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Some Observations on the Reactive Sulphydryl Groups in Haemoglobin

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The SH groups in a protein are generally described as either 'reactive' or 'unreactive'. The reactive SH groups are readily titrated with heavy-metal reagents and have properties similar to cysteine, whereas the unreactive SH groups in the native protein combine slowly with heavy-metal reagents. In a comparative study of the haemoglobins of various mammalian species, Riggs (1959, 1960) found that the number of Hg^{2+} ions which are bound by the deoxygenated haemoglobins is approximately equal to the number of protons liberated during oxygenation, and he suggested that the reactive SH groups may be the oxygen-linked acidic groups. He also found that the number of protons liberated during oxygenation is inversely related to the body weight of the animal. This implies that there is a relation between the reactive SH group content of the haemoglobin and body weight. However, Benesch & Benesch (1960, 1961) showed that the addition of heavy-metal ions to human haemoglobin does not prevent the liberation of protons during oxygenation. They

concluded that the reactive SH groups are not the oxygen-linked acidic groups.

Allison & Cecil (1958) showed that high values for the SH group content of human haemoglobin are obtained unless precautions are taken to prevent the combination of heavy-metal ions with groups other than SH in the protein. They concluded that the SH groups of human haemoglobin can be estimated by amperometric titration with phenylmercuric hydroxide in the presence of sulphite, with the dropping-mercury electrode as the end-point indicator. Cecil & Snow (1962) showed that the rate of combination of heavy-metal reagents with the unreactive SH groups of human haemoglobin was faster in deoxygenated haemoglobin than in carboxyhaemoglobin. Consequently, carboxyhaemoglobin should be used for the estimation of reactive SH groups to reduce the possibility of heavy-metal reagent combining with unreactive SH groups during the course of the titration. For his comparative study of the reactive SH groups Riggs (1959, 1960) used deoxygenated haemoglobin and did not take precautions against the combination of Hg^{2+} ions with groups other than SH. In the light of the results of Allison & Cecil (1958) and

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Cecil & Snow (1962) it seemed possible that the opposing conclusions of Riggs (1959, 1960) and of Benesch & Benesch (1960, 1961) on the relationship between the reactive SH groups and the oxygen-linked acidic groups could be attributed to incorrect estimates of the SH group contents in Riggs's (1959, 1960) comparative study. The numbers of both the reactive and unreactive SH groups in the haemoglobins of several mammals, as estimated by the method of Allison & Cecil (1958) and by using carboxyhaemoglobin, are reported in this paper.

Acid-base titrations have been carried out on human haemoglobin and oxyhaemoglobin in the presence and absence of phenylmercuric hydroxide, as a further check on the relation between the reactive SH groups and the Bohr effect.

EXPERIMENTAL

Haemoglobin. Solutions of oxyhaemoglobin were prepared as described by Allison & Cecil (1958). With the exception of the human haemoglobin used for the acid-base-titration curves, the haemoglobins were converted into carboxyhaemoglobin. The concentrations were estimated by taking ϵ to be 59.6×10^3 for 4 iron atoms at 540 m μ for carboxyhaemoglobin.

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

Apparatus. The polarographic apparatus described by Cecil & Snow (1962) was used for amperometric titrations and current-voltage measurements.

Acid-base-titration curves were made with a Radiometer TTT1 Titrator and SBR2 Recorder. The haemoglobin solutions were saturated with O_2 or deoxygenated by bubbling N_2 through them before titration. Frothing was reduced by coating the walls of the titration vessel with silicone high-vacuum grease.

Measurements were made at room temperature unless otherwise stated.

Analytical methods. The reactive SH groups in the haemoglobins were determined by amperometric titration with phenylmercuric hydroxide in the presence of sodium

sulphite, as described by Allison & Cecil (1958). Sulphite has the added advantage of combining with O_2 and reduces the time needed to deoxygenate the solution. Titration in borate buffer in the absence of sulphite gave slightly higher values for all haemoglobins studied, indicating that there are no disulphide bonds in these haemoglobins. The total number of SH groups in the haemoglobins was determined by titration in the presence of sodium dodecyl sulphate as described by Allison & Cecil (1958).

Unless otherwise stated, the supporting electrolyte consisted of KCl (0.15M), $Na_2B_4O_7$ (0.02M) and Na_2SO_3 (0.08M). The titrations were carried out at a potential of -0.6V versus the saturated calomel electrode.

RESULTS

Sulphydryl group content of the various haemoglobins. The titration values for native and denatured carboxyhaemoglobin with phenylmercuric hydroxide in the presence of sulphite are given in Table 1. The species are listed in order of decreasing body weight and it is clear that there is no correlation between the reactive, unreactive or total SH group content and the weight of the animal. Under these titration conditions the number of reactive SH groups/haemoglobin molecule approximated to either 2 or 4, and the total number of SH groups/haemoglobin molecule ranged from 2 to 10. The ox and sheep haemoglobins did not show any appreciable increase in the number of SH groups when the protein was denatured with sodium dodecyl sulphate.

The same titres were obtained when a supporting electrolyte consisting of ammonium hydroxide (0.2M) and ammonium chloride (0.2M) was substituted for the potassium chloride-sodium tetraborate-sodium sulphite mixture, except for the shrew when the titre increased from 1.6 to 1.9.

Allison & Cecil (1958) found that, in human carboxyhaemoglobin, thiosulphate could be substituted for sulphite as a complexing agent for phenylmercuric hydroxide and the same titre ob-

Table 1. *Sulphydryl group content of the haemoglobins of various mammalian species*

Experimental details are given in the text.

Species	Native protein (SH groups/molecule)		Denatured protein (SH groups/ molecule)
	This investigation	Riggs (1960)	
Ox	2.0	1.93	2.2
Horse	2.1	1.73	4.0
Sheep	4.0	—	4.1
Human	2.2	2.35	6.0
Dog	4.0	2.60	8.1
Rabbit	2.3	1.82, 1.96	4.3
Rat	3.8	—	10.0
Mouse	4.0	3.66	8.0
Shrew (<i>Sorex araneus</i>)	1.6	—	5.9

tained. However lower titres for the reactive SH groups were obtained with some of the species listed in Table 1. For example, the ox-haemoglobin titre decreased from 2.0 to 1.8 in the presence of 0.02 M-sodium thiosulphate, and the dog-haemoglobin titre decreased from 4.0 to 1.8. The remaining reactive SH groups in dog carboxyhaemoglobin reacted slowly with the phenylmercuric hydroxide-thiosulphate complex. It therefore seemed likely that thiosulphate interferes with the reaction between the reactive SH groups and phenylmercuric hydroxide in some haemoglobins.

Titration of human carboxyhaemoglobin with silver nitrate in the presence of thiosulphate. Human carboxyhaemoglobin was titrated with silver nitrate containing sodium thiosulphate. The effect of various concentrations of thiosulphate on the end point is shown in Fig. 1. The titre fell from 10 in the absence of thiosulphate to zero with a $S_2O_3^{2-}$ ion: Ag^+ ion ratio of 100:1. The large end point (approx. 10 equiv. of Ag^+ ion/mole of protein) observed in the absence of thiosulphate is probably due to additional Ag^+ ions complexed with protein mercaptide groups (Cecil & McPhee, 1959).

When 0.3 mm-cysteine hydrochloride was titrated at -0.4 v with a mixture containing a $S_2O_3^{2-}$ ion: Ag^+ ion ratio of 1000:1 under the same conditions, an end point of 1 equiv. of Ag^+ ion/mole of cysteine was obtained, indicating that thiosulphate does not affect the formation of silver cysteine mercaptide.

Effect of phenylmercuric hydroxide on the acid-base titration curves of human haemoglobin. Dissociation of the SH groups in a protein:

cannot occur when the SH groups have formed mercaptides with phenylmercuric hydroxide:

No difference in the acid-base titration curve of the protein treated with phenylmercuric hydroxide compared with that of the untreated protein would therefore be expected at pH values below that at which the SH groups have a measurable degree of dissociation. At more alkaline pH values a maximum difference in the curves of 2.2 equiv. of H^+ ion/mole of protein should be observed for human haemoglobin. The apparent dissociation constant of the SH groups, pK'_{SH} , is equivalent to the pH at which the degree of dissociation is 0.5, corresponding to a difference in the curves of 1.1 equiv. of H^+ ion/mole of human haemoglobin. However, no significant difference in the acid-base titration curves of either deoxygenated haemoglobin or oxyhaemoglobin in the presence and absence of 2.2 moles of phenylmercuric hydroxide/mole of haemoglobin was observed in the pH range

Fig. 1. Effect of thiosulphate on the amperometric titration of human carboxyhaemoglobin with silver nitrate. The solution consisted of 2 ml. of 0.13 mM-carboxyhaemoglobin in sodium tetraborate (0.05 M) and sodium nitrate (0.1 M), pH 9, at 20°. It was titrated with 10 mM-silver nitrate containing various amounts of sodium thiosulphate as follows: O, no thiosulphate; the end point corresponds to approx. 10 equiv. of Ag^+ ion/mole of protein. x, $S_2O_3^{2-}$ ion: Ag^+ ion ratio of 3:1; the end point corresponds to approx. 2.2 equiv. of Ag^+ ion/mole of protein. Δ , $S_2O_3^{2-}$ ion: Ag^+ ion ratio of 10:1; the end point corresponds to approx. 1 equiv. of Ag^+ ion/mole of protein. $\bullet$, $S_2O_3^{2-}$ ion: Ag^+ ion ratio of 100:1. The solutions were deoxygenated with nitrogen, and the currents were measured at -0.9 v.

Fig. 2. Difference in the acid-base titration curves of oxyhaemoglobin and deoxygenated haemoglobin (Bohr effect) in the presence and absence of 2.2 moles of phenylmercuric hydroxide/mole of protein. $\bullet$, (Equiv. of OH^- ion/mole of oxyhaemoglobin) - (equiv. of OH^- ion/mole of deoxygenated haemoglobin); $\circ$, as $\bullet$, but in the presence of 2.2 moles of phenylmercuric hydroxide/mole of protein. The acid-base titration curves were determined by titrating 5 ml. of 0.5 mM-haemoglobin in 0.7 M-potassium chloride with 0.15 N-hydrochloric acid or potassium hydroxide.

studied (pH 5–9.6). If the pK'_{SH} of the reactive SH groups were outside this pH range at, say, pH 10, the degree of dissociation expected at pH 9.6 would be 0.3. This would result in a difference of 0.66 equiv. of H^+ ion/mole of protein in the acid–base–titration curves of human haemoglobin in the presence and absence of phenylmercuric hydroxide. As no significant difference in the curves was observed it appears that the pK'_{SH} of the reactive SH groups is greater than 10.

The differences in the number of H^+ or OH^- ions/protein molecule required to bring oxyhaemoglobin and deoxygenated haemoglobin to a given pH are plotted in Fig. 2. Also shown are the results when the oxyhaemoglobin and deoxygenated haemoglobin were first treated with 2.2 moles of phenylmercuric hydroxide/mole of protein. There are minor differences in the curves in the acidic range; nevertheless the dissociation of protons during oxygenation occurs in the presence of phenylmercuric hydroxide. This is in agreement with the finding of Benesch & Benesch (1960, 1961) that the Bohr effect is observed after the reactive SH groups have combined with heavy-metal ions.

DISCUSSION

A potential source of error in relating the number of reactive SH groups in haemoglobin to the amount of heavy-metal ions bound by the protein is the combination of heavy-metal ions with groups other than SH in the protein. This error can be avoided by the use of sulphite (Allison & Cecil, 1958). Under some conditions the rate of reaction of heavy-metal ions with the unreactive SH groups can be rapid (Cecil & Snow, 1962). An example of this is the reaction between human deoxygenated haemoglobin and Hg^{2+} ions. Errors from this cause can be reduced by titrating carboxyhaemoglobin with phenylmercuric hydroxide in the presence of sulphite, when the rate of reaction with the unreactive SH groups is much slower.

Riggs (1959, 1960) estimated the number of reactive SH groups by titrating deoxygenated haemoglobin with Hg^{2+} ions, whereas in this investigation carboxyhaemoglobin was titrated with phenylmercuric hydroxide in the presence of sulphite. It might therefore be expected that Riggs's values would be higher than those determined under the conditions used in this investigation. Table 1 shows that Riggs's values are in fact lower, except for human haemoglobin. The explanation for Riggs's generally lower results could be the time taken to complete a titration. Riggs used the rotating platinum electrode as the end-point indicator and completed a titration in 1 min., but noted that more Hg^{2+} ions were removed from

solution after this time. In this investigation, the use of the dropping-mercury electrode increased the titration time to 10–15 min. It seems likely therefore that Riggs's low results can be attributed to incomplete reaction of all the reactive SH groups. This is of interest since it implies that some of the 'reactive' SH groups in many of the haemoglobins investigated react more slowly than low-molecular-weight thiols.

Table 1 shows that there is no correlation between the number of reactive, unreactive or total SH groups and the weight of the animal. Further, the number of reactive SH groups/haemoglobin molecule in the species examined approximates to either 2 or 4. Although the reactive SH groups are not concerned with the Bohr effect they do appear to be concerned, directly or indirectly, with the oxygenation process. For example, Riggs & Wolbach (1956) have shown that the haem–haem interaction of horse haemoglobin is decreased when the reactive SH groups are combined with heavy-metal ions.

In addition to the lower rate of reaction with heavy-metal ions, the behaviour of some of the reactive SH groups in haemoglobin differs from cysteine in two other respects: (a) The formation of $Ag(S_2O_3)_2^{3-}$ ions interferes with the reaction between Ag^+ ions and the reactive SH groups of human carboxyhaemoglobin, but not with cysteine. Moreover, in some haemoglobins, thiosulphate interferes with the reaction with $C_6H_5Hg^+$ and Hg^{2+} ions. At first sight this suggests that the protein mercaptide dissociation constant is higher than that of silver cysteine mercaptide. Allison & Cecil (1958), however, showed that an exchange reaction occurs between mercuric dicycysteine mercaptide and the reactive SH groups of human haemoglobin to give the mixed dimercaptide and free cysteine. The lower reactive SH titre observed in the presence of thiosulphate may therefore be due to a specific effect of thiosulphate and not to a higher dissociation constant of the mercaptide. (b) The acid–base–titration curves suggest that the reactive SH groups of human haemoglobin are not ionized below pH 10, whereas the pK'_{SH} of cysteine is 8.5 (Benesch & Benesch, 1955).

The explanation of the difference in behaviour of the reactive SH groups of haemoglobin and cysteine must await more detailed knowledge of the environment of the SH groups within the protein molecule.

SUMMARY

1. A comparative study of the haemoglobins of several mammalian species has shown no correlation between the reactive, unreactive, or total SH group content and the weight of the animal.

2. The number of reactive SH groups/molecule in the haemoglobins studied approximate either to 2 or to 4.

3. The behaviour of the reactive SH groups differs from that of cysteine in three ways: (a) the rate of reaction of heavy-metal ions with the reactive SH groups is slower than with cysteine; (b) thiosulphate interferes with the reaction between Ag^+ ions and the reactive SH groups of human haemoglobin, but not with cysteine; (c) the acid-base-titration curves of human haemoglobin in the presence and absence of phenylmercuric hydroxide suggest that the reactive SH groups are not ionized at pH values less than 10.

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Acute Toxic Liver Injury

NICOTINAMIDE-ADENINE DINUCLEOTIDE-PYROPHOSPHORYLASE ACTIVITY OF NUCLEI ISOLATED FROM RAT LIVER IN HELIOTRINE AND IN DIMETHYLNITROSAMINE POISONING

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Manometric studies on homogenate and mitochondrial preparations from the livers of rats poisoned with heliotrine (Christie, 1958) and with dimethylnitrosamine (Bailie & Christie, 1959) showed that an apparent deficiency of NAD developed during the course of the toxic process. Decreased concentrations of both NAD and NADP have since been demonstrated in homogenates and in the mitochondrial and supernatant fractions from livers of rats treated with heliotrine for 17–20 hr. (Christie, Le Page & Bailie, 1961; Christie & Le Page, 1962*a*) and with dimethylnitrosamine for 14 hr. (Bailie, 1959; M. J. Bailie, G. S. Christie & R. N. Le Page, unpublished work). Also the capacity of the liver to synthesize NAD *in vivo* in 'response' to a single large dose of nicotinamide appeared to decrease with increasing duration of pretreatment of the rats with heliotrine (Christie & Le Page, 1962*b*); the possibility remained, however, that the apparent decrease of synthesis was not real but arose from losses of liver coenzymes which were known to be occurring. It seemed that measurements of the NAD-pyrophosphorylase activity of nuclei isolated from the livers of rats poisoned with heliotrine or with dimethylnitros-

amine might help to show whether a real interference with NAD synthesis occurred.

NAD pyrophosphorylase (Kornberg, 1950) converts NMN into NAD and appears to be indistinguishable from the enzyme which converts nicotinic acid mononucleotide into nicotinic acid-adenine dinucleotide in the three-step pathway of NAD synthesis proposed by Preiss & Handler (1958*a, b*). The enzyme is localized in the nucleus (Hogebom & Schneider, 1952). Low activities of the enzyme have been observed in nuclei isolated from certain rapidly-growing tissues, e.g. spontaneous and induced tumours, foetal-mouse liver and regenerating rat liver (Branster & Morton, 1956; Morton, 1958; Stirpe & Aldridge, 1961); but 'little change was found' in preparations from livers of rats treated with the liver poisons carbon tetrachloride, dimethylnitrosamine or ethionine (Stirpe & Aldridge, 1961). However, Stirpe & Aldridge (1961) observed a significant fall of activity with beryllium.

The observations presented below show that NAD-pyrophosphorylase activity was significantly decreased 18 hr. after administration of heliotrine, but not 19 hr. after administration of dimethylnitrosamine.