Further Studies on the Isolation and Properties of α -Chain Sub-units of Haemoglobin

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1. α^A -Chain sub-units have been isolated from neutral haemolysates containing certain unstable β -chain haemoglobin variants. They are identical, in electrophoretic properties, tryptic peptide analyses and their ability to recombine with Hb- β_4^A at neutral pH, with the α^A -chain sub-units isolated from Hb-A at acid pH. Abnormal α^G -chain sub-units have been prepared from haemolysates containing the α -chain variant Hb-G. 2. α^A -Chain sub-units prepared by either method are eluted from Sephadex at low haemoglobin concentration as monomer sub-units. The elution volume, sedimentation coefficient and apparent molecular weight are, however, concentration-dependent, suggesting an equilibrium between monomers and higher polymers with the former as the predominant species. Elevated temperatures cause the α^A -chain sub-unit to aggregate and denature. 3. The product of the reaction between the α^A -chain sub-unit and Hb- β_4^A has been characterized as Hb-A. Only Hb-A and Hb-G can be detected as the products when a mixture of α^A - and α^G -chain sub-units reacts with Hb- β_4^A . The absence of the species $\alpha^G\alpha^G\beta_2^A$ can be explained in terms of the rapid equilibrium between whole and half haemoglobin molecules and the reassortment of $\alpha\beta$ sub-units during the separation process.

Interest in the sub-unit structure of proteins has been greatly stimulated by the realization that haemoglobin is not the only protein that mediates interaction between different binding sites. It is now recognized that a number of enzymes owe their metabolic regulatory function to such interactions or allosteric effects (Monod, Changeux & Jacob, 1963). For one enzyme, aspartate transcarbamoylase, the distinction between catalytic and regulatory functions has been delineated at the sub-unit level, the enzyme molecule containing two sub-units solely responsible for its catalytic activity and four sub-units with regulatory function (Gerhardt & Schachmann, 1965). The binding of the inhibitor CTP to the regulatory sub-unit alters the conformation of the enzyme, stabilizing the less-active form (cf. Changeux, 1964; Monod, Wyman & Changeux, 1965). With haemoglobin, X-ray studies have shown a considerable shift in the positions of the β -chains relative to each other during oxygenation (Muirhead & Perutz, 1963). The interactions between binding sites in these

proteins are therefore a function of the conformation and arrangement of the sub-units (a hypothesis originally formulated by Wyman & Allen, 1951), and consequently studies of the properties, conformation and interactions of the sub-units themselves offer one approach to an understanding of these problems. Haemoglobin is a model protein for such studies because a number of methods are now available for the isolation of the haem containing α^A - and β^A -chain sub-units.

The original method for the α^A -chain sub-units (Huehns, Dance, Shooter & Beaven, 1962) relied on acid dissociation of normal adult and foetal haemoglobin and subsequent isolation of the sub-unit by chromatography on CM-cellulose. The complementary β^A - or γ^F -chain sub-units can also be isolated from these solutions and are obtained in forms that are indistinguishable from naturally occurring Hb- β_4^A and Hb- γ_4^F respectively (Dance & Huehns, 1962; Huehns, Dance, Jacobs, Beaven & Shooter, 1965). Small yields of the α^A - and γ^F -chain sub-units result if Hb-F is simply exposed to acid

pH for periods longer than 12 hr. (Huehns *et al.* 1965), a method that can be applied to oxyhaemoglobin solutions. Ranney, Briehl & Jacobs (1965) have also prepared oxy- α^A -chain sub-units from Hb-A by a similar procedure. An alternative method, involving the separation of globin chains by selective precipitation and subsequent recombination of the individual chains with haem, has been reported by Satake & Take (1962) and Take (1964). Bucci & Fronticelli (1965) have described an effective method for obtaining α^A - and β^A -chain sub-units by dissociating haemoglobin, either the oxy or carbonmonoxy derivative, with an excess of *p*-chloromercuribenzoate. A source of α^A -chain sub-units that does not involve recourse to either acid or mercurial treatment has also been discovered (Huehns & Shooter, 1964a) and is now reported in detail.

Preliminary results (Huehns, Dance, Shooter, Beaven & Shooter, 1961) indicated that the α^A -chain sub-unit did not have the same tendency to polymerize as the non- α^A -chain sub-units. Further experiments, given below, confirm this finding, as do the results reported by Ranney *et al.* (1965) and Bucci *et al.* (1965). These sub-units are, however, functional in the sense that they recombine readily with β^A -chain sub-units (Huehns & Shooter, 1962), and further characterization of the product of this reaction is described. The preparation of an abnormal α^G -chain sub-unit and of its reaction, in mixture with α^A -chain sub-units, with Hb- β_4^A is also reported. A brief account of this work has been given elsewhere (Huehns & Shooter, 1964b).

MATERIALS AND METHODS

α -Chain sub-units. α^A -Chain sub-units (Hb- α^A) were isolated in the cyanmet or carbonmonoxy form from acid solutions of Hb-A or cord-blood haemoglobin by the method of Huehns *et al.* (1962). This method has been extended to the preparation of abnormal α^G -chain sub-units (Hb- α^G) as follows. The starting haemolysate contained the four major haemoglobins Hb-A, Hb-G_{Br}, Hb-C and Hb-G_{Br}/C (Raper, Gammack, Huehns & Shooter, 1960). The α -chain variant Hb-G_{Br} is identical with Hb-G_{Phl}, $\alpha_2^{S81V}\beta_2^A$ (Baglioni & Ingram, 1961; Dance, Huehns & Shooter, 1964), in which a lysine residue replaces the normal α -68 asparagine residue. The abnormal chain is referred to below simply as the α^G -chain. The method, with the carbonmonoxy derivatives, was identical with the Hb- α^A procedure up to the stepwise elution with 0.3M-sodium acetate buffer, pH 4.7. In this new preparation starch-gel analyses showed that the first 70 ml. of the 0.3M-acetate eluate contained mainly Hb- α^A with only traces of Hb-A (Fig. 1a). The remaining 0.3M-acetate eluate contained much smaller amounts of haemoglobin than in the usual preparation and consisted of Hb-A and Hb- α^A (Fig. 1b).

No Hb-G, Hb-C, Hb-G/C or α^G -chain sub-units were found in any of the fractions obtained during elution with

Fig. 1. Preparation of Hb- α^A and Hb- α^G : analyses of fractions from CM-cellulose chromatography at pH 4.7 of a mixture of Hb-A, Hb-G, Hb-C and Hb-G/C as described in the Materials and Methods section. Starch-gel electrophoresis was carried out in the discontinuous buffer system; the migration time was 3 hr. The bands were stained with *o*-dianisidine. (a) First part of 0.3M-acetate eluate; (b) remaining 0.3M-acetate eluate; (c) first part of 0.6M-acetate eluate; (d) remaining 0.6M-acetate eluate.

the 0.3M-acetate buffer. Elution with 0.6M-acetate buffer, pH 4.7, rapidly displaced a major amount of haemoglobin from the column; this fraction contained the six haemoglobins A, G, C, G/C, α^A and α^G (Fig. 1c). Subsequent fractions of the 0.6M-acetate eluate contained only the four haemoglobins A, G, C and G/C in almost equal proportions, as did eluates obtained with 2M-acetate buffer, pH 4.7, at room temperature (Fig. 1d).

The first 200 ml. of the 0.6M-acetate eluate was concentrated and dialysed against 0.04M-phosphate buffer, pH 7.0, and then subjected to starch-grain electrophoresis in the same buffer. A double but poorly resolved minor zone was observed, migrating nearest to the cathode, that on subsequent examination by starch-gel electrophoresis proved to contain both Hb- α^A and Hb- α^G with the former in slight excess (Fig. 8). The haemoglobin eluted from this zone was concentrated by pressure dialysis in $\frac{1}{4}$ in. Visking tubing that had previously been boiled in water for 30 min. (Huehns & Shooter, 1961).

Hb- β_4^A and Hb-I. Hb- β_4^A was isolated from appropriate haemolysates by the methods described by Dance, Huehns & Beaven (1963). Hb-I was separated from the haemolysate of the individual with Hb-I thalassaemia (Atwater, Schwartz, Erslev, Montgomery & Tocantins, 1960) by starch-grain electrophoresis.

Sedimentation-velocity experiments. These were carried out in a Spinco model E ultracentrifuge equipped with the temperature-control device at 5° and 59780 rev./min. Sedimentation-equilibrium experiments were made by the method of Van Holde & Baldwin (1958) by using 3 mm. columns in double-sector cells at 14290 rev./min. for 24 hr. at 5°. A Wratten 29 filter and Kodak 103-F photographic plates were used. Haemoglobin solutions were in 0.04M-phosphate-0.01M-KCN buffer, pH 7.0.

Sephadex-gel filtration experiments. These were carried out at 2° with Sephadex G-75 or G-100 (Pharmacia, Uppsala,

Sweden) in 150 cm. $\times$ 1.5 cm. columns with either 0.1 M-phosphate-0.1 M-NaCl-0.01 M-KCN buffer, pH 6.92, or 0.1 M-sodium borate-0.1 M-NaCl-0.01 M-KCN buffer, pH 8.30. Bovine γ -globulin, bovine serum albumin (fraction V, crystalline: Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex), ovalbumin (Seravac Laboratories Ltd., Maidenhead, Berks.) and sperm-whale myoglobin (a gift from Dr A. Edmundsen) were used as molecular-weight markers. Flow rates were approx. 7 ml./hr.; 0.7 ml. fractions were collected and their extinctions determined at 380 and 415 m μ .

Starch-grain and starch-gel electrophoresis. These were carried out as described by Huehns *et al.* (1962). An additional buffer system for starch-gel electrophoresis (O. Smithies, unpublished work) had the following composition. Gels were made from a 1:20 dilution of a solution containing 109 g. of tris, 5.84 g. of EDTA (disodium salt) and 30.9 g. of boric acid/l., and the electrode solution comprised a 1:7 dilution of the same buffer. This buffer had pH 8.54 at 25°. A second buffer, with 69.9 g. instead of 109 g. of tris, had pH 8.32 and in this system Hb- α^A migrated to the cathode.

Recombination experiments at acid pH. These were carried out as described by Gammack, Huehns, Shooter & Gerald (1960).

RESULTS

Properties of the α^A -chain sub-unit. Starch-gel analyses of the neutralized eluates obtained when Hb-A or Hb-F is eluted from CM-cellulose columns or of the neutralized solutions of Hb-F that have been exposed to acid pH showed a single well-defined zone for Hb- α^A (Fig. 2a).

Fig. 2. Comparison of α^A -chain sub-units isolated by acid treatment from Hb-F and from neutral haemolysates of Hb-Seattle. Starch-gel electrophoresis was carried out in the tris-EDTA-borate system, pH 8.32; the migration time was 4 hr. The bands were stained with *o*-dianisidine. (a) Cord-blood haemolysate (2 g./100 ml.) exposed to 0.1 M-sodium acetate-0.01 M-KCN buffer, pH 4.7, for 48 hr. and then dialysed against 0.04 M-sodium phosphate-0.01 M-KCN buffer, pH 7.0; (b) α^A -chain sub-unit isolated by starch-grain electrophoresis from cord-blood haemolysate treated as in (a); (c) α^A -chain sub-unit isolated by starch-grain electrophoresis from haemolysate containing Hb-Seattle.

This zone remained at the origin in this buffer at pH 8.55 but migrated cathodically at lower pH values. The isolated Hb- α^A has the same mobility properties (Fig. 2b) but shows in addition very small amounts (less than 1% of the haemoglobin in the isolation fraction) of minor haemoglobin components that migrate anodically in this system. (The concentration of these components is so low that they are only just visible after staining with *o*-dianisidine and reproduce poorly, if at all, in photographs of the stained gel; see also Fig. 7.) The most prominent member of the minor components has an anodic mobility equal to that of α^A -chain sub-units treated with *p*-chloromercuribenzoate (α^{PMB} in the terminology of Bucci *et al.* 1965); the least prominent has the mobility of Hb-A and probably represents contamination of Hb- α^A with Hb-A, whereas the third migrates between the other two.

The isolated Hb- α^A was eluted from Sephadex G-75 or G-100 at a volume close to that for myoglobin (Fig. 3). The plot of the ratio of elution to void volume against logarithm of the molecular weight (Whitaker, 1963) gave a straight line for the known molecular-weight markers. The molecular weight of Hb- α^A derived from these calibration data showed some dependence on concentration, having a value of 16000 (Sephadex G-75 column) when the initial concentration of the material applied to the column was 0.35 g./100 ml. and 19000 (Sephadex G-100 column) at 0.5 g./100 ml.

The haemoglobin concentrations in the middle of the eluted haemoglobin zones were approx. 0.02 and 0.03 g./100 ml. in the two experiments so that the observed molecular weights refer to concentrations that varied from the initial to the final lower value during the elution on the Sephadex column. The computed molecular weight for myoglobin from these experiments was 18000, which is in close agreement with the known value for this protein. No dependence of molecular weight on concentration was observed for myoglobin. Under similar buffer conditions Hb-A or Hb-F was eluted just behind the ovalbumin zone.

Other haem-containing material, comprising about 6% of the total haemoglobin, was eluted before Hb- α^A in a relatively broad zone with a maximum at a volume corresponding to a molecular weight of approx. 60000. This fraction was reduced to 3% of the total haemoglobin if the main Hb- α^A zone was isolated, concentrated and analysed a second time on the Sephadex column. Such Hb- α^A preparations showed no change in composition if stored at 2° and pH between 6.9 and 8.3 for periods of 2 weeks. However, aggregation of Hb- α^A was observed at higher temperature, as shown in Fig. 4, some 20% of the monomer sub-unit being converted into higher-molecular-weight material after

Fig. 3. Gel filtration of Hb- α^A on Sephadex G-100. A 0.2ml. volume of solution containing 10mg. of γ -globulin (A), 10mg. of bovine serum albumin (B), 10mg. of ovalbumin (C), 20mg. of ribonuclease (dimer D, monomer E) and either (a) 1.0mg. of myoglobin (F) or (b) 1.0mg. of Hb- α^A (G) was applied to and eluted from a 150cm. $\times$ 1.15cm. column of Sephadex G-100 at 2°. The column was equilibrated and eluted with 0.1M-sodium phosphate-0.1M-NaCl-0.01M-KCN buffer, pH 6.92. The flow rate was 7ml./hr.; 0.7ml. fractions were collected and their extinctions determined at 280 and 415m μ . O, E_{280} ; ●, E_{415} .

24hr. at 20°. The aggregation appears to be a non-specific denaturation because isolated and concentrated aggregate material gave only a trail with no defined zones on starch-gel electrophoresis. Electrophoresis of the main Hb- α^A zone from Sephadex showed the same minor haemoglobin components as the original preparation.

The original sedimentation data (Huehns *et al.* 1961) gave an $S_{20,w}$ value of 1.7s and an apparent weight-average molecular weight of 17000 ± 1000 at a concentration of 0.04g./100ml. At higher concentrations the sedimentation coefficient and apparent molecular weight were both significantly higher than the values expected for single α^A -chains (Table 1). These results fit well with the values observed by Bucci *et al.* (1965) and Ranney *et al.* (1965), which are also collected in Table 1.

Fig. 4. Aggregation of Hb- α^A at 20°: gel filtration on Sephadex G-75 of an Hb- α^A preparation before and after standing at 20° for 24hr. A 0.2ml. volume of solution containing 0.6mg. of Hb- α^A was applied to and eluted from a 150cm. $\times$ 1.15cm. column of Sephadex G-75 at 2°. The column was equilibrated and eluted with 0.1M-sodium borate-0.1M-NaCl-0.01M-KCN buffer, pH 8.3. The flow rate was 5ml./hr.; 0.7ml. fractions were collected and their extinctions determined at 415m μ . ●, Original Hb- α^A preparation; O, after exposure to 20° for 24hr.

The dependence of sedimentation coefficient, molecular weight and elution volume from Sephadex on concentration suggests that the single α^A -chain sub-units are in an interacting equilibrium with higher polymeric forms. At low protein concentration the equilibrium lies entirely in favour of the monomer sub-unit. Assuming that higher polymers are dimers then the proportion of dimers at the highest concentration (1.03g./100ml.) is somewhat less than 40% of the total haemoglobin.

Reaction of the α^A -chain sub-unit with Hb- β_4^A . The α^A -chain sub-units exchange with other α -chain sub-units in recombination experiments at acid pH (Huehns *et al.* 1962). In contrast, they react with β^A -chain sub-units at neutral pH. The product of this reaction is a haemoglobin species indistinguishable from Hb-A. It had the same electrophoretic mobility as Hb-A at both pH 7.4 (Fig. 5) and 8.5.

Unlike either of the two reactants, the reconstituted haemoglobin was stable in solution at 4° and was not precipitated by incubation at 37°, conditions that precipitate the α^A -chain sub-units, or by freezing and thawing, which is known to precipitate Hb- β_4^A . The isolated haemoglobin gave the same tryptic peptide pattern and had the same sedimentation properties as Hb-A (Table 2).

Table 1. *Sedimentation properties of the α^A -chain sub-unit*

Experimental conditions were as follows: 1 (Huehns *et al.* 1961), 0.01M-phosphate-0.2M-NaCl buffer, pH6.8; 2 (Ranney *et al.* 1965), 0.1M-phosphate buffer, pH7.0, at 10° and 20°; equilibrium experiments were carried out at pH6.3, 7.0 and 7.7 at 15° and 18°; 3 (Bucci *et al.* 1965), 0.05M-phosphate buffer, pH7.0, at 4–7°; 4 (this paper), 0.04M-phosphate-0.01M-KCN buffer, pH7.0, at 5°.

Concn. of haemoglobin (g./100ml.)	$S_{20,w}$ (s)	M_w (app.)	Reference
1.03	2.31	25000	4
0.80	2.30	25000	4
0.46	2.4		2
0.1–0.55	1.7–2.2		3
0.06		21000 $\pm$ 1500	2
0.04	1.7	17000 $\pm$ 1000	1
< 0.03		18000	2
Myoglobin			
0.04	1.8	18000 $\pm$ 2000	1

Fig. 5. Recombination of α^A -chain sub-units with Hb- β^A . Starch-gel electrophoresis was carried out in phosphate buffer, pH7.4; the migration time was 1.25 hr. The bands were stained with *o*-dianisidine. (a) Hb-A; (b) 0.1 ml. of Hb- α^A solution (0.42 g./100 ml.) and 0.1 ml. of Hb- β^A solution (1.1 g./100 ml.) mixed and kept at 20° for 5 min. and then applied to the gel; (c) original solution of Hb- β^A ; (d) original solution of Hb- α^A .

Ranney *et al.* (1965) obtained similar results and have also shown that the reconstituted Hb-A binds oxygen reversibly, has an oxygen affinity much lower than that of the individual chains but identical with that of Hb-A, and gives a Bohr effect.

If more Hb- β^A was present in the reaction mixture than α^A -chain sub-unit, all the latter was utilized in the production of Hb-A. Provided that fresh Hb- β^A was used the converse was found to hold when an excess of the α^A -chain sub-unit was present. Equal amounts of the two sub-units reacted in stoichiometric manner and Hb-A was then virtually the only species remaining in the reaction mixture. The reaction proceeded sufficiently fast at room temperature for it to be 73% complete within the time required to insert the sample into the starch gel and achieve resolution of the Hb-A zone, usually about 15 min. Lower rates of reaction were found with Hb- β^A preparations that were not fresh (cf. Huehns, Beaven & Stevens, 1964).

Table 2. *Sedimentation properties of Hb-A reconstituted in the reaction of α^A -chain sub-units with Hb- β^A*

Equal volumes of solutions containing Hb- β^A (1.2 g./100 ml.) and Hb- α^A in cyanmet form (0.4 g./100 ml.) in 0.04M-phosphate buffer, pH7.0, were mixed at 20° and then kept for 24 hr. at 2°. The resultant solution was subjected to starch-grain electrophoresis and the zone migrating in the position of Hb-A (cf. Fig. 5) was eluted, concentrated and dialysed against 0.10M-phosphate-0.01M-KCN buffer, pH7.0. Hb-A was isolated from fresh normal adult haemolysate in the same manner and dialysed against the same buffer.

Haemoglobin	Concn. (g./100 ml.)	$S_{20,w}$ (s)
Hb-A	0.62	4.25
Reconstituted Hb-A	0.57	4.33

The minor components in the α^A -chain sub-unit preparation also reacted with Hb- β^A to form Hb-A; with an excess of Hb- β^A all the haem species in the preparation were converted into Hb-A (see Fig. 7).

Isolation of Hb- α^A from haemolysates containing certain unstable haemoglobins. Hb-Seattle has been found in three related individuals with a mild non-spherocytic anaemia (E. R. Huehns, J. Hartmann, F. Hecht & A. G. Motulsky, unpublished work). It amounts to 40% of the total haemoglobin of the erythrocytes. The amino acid substitution is in the β -chains, an alanine residue being replaced by glutamic acid at position β -70 or β -76. The abnormal haemoglobin is less stable than Hb-A to heat denaturation. Analysis of the haemolysate from one individual revealed a haemoglobin zone migrating in the position of Hb- α^A and accounting for 0.5% of the total haemoglobin. Hb-Seattle fractions isolated by starch-grain electrophoresis

Fig. 6. Gel filtration of isolated Hb-Seattle fraction on Sephadex G-100. A 0.3 ml. volume of solution containing approx. 20 mg. of isolated Hb-Seattle was applied to and eluted from a 130 cm. $\times$ 1.5 cm. column of Sephadex G-100 at 2°. The column was equilibrated and eluted with 0.1 M-sodium phosphate-0.1 M-NaCl-0.01 M-KCN buffer, pH 6.92. $\circ$, E_{540} ; $\bullet$, E_{415} .

showed the same zone when examined by starch-gel electrophoresis. Gel filtration of the isolated Hb-Seattle fraction revealed a minor zone eluted after the main Hb-Seattle zone and in the position expected for the α^A -chain sub-unit (Fig. 6).

This low-molecular-weight material, isolated by gel filtration, had the mobility of the α^A -chain sub-units isolated from the acid column eluates (Fig. 1) as well as showing the three anodically migrating minor components characteristic of the acid preparations. The tryptic peptide analysis was identical with that of the α^A -chain and, like the α^A -chain sub-unit prepared from acid solutions, it reacted rapidly at neutral pH with Hb- β_4^A to form a new species with the mobility of Hb-A (Fig. 7). The time-course of this reaction was similar to that of Hb- β_4^A with acid-prepared α^A -chain sub-units.

Another unstable haemoglobin, Hb-St Mary's (A. Buchanan, P. Barkhan, P. E. Crome, P. L. Mollison & E. R. Huehns, unpublished work), showed the same phenomenon, about 1% of the haemoglobin of the haemolysate consisting of α^A -chain sub-units. The gel-filtration analysis of the Hb-Seattle fraction also showed some haemoglobin material of high molecular weight eluted at the solvent front, and this fraction was present in the corresponding analysis of the Hb-St Mary's.

Examination of haemolysates of patients with β -thalassaemia major confirmed the finding of Fessas & Loukopoulou (1964) that some α^A -chain

Fig. 7. Recombination of α^A -chain sub-units isolated from purified Hb-Seattle fractions with Hb- β_4^A . Starch-gel electrophoresis was carried out in tris-EDTA-borate buffer, pH 8.32; the migration time was 2 hr. The bands were stained with *o*-dianisidine. (a) Hb- β_4^A ; (b) Hb-A; (c) 0.05 ml. of neutral α^A -chain sub-unit solution (1.5 g./100 ml.) and 0.05 ml. of Hb- β_4^A solution (6.0 g./100 ml.) mixed and kept for 16 hr. at 20°; (d) neutral α^A -chain sub-unit.

Fig. 8. Recombination of a mixture of α^A - and α^G -chain sub-units with Hb- β_4^A . Starch-gel analysis was carried out in the discontinuous buffer system; the migration time was 3 hr. The bands were stained with *o*-dianisidine. (a) Original preparation of α^A - and α^G -chain sub-units; (b) mixture of (a) with an excess of Hb- β_4^A kept at 20° for 1 hr.; (c) Hb-A, Hb-G, Hb-C and Hb-G/C marker; (d) Hb- β_4^A .

sub-units were present. Trace amounts (less than 0.1% of the total haemoglobin) of α^A -chain sub-units were also detected in stored haemolysates containing Hb-S or Hb-C.

Reaction of Hb- α^A and Hb- α^G with Hb- β_4^A . The α -chain sub-unit preparation gave two new haemoglobin species when the starting haemolysate contained the abnormal α -chain haemoglobin $\alpha_2^G\beta_2^A$ as well as Hb-A (Fig. 8).

One of them, the one migrating to the anode, has already been identified as Hb- α^A (Huehns *et al.* 1962). The other new species migrated towards the cathode at pH 8.5 in the discontinuous system. Since the α^G -chain has a lysyl residue replacing a normal asparaginyl residue in the α^A -chain, it carries one extra positive charge compared with an

α^A -chain sub-unit. This charge difference between single-chain sub-units is roughly equal, in terms of the mobility difference it will produce, to a charge difference of 4 between tetramer haemoglobin molecules. Thus the expected mobility difference between Hb- α^A and Hb- α^G is approximately that between Hb-G/C and Hb-G. The actual mobility difference between Hb- α^A and the cathodically moving zone in Fig. 8(a) is very close to this value. Huehns *et al.* (1962) noted that α^A -chain sub-units will exchange with other α -chains and showed that the α^G -chains in the species $\alpha_2^G\beta_2^G$ could be replaced with α^A -chains to form Hb-C, $\alpha_2^G\beta_2^C$. The displaced α^G -chain sub-units migrated to the cathode in exactly the position found for the second of the new species described above. The cathodically migrating haemoglobin zone can therefore be identified as the α^G -chain sub-unit.

When the mixture of α^A - and α^G -chain sub-units was treated with an excess of Hb- β_4^A both sub-units disappeared and two new zones with mobilities equal to those of Hb-A and Hb-G were formed (Fig. 8b). The same result was obtained when the mixture of α^A - and α^G -chain sub-units was dissociated at acid pH and recombined with an excess of Hb-I, $\alpha_2^I\beta_2^A$. No haemoglobin zone was observed between Hb-A and Hb-G in either of these two experiments.

DISCUSSION

α^A -Chain sub-units. The α^A -chain sub-unit isolated at neutral pH from haemolysates containing unstable β -chain variants is identical in all the properties studied here with the α^A -chain sub-unit isolated from acid solutions of normal adult or foetal haemoglobin, thereby strengthening the original conclusion (Huehns *et al.* 1962) that the latter are not denatured through exposure to acid. Moreover, the high rate of recombination of the α^A -chain sub-units with Hb- β_4^A and the fact that they combine reversibly with oxygen (Ranney *et al.* 1965) also support the previous contention (Huehns *et al.* 1961) that the α^A -chain sub-units retain a compact and probably specific conformation. Indeed, Breslow, Beychok, Hardman & Gurd (1965) and Harrison & Blout (1965) have shown that even apomyoglobin retains much of the conformation of myoglobin. The release of α^A -chain sub-units from the unstable β -chain haemoglobin variants presumably arises because the abnormal β -chain sub-units differ sufficiently in conformation from the normal sub-units for the interaction between α - and β -chains to be significantly decreased.

The appearance of trace amounts of α^A -chain sub-units in stored samples of Hb-S or Hb-C but

not of Hb-A is probably an indication of a slight decrease in the interaction between α - and β -chain sub-units in these abnormal haemoglobins. The significance of the α^A -chain sub-units found in the erythrocytes in β -thalassaemia is apparently different. Huehns & McLoughlin (1966) have shown that this fraction is probably an intermediate in the formation of Hb-A in this condition and that the newly synthesized α -chain sub-units released from their ribosomal sites of synthesis in this condition behave similarly to the α -chain preparations described above on starch-gel electrophoresis and Sephadex chromatography.

The α^A -chain sub-units regenerated from the sub-units prepared by treating Hb-A with an excess of *p*-chloromercuribenzoate (Bucci *et al.* 1965) also have the same properties as the sub-units described above. Thus a variety of dissociation procedures produce the same stable and functional sub-unit.

The gel-filtration experiments confirm previous observations (Huehns *et al.* 1961) that the α^A -chain sub-unit is a monomer at low concentration. Sedimentation data at higher concentration indicate, as do the results reported by Ranney *et al.* (1965) and Bucci *et al.* (1965), that higher polymers are present, and the dependence of molecular properties on concentration suggests an equilibrium between monomer and polymer forms with, however, the monomer as the predominant species. These findings contrast with the properties of the α^A -chain sub-unit isolated by Chernoff (1964) from normal adult haemolysates by ion-exchange chromatography. The latter had a sedimentation coefficient of 2.86S and a molecular weight of approx. 66 000. Molecular weights of this order have only been obtained in the present investigation with the aggregated material that appears after the sub-unit preparation has stood at 20°. It should be noted, moreover, that the α^A -chain sub-units isolated directly from the Hb-Seattle-containing haemolysate as well as the newly synthesized α -chains in β -thalassaemia are eluted from Sephadex as monomers.

The physical properties of the α^A -chain sub-unit support the conclusion that haemoglobin dissociates into symmetric $\alpha\beta$ sub-units and subsequently into α - and β -chain sub-units. The α^A -chain sub-units do not have, under any of the conditions studied here or by other workers, the properties of a stable α_2^A dimer required for an asymmetric mode of dissociation.

The rapid re-formation of Hb-A when solutions containing α^A -chain sub-units and Hb- β_4^A are mixed demonstrates that the interactions between unlike sub-units are considerably greater than between like β^A -chain sub-units. It is precisely these interactions between unlike sub-units that determine the characteristic oxygen-binding properties

of Hb-A, for neither Hb- β_4^A nor the α^A -chain sub-units show haem-haem interaction or a Bohr effect (Benesch, Ranney, Benesch & Smith, 1961; Ranney *et al.* 1965). The high affinity of the two types of sub-units for each other shows that the coiling of the peptide chains to produce the individual sub-units also generates the binding sites responsible for their interaction. This has been recognized in a number of proteins where regeneration of a functional polymeric structure from identical or dissimilar sub-units has been obtained (cf. Gerhardt & Schachmann, 1965). Since it is recognized that the sequence of amino acid residues in a peptide chain determines the conformation the chain adopts (White, 1961), then it is apparent that the sequence also determines the higher (quaternary) structure in polymeric proteins. However, in some proteins the quaternary structure is also affected by the attachment of other groups, e.g. the formation of the haemoglobin tetramer from the $\alpha\beta$ -globin dimer when the haem groups are attached (Winterhalter & Huehns, 1965).

Finally, the similarity between the two proteins haemoglobin and aspartate transcarbamoylase is striking. Stable and functional sub-units can be isolated from either protein. The two types of aspartate-transcarbamoylase sub-units and the α^A -chain sub-units remain essentially unaggregated. The β^A -chain sub-units differ in that they polymerize in a specific manner to form the tetramer Hb- β_4^A . The two haemoglobin sub-units have higher oxygen affinities than does haemoglobin and the catalytic sub-unit of aspartate transcarbamoylase has a higher activity than that of the whole enzyme, and therefore in both instances the catalytic activity of the sub-units is decreased by their interactions in the whole protein molecule. The similarity extends also to the effectiveness with which excess of *p*-chloromercuribenzoate dissociates both proteins into their respective sub-units.

Minor components in the α^A -chain sub-unit preparations. Additional minor haem components are detected in the sub-unit preparation whether it is obtained from acid solutions, neutral haemolysates or by the *p*-chloromercuribenzoate method. These components are more negatively charged than the α^A -chain sub-unit at alkaline pH (Fig. 7) and in this respect resemble the minor components noted by Perkoff, Hill, Brown & Tyler (1963) in solutions of human ferrimyoglobin. Unlike the latter, however, they can still be distinguished when the haemoglobin is converted into the cyanide derivative of the ferri form. The minor components can, however, be identified as α^A -chain sub-units (with the possible exception of the component with the mobility of Hb-A) because they react with β^A -chain sub-units to form Hb-A. Their

appearance in starch-gel analyses of the isolated monomer peak from the gel-filtration experiments suggests that they are also single-chain sub-units. The alternative explanation that they are aggregated species re-formed in small yield after isolation of the monomer seems unlikely, since in the preparations obtained by Bucci *et al.* (1965) one of these minor components was present in much larger amounts and accounted for about one-half of the total haemoglobin and yet the preparation as a whole behaved as a monomer on gel filtration and during sedimentation. A comparison of the electrophoretic properties of the minor components in the α^A -chain sub-unit preparation with those of the mercurial sub-unit obtained in the *p*-chloromercuribenzoate method provides a possible clue to the identity of at least one of them.

The α^A -chain sub-unit in which the thiol group carries the mercuribenzoate residue has a greater negative charge than the normal sub-unit. If it is assumed that reaction with the mercurial does not significantly alter the pK values of other ionizable groups in the sub-unit then the extra charge can be attributed to the replacement of an un-ionized thiol group by the substituted $-S \cdot Hg \cdot C_6H_4 \cdot CO_2^-$ group. Since the most prominent of the minor components has a mobility close to that of the reacted sub-unit it is possible that in this component the thiol group is ionized. To account for the fact that there are two separable forms of the α^A -chain sub-unit possibly differing only in the ionization of the thiol group probably requires that they have different conformations.

Reaction of a mixture of unlike α -chain sub-units with Hb- β_4^A . The separable products of the reaction of a mixture of α^A - and α^G -chain sub-units with Hb- β_4^A , under conditions where substantial amounts of monomer sub-units are present, are only Hb-A and Hb-G. It is now recognized that the failure to detect the asymmetric species $\alpha^A\alpha^G\beta_2^A$ is a consequence of the rapidly adjusting equilibrium between whole and half haemoglobin molecules rather than of any inherent specificity in the association of $\alpha\beta$ sub-units (Guidotti, Konigsberg & Craig, 1963). Experiments by Guidotti (1964) on the elution behaviour from Sephadex of a mixture of normal adult and two alkylated haemoglobins and by Benesch, Benesch & Macduff (1965), who have shown that under certain conditions the oxygen equilibria of an equimolar mixture of ferro- and ferri-haemoglobin is the same as that of a haemoglobin solution in which half the haem groups have been oxidized to the ferri form, provide considerable evidence in favour of this interpretation.

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