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The SH-Group Content of Human Foetal and Sickle-Cell Haemoglobins

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In a previous paper (Allison & Cecil, 1958) it was shown that the values obtained by various workers for the SH-group content of normal adult human haemoglobin (Hb A) by amperometric titration with silver nitrate were too high. Moreover, the high values obtained with silver nitrate had led to an incorrect interpretation of the results obtained with mercuric chloride (the interpretation depends on a knowledge of which of the possible mercaptides has been formed). The matter was resolved by using a univalent organic mercurial, phenyl mercuric hydroxide, as the titrating agent.

In this paper estimations of the SH content of human foetal haemoglobin (Hb F) and sickle-cell haemoglobin (Hb S) are reported. In the light of the results obtained by Allison & Cecil (1958) with Hb A it was considered sufficient to use only the method of amperometric titration with phenyl mercuric hydroxide.

Hommès, Santema-Drinkwaard & Huisman (1956) found six half-cysteine residues per mole of Hb F by amino acid analysis. Hommès, Dozy & Huisman (1958), using amperometric titration with silver nitrate, concluded that there are four titratable SH groups in both the native and denatured protein. They also found that after treatment with thioglycolic acid, the protein gave two components by paper electrophoresis, whereas the untreated protein gave only one. They interpreted this observation as meaning that the protein consists of two peptide chains joined by one disulphide

bond and that this was reduced by the thioglycolic acid.

When blood from patients suffering from homozygous sickle-cell anaemia is deoxygenated the haemoglobin forms liquid crystals within the erythrocytes and gives them the characteristic sickle shape (Pauling, Itano, Singer & Wells, 1949; Perutz & Mitchison, 1950; Perutz, Liquori & Eirich, 1951). It has been shown (Thomas & Stetson, 1948; Ingbar & Kass, 1951; Riggs, 1953) that the addition of reagents that react with SH groups prevents this behaviour. Similarly, Allison (1957) found that the increase in viscosity associated with deoxygenation of solutions of Hb S is prevented by the addition of 2 moles of *p*-chloromercuribenzoate per mole. This effect of SH-group reagents has given rise to the idea that the difference in properties of Hb A and Hb S might be due to differences in the SH-group content.

Ingbar & Kass (1951) found three SH groups per mole of native Hb S by amperometric silver titration as compared with two for Hb A. Ingram (1957a) found that four silver atoms and 2.2 mercury atoms were bound per mole of native protein, the same result as he had previously obtained for Hb A (Ingram, 1955). Hommès *et al.* (1956) found that denatured Hb S and Hb A both bound eight atoms of silver per mole. Murayama (1957) reported that native Hb S bound four atoms of silver per mole at 0° and 1.9 at 38° but that 2.9 moles of *p*-chloromercuribenzoate were bound at both temperatures.

EXPERIMENTAL

Preparation of materials

Foetal haemoglobin (Hb F). Solutions of carboxyhaemoglobin were prepared from six samples of human umbilical-cord blood by the method used by Allison & Cecil (1958). The proportions of Hb F and Hb A in these samples were determined in two ways: (i) by measuring the rate of denaturation in alkaline solution (Jonxis, 1949); (ii) by measuring the shift in the tryptophan fine-structure band (Beaven, Hoch & Holiday, 1951). We are indebted to Dr G. H. Beaven for these determinations. As there is no reason for favouring either of these methods mean values were used.

Sickle-cell haemoglobin (Hb S). Samples of blood were obtained from two patients suffering from sickle-cell anaemia. Solutions of carboxyhaemoglobin were prepared as described above.

Analytical methods

The method and apparatus used for the titrations with phenylmercuric hydroxide, the dropping-mercury electrode being used, were described by Allison & Cecil (1958). In that work a potential of -0.5 v versus the saturated-calomel electrode was used for the titrations. It has since been found that a potential of -1.1 v can be used with advantage. At this potential, current due to both the reduction waves of phenylmercuric hydroxide, instead of only the first, is recorded (see Benesch & Benesch, 1951) and the slope of the excess of reagent line is therefore greater.

RESULTS

Human foetal haemoglobin. The results show a considerable variation in the SH-group content of the different samples. In order to make sure that this variation is a true property of the haemoglobins, titrations were carried out under a range of conditions with both the native and denatured proteins. Two buffers were used, 0.25 M- NH_4Cl -aq. 0.25 M- NH_3 soln. and 0.025 M- $\text{Na}_2\text{B}_4\text{O}_7$ - 0.25 M-KCl. Both buffers were used with concentrations of sodium sulphite from zero to 0.15 M and of sodium thiosulphate from zero to 0.05 M.

Both these substances form complexes with phenylmercuric hydroxide which are reducible at the dropping-mercury electrode. Constant titres over a range of concentration indicate that all the phenylmercuric hydroxide, except that bound as mercaptide, is either free or bound as a sulphite complex. This is necessary if the titrations are to give a true measure of the SH-group content of the protein.

Sulphite reacts with disulphide bonds and thio-sulphate does not. If the same titres are obtained in the presence of each substance it is evidence that there are no disulphide bonds present.

Denaturation was effected by the addition of sodium dodecyl sulphate at a molar concentration of approximately 400 times that of the protein.

That the same results were obtained from any one sample under these different conditions shows that the variations are due to differences in the samples of Hb F and not to the conditions of titration.

The results obtained are summarized in Table 1. Whereas the mean figures for the six samples are 2.0 SH groups/mole for the native protein and 4.2 SH groups/mole for the denatured protein, the values range from 1.8 to 2.2 and 3.6 to 4.5 respectively. No such variation between samples was found by Allison & Cecil (1958) for Hb A.

Sickle-cell haemoglobin. Two samples were used. Titrations were carried out in the borate buffer only, otherwise the conditions were the same as those used for Hb F. The uptake of phenylmercuric hydroxide corresponded to 2.2 SH groups/mole in the native protein and 6.0 SH groups/mole in the denatured protein. This result is the same as that obtained by Allison & Cecil (1958) for Hb A.

DISCUSSION

The tendency of silver nitrate titrations to give high results for the SH-group content of haemoglobins was discussed by Allison & Cecil (1958).

Table 1. *SH-groups content of human foetal haemoglobin*

a, Spectroscopic method; b, alkaline denaturation method.

Sample no.	Percentage of Hb F			Native protein (SH groups/mole)		Denatured protein (SH groups/mole)	
	a	b	Mean of a and b	Observed	Calc. for 100% Hb F	Observed	Calc. for 100% Hb F
1	70	82	76	2.0	1.9	4.9	4.6
2	66	76	71	2.1	2.1	4.9	4.5
3	70	82	76	2.1	2.1	4.5	4.0
4	82	70	76	2.0	1.9	4.4	3.9
5	75	73	74	1.9	1.8	4.2	3.6
6	81	75	78	2.2	2.2	4.8	4.5
				Mean	2.0	—	4.2

Many silver mercaptides form stable complexes with additional silver ion. In such cases titrations interpreted on a one silver atom to one sulphur atom basis give too high a value for the number of SH groups present. A detailed discussion of the various methods of estimating SH groups is given by Cecil & McPhee (1960).

Foetal haemoglobin. The variation in the SH-group content of different samples of Hb F (1.8–2.2 for the native protein and 3.6–4.5 for the denatured protein) is much greater than that found by Allison & Cecil (1958) for Hb A and that now found for Hb S. Part of this variation could be due to the insensitivity of the methods for estimating the proportion of Hb F in mixtures, the probable accuracy of which is $\pm 7\%$ in the range used. Errors from this source, however, are insufficient to account for all the variations observed.

Another possibility is that there are two forms of Hb F which are replaced independently, i.e. at different rates, by Hb A. Heterogeneity of Hb F has been reported by Roche, Derrien & Roques (1952). Further work is necessary to establish the existence of independently synthesized foetal-haemoglobin components and to ascertain whether they have different SH-group contents. This will be difficult technically since most fractionation procedures are likely to result in some denaturation and some oxidation.

The suggestion of Hommes *et al.* (1958) that the molecule consists of two polypeptide chains joined by one disulphide bond is not substantiated by the present work, since no evidence could be found for the existence of any disulphide. Nor have disulphide bonds been reported in any other haemoglobins. However, other explanations are possible.

It has been shown by Gutter, Sober & Peterson (1956) that Hb A and horse haemoglobin are dissociated not only by urea but also by 2-mercaptoethanol. Urea is presumed to act in such circumstances by competitive breaking of the N-H-N and N-H-O hydrogen bonds in the proteins. The effect of 2-mercaptoethanol in dissociating these haemoglobins suggests a similar effect and implies that some at least of the SH groups may be involved in some form of labile interchain bond. The observation of Hommes *et al.* (1958) on the effect of thioglycolic acid in causing Hb F to dissociate into two components could be explained in the same way.

The supposition that Hb F consists of only two polypeptide chains was based on the observations of Porter & Sanger (1948) and Huisman & Drinkwaard (1955) that there are two valyl end groups. Since then Schroeder & Matsuda (1958) have shown that there are in fact four *N*-terminal groups, two valyl and two glycyl. Thus Hb F

falls into line with the other mammalian haemoglobins that have been studied in having four haem groups, four polypeptide chains and no disulphide bonds.

Hunt (1959) has shown that the α chains in Hb A and Hb F are identical. Since Hill & Craig (1959) have shown, by amino acid analysis, that the α chains contain one SH group it follows that the γ chain in Hb F must also contain one SH group. The four SH groups in Hb F are therefore likely to be distributed symmetrically between the four chains.

Sickle-cell haemoglobin. Although the results obtained by other workers have shown considerable variation they have generally been the same as those obtained with Hb A. This work confirms that they have the same SH-group content.

Murayama (1957) found that the titre with silver nitrate fell as the temperature was raised (four atoms of silver per mole at 0° and 1.9 at 38°). His results with *p*-chloromercuribenzoate, although different from those of the present work, were the same at both temperatures. This, together with the fact that his silver titrations are high at 0° , suggests that the explanation lies not in difference in SH-group content between Hb S and Hb A but in an increase in the dissociation constant of the silver mercaptide-silver ion complex with increasing temperature.

The only difference so far observed between Hb S and Hb A is the substitution of two glutamyl residues in Hb A for two valyl residues in Hb S (Ingram, 1957*b*, 1958; Hunt & Ingram, 1958). The action of SH-group reagents in preventing sickling cannot be ascribed to a difference in SH-group content between Hb S and Hb A. However, the fact that there is so small a difference in the constitution of the two proteins suggests that the steric effect of substitution of the SH groups might be sufficient to prevent sickling (see Allison, 1957).

SUMMARY

1. The SH-group contents of human foetal and sickle-cell haemoglobins have been determined by amperometric titration with phenyl mercuric hydroxide.

2. Some variation was found in the SH-group content of different samples of foetal haemoglobin. The mean values from six samples was 2.0 SH groups per mole in the native protein and 4.2 SH groups per mole in the denatured protein. Possible reasons for this variation are discussed.

3. Sickle-cell haemoglobin was found to have the same SH-group content as normal human adult haemoglobin, namely 2.2 SH groups per mole in the native protein and 6.0 SH groups per mole in the denatured protein.

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The Relationship between the Synthesis of Protein and the Synthesis of Ribonucleic Acid in the Connective-Tissue Cell

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In a previous paper (Harris, 1959) the use of quantitative radioautographic techniques for the study of ribonucleic acid turnover in the nucleus and cytoplasm of animal cells was described. These techniques were used in the present experiments to examine some aspects of the relationship between the synthesis of ribonucleic acid and the synthesis of protein in the connective-tissue cell of the rat. The synthesis of protein was measured by the incorporation of tritium-labelled valine and methionine, the synthesis of ribonucleic acid by the incorporation of tritium-labelled adenosine and cytidine. The cells were cultivated in media containing β -2-thienylalanine and 8-azaguanine and the effect of these compounds on the rates of synthesis of both nuclear and cytoplasmic ribonucleic acid and protein were measured.

EXPERIMENTAL

The connective-tissue cells were obtained from the hearts of 5-day-old rats and were cultivated as dissociated cells in small coverslip chambers (Harris, 1955a, b, 1956). The

culture medium consisted of 80% (v/v) of Eagle's soln. (Eagle, Oyama, Levy, Horton & Fleischman, 1956) and 20% (v/v) of bovine serum which had been dialysed for 18 hr. against an excess of 0.85% NaCl soln. When β -2-thienylalanine was added to the medium, phenylalanine was omitted from the Eagle's soln. The β -2-thienylalanine was added to make a final concentration of 5 mM, the 8-azaguanine to make a final concentration of 2 mM.

The tritium-labelled adenosine, cytidine, thymidine and valine were used at a concentration of 5 μ C/ml. of medium; the tritium-labelled methionine was used at a concentration of 1 μ C/ml. The tritium-labelled adenosine, cytidine and thymidine were obtained from Schwarz Laboratories Inc., Mt. Vernon, N.Y., U.S.A., and had specific activities of 1.0, 0.36 and 1.9 C/m-mole respectively. These compounds were labelled in the purine or pyrimidine ring. Tritium-labelled methionine was obtained from The Radiochemical Centre, Amersham, Bucks.; this compound was generally labelled and had a specific activity of 28 mC/m-mole. Tritium-labelled valine was prepared by an exchange reaction between tritium gas and the vapour of the ethyl ester of valine on a platinized platinum electrode at 50°. The tritium gas was obtained from the Atomic Energy Research Establishment, Harwell, and contained a maximum of 95% of tritium in hydrogen. The