Sodium cyanide samples which gave different colour yields with a given amount of uric acid were assayed for total cyanide and titratable alkalinity, but little consistent agreement was obtained. One commercial grade giving a low colour yield had a low assay and revealed traces of iron on analysis, the latter in the ferric form considerably reduces the colour yield of uric acid. Changes in alkalinity of the reaction mixture influence considerably the maximum optical density produced by a fixed quantity of uric acid.

Several substances modify the colour reaction; the most significant in the assay of serum are the amino acids. Dilution of the test solutions has been shown to reduce this effect and to yield higher recoveries and subsequent higher assays.

SUMMARY

1. In the reaction between uric acid and phosphotungstic acid in the presence of sodium cyanide, the effects of time, temperature, concentration of reactants and alkalinity of the reaction mixture have been investigated.

2. A method is described which is suitable for the determination of uric acid in as little as 0.05 ml. of serum containing up to 25 mg. of uric acid/100 ml.

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Further Studies on the Cysteine-Cystine Content of the Foetal-Human Haemoglobin

By F. A. HOMMES, ANDRÉE DOZY AND T. H. J. HUISMAN
Department of Paediatrics, State University of Groningen, The Netherlands

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In a previous paper (Hommes, Santema-Drinkwaard & Huisman, 1956) we described analyses of the total half-cystine content and the number of free sulphydryl groups in foetal-human haemoglobin. With a chromatographic procedure (Schram, Moore & Bigwood, 1954; Kimmel, Thompson & Smith, 1955), a total number of six half-cystine residues was found, whereas with an amperometric-titration method with silver nitrate only four free -SH groups could be demonstrated. These data suggest the existence of one disulphide bridge in the foetal haemoglobin.

In this paper the total number of free -SH groups in this protein are given, as established by two other techniques. In addition, an attempt was made to divide the foetal haemoglobin into two different parts by reduction of the disulphide bridge which should be present. It should thus be possible to get indications if the disulphide linkage is present in one polypeptide chain or between two

polypeptide chains. In this study the results obtained with foetal haemoglobin are compared with those for the adult component.

METHODS

Normal adult haemoglobin, obtained from healthy adults (laboratory workers) and pure foetal haemoglobin were prepared as described earlier (van der Schaaf & Huisman, 1955). The carboxyhaemoglobins were stored at 0° for no longer than 1 week. No experimental difference was found between this preparation of adult haemoglobin and that obtained by starch electrophoresis according to Kunkel & Wallenius (1955). Amperometric titrations with AgNO_3 and 0.05M- NH_4NO_3 -aq. 0.1M- NH_3 soln. as supporting electrolyte [Hommes *et al.* (1956) described in detail in the Addendum] followed the method of Benesch, Lardy & Benesch (1955). In this amperometric method a titration mixture of 0.133M-2-amino-2-hydroxymethylpropane-1:3-diol, 0.1133M- HNO_3 and 0.01M-KCl was used in all experiments. The titrimetric experiments were carried out at room temperature. No change was found on lowering the

temperature to 2° (Murayama, 1956, 1957). The results given by these two amperometric techniques were also compared with those obtained with the spectrophotometric method with *p*-chloromercuribenzoate (PCMB) (Boyer, 1954). The increase in optical density caused by the interaction of the reagent with the -SH groups of the haemoglobin (in a 0.2% solution in 0.1M-acetate, pH 4.6) was measured at 255 m μ . This increase was obtained by subtracting the optical densities of a haemoglobin solution and of a PCMB solution of corresponding concentrations from the optical density of a mixture of haemoglobin and PCMB. The number of -SH groups was estimated from this calculated extinction increment by using 6000 as the molar-extinction increment for the mercaptide formed at 255 m μ (Boyer, 1954). In some experiments denaturing agents such as a polyoxyethylene lauryl alcohol (Brij 35) in a final concentration of 1% and urea (4M) were used.

For the reduction of the -S-S- bridge, possibly present in the foetal protein, the method of Lindley (1955) was used. Small amounts (3-5 mg.) of protein were incubated for 4 hr. with 0.5M-lithium thioglycollate soln. After addition of a mixture of 39 ml. of acetone and 1 ml. of N-HCl, the precipitate (consisting only of the globin part of the haemoglobin) was removed by centrifuging. This precipitate was treated as for other proteins (Lindley, 1955) by coupling it with iodoacetamide, and was finally dissolved in a small amount of 0.1M-thioglycolic acid soln. This protein solution was submitted to paper electrophoresis (Whatman no. 3MM) for 16 hr. with an 0.1M-buffer solution of lithium thioglycollate, pH 5.1, with addition of 4M-urea (300 v at 10 mA). Colour development was obtained with ninhydrin after drying at 120° and washing the paper with ethanol to remove the urea. Larger amounts of this protein solution were prepared and investigated by moving-boundary electrophoresis with the same buffer solution. Control experiments with globin solutions were carried out, 0.1M-glycine buffer, pH 5.1, with addition of 4M-urea, being used.

RESULTS

The results obtained with the two amperometric-titration methods and the spectrophotometric method (PCMB method) are given in Table 1. These analyses were carried out both for normal adult haemoglobin and the foetal pigment. The

data are compared with those reported in an earlier paper (Hommès *et al.* 1956). First the results obtained with the amperometric-titration method, with NH₄NO₃-aq. NH₃ soln. as supporting electrolyte, were confirmed. For both haemoglobins the results with the method of Benesch *et al.* (1955) are 10-20% higher than those with the first titration method, which is in accordance with the data given by Benesch *et al.* (1955). No clear explanation can be given for this phenomenon. It may be due to the formation of complexes of protein-silver mercaptides with additional Ag⁺ ions, which are less stable after denaturation. Addition of a high concentration of urea decreases the number of titratable -SH groups. The values obtained with the tris method, in 4M-urea, are identical with those found with the NH₄NO₃-aq. NH₃ soln. method. The data obtained with the PCMB method are in poor agreement. The total numbers of -SH groups for adult and foetal haemoglobins were found to be 6.7 and 2.8 respectively, whereas amperometric-titration methods gave 7.6 and 4.1. Addition of a denaturing agent (1% of Brij 35) resulted in a decrease of the reactive -SH groups. The difference between the results obtained with the PCMB method and with the amperometric techniques may be due to the choice of a molar-absorption increment of 6000. According to Boyer (1954) the molar-absorption increment is not identical for mercaptides in different proteins. The method is therefore suitable mainly for comparative studies. A similarly lower amount of reactive -SH groups was found to be present in the foetal haemoglobin compared with the adult component as estimated with both amperometric-titration techniques.

In order to prove the hypothesis of the existence of a disulphide bridge in the foetal haemoglobin more directly some investigations were carried out by reducing this linkage with thioglycolic acid according to the method of Lindley (1955). The reduced protein was studied, after coupling of the

Table 1. *Sulphydryl content of normal adult and foetal-human haemoglobin*

Values are given as half-cystine residues/mole of haemoglobin, mol.wt. 68 000. *n* = Number of determinations.

Method	Haemoglobin					
	Adult			Foetal		
	<i>n</i>	Found	Assumed	<i>n</i>	Found	Assumed
Chemical	15	8.28 ± 0.17	8	15	5.67 ± 0.15	6
Amperometric titration*	10	7.6 ± 0.10	8	10	4.0 ± 0.10	4
Amperometric titration*	5	7.5 ± 0.06	8	12	4.1 ± 0.08	4
Benesch <i>et al.</i> (1955)†	2	9.6	—	3	4.4	—
Benesch <i>et al.</i> (1955)† + 4M-urea	2	7.6	8	3	4.1	4
PCMB	10	6.7 ± 0.3	—	10	2.8 ± 0.4	—
PCMB + 1% Brij 35	10	2.7	—	10	2.4	—

* Supporting electrolyte: 0.05M-NH₄NO₃-aq. 0.1M-NH₃ soln.

† Supporting electrolyte: 0.133M-tris-0.1133M-HNO₃-0.01M-KCl.

free -SH groups with iodoacetamide, both by paper electrophoresis and by the moving-boundary technique. If a disulphide linkage were present between two polypeptide chains two different components might be formed, which could be separated by these techniques. The paper-electrophoretic patterns of the adult and foetal haemoglobins, given in Fig. 1, show two well-marked spots (in addition to a small amount at the

Fig. 1. Paper-electrophoretic pattern of adult (A) and foetal (F) haemoglobin after reduction with thioglycolic acid and coupling with iodoacetamide. Whatman no. 3MM; 0.1M-lithium thioglycollate, pH 5.1, with addition of 4M-urea.

Fig. 2. Electrophoretic pattern of adult and foetal haemoglobins after reduction with thioglycolic acid and coupling with iodoacetamide. Buffer solution: 0.1M-lithium thioglycollate, pH 5.1, with 4M-urea (300v at 10 mA). (A) and (B) normal adult haemoglobin: (A) after 2½ hr.; (B) after 4½ hr. (C), (D) and (E) foetal haemoglobin: (C) after 3 hr.; (D) after 4½ hr.; (E) after 5 hr.

starting-point) with foetal haemoglobin, whereas only one spot was found for the adult haemoglobin.

Similar results were obtained with moving-boundary electrophoresis. Fig. 2 shows only one component (marked 1) for the adult haemoglobin, whereas two distinct components (marked 2 and 3) are present with the foetal haemoglobin. Without reduction with thioglycolic acid no splitting of the foetal component occurred (Fig. 3) when this protein was studied by the moving-boundary electrophoresis with a glycine-buffer solution of the same pH (5.1) and with the same high concentration of urea (4M).

DISCUSSION

The results of the different methods for the estimation of the total numbers of free -SH groups in the foetal haemoglobin support the previous finding (Hommes *et al.* 1956) that only four of the six half-cysteine residues present in foetal haemoglobin are reactive sulphhydryl groups. These analyses suggest the presence of a disulphide bridge in this protein. As all the eight half-cysteine residues in the normal adult haemoglobin are found to be present as free -SH groups no indication of such a linkage in this protein was obtained. The electrophoretic studies show that the foetal haemoglobin is divided into two different components by a reducing agent, probably by reduction of a disulphide bridge. It is therefore likely that the disulphide linkage is present between two polypeptide chains rather than in one polypeptide chain only. These results and the findings that only two valyl residues are present in *N*-terminal position (Porter & Sanger, 1948 and Huisman & Drinkwaard, 1955), and probably one histidine and one tyrosine in *C*-terminal position (Huisman & Dozy, 1956), support the hypothesis (Hommes *et al.* 1956) that foetal haemoglobin is built up by two large and different polypeptide chains, connected at least to each other by one disulphide bridge. Further studies,

Fig. 3. Electrophoretic pattern of the globins of adult (A) and foetal (F) haemoglobins with 0.1M-glycine buffer soln., pH 5.1, with addition of 4M-urea.

such as a more direct investigation of the chemical characteristics (*N*- and *C*-terminal residues) of the two different parts, will be valuable in confirming this structure of the foetal haemoglobin.

SUMMARY

1. Previous reports that only four of the total number of six half-cystine residues present in foetal-human haemoglobin are reactive sulphydryl groups were confirmed by two other techniques.

2. After reduction with thioglycolic acid reduced foetal haemoglobin was studied by paper and moving-boundary electrophoresis. With both techniques two different components were found.

3. These results support the hypothesis that foetal-human haemoglobin is built up of two different polypeptide chains connected at least to each other by one disulphide bridge.

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ADDENDUM

Amperometric Titration of Sulphydryl Groups in Human Haemoglobin with Silver Nitrate

BY F. A. HOMMES AND T. H. J. HUISMAN

Department of Paediatrics, University of Groningen, The Netherlands

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The slightly different amperometric-titration methods with silver, used for the estimation of sulphydryl groups in haemoglobins, have not always given identical results. Ingbar & Kass (1951), for instance, employing a titration medium of 0.85% NaCl found 2 moles of $-SH$ /mole of normal adult haemoglobin and 3 moles of $-SH$ /mole of sickle-cell haemoglobin. Addition of 20% methanol increases this number to 4–5 moles/mole of haemoglobin. Benesch, Lardy & Benesch (1955), using a 2-amino-2-hydroxymethylpropane-1:3-diol (tris) solution as supporting medium, obtained 9.6 moles of $-SH$ /mole of native normal adult haemoglobin, whereas after denaturation with 8M-urea this number was reduced to 8 moles of $-SH$ /mole of adult haemoglobin.

With NH_4NO_3 -aq. NH_3 soln. buffers Ingram (1955) has found that the number of titratable $-SH$ groups in this protein decreases with increasing concentration of ammonia. He concluded that there are 4 moles of $-SH$ /mole of native adult human haemoglobin, a number which increased to 8 after denaturation with sodium dodecyl sulphate. In previous papers (Hommes, Santema-Drink-

waard & Huisman, 1956; Hommes, Dozy & Huisman, 1958) we reported the presence of 8 $-SH$ groups/molecule of native adult human haemoglobin. The method used was similar to that of Ingram (1955) except that it was found necessary to pretreat the electrode by immersing it in an ammoniacal $HgCl_2$ solution. As Ingram has recently (1957) described different results obtained with this modified method, it is important to describe our technique in fuller detail.

METHODS AND RESULTS

The haemoglobin solutions were prepared as described earlier (van der Schaaf & Huisman, 1955). Washed red-blood cells were haemolysed by the addition of 1 vol. of water and 0.4 vol. of toluene. This mixture was stored for 24 hr. at 0°. After centrifuging and removal of the toluene layer the haemoglobin solution was shaken for 1 min. with Celite 535 (1 g./100 ml. of haemoglobin soln.) and slowly filtered with suction on a Büchner funnel. The clear haemoglobin solution was finally saturated with carbon monoxide and dialysed

against twice-distilled water for 48 hr. at 4° with eight changes of the dialysing fluid. A haemoglobin solution prepared in this way showed no denaturation of the protein, as measured by electrophoretic, chromatographic and salting-out experiments (Huisman & Schreuder, 1957).

The argentometric titration was first performed in 50 ml. of solution with a dropping-mercury electrode. The solution contained 0.05M-NH₄NO₃ and different concentrations of aq. NH₃ soln. (0.02M, 0.10M and 0.25M), with $0.4\text{--}0.7 \times 10^{-5}$ mole of carboxyhaemoglobin. The haemoglobin concentration was estimated spectrophotometrically as cyanmethaemoglobin at 540 and 544 mμ. The molar-extinction coefficient at both wavelengths is 1.15×10^4 , calculated on a molecular weight of 16 700.

The titrations were carried out with a 20 mm-AgNO₃ solution (Merck and Co. Ltd). The diffusion current to the dropping-mercury electrode was

Table 1. *Sulphydryl content of normal adult human haemoglobin estimated by the argentometric-titration method with a dropping-mercury electrode*

Concn. of NH ₃ (M)	Number of titrations	Moles of -SH/mole of adult haemoglobin
0.02	7	8.7 ± 0.2
0.10	7	8.7 ± 0.2
0.25	7	8.5 ± 0.2

Fig. 1. Argentometric-titration curves of adult human haemoglobin under different conditions (for explanation see text).

measured with a Kipp galvanometer (A70). A potential of -0.3 v was applied. The results of the titration of the normal adult haemoglobin are given in Table 1.

Argentometric titrations were next undertaken with an untreated, clean, rotating platinum-wire electrode (900 rev./min.). The supporting electrolyte contained 0.05M-NH₄NO₃-aq. 0.1M-NH₃ soln., and the volume in the titration vessel was again 50 ml., containing the same amount of $0.4\text{--}0.7 \times 10^{-5}$ mole of carboxyhaemoglobin. The same potential of -0.3 v was applied. The number of titratable -SH groups/mole of haemoglobin was now found to be 3.6 ± 0.15 (eight titrations). Pretreatment of the platinum wire with an ammoniacal HgCl₂ solution (30–45 min. at -0.2 v versus a standard calomel electrode) resulted in an increase of the titratable -SH groups to 7.6 ± 0.1 (ten titrations). Fig. 1 shows some titration curves without pretreatment (curve 1) and after pretreatment of the platinum electrode with the ammoniacal HgCl₂ solution (curve 2). Repetition of the titration experiments with the treated electrode, but without repeating the pretreatment of the platinum wire, resulted in a gradual shift of the titration curves to the left (for instance, Fig. 1, curve 3) and therefore to a smaller number of titratable -SH groups. Finally, a value of 3.6 -SH groups/mole of haemoglobin was reached (Fig. 1, curve 1), which did not change in further titration experiments. It will be noted that in these titration curves only one sharp endpoint was obtained.

DISCUSSION

Ingram (1955) described a marked influence of the ammonia concentration with an untreated rotating platinum-wire electrode. With the dropping-mercury electrode, however, no distinct effect of ammonia concentration was found (Table 1).

Fig. 2. Argentometric-titration curve of adult human haemoglobin with the dropping-mercury electrode and 0.05M-NH₄NO₃-aq. 0.1M-NH₃ soln. as supporting electrolyte.

Although the end-point of the titration curve (Fig. 2) is not very sharp, it offers no explanation for this difference. It seems likely that the ammonia effect has to be explained by the difference in the electrode used. In our comparative studies on the determination of -SH groups in haemoglobins we therefore used a median ammonia concentration (0.1M) for both untreated and treated platinum-wire electrodes.

An important finding is the effect of the pre-treatment of the platinum electrode with ammoniacal mercuric chloride solution before use. The final results obtained with such a system for studying native human haemoglobin are in agreement with the data obtained by Ingram (1957), except that only one end-point was found in our experiments. The differences between the results in titrations with the untreated platinum electrode and with the mercury-coated electrode must be explained rather by the different experimental conditions than by a different reactivity of some of the sulphhydryl groups in the haemoglobin, as examination of the current-voltage curves has shown an increased sensitivity of the mercury-coated electrode. Moreover, the results of repeated titrations with a mercury-coated platinum electrode show a gradual decrease in the amount of silver bound. We therefore believe that the number of reactive -SH groups of different haemoglobins, and perhaps of other proteins, can be studied in the native state and after denaturation very clearly by the procedure using a mercury-coated platinum-wire electrode.

SUMMARY

1. A detailed description of the argentometric-titration procedure with a rotating mercury-coated platinum electrode for the estimation of the number of reactive sulphhydryl groups in normal adult human haemoglobin is given.

2. A comparison of this method with the technique using an untreated platinum electrode revealed that with the latter procedure lower amounts of titratable -SH groups were found to be present in native adult human haemoglobin. It is suggested that this decrease is caused by the different experimental conditions.

3. The titration curves obtained by amperometric titration of native normal adult human haemoglobin with silver nitrate at the mercury-treated platinum electrode show only one sharp end-point.

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The Determination of Phylloerythrin in Blood

By D. D. PERRIN*

Ruakura Animal Research Station, Hamilton, Department of Agriculture, New Zealand

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Diseases of livestock in which there is an acute sensitivity of exposed parts to sunlight occur in many parts of the world. The need to identify the photodynamic agents responsible has been stressed (Clare, 1952). Photosensitivity directly due to components of the diet has been shown to occur in some instances, such as in poisoning by plants of the genus *Hypericum* (Pace, 1942), by *Fagopyrum esculentum* (Wender, Gortner & Inman, 1943), and after administration of phenothiazine to calves (Clare, 1947) and lambs (Gordon & Green, 1951).

* Present address: The John Curtin School of Medical Research, The Australian National University, Canberra, Australia.

More commonly, where liver damage is also found, the pigment phylloerythrin may be responsible (Clare, 1952). Phylloerythrin has been shown conclusively to be the photodynamic agent in geeldikkop (Rimington & Quin, 1934), in facial eczema (Clare, 1944), in congenital photosensitivity in Southdown sheep (Clare, 1945), in bile-duct ligation in sheep (Rimington & Quin, 1934) and in poisoning of sheep by *Lippia rehmanni*, *Panicum miliaceum*, *Myoporum laetum* and copper (Clare, 1952).

Phylloerythrin, a normal component of the bile and faeces of herbivores, is formed by microbial degradation of chlorophyll and related pigments in