particular pH owing to the screening of the haematin iron atom from the influence of the various ionizing groups. On this basis a larger critical I would signify that a larger concentration of salt ions is required for complete screening, and hence that there is a stronger interaction between the iron and the ionizing groups in MetHb.

In conclusion, although the mechanism may be different it may be noted that the Bohr effect, which is due to interaction between the iron and the specific haem-linked ionizing group, is greater in haemoglobin than in myoglobin.

SUMMARY

1. The ionization constant of the iron-bound water molecule in acidic metmyoglobin has been measured from about pH 7 to 10 at eight ionic strengths of buffer solution ranging from 0.40 to 0.002. The values confirm the conclusion suggested by the measurements reported in paper 1 (George & Hanania, 1952) that the ionization constant is independent of pH only in the presence of appreciable concentrations of neutral salt.

2. At $I = 0.40$ at 20° , pK' is 8.98 throughout the pH range 7.5–9.8, but at $I < 0.02$, pK' decreases as pH is decreased below 8.6, and it increases as pH is increased above 9.3.

3. The extrapolation of pK' values plotted against $\sqrt{I}/(1 + \sqrt{I})$ to zero ionic strength gives pK_0 values of 8.81, 9.07 and 9.25 at pH 7.8, between pH 8.6 and 9.3 and at pH 9.8 respectively.

4. It is suggested that this variation of the free energy of ionization with pH is due to an unspecific interaction between charged groups on the protein

moiety and the haematin iron atom, which is observable only at low I because of the screening effect of neutral salt ions. In contrast, the effect of a specific haem-linked ionizing group is observable at all ionic strengths.

5. The limiting slopes of the pK' plots are approximately +0.5, -2.5 and -4.5 at pH 7.8, between pH 8.6 and 9.3 and at pH 9.8, which, on the basis of a simple Debye-Hückel model may be attributed to the effective charge of the haematin iron atom changing upon ionization from +1 to 0, from -2 to -3 and from -4 to -5 respectively.

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The Sulphydryl Groups of Sick-cell Haemoglobin

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In patients with homozygous sickle-cell anaemia the red blood corpuscles become distorted into sickle shapes at low oxygen pressure, apparently due to the formation of liquid crystals of reduced haemoglobin. Such liquid crystals can also be formed *in vitro* by reduction of purified solutions of sickle-cell haemoglobin, a phenomenon which is apparently due to the abnormally low solubility of sickle-cell haemoglobin in the reduced state (Perutz & Mitchison, 1950; Perutz, Liquori & Eirich, 1951). The formation of liquid crystals can be inhibited

and the solubility restored to normal by reaction with SH group inhibitors such as silver and mercury ions and mercury derivatives (Thomas & Stetson, 1948; Ingbar & Kass, 1951). Presumably the sulphydryl groups are closely involved in the 'sickling' process. Ingbar & Kass (1951) made a study of the number of available SH groups with amperometric silver nitrate titrations and made the significant observation that there were apparently two SH groups in normal but three in sickle-cell haemoglobin (haemoglobin A and S respectively).

Some years later Ingram (1955) studied the SH groups of several species of haemoglobins, including human, by a similar technique and found native human haemoglobin A to have not two, but four, available sulphhydryl groups, a number which increased to eight on denaturation with sodium dodecyl sulphate. In view of the contradiction between the two sets of results it seemed desirable to repeat the measurements of Ingbar & Kass on sickle-cell haemoglobin. Furthermore, although Benesch, Lardy & Benesch (1955) found eight SH groups after denaturation with 8M urea, in agreement with my own results, Hommes, Santema-Drinkwaard & Huisman (1956) found eight SH groups in the native haemoglobins A and S and an uptake of 5.4 and 7.0 molecules of HgCl_2 respectively per native haemoglobin molecule, whereas the corresponding numbers found by myself had been four SH groups and 2 molecules of HgCl_2 in haemoglobin A. Closely related methods were used by all the different investigators. As this paper shows, I was able to confirm my previous results.

Murayama (1956) has titrated deoxygenated haemoglobins A and S at 0° and found four SH

groups, in agreement with the values reported in this paper for oxyhaemoglobins A and S at room temperature. However, at 38° he finds a difference between the two proteins, namely three and two SH groups per molecule of haemoglobin A and S respectively, i.e. results which are the exact opposite to those reported by Ingbar & Kass (1951).

METHODS AND RESULTS

The methods previously described were used with only minor modifications (Ingram, 1955).

Mercurimetric titrations were first performed in 5 ml. of solution with a rotating Pt-wire electrode in 0.05M- NH_4NO_3 and 0.1M- NH_3 soln., $\text{Hg}(\text{NO}_3)_2$ being the titrating agent. The haemoglobin solutions were prepared as previously described and dialysed as indicated in Table 1, which gives the results obtained. Fig. 1 shows examples of the Hg-titration plots. Such end points leave no room for uncertainty about the number of atoms of Hg bound by a molecule of protein, whether native or denatured. No difference could be detected between haemoglobins A and S.

Argentometric titrations were next undertaken, a normal, clean Pt-wire electrode being used. The supporting electrolyte was again 0.05M- $\text{NH}_4(\text{NO}_3)$ with NH_3 of the strength indicated in Table 1. These titrations gave lower results

Table 1. *Reactions of metal ions with haemoglobin*

<i>Mercury titrations</i>				Hg bound/4Fe	
	Dialysis	Storage time* (days)	NH ₃ concn. (M)	Native	Sodium dodecyl sulphate denatured (400 moles/4Fe)
Haemoglobin A	Saline	3	0.10	2.2	5.9
	Saline	19	0.10	2.1	5.6
Haemoglobin S	Saline	10	0.10	2.1	6.1
<i>Silver titrations, normal platinum electrode</i>				Ag bound/4Fe	
	Dialysis	Storage time (days)	NH ₃ concn. (M)	Native	Sodium dodecyl sulphate denatured (400 moles/4Fe)
Haemoglobin A	Saline	6	0.03	2.9	—
			0.10	2.4	—
			0.25	1.8	—
Haemoglobin S	Saline	10	0.03	2.8	—
			0.25	1.8	—
			0.10	—	8.2
<i>Silver titrations, mercury-treated platinum electrode</i>				Ag bound/4Fe	
	Dialysis	Storage time (days)	NH ₃ concn. (M)	1st end point	2nd end point
Haemoglobin A	Saline	2	0.03	4.2	—†
	Saline	4	0.03	3.0	8.0
	Saline	7	0.03	2.8	7.9
	Water	2	0.03	3.9	—†
	Water	4	0.03	2.9	7.7
Haemoglobin S	Saline	1	0.03	3.8	8.4

* Days counted from thawing the washed and frozen red cells.

† The second end point was not suspected when these titrations were performed, although it was probably present.

than those previously reported, but the samples of both proteins happened to be considerably more than 2 days old. As is shown in the table the number of available SH groups decreased rapidly when the sample was stored, even at 4°. Again normal and sickle-cell haemoglobins gave identical results, showing a similar decrease of detectable SH with rising NH_3 concentration, as reported earlier (Ingram, 1955).

Fig. 1. Titration with mercuric nitrate of (a) native haemoglobin, and (b) haemoglobin denatured with sodium dodecyl sulphate.

Fig. 2. Titration with silver nitrate of native haemoglobin S. The electrode was treated with ammoniacal mercuric nitrate solution before use.

The finding of eight SH groups in the sickle-cell haemoglobin denatured with sodium dodecyl sulphate agrees with the number found previously for the normal protein.

Hommes *et al.* (1956) had found it necessary to pretreat their electrode by immersing it in an ammoniacal HgCl_2 solution. Therefore for the final set of titrations a rotating platinum-wire electrode was used which had previously been employed for $\text{Hg}(\text{NO}_3)_2$ titrations in dilute NH_3 soln. Such a system did indeed give different titration plots with native haemoglobin when AgNO_3 was used; it can be seen that two end points (Fig. 2) were obtained. The table shows that the first end point of four SH groups corresponds very closely to the number of SH groups found in the native protein with the untreated platinum electrode. The second break in the titration curve occurs at $8\text{Ag}:4\text{Fe}$, the number found previously in the denatured haemoglobin. Presumably this is the end point observed by the Dutch workers with their mercury-treated electrode.

In the present experiments fresh sickle-cell haemoglobin has given values for the SH groups which are almost identical with those for the normal protein, but both human haemoglobins show a pronounced storage effect. Frozen solution also deteriorates, but frozen red cells do not, perhaps because the presence of glutathione in the latter has a stabilizing effect. Horse haemoglobin, on the other hand, is quite stable for at least a week at 4°. The change is unlikely to be due simply to the formation of disulphide bonds since the older samples still show eight SH groups when denatured.

DISCUSSION

It has been known since 1949 (Pauling, Itano, Singer & Wells, 1949; Scheinberg, Harris & Spitzer, 1954) that there is a difference in the electrophoretic mobilities, and therefore in the number of charged groups, of normal human and sickle-cell haemoglobin. The only other definite evidence of a chemical difference between the proteins is a change in the amino acid sequence of one small portion of a polypeptide chain (Ingram, 1956).

The most important though disappointing result of the present titration experiments is that the numbers of SH groups in the native and denatured forms of normal and sickle-cell haemoglobin are the same. Shortage of material prevented further examination of the sulphydryl groups of the abnormal haemoglobin by the blocking technique with mercury derivatives (Ingram, 1955). The present results leave no doubt that haemoglobin S shows the same pattern of heavy-metal uptake as normal haemoglobin. The four available SH groups of the native haemoglobin S combine with four silver atoms, but only two mercury atoms. On denaturation with sodium dodecyl sulphate another four SH groups are revealed, making a total of eight, whilst at the same time four additional mercury atoms are bound, making six in all. This similarity in behaviour suggests strongly that the arrangement of these groups, as well as their numbers, is the same in the two haemoglobins, i.e.

two equivalent clusters of three SH groups and a further two SH groups in separate but equivalent positions.

My results agree with those of Murayama obtained at 0°, but not with those of Huisman and his collaborators, except that they also were unable to find a difference between the two haemoglobins. Their figures for the silver and mercury uptake of native haemoglobin A and S are very close indeed to those obtained in the present, and previous, experiments with samples fully denatured by sodium dodecyl sulphate. Their method of preparing the haemoglobin is a different and perhaps a harsher one than the method used in this work; this might explain the high uptake of heavy metal without previous denaturation. With respect to the first of the two breaks in the titration plot obtained with a mercury-coated electrode, it is possible that rather small changes in the titration conditions might make it almost invisible.

SUMMARY

1. The numbers of sulphydryl groups in native and sodium dodecyl sulphate-denatured sickle-cell haemoglobin are the same as in the normal haemoglobin. Their configuration is probably also the same.

2. Two kinds of sulphydryl groups, which have different affinities for silver, can be estimated by amperometric titration with silver nitrate at the

mercury-treated platinum electrode estimate. The groups correspond apparently to the sulphydryl groups available to silver at the untreated electrode in native and in denatured human haemoglobin.

3. Storage at 4° leads to a loss in sulphydryl groups available in the native protein.

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The Oxidation of Tryptamine to 3-Indolylacetaldehyde by Plant Amine Oxidase

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3-Indolylacetic acid (IAA) is believed to be a principal auxin of higher plants. There is much evidence that it is formed in plants from tryptophan but the metabolic route is not yet established. It has been suggested that the route is by way of tryptamine and 3-indolylacetaldehyde (IAc) or 3-indolylpyruvic acid and IAc. Two oxidases, or groups of oxidases, catalysing the oxidation of amines have been found in higher plants. The presence of a monoamine oxidase was shown by Werle & Roewer (1950, 1952), and that of a histaminase or diamine oxidase by Werle & Zabel (1948) and Werle & Pechmann (1949). Kenten & Mann (1952a) found that the diamine oxidase

catalysed the oxidation of both mono- and diamines and preferred the name plant amine oxidase. It was shown that the oxidation of tryptamine is catalysed by the enzyme and this reaction, followed by the action of an aldehyde oxidase on the product, was suggested as a possible mechanism of formation of IAA in higher plants. Subsequent attempts to prove that IAc is the product of the oxidation of tryptamine catalysed by plant amine oxidase, with the relatively crude preparations of the enzyme described by Kenten & Mann (1952a), were unsuccessful. In these experiments the observed oxygen uptake was much greater than that required for oxidation of tryptamine to the corresponding