The Abnormal Haemoglobins in Haemoglobin-H Disease

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Two haemoglobins have been described that do not contain α -chains: haemoglobin H (Rigas, Koler & Osgood, 1955, 1956; Gouttas, Fessas, Tsevremis & Xefteri, 1955) consists of four β^A -polypeptide chains (Jones, Schroeder, Balog & Vinograd, 1959), and haemoglobin Bart's (Ager & Lehmann, 1958) consists of four γ^F -polypeptide chains (Hunt & Lehmann, 1959). These two abnormal haemoglobins are now referred to as Hb- β^A_4 and Hb- γ^F_4 respectively.

In a number of carriers of Hb- β^A_4 a second abnormal haemoglobin has been detected and this usually resembles Hb- γ^F_4 (cf. Tuchinda, Vareenil, Bhanchit & Minnich, 1959; Ramot, Sheba, Fisher, Ager & Lehmann, 1959; Fessas & Mastrokalos, 1959; Fessas, 1960; Silvestroni, Bianco & Muzzolini, 1960; Huehns, Flynn, Butler & Shooter, 1960). In other cases a second abnormal haemoglobin was found that differed from Hb- γ^F_4 (Fessas, 1960; Koler & Rigas, 1961). However, in all these reports the composition of the second abnormal haemoglobin was not determined. Jones & Schroeder (1960) examined the red cells from three carriers of Hb- β^A_4 and found a second abnormal haemoglobin in each, but in only one case did this have the composition γ^F_4 . Recently, it has been shown that Hb-H can exist in two electrophoretically separable isomeric forms (Benesch, Benesch, Ranney & Jacobs, 1962), and these authors suggest that the second abnormal haemoglobin reported by other workers to occur in association with Hb- β^A_4 might be the isomeric form of this pigment.

It has been postulated that Hb- β^A_4 occurs as a result of an imbalance in the rates of formation of α - and β -chains (Jones *et al.* 1959). The idea that the α -chains of Hb-A ($\alpha^A_2\beta^A_2$), Hb-F ($\alpha^A_2\gamma^F_2$) and Hb-A₂ ($\alpha^A_2\delta^A_2$) arise from the same metabolic pool would then imply that individuals who carry Hb- β^A_4 might also carry haemoglobins consisting solely of γ - or δ -chains.

It had already been suggested that the individuals with Hb-H disease previously studied (Bingle, Huehns & Prankerd, 1958) might also carry Hb- γ^F_4 (Huehns *et al.* 1960), and more recently a third

abnormal haemoglobin was detected in their red cells (Huehns, 1962). Three of these Hb- β^A_4 carriers have been re-examined and a further case of Hb-H disease has been studied. It has now been shown that all four individuals carry three abnormal haemoglobins not containing any α -chains, namely Hb- β^A_4 , Hb- γ^F_4 and Hb- δ^A_2 . A preliminary report of the identification of Hb- δ^A_2 has been made (Dance & Huehns, 1962).

METHODS

Abbreviation. HiCN, Cyanide form of methaemoglobin.

Preparation of the haemolysate. The haemolysates were prepared from the red cells of four patients with Hb-H disease. The red cells from approx. 40 ml. of blood were washed four times with iso-osmotic NaCl soln., and lysed by shaking for 5 min. with 1 vol. of water and 0.5 vol. of toluene; the mixture was centrifuged and the clear haemoglobin solution pipetted off. The haemoglobin in the haemolysate was converted into HiCN by the addition of an excess of a solution containing 0.75 g. of potassium ferricyanide and 0.15 g. of KCN in 10 ml. of sodium phosphate buffer, pH 6.8, *I* 0.10. The excess of ferricyanide was removed by dialysis for 24 hr. against three separate 1 l. volumes of a buffer containing 0.1 M- NaH_2PO_4 and 0.01 M- KCN adjusted at room temperature to pH 7.0 with conc. H_3PO_4 (phosphate-KCN buffer, pH 7.0) over a period of 24 hr.

Purification of the three abnormal haemoglobins. This was carried out by chromatography followed by starch-block electrophoresis at two different pH values, as outlined in Scheme 1.

Chromatography. This was carried out on carboxymethylcellulose (Whatman CM. 70) at 4° (Huisman, Martis & Dozy, 1958). The carboxymethylcellulose powder was rubbed through a 60-mesh sieve and suspended in the phosphate-KCN buffer, pH 7.0. The pH of the slurry was then re-adjusted to 7.0 by the addition of 40% NaOH. A column, 90 cm. $\times$ 2 cm., was packed with the prepared carboxymethylcellulose and washed with 2 l. of the above-mentioned buffer. About 40 ml. of the prepared haemoglobin solution, containing about 4 g. of pigment, was applied to the column, care being taken to avoid dilution of the sample. Elution was carried out with the same buffer at a rate of 20 ml./hr. At pH 7.0 Hb-A is adsorbed by carboxymethylcellulose and the three abnormal haemoglobins and non-haem proteins are eluted. Because the large amount of haemoglobin applied to the column saturates its adsorptive capacity, the eluate still contained about 30% of

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Scheme 1. Flow sheet for the isolation of Hb- β_4^A , Hb- γ_4^F and Hb- δ^{A_2} from haemolysates of individuals with Hb-H disease.

Hb-A. The eluate was finally concentrated with concurrent dialysis against the phosphate-KCN buffer as described by Huehns & Shooter (1961).

Starch-block electrophoresis. The method outlined by Kunkel (1954), with maize starch B.P. (British Drug Houses Ltd.) as the supporting medium, was used.

The mixture of haemoglobins obtained on chromatography was first separated on a starch block made with a sodium phosphate buffer, pH 7.0, Na^+ ions 0.08M. A current of 60 mA passed through a block 25 cm. long, 28 cm. wide and 0.5 cm. deep, giving a voltage gradient of 5 v/cm., produced adequate separation overnight. The three 'fast' haemoglobins were eluted together, concentrated and subjected to starch-block electrophoresis in barbiturate buffer, pH 8.6, I 0.05, voltage gradient 8 v/cm. With this buffer three pigment zones formed on electrophoresis (Fig. 1*a*) and each was eluted separately. As the minor haemoglobin fractions were still contaminated with Hb- β_4^A and Hb-A, they were concentrated and separately submitted to electrophoresis on starch block, again using the phosphate buffer, pH 7.0 (Fig. 1*b*). After elution, concentration and dialysis against the phosphate-KCN buffer, pH 7.0, the isolated haemoglobins were used for the studies described below.

The proportions of Hb-A₂ and of Hb-($\beta_4^A + \gamma_4^F$) were estimated in the total haemolysates by starch-block electrophoresis in barbiturate buffer, pH 8.6, followed by spectrophotometry of the eluates. The separate proportions of Hb- β_4^A , Hb- γ_4^F and Hb- δ^{A_2} were obtained by measuring the relative amounts of the pure haemoglobins obtained after their final isolation.

Dissociation and recombination experiments. Recombination experiments of the isolated haemoglobins with canine haemoglobin were made as described by Huehns, Shooter & Beaven (1962*a*). The isolated abnormal haemoglobin was mixed with an equal amount of canine haemoglobin, dissociated by overnight dialysis at pH 4.7 and recombined by dialysis to pH 7.0. The resulting haemoglobin mixtures were analysed by starch-gel electrophoresis.

Fig. 1. Starch-block electrophoresis, 18 hr. (a) Barbiturate buffer, pH 8.6: isolated 'fast' haemoglobins. (b) Phosphate buffer, pH 7.0: (i) Hb- β_4^A ; (ii) Hb- γ_4^F ; (iii) Hb- δ^{A_2} (+ Hb-A).

Tryptic peptide mapping. A modification of the method of Baglioni (1961) was used.

The haem group was removed from the isolated haemoglobin with 0.15N-HCl in acetone at -15° and the resulting

globin hydrolysed at pH 9.5 and 37°, with trypsin, the pH being kept constant by the automatic addition of 0.1N-NaOH. After 90 min. the pH was adjusted to 6.5 with 0.1N-HCl, which precipitated the so-called 'core' peptides; these were removed by centrifuging.

The soluble tryptic peptides were separated by high-voltage electrophoresis in a pyridine-acetate buffer, pH 6.4 (Michl, 1951) under White Spirit 100, followed by chromatography at right angles in pyridine-3-methylbutan-1-ol-water (35:35:27, by vol.) (Heyns & Walter, 1951). The resulting peptide pattern was stained with ninhydrin (0.2%, w/v, in acetone) to give a peptide map ('fingerprint'). The presence of arginine, histidine, methionine, tryptophan and tyrosine in the peptides was detected by specific staining reactions (Smith, 1960).

Haemoglobin α^A . This haemoglobin was prepared as the HiCN derivative by a modification (Huehns, Dance, Shooter & Beaven, 1962*b*) of the original method (Huehns, Shooter & Dance, 1961).

Alkaline denaturation rate. This was carried out as described by Huehns, Dance, Shooter, Beaven & Gratzel (1962*c*).

Absorption spectra. These were measured on various automatic recording spectrophotometers over the wavelength range 2500–6500 Å. The position of the tryptophan fine-structure band was determined by the moving-plate method of Holiday (1937), as described in detail for haemoglobins by Beaven, Ellis & White (1960).

RESULTS

Analysis of the haemolysate of case I (Bingle et al. 1958)

Haemoglobin β^A . The abnormal haemoglobin migrating most rapidly towards the anode on starch-block electrophoresis in barbiturate buffer (Fig. 1*a*) is shown to be Hb- β^A .

Tryptic peptide mapping. The peptide map of this haemoglobin is compared with that of Hb-A in Fig. 2. The number of peptides produced by tryptic hydrolysis of Hb- β^A is less than from Hb-A, and those peptides in the map of Hb-A that are known to arise solely from the α^A -chain are absent. The peptides present correspond to those known to arise from the isolated β -chain of Hb-A. Two discrepancies can be seen on the 'fingerprints'. Some unidentified ninhydrin-staining material appears in the neutral peptide band in the position of peptide $\alpha T-9$, and the relative positions of peptides $\beta T-2$ and $\beta T-15$ appear to be altered. The appearance of unexplained ninhydrin-staining material in the neutral region of haemoglobin 'fingerprints' is well-recognized and need have no special significance. The difference in relative positions of peptides $\beta T-2$ and $\beta T-15$ of Hb-A and Hb- β^A can be accounted for by the presence of α -chain peptides in the same region of the peptide map of Hb-A. This analysis therefore indicates that this sample of Hb-H, like those of Jones *et al.* (1959) and Benesch *et al.* (1962), consists solely of β^A -chains.

Properties of Hb- β^A . (a) Absorption spectrum. Comparison of the absorption spectrum of Hb- β^A with Hb-A, both in the HiCN form, showed that the haem absorption maxima and minima occurred at the same wavelengths and had the same relative intensities. The tryptophan fine-structure band is an unresolved inflexion at 2910 Å, similar to that of Hb-A (Fig. 3).

Fig. 2. Tracing of tryptic peptide map of Hb- β^A (a) compared with that of Hb-A (b). Electrophoresis in pyridine-acetic acid-water (10:0.4:90, by vol.), pH 6.4, at 40 v/cm. for 75 min., followed by ascending chromatography with pyridine-3-methylbutan-1-ol-water (35:35:27, by vol.) overnight. α -Chain peptide areas are filled in; β -chain peptide areas are open. Peptides are numbered according to Baglioni (1961).

Fig. 3. Absorption spectrum of various haemoglobin species in HiCN form in 2800–3000 Å region. (i) Hb-A (and Hb- β^A); (ii) Hb-F; (iii) Hb- γ^F .

(b) Alkaline denaturation rate. Hb- β_4^A (as HiCN) is rapidly denatured by alkali, as an apparent first-order process with $t_{\frac{1}{2}}$ approx. 15 sec. (Fig. 4).

(c) Electrophoretic mobility. This has previously been described in detail by Rigas *et al.* (1956). On starch-gel electrophoresis the abnormal haemoglobin migrates with Hb-I in the discontinuous buffer system (Fig. 5a), and in phosphate buffer, pH 7.4 (Fig. 5b), it migrates more rapidly towards the anode than any other known haemoglobin variant.

(d) Formation of inclusion bodies. The presence of Hb-H in the original red cells was confirmed by the formation of inclusion bodies on incubation with brilliant cresyl blue at 37° (Rigas *et al.* 1956).

Haemoglobin γ_4^F . The abnormal haemoglobin with intermediate mobility on starch-block electrophoresis in barbiturate buffer (Fig. 1a) is shown to be Hb- γ_4^F .

Composition of Hb- γ_4^F . The peptide map of this haemoglobin compared with that of Hb-F is shown in Fig. 6. The number of peptides produced by tryptic hydrolysis of this haemoglobin is less than from Hb-F and the pattern corresponds to that expected for the tryptic peptides of the isolated γ -chain of Hb-F (N. Dance, E. R. Huehns & G. H. Beaven, unpublished work).

Dissociation and recombination of this haemoglobin with canine haemoglobin produced one new species, which migrated on electrophoresis close to the species $\alpha_2^{\text{Can.}}\beta_2^A$ and in the known position of the species $\alpha_2^{\text{Can.}}\gamma_2^F$ (Fig. 7; cf. Huehns *et al.* 1962c); the species $\alpha_2^A\beta_2^{\text{Can.}}$ was not formed.

Fig. 4. Alkaline denaturation rate plots of various haemoglobin species in the HiCN form at 25° in 38 mN-NaOH.

The peptide map indicates that this haemoglobin consists solely of γ^F -chains. The recombination experiment confirms the absence of α^A -chains in this haemoglobin, as well as indicating the presence of a polypeptide chain with the same net charge as the γ^F -chain. These results indicate that this haemoglobin, like that reported by Hunt & Lehmann (1959), consists solely of γ^F -chains.

Properties of Hb- γ_4^F . (a) Electrophoretic mobility. On starch-gel electrophoresis in the discontinuous buffer system Hb- γ_4^F migrates slightly more slowly towards the anode than Hb- β_4^A ; in phosphate buffer, pH 7.4, it migrates more slowly than Hb- β_4^A (Fig. 8) but more rapidly than Hb-I. On starch-block electrophoresis, both in barbiturate buffer, pH 8.6, and phosphate buffer, pH 7.0, Hb- γ_4^F migrates between Hb-A and Hb- β_4^A (Fig. 1).

(b) Absorption spectrum. The absorption spectrum of the HiCN derivative in the visible spectrum was the same as that of Hb-F. The spectrum from

Fig. 5. Starch-gel electrophoresis. (a) Tris-citrate-borate system; 90 min., o-dianisidine stain: (i) Hb-J; (ii) Hb- β_4^A ; (iii) Hb-A + Hb-I marker. (b) Phosphate buffer, pH 7.4; 2 hr., o-dianisidine stain: (i) Hb-A + Hb- β_4^A ; (ii) Hb-A + Hb-I.

2800 to 3000 Å is shown in Fig. 3. The tryptophan fine-structure band, at 2896 Å, is in the same position as for pure Hb-F, but is distinctly more resolved.

(c) Alkaline denaturation. Hb- γ_4^F is more resistant to alkali than Hb-A. The rate of denaturation of Hb- γ_4^F does not, however, proceed by the usual apparent first-order process. Even when denaturation is continued for 1½ hr. a linear semi-logarithmic plot is not obtained (Fig. 4). Three different samples of natural Hb- γ_4^F showed this behaviour. Under identical conditions the alkaline denaturation of Hb-F follows apparent first-order

kinetics, and the time for 50% denaturation is approx. 800 sec.

Haemoglobin δ^{A_2} . The 'fast' haemoglobin that migrates with Hb-A on starch-block electrophoresis in barbiturate buffer (Fig. 1a) is shown to consist solely of δ^{A_2} -chains.

Composition of Hb- δ^{A_2} . Analysis of the tryptic peptides by high-voltage electrophoresis followed by chromatography is shown in Fig. 9. Comparison with the tryptic peptide map of the β^A -chain or of Hb- β_4^A (Fig. 2) shows the presence of two extra peptides. These correspond in position to peptides $A_2\alpha$ and $\delta T-3$ described by Ingram & Stretton (1961). Methionine is not present in peptide $\beta T-13$

Fig. 6. Tracing of tryptic peptide map of Hb- γ_4^F (a) compared with that of Hb-F (b); conditions were as described in Fig. 2. α -Chain peptide areas are shown filled in; γ -chain peptide areas are open.

Fig. 7. Starch-gel electrophoresis in phosphate buffer, pH 7.4; migration time 2 hr., o-dianisidine stain. (i) Mixture of Hb-A with Hb-Canine dissociated and recombined; (ii) same mixture as (i), dissociated and recombined; (iii) mixture of Hb- γ_4^F with Hb-Canine.

Fig. 8. Starch-gel electrophoresis in phosphate buffer, pH 7.4; migration time 2 hr., o-dianisidine stain; same samples as Fig. 1b. (i) Hb- β_4^A ; (ii) Hb- γ_4^F ; (iii) Hb- δ^{A_2} ; (iv) haemolysate containing Hb- β_4^A . (Bubbles in the gel are caused by the release of oxygen from H_2O_2 in the staining reagent by catalase.)

Fig. 9. Tracing of tryptic peptide map of Hb- δ^{A_2} (a) compared with that of Hb- A_2 (b); conditions were as described in Fig. 2. α -Chain peptide areas are shown filled in; δ -chain peptide areas are open. Peptide notation of Ingram & Stretton (1961).

but is present in the corresponding peptides from Hb-A₂ (A. O. W. Stretton, personal communication; Dance & Huehns, 1962) and Hb- δ^A . Peptides β T-2 and β T-5 are known to differ chromatographically when compared with the corresponding δ^A -chain peptides in butanol-acetic acid-water (67:10:23, by vol.) (Ingram & Stretton, 1961). The two corresponding peptides from Hb- δ^A behave similarly to the Hb-A₂ peptides. No peptides derived solely from the α^A -chain are visible in the peptide map of Hb- δ^A . These results indicate that this haemoglobin consists solely of δ^A chains. The appearance of small amounts of peptide β T-3 indicate that the preparation is still contaminated with some Hb- β^A .

Reaction of Hb- δ^A with Hb- α^A . An electrophoretic analysis of a neutral mixture of haemoglobins δ^A and α^A is shown in Fig. 10. It can be seen that a new haemoglobin species is formed, which migrates in the Hb-A₂ position. The formation of a new haemoglobin species with the electrophoretic mobility of Hb-A₂ shows that Hb- δ^A contains some polypeptide chains with the same net charge as δ^A -chains. The formation of this new species at neutral pH is analogous to the formation of Hb-A in mixtures of Hb- α^A with Hb- β^A (Huehns & Shooter, 1962) and of Hb-F in mixtures of Hb- α^A with Hb- γ^F (Huehns & Beaven, 1962), and lends strong support to the idea that this haemoglobin consists solely of δ^A -chains. It is therefore called Hb- δ^A .

Properties of Hb- δ^A . (a) Absorption spectrum. Comparison of the absorption spectrum of Hb- δ^A with Hb-A, both in the HiCN form, showed that the haem absorption maxima and minima occurred at the same wavelengths and had the same relative intensities. The tryptophan fine-structure band is an unresolved inflexion at 2910 Å, like that of Hb-A (Fig. 3).

(b) Alkaline denaturation rate. The HiCN form of Hb- δ^A is rapidly denatured by alkali at a similar rate to Hb-A (Fig. 4).

(c) Electrophoretic mobility. On starch-block electrophoresis in barbiturate buffer, Hb- δ^A migrates with Hb-A (Fig. 1a), and in phosphate buffer, pH 7.0, it migrates between Hb- β^A and Hb-A, but slightly more slowly towards the cathode than Hb- γ^F (Fig. 1b). On starch-gel electrophoresis in phosphate buffer, pH 7.4, Hb- δ^A migrates between Hb-A and Hb- γ^F (Fig. 8), and in the discontinuous buffer system it migrates slightly more slowly towards the anode than Hb-A (Fig. 10).

(d) Molecular weight. The amounts of Hb- δ^A isolated in the present work were insufficient for molecular-weight studies. In its behaviour during concentration dialysis Hb- δ^A resembles the tetramer haemoglobins (mol.wt. approx. 68000) rather than the predominantly monomeric species Hb- α^A (Huehns, Shooter, Dance, Beaven & Shooter, 1962d).

The proportions of the various haemoglobins found were: Hb-A, 91%; Hb- β^A , 7.5%; Hb-A₂, 1%; Hb- δ^A , 0.3%; Hb- γ^F , 0.2%.

Analysis of other cases of Hb-H disease

Case II (Bingle et al. 1958). The three 'fast' haemoglobins from this individual were isolated as described above. Hb- β^A and Hb- γ^F were identified by peptide mapping, recombination experiments, absorption spectra and alkaline denaturation studies. Hb- δ^A was isolated and characterized by electrophoresis on starch block and starch gel, as well as by absorption spectra and alkaline denaturation studies. The proportions of the various haemoglobins in this individual were somewhat different: Hb-A, 83%; Hb- β^A , 14%; Hb- γ^F , 2%; Hb-A₂, 1%; Hb- δ^A approx. 0.1%.

Case III (sister of case II; Bingle et al. 1958). The presence of Hb- β^A , Hb- γ^F and Hb- δ^A was shown by isolation of the abnormal haemoglobins by starch-block electrophoresis. The haemoglobin corresponding to Hb- γ^F was relatively resistant to alkali and had a well-resolved tryptophan fine-structure band at 2896 Å. No analytical studies on the haemoglobin of this individual were made. The proportions found were comparable with those of her sister, with a relatively low proportion of Hb-A₂ and more Hb- γ^F than Hb- δ^A .

Case IV (previously unreported). Only one sample has so far been obtained from this patient. The 'fast' haemoglobins of this haemolysate were isolated by starch-block electrophoresis in phosphate buffer, pH 7.0. Further examination of the 'fast' fraction showed that it contained approx. 10% of alkali-resistant pigment. The position of the tryptophan fine-structure, at 2905 Å, also confirmed the presence of a haemoglobin species containing γ -chains in this fraction. Electrophoresis in starch gel, with phosphate buffer, pH 7.4, revealed three

Fig. 10. Starch-gel electrophoresis in the tris-citrate-borate system; migration time 90 min., o-dianisidine stain. (i) Hb-A, Hb-A₂; (ii) Hb- δ^A and Hb- α^A , mixed at room temperature immediately before insertion into gel; (iii) Hb- δ^A ; (iv) Hb- α^A .

'fast' haemoglobins in the known positions for Hb- β_4^F , Hb- γ_4^F and Hb- δ^A when stained with benzidine and hydrogen peroxide.

DISCUSSION

The haemoglobins present in the red cells from four individuals with Hb-H disease have been examined. It has been shown that in each individual three abnormal haemoglobins are present, and that the proportion of Hb- A_2 is, in each case, less than in normal red cells. Analyses of the abnormal haemoglobins show that none of these contains α -chains, but all consist solely of β^A -, γ^F -, or δ^A -chains respectively. The concurrent appearance of these three non- α -chain haemoglobins in four individuals with Hb-H disease is consistent with the present concept of the final stages of haemoglobin synthesis. A number of workers have presented evidence that the α -chains of Hb-A, Hb-F and Hb- A_2 are identical, and it has been postulated that the final stage in their formation is the combination of independently synthesized α -chains with β -, γ - and δ -chains to form the haemoglobins normally found *in vivo*. It has been suggested that the appearance of Hb-H is due to a reduced rate of synthesis of α -chains (Jones *et al.* 1959). It might therefore be expected that these individuals would also carry some Hb- γ_4^F and Hb- δ^A . The relative amounts of the three non- α -chain haemoglobins found presumably depend on the relative rates of synthesis and combination of the various polypeptide chains *in vivo*.

A second abnormal haemoglobin has been reported in a number of cases of Hb-H disease. This usually appears to resemble Hb- γ_4^F (cf. Tuchinda *et al.* 1959; Fessas & Mastrokalos, 1959; Ramot *et al.* 1959; Fessas, 1960; Silvestroni *et al.* 1960). In other cases, it resembles Hb-A in its alkaline denaturation rate and ultraviolet-absorption spectrum (Fessas, 1960; Koler & Rigas, 1961); it is possible that these are examples of Hb- δ^A . Huisman *et al.* (1958) analysed the haemolysates from an individual with Hb-H disease, and one of the chromatograms showed Hb-A, Hb- A_2 and Hb- β_4^A , as well as two minor fractions eluted between Hb-A and Hb- β_4^A , possibly Hb- γ_4^F and Hb- δ^A . The finding that the proportion of Hb- A_2 is reduced in Hb-H disease (Gerald & Diamond, 1958; Ramot *et al.* 1959; Dittman, Haut, Wintrobe & Cartwright, 1960; Koler & Rigas, 1961) implies either that δ^A -chain synthesis is impaired, or more probably, that some δ^A -chains are not used for the formation of Hb- A_2 and presumably form Hb- δ^A . The finding that Hb- δ^A is present in all four cases of Hb-H disease examined here, in association with a low Hb- A_2 , suggests that Hb- δ^A may always be present in Hb-H disease.

The fact that some authors have found Hb- γ_4^F , whereas others have reported only a haemoglobin resembling Hb- δ^A , suggests that the relative proportions in which these two species occur may vary. In case I the proportion of Hb- δ^A exceeds that of Hb- γ_4^F , whereas in cases II, III and IV more Hb- γ_4^F than Hb- δ^A is found. This situation is similar to the variations in the relative proportions of Hb-F and Hb- A_2 found in thalassaemia minor (Went & MacIver, 1961; Zuelzer, Robinson & Booker, 1961; Gouttas, Tsevremis, Papaspyrou, Fertakis & Vorias, 1961; Beaven & White, 1962); these separate observations, although at present unexplained, may be related.

The electrophoretic mobilities of Hb- γ_4^F and Hb- δ^A in phosphate buffers are very similar, and any second abnormal haemoglobin found in Hb-H disease must be further characterized to establish its identity. This is relatively easy for Hb- γ_4^F , for which the typical ultraviolet absorption of the isolated haemoglobin and its alkaline denaturation rate confirm the presence of γ -chains. Hb- δ^A resembles the isomer of Hb-H described by Benesch *et al.* (1962) in its ultraviolet-absorption spectrum rate of alkaline denaturation and at least some of its electrophoretic properties. The two species can, however, be differentiated by peptide mapping.

The specific spectroscopic features of Hb-F and Hb- γ_4^F require further comment, in view of certain factors which influence the quantitative evaluation of these features, and which have not been mentioned by previous workers. Although the increased resolution of the tryptophan fine-structure band in Hb- γ_4^F , as compared with Hb-F ($\alpha_2^F\gamma_2^F$), is a possible criterion for distinguishing between these two haemoglobin species, it must be used with some care. The experimental data can be placed on a uniform basis by expressing the observed resolution in terms of a 'fractional resolution' (R) defined as $\Delta E/E_{\max}$, where ΔE is the extinction difference ($E_{\max} - E_{\min}$) between the maximum and minimum of the resolved fine-structure band, and $E_{\max}$ is the extinction at the maximum. The fractional resolution is thus the value of ΔE that would be observed for a haemoglobin solution having E 1.000 at the fine-structure band maxima. For the purest samples of Hb- γ_4^F studied the values of R , measured at an appropriate spectral band width (see below), are in the range 5.6×10^{-2} – 6.1×10^{-2} ; thus for a solution with $E_{\max}$ 0.5 (at the fine-structure band) the observed value of ΔE (approx. 0.03) is quite small and must be measured with some care; for more dilute solutions the observed values of ΔE are reduced in proportion. With less pure samples of Hb- γ_4^F lower values of R , down to 2×10^{-2} , have been obtained. It seems likely that this is due to contamination with Hb- β_4^A or with ultraviolet-

Table 1. *Dependence of resolution and R on spectral band width*These results are for a sample of Hb- γ_4^F .

Spectral band width ($\text{\AA}$)	ΔE	$10^2 R$
14	0.009	1.8
7	0.018	3.7
5	0.019	3.9

absorbing impurities, either of which would reduce the magnitude of ΔE for a given value of $E_{\max}$.

For a given sample of Hb- γ_4^F the observed resolution ΔE , and hence R , depends on the nominal spectral band width used, as shown by the results in Table 1 for a sample of Hb- γ_4^F of moderate (spectrophotometric) purity. These figures in Table 1 and results of similar tests on other samples of Hb- γ_4^F show that for maximum resolution the spectral band width used should not exceed 10 $\text{\AA}$; trials with two recording spectrophotometers and a manual instrument indicate that a value of 5–7 $\text{\AA}$ is suitable.

Measurements at the same spectral band width on samples of pure Hb-F gave R approx. 2×10^{-2} , with slightly lower values for cord-blood haemolysates (approx. 80 % Hb-F). The fractional resolution of the tryptophan fine-structure band in Hb-F is thus less than half that found for Hb- γ_4^F , and these two haemoglobin species could be distinguished by this feature alone, in the absence of ultraviolet-absorbing impurities, if measured at an appropriate constant spectral band width.

A previous spectrophotometric study of the fine-structure band (Fessas, 1960; cf. Choremis, Zannos-Mariolea, Ager & Lehmann, 1959) clearly shows the increased resolution in a fraction containing Hb- γ_4^F , when compared with Hb-F, but is inadequate to calculate R for the two species. The more precise data of Silvestroni *et al.* (1960) and of Silvestroni & Bianco (1961) can be used to derive R 0.4×10^{-2} and 0.8×10^{-2} – 3.2×10^{-2} for Hb-F and various samples of Hb- γ_4^F respectively, but the spectral band width used was not stated.

SUMMARY

1. The haemolysates from four individuals with Hb-H disease have been examined and each has been shown to contain three abnormal haemoglobins not containing α -chains, namely Hb- β_4^A , Hb- γ_4^F and Hb- δ^A . These three haemoglobins have been isolated, characterized by electrophoresis in starch gel and starch block, peptide mapping, ultraviolet- and visible-absorption spectra and alkaline denaturation rate.

2. Hb- β_4^A gives a peptide map identical with that of the β^A -chains of normal Hb-A. Its tryptophan

fine-structure band is an unresolved inflexion in the adult position, and its alkaline denaturation rate is fast and comparable with that of Hb-A.

3. Hb- γ_4^F gives a peptide map identical with that of the γ^F -chains of normal Hb-F. Its tryptophan fine-structure band is in the foetal position, but is resolved to a greater degree than in Hb-F. It is relatively resistant to alkaline denaturation, but its behaviour in this respect is not identical with Hb-F.

4. Hb- δ^A gives a peptide map identical with that of the δ^A -chains of Hb-A₂. Its tryptophan fine-structure band is an unresolved inflexion in the adult position, and its alkaline denaturation rate is fast and of the same order as the rates for Hb-A and Hb-A₂.

5. The proportions of Hb- β_4^A , Hb- γ_4^F and Hb- δ^A vary in the four cases studied.

6. The findings are discussed in relation to present concepts of the final stages of normal haemoglobin synthesis and their disturbance in Hb-H disease.

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The Distribution of Adenosine Triphosphate in Subcellular Fractions of Brain Tissue

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Most of the bound acetylcholine (Whittaker, 1959), hydroxytryptamine (Whittaker, 1959; Michaelson & Whittaker, 1962) and noradrenaline (Chruściel, 1960) of brain tissue are recovered in a subcellular fraction which consists mainly of pinched-off nerve endings (Gray & Whittaker, 1960, 1962). The work now to be described originated in an attempt to see whether ATP was involved in the binding of the pressor amines to the particles of this fraction, as it seems to be in the denser and morphologically different type of storage granule found in adrenal medulla, platelets, duodenal mucosa and adrenergic nerves (for reviews see Hagen & Barrnett, 1960; Blaschko, 1959).

A brief account of this work has been given by Whittaker (1961).

METHODS

Preparation of subcellular fractions from brain tissue. Fractions were prepared at 0–4° from guinea-pig and dog

brain essentially as described by Whittaker (1959) and Gray & Whittaker (1962) and as summarized in Scheme 1. Unless otherwise stated adult guinea pigs (400–500 g. body wt.) were killed by decapitation, with or without a preceding blow at the base of the skull. Dogs were killed under ether or nembutal anaesthesia by bleeding or by sectioning the brain stem. The brains were removed as rapidly as possible and chilled before dissection and homogenization. Factors making for satisfactory recoveries were as follows. (1) All traces of detergent were excluded from the apparatus used in the experiment, otherwise losses of ATP of up to 90% could occur. Glassware was cleaned by brushing in hot water and rinsing in dilute HCl, followed by glass-distilled water. (2) Adequate refrigeration was maintained, especially during centrifuging. The use of the Servall refrigerated automatic centrifuge type SS-34 in place of the bench model (SS-1) in the cold room for the preparation of the P_2 fraction doubled the ATP content of this fraction. Density-gradient separations at 39 000 rev./min. for 45 min. instead of at 25 000 rev./min. for 2 hr. increased the recovery of ATP in the A, B and C subfractions by over 25%. (3) Pellets were resuspended with as little mechanical disturbance as possible. Even so, 15–20% of 'stable' ATP was lost during the preparation of P_2 , and a further 10% during high-speed centrifuging.

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