimation of disulphides with the dropping mercury electrode. Apart from the magnitude of the diffusion currents obtained, there are two important differences in the behaviour of the dropping mercury and

rotating platinum electrodes. One is that the sulphito-mercury complex is reduced at the mercury but not at the platinum electrode, so that if titrations are to be carried out in the presence of sulphite the mercury electrode must be used. HbCO has a considerable affinity for O_2 , and N_2 bubbling alone may take 30 min. or more to remove the last traces. The presence of sulphite reduces this time to 5–10 min. Since HbCO does not appear to contain any disulphide bonds (see below) the use of sulphite is permissible.

The second difference is that with simple thiols the endpoint occurs when the Hg to SH ratio is 1:2, corresponding to the compound $(RS)_2Hg$. This is because the loosely bound atom of mercury in the compound $(RS)_2Hg_2$ is reduced at the same potential as free Hg^{2+} ions at the dropping mercury electrode but not at the rotating platinum electrode. With proteins, however, the probability is that the half mercaptide $Prot \cdot S \cdot HgCl$ will be formed and the endpoint will occur when the Hg to SH ratio is 1:1. On the other hand, if the thiols are in close proximity, or if the protein has been denatured, the 1:2 ratio may obtain. Because of this possible ambiguity $HgCl_2$ titrations cannot by themselves give the required answer.

Unless otherwise stated, the supporting electrolyte consisted of: KCl, 0.15M; $Na_2B_4O_7$, 0.02M; Na_2SO_3 , 0.04–0.1M. The bottom of the titration vessel was covered with a layer of $CHCl_3$ to prevent reaction between Hg^{2+} ions and Hg from the dropping electrode. Titrations were normally carried out at -0.25 v.

Titrations with phenylmercuric hydroxide. In view of the possible ambiguity in the $HgCl_2$ titrations another reagent was needed which is univalent towards thiol. Silver nitrate is unsuitable for the reasons already given, but organic mercurials of the type $R \cdot Hg \cdot X$ are suitable. Phenylmercuric hydroxide was chosen because it is easily prepared and estimated, and moderately soluble in water. The reaction with thiols is as follows:

It is the same with simple thiols and with proteins. The polarographic behaviour of $PhHgOH$ and its mercaptides has been described by Benesch & Benesch (1951, 1952). $PhHgOH$ has also been used by Cecil & Loening (1957) in work on the disulphide bonds of insulin. The apparatus and supporting electrolyte was the same as that for the $HgCl_2$ titrations except that $CHCl_3$ is not used as there is no interaction between $PhHgOH$ and mercury. The potential was normally -0.5 v.

RESULTS

Native carboxyhaemoglobin

Titrations with mercuric chloride. The relevant current-voltage curves are shown in Fig. 1. The titrations were carried out at -0.25 v and a typical titration curve is shown in Fig. 2. Constant titres were obtained when the sulphite concentration was varied from 0.02 to 0.1 M. In the absence of sulphite the titres were 5% higher. These results are summarized in Table 1. If caprylic alcohol is used to prevent frothing the Hg^{2+} ion wave shows an

anomalous dip (see Fig. 5, curve c) similar to that found by Stricks *et al.* (1954) when they titrated glutathione (GSH) with $HgCl_2$ in an ammonia buffer.

Fig. 1. Current-voltage curves of Hg^{2+} ion in the presence of native HbCO; (a) 5 ml. of 9.4×10^{-5} M-HbCO in 0.15M-KCl, 0.02M- $Na_2B_4O_7$, 0.08M- Na_2SO_3 ; (b) +0.125 ml. of 10 mM- $HgCl_2$, showing the free Hg^{2+} ion wave. This corresponds to point A in Fig. 2.

Fig. 2. Amperometric titration of native HbCO with 10 mM- $HgCl_2$ at -0.25 v; 5 ml. of 9.4×10^{-5} M-HbCO in 0.15M-KCl, 0.02M- $Na_2B_4O_7$, 0.08M- Na_2SO_3 .

Table 1. Titration of native carboxyhaemoglobin with mercuric chloride and phenylmercuric hydroxide

Concn. of Na_2SO_3 in supporting electrolyte (M)	Hg^{2+} ion bound/mol. of HbCO	$PhHgOH$ bound/mol. of HbCO
0.02–0.1	2.14	2.20
0	2.24	2.30

Titration with phenylmercuric hydroxide. The relevant current-voltage curves are shown in Fig. 3. The half-wave potentials of both waves of PhHgOH are slightly affected by the presence of HbCO (cf. Benesch & Benesch, 1951). Fig. 4 shows a typical titration carried out at a potential of -0.5 v. The results, summarized in Table 1, are slightly higher than those obtained with HgCl_2 . This difference between the PhHgOH and HgCl_2 titres was observed with all the samples of HbCO and is probably just significant. It could be due to the formation of a small amount of $(\text{RS})_2\text{Hg}$ as well as RSHgCl .

Effect of mercaptides. Stricks *et al.* (1954) found that the anomalous dip in the Hg^{2+} ion wave mentioned earlier could be removed by the addition of a small amount of cystine, and it was of

interest to see whether the same applied here. Since sulphite and Hg^{2+} ion were present the effective agent was the mercaptide $(\text{CyS})_2\text{Hg}$. Solutions of this were prepared by mixing equivalent quantities of cysteine hydrochloride and HgCl_2 , and varying amounts of the mixture were added to the haemoglobin titration solutions. The dip in the wave was removed, but there were two other effects. An anodic wave appeared in the current-voltage curve, indicating the presence of a low-molecular-weight thiol, and the HgCl_2 titre decreased until, in the presence of an excess of mercaptide, it was halved. If the titration was carried out with PhHgOH the titre was not altered. These observations can be accounted for by the following reaction:

so that the protein thiol is replaced by free cysteine. Similar results were obtained with the mercaptides of cysteamine and thioglycolic acid.

As most thiols are oxidized rapidly at pH 9, O_2 should be removed from the solution before adding the mercaptide.

The half-wave potential of the wave of the cysteine produced by the addition of $(\text{CyS})_2\text{Hg}$ is -0.45 v, whereas the E_1 of the cysteine wave at pH 9 is normally -0.53 v. However, it was found that the addition of a small amount of haemoglobin to a solution of cysteine shifted the half-wave potential from -0.53 to -0.45 . This shift is most likely due to absorption of haemoglobin on the Hg drop.

Denatured carboxyhaemoglobin

Two methods of denaturation were tried and the results are summarized in Table 2.

It was found that although the thiol groups in the native protein are stable to oxidation those in the denatured protein are not. Unless precautions are taken to avoid oxidation, considerable errors may result. The procedure adopted in all these experiments was as follows. The supporting electrolyte was made up in the first instance without the denaturing agent and the native HbCO added, followed by a slight excess of PhHgOH.

Fig. 3. Current-voltage curves of PhHgOH in the presence of native HbCO; (a) 5 ml. of 7.4×10^{-5} M-HbCO in 0.15 M-KCl, 0.02 M- $\text{Na}_2\text{B}_4\text{O}_7$, 0.08 M- Na_2SO_3 ; (b) + 0.13 ml. of 10 mM-PhHgOH. This corresponds to point A in Fig. 4.

Fig. 4. Amperometric titration of native HbCO with 10 mM-PhHgOH at -0.5 v; 5 ml. of 7.4×10^{-5} M-HbCO in 0.15 M-KCl, 0.02 M- $\text{Na}_2\text{B}_4\text{O}_7$, 0.08 M- Na_2SO_3 .

Table 2. Titration of denatured carboxyhaemoglobin with mercuric chloride and phenylmercuric hydroxide

Denaturing agent	Concn. of Na_2SO_3 in supporting electrolyte (M)	Hg^{2+} ion bound/mol. of HbCO	PhHgOH bound/mol. of HbCO
SDS (pH ≈ 9)	0.04-0.1	3	6.0
Acetic acid (pH ≈ 3)	0.04-0.1	3.0	7.2-6.5

Nitrogen was bubbled through to mix the solution and remove O_2 , and the denaturing agent was then added. Thiol groups liberated by denaturation react immediately with the $PhHgOH$ and oxidation is avoided. The residual current was determined in a separate experiment.

Denaturation with sodium dodecyl sulphate. The molar concentration of SDS used was 150–200 times that of the $HbCO$. The protein was buffered at pH 9 and the titration carried out at the same pH.

(i) Titrations with $PhHgOH$: The titration conditions used were the same as with the native

protein, namely pH 9 and -0.5 v. The results were constant over a range of sulphite concentration of 0.04 – 0.1 M: in the absence of sulphite they were 6 – 8% higher. A figure of 6.0 ± 0.1 thiol groups/mol. was obtained for all three samples of $HbCO$.

(ii) Titrations with $HgCl_2$: The normal practice of having a layer of $CHCl_3$ at the bottom of the cell to prevent reaction between Hg and Hg^{2+} ion could not be followed because of the presence of SDS. Instead, a cell was used with a tube of 2 mm. diameter in the bottom into which the drops of Hg fell. A layer of saturated KCl above the Hg prevented the protein solution from entering the tube. In the presence of SDS and $HbCO$ the current-voltage curve of Hg^{2+} ion shows the same anomalous dip as it does with native $HbCO$, and with SDS, $HbCO$ and caprylic alcohol, the curve of Hg^{2+} ion shows two separate waves. These current-voltage curves, together with a normal one, are reproduced in Fig. 5. Titrations were tried at -0.2 v without caprylic alcohol and at -1.0 v with it. In both the current was proportional to only a small range of Hg^{2+} ion concentration and the titration curves were of the shape shown in Fig. 6. The accuracy of such titrations is limited but they serve to show that three atoms of mercury, and not six, combine with one mol. of $HbCO$ denatured with SDS.

Denaturation with acid. Sufficient m -acetic acid was added to bring the protein solution to about pH 3. The solutions were allowed to stand at this pH for 5 min. before titration. The $HbCO$ is precipitated by this treatment and caprylic alcohol had to be used as the anti-frothing agent in order to keep the precipitate finely dispersed. If silicone grease is used the precipitate forms a clot on the grease.

(i) Titrations with $PhHgOH$: as satisfactory titrations could not be obtained at pH 3 they were carried out at pH 9 and -0.5 v. After denaturation was complete sufficient 0.1 M- $Na_2B_4O_7$ and 0.5 N- $NaOH$ were added, together with the required amount of sulphite, to bring to pH 9. The excess of reagent lines were straight but the titres continued to decrease with increasing sulphite concentration. This is an indication that some $PhHgOH$ is being bound by groups other than thiol. The lowest titre obtained was 6.5 mol. of $PhHgOH$ /mol. of $HbCO$ and it must be assumed that this figure is high.

(ii) Titrations with $HgCl_2$: the protein was denatured at pH 3 and the titrations were carried out at this pH and -0.25 v. The relevant current-voltage curves are given in Fig. 7. The large wave starting at -0.35 v is due to the presence of HSO_3^- ion. Normal titration curves were obtained. The titres were constant over a range of sulphite concentration (0.04 – 0.1 M) and three atoms of Hg

Fig. 5. Current-voltage curves of Hg^{2+} ion under various conditions. (a) Residual-current curve of buffer of composition 0.15 M- KCl , 0.02 M- $Na_2B_4O_7$, 0.04 M- Na_2SO_3 . (b) Buffer + 10^{-4} M- $HgCl_2$ showing normal Hg^{2+} ion curve. (c) Buffer + 4×10^{-5} M- $HbCO$ denatured with 0.4% SDS + 3×10^{-4} M- $HgCl_2$ (excess = 6×10^{-5} M), showing the anomalous dip in the Hg^{2+} ion curve. A similar effect is obtained with native $HbCO$ in the presence of caprylic alcohol. (d) Buffer + 4×10^{-5} M- $HbCO$ denatured with 0.4% SDS + 2.8×10^{-4} M- $HgCl_2$ (excess = 4×10^{-5} M) + 2 drops of caprylic alcohol. Instead of a single curve there are now two waves with $E_{1/2}$ of -0.7 v and -1.3 v respectively.

Fig. 6. Amperometric titration of $HbCO$, denatured with SDS with 10 mM- $HgCl_2$ at -0.2 v; 5 ml. of 4.7×10^{-5} M- $HbCO$ in 0.4% SDS, 0.15 M- KCl , 0.02 M- $Na_2B_4O_7$, 0.04 M- Na_2SO_3 .

were bound/mol. of HbCO. This means that each Hg atom binds two thiol groups. It confirms the figure of six thiol groups/mol. found with SDS-denatured HbCO and is further evidence that the figure of 6.5 obtained with PhHgOH on acid-denatured HbCO is high.

Test for disulphide bonds. Sodium sulphite was used in the supporting electrolyte for the titrations both to expedite the removal of O_2 and to prevent binding of PhHgOH by groups other than thiol. If any disulphide bonds were present they would be expected to react with sodium sulphite according to the equation

resulting in an increased thiol titre in the presence of sulphite. In fact, lower titres were always obtained in the presence of sulphite than in its absence. Sodium thiosulphate can also be used to prevent non-thiol binding of PhHgOH although it does not help to remove O_2 . Unlike sodium sulphite it does not react with the disulphide bond of cystine (R. Cecil, unpublished work). If thiosulphate is substituted for sulphite in the titrations described, then, apart from the longer time taken to

remove O_2 , the same results are obtained. It may be assumed from these two lines of evidence that there are no disulphide bonds in the HbCO that react with sulphite.

Cecil & Loening (1957) have shown that one of the disulphide bonds in insulin does not react with sulphite alone but does react with a mixture of sulphite and PhHgOH at pH 9. Accordingly, a solution of HbCO denatured with SDS as before, containing sulphite (0.04M) and twice the amount of PhHgOH required to react with the thiol groups present, was incubated for 30 min. at 25°. The remaining excess of PhHgOH was then back-titrated with cysteine at -0.5v. The amount of PhHgOH that had reacted with the HbCO corresponded with the known thiol content and there was no evidence of disulphide reactivity.

Titration of 2:3-dimercaptopropanol with silver nitrate in the presence of phenylmercuric hydroxide. Ingram (1955) used a mercurial, *p*-carboxyphenylmercuric chloride, to block the thiol groups of haemoglobin against titration with $AgNO_3$. He suggested that 1 mol. of mercurial, although it can combine only with one thiol group, can block two such groups against titration with $AgNO_3$ provided that they are in close proximity.

An aqueous solution of 2:3-dimercaptopropanol (BAL) was titrated potentiometrically at pH 2 with $AgNO_3$ (Cecil & McPhee, 1955). Half an equivalent of PhHgOH was added to another sample and it was found that the remaining half equivalent of free thiol could be titrated with $AgNO_3$.

Coal gas as a source of carbon monoxide. It is common practice to use coal gas for preparing HbCO. This practice was originally adopted in the present work. It was then found that solutions of HbCO thus prepared contained some low-molecular-weight thiol which was partly liberated in the presence of a neutral salt. When separated by dialysis against KCl solution this thiol was found to have an ultraviolet-absorption spectrum closely resembling that of ergothioneine, although it did not give the Hunter (1928) diazo test. It was only later that a thiol with the same properties was isolated from Oxford coal gas. Thereafter pure CO was used.

DISCUSSION

It was suggested in the introduction that previous results could best be explained on the assumption that the reaction of mercuric chloride with haemoglobin results in a compound of the type $Prot \cdot S \cdot HgCl$ rather than the dimercaptide $(Prot \cdot S)_2Hg$. This is confirmed by the finding that native carboxyhaemoglobin gives the same titre (within 3%) with mercuric chloride and phenylmercuric hydroxide. Phenylmercuric hydroxide can combine only with one equivalent of thiol unless, of

Fig. 7. Current-voltage curves of Hg^{2+} ion in the presence of HbCO denatured with acetic acid at pH $\approx$ 3; (a) 5 ml. of $7.5 \times 10^{-5}M$ -HbCO in 0.2M- KNO_3 , 0.4M-acetic acid, 0.04M- Na_2SO_3 + 1 drop of caprylic alcohol; (b) +0.06 ml. of 10 mM- $HgCl_2$.

course, the mercury-carbon bond splits. Although this is a weak bond and can be split by reagents such as bromine, or more slowly by dithizone (Webb, Bhatia, Corwin & Sharp, 1950), there is no evidence that this splitting occurs under the conditions used for titration.

The situation with the denatured protein is different in that the number of molecules of phenylmercuric hydroxide bound is twice the number of mercury atoms bound. Presumably therefore the structure of the denatured protein is sufficiently disorganized for the dimercaptide $(RS)_2Hg$ to be formed with Hg^{2+} ion. The fact that the thiol groups in the denatured protein are easily oxidized, whereas those in the native protein are stable, is another indication of the same effect.

It is interesting to note here that Ingram (1955), using the rotating platinum electrode, found that six atoms of mercury were bound/mol. of carboxyhaemoglobin denatured with SDS. This fits with the assumption that the denatured protein is behaving in the same way as a low-molecular thiol and that Ingram's endpoint corresponds to the compound $(RS)_2Hg_3$.

We have now to consider whether these titrations give a true measure of the number of thiol groups in haemoglobin. If either phenylmercuric hydroxide or Hg^{2+} ion was being bound by groups other than thiol under the conditions used for titration, then:

(i) The amount of such binding, and therefore the apparent titre, would be expected to decrease as the concentration of sulphite, which forms a low-molecular-weight reducible complex with phenylmercuric hydroxide and Hg^{2+} ion, is increased. This occurs in one case, the titration of acid-denatured carboxyhaemoglobin with phenylmercuric hydroxide, when it is known that the results are high. In all the other titrations, however, there is a wide range of sulphite concentration over which constant titres are obtained.

(ii) With the exception of the titration of acid-denatured carboxyhaemoglobin with phenylmercuric hydroxide the agreement between the phenylmercuric hydroxide and mercuric chloride titrations is good. Since it is most unlikely that Hg^{2+} ions and phenylmercuric hydroxide would be bound by non-thiol groups to exactly the same extent, the probability is that such binding is negligibly small in both cases.

In view of this there is little doubt that the figures of four thiol groups/mol. of native haemoglobin and eight thiol groups/mol. of denatured haemoglobin, obtained variously by Ingram (1955), Benesch *et al.* (1955), Hommes *et al.* (1956) and Murayama (1956), are too high. It has been shown by various techniques (Kunkel & Wallenius, 1955; Morrison & Cook, 1955; Allison & Tombs, 1957) that normal adult haemoglobin as usually pre-

pared is not homogeneous but consists of one major and two minor components. Hence an integral number of thiol groups/mol. would not necessarily be expected. Stein, Kunkel, Cole, Speakman & Moore (1957) have found four to five half-cysteine residues/mol. for the major component of haemoglobin, which suggests that one or both of the minor components may have a thiol content greater than six groups/mol.

An attempt was made to analyse the different fractions obtained by the method of Allison & Tombs (1957). However, these fractions gave anomalous titrations for reasons which have yet to be established. It was thought to be worth while publishing the results on unfractionated haemoglobin as most of the earlier work has been done with this material.

The figure of 2.2 thiol groups/mol. of native haemoglobin could represent the mean for the different components. On the other hand, the reason for the difference in reactivity between native and denatured protein is not known. It may be that certain thiol groups in the native protein react whereas the others do not. Alternatively, there may be an equilibrium in which all the thiol groups react to some extent. Thus even if there is an integral number of total thiol groups in a single protein component there will not necessarily be an integral number of reactive thiol groups in the native protein. This subject is being investigated further.

Ingram (1955) postulated that native human haemoglobin has four thiol groups/mol., arranged in two pairs. He also suggested that if one thiol group in either pair was combined with a mercurial (*p*-carboxyphenylmercuric chloride) then the other thiol group of the pair was prevented from reacting with Ag^+ ion. It has been shown in this work that there are in fact only two reactive thiol groups/mol. of native haemoglobin, and there is no evidence to suggest that they are in close proximity. Nevertheless the question of whether or not one mol. of mercurial can block two closely spaced thiol groups is an important one, and it was thought worth while trying an experiment with a simple model system. Although model systems do not necessarily reproduce the conditions found in a protein the information obtained is frequently of value in interpreting experiments with proteins.

BAL was chosen as the model because its thiol groups are on adjacent carbon atoms. It reacts with heavy metals to give ring compounds of the type (Stocken & Thompson, 1946)

although free rotation about the C—C bonds is possible in the free thiol. The experiment showed that combination of one thiol group with phenylmercuric hydroxide leaves the other group free to react with Ag^+ ion in the normal way.

The reaction of the mercuric mercaptide of cysteine and other thiols with haemoglobin did not contribute to the analytical results but it is interesting as an example of an exchange reaction. A comparable reaction is the interchange that takes place between different disulphides (Sanger, 1953; Ryle & Sanger, 1954). The mercaptide reaction is rapid and gives the mixed mercaptide and free cysteine, or other thiol, in quantitative yield.

No evidence could be found for the existence of disulphide bonds. This agrees with the findings of other workers (Benesch *et al.* 1955; Stein *et al.* 1957).

SUMMARY

1. In view of discrepancies in earlier work the thiol content of normal adult human haemoglobin has been reinvestigated. Both the native and denatured protein have been titrated amperometrically with mercuric chloride and with phenylmercuric hydroxide.

2. Native carboxyhaemoglobin binds 2.2 Hg^{2+} ions and 2.2 molecules of phenylmercuric hydroxide/molecule. Reasons are given for concluding that the two mercaptides formed are $\text{Prot}\cdot\text{S}\cdot\text{HgCl}$ and $\text{Prot}\cdot\text{S}\cdot\text{HgPh}$. The figure of 2.2 therefore represents the reactive thiol groups/molecule of native protein, as compared with four given by previous workers.

3. Denatured carboxyhaemoglobin binds three Hg^{2+} ions and six molecules of phenylmercuric hydroxide/molecule. In this case the two mercaptides formed are assumed to be $(\text{Prot}\cdot\text{S})_2\text{Hg}$ and $\text{Prot}\cdot\text{S}\cdot\text{HgPh}$. The total number of thiol groups/molecule of carboxyhaemoglobin is therefore six, as compared with eight given by previous workers.

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