The Properties and Reactions of Haemoglobin F_I and their Bearing on the Dissociation Equilibrium of Haemoglobin

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(Received 27 April 1966)

1. Hb-F_I, with the previously proposed structure $\alpha_2^A\gamma^F\gamma^{A\alpha F}$, would be an exception to the general hypothesis that haemoglobins are in mobile equilibrium with their sub-units at all pH values. However, studies presented in this paper suggest that this is not so. 2. Gel-filtration and sedimentation analyses show that Hb-F_I dissociates at acid pH and gives only the usual types of hybrids in recombination experiments. When self-hybridized, Hb-F_I is the main haemoglobin species re-formed, although small but increasing amounts of Hb-F_{II} appear on prolonged exposure to acid. 3. Exchange experiments with isotopically labelled Hb-F_{II} and unlabelled Hb-F_I show no exchange of sub-units at neutral pH or after brief exposure to acid pH. Under equilibrium conditions at acid pH non- α^A -chains do not exchange, although α^A -chains equilibrate completely between the two species. 4. These results indicate that Hb-F_I does not contain γ^F -chains and its possible structure is discussed on this basis. Since the dissociation properties of Hb-F_I are not markedly different from those of Hb-A or Hb-F_{II} it is concluded that Hb-F_I, like other haemoglobins, is an equilibrium system.

One of the striking aspects of the problem of the assembly of the sub-units in the haemoglobin molecule is the failure, with two possible exceptions noted below, to isolate from either erythrocyte haemolysates or recombination systems *in vitro* molecules containing either unlike α - or unlike β -chains. Moreover, attempts to produce such species by a number of different approaches have not been successful. These attempts have included the recombination of a mixture of largely monomeric α^A - and α^G -chain sub-units with β^A -chain sub-units (Huehns, 1962; Huehns & Shooter, 1966), the recombination of a mixture of globin A, $\alpha^A\beta^A$, and globin C, $\alpha^A\beta^C$, with haem (Winterhalter & Huehns, 1964) and the partial digestion of Hb-A with carboxypeptidase (Bucci, Fronticelli, Dance & Shooter, 1965). Guidotti, Konigsberg & Craig (1963) also noted that a haemoglobin species with different substituents on each of its two β^A -chains was not observed when Hb-A was treated simultaneously with two thiol-alkylating agents. They suggested, however, that such a species was produced in the reaction but remained undetected in the chromatographic separation because of the reassortment of $\alpha\beta$ sub-units resulting from a rapid

equilibrium between whole and half haemoglobin molecules. They argued that provided the rate of separation of the haemoglobin species is significantly lower than the rates of interconversion in the equilibrium:

a species Hb- $\alpha_2^A\beta^A\beta^C$, for example, would be converted during the separation process into the species Hb- $\alpha_2^A\beta_2^A$ and Hb- $\alpha_2^A\beta_2^C$ via its $\alpha\beta$ sub-units. To make this explanation acceptable, it has to be shown that haemoglobin is in the above equilibrium at neutral pH and either that there are no isolatable species with the structure $\alpha_2\beta^X\beta^Y$ or, if such haemoglobins exist, that these do not dissociate at neutral pH.

The existence of an equilibrium between the whole haemoglobin molecule and its sub-units was observed many years ago by Lamm & Polson (1936) and Pedersen (1940) and demonstrated more recently by thin-film dialysis studies (Guidotti & Craig, 1963). The finding that hybrid haemoglobins form in haemoglobin mixtures at neutral (as well as acid or alkaline) pH directly confirmed the presence of an equilibrium involving sub-units

at those pH values (Drabkin & Wise, 1962; Huehns, Beaven & Stevens, 1964b). Guidotti (1964) has now also shown that the elution behaviour from Sephadex G-200 at neutral pH of a mixture of normal adult and the two alkylated haemoglobins indicates interaction between all the different types of $\alpha\beta$ sub-units. Further, Benesch, Benesch & Macduff (1965) have found that at 30° and I 0.25 the oxygen equilibrium curve of an equimolar mixture of ferro- and ferri-haemoglobin coincides with that of a haemoglobin solution in which half the haem groups have been oxidized to the ferri form, indicating a rapid exchange of $\alpha\beta$ sub-units. The evidence in favour of the mobile equilibrium between whole molecules and $\alpha\beta$ sub-units at neutral pH is therefore convincing.

However, two haemoglobin species in which unlike non- α -chains are present have been reported. These are Hb- F_I , a normal minor component found in all cord-blood samples (Allen, Schroeder & Balog, 1958), with the structure $\alpha_2\gamma^F\gamma^{AcF}$ (Schroeder, Cua, Matsuda & Fenninger, 1962), and Hb- A_{10} , a normal minor component, with the structure $\alpha_2^A\beta^A\beta^{XA}$ (Holmquist & Schroeder, 1964). In Hb- F_I one γ^F -chain is acetylated at the N -terminal residue, whereas in Hb- A_{10} the N -terminal amino group of one β^A -chain carries an unknown substituent. If the mobile-equilibrium idea outlined above is accepted, then it should not be possible to isolate either of the above two haemoglobins unless they differ significantly from other haemoglobins in their dissociation properties. The present paper reports a detailed study of the dissociation and recombination reactions of Hb- F_I and concludes that these properties are not unusual. On this basis, as well as on the results of a number of sub-unit-exchange experiments with radioactive tracers, it would appear that the structure of Hb- F_I is more complex than that proposed by Schroeder *et al.* (1962).

MATERIALS AND METHODS

Haemoglobins. Hb- F_I and Hb- F_{II} were isolated from normal cord-blood haemolysate by column chromatography on Amberlite IRC-50 as described by Allen *et al.* (1958), with their developer 4 throughout. Each haemoglobin was further purified by rechromatography.

Radioactive Hb- F_{II} was made by incubating reticulocyte-rich cord blood (obtained from patients with haemolytic disease of the newborn) with either ^{59}Fe -labelled transferrin or ^{14}C -labelled amino acid. After incubation at 37° for 8 hr. the cells were washed five times with NaCl (0.9 g./100 ml.) and the haemolysates prepared in the usual way. The haemolysates were centrifuged to give a clear solution and dialysed against developer 4. The radioactive Hb- F_{II} was then isolated by chromatography on Amberlite IRC-50 (Allen *et al.* 1958).

Hb- β_4^A was isolated from a patient with Hb-H disease. The structure of this haemoglobin has previously been determined (Dance, Huehns & Beaven, 1963).

Hb- α -rabbit was prepared by exposing rabbit haemolysate to 1.0M-sodium acetate buffer, pH 4.7, for 48 hr., dialysing to pH 8.6 against 0.05M-sodium diethylbarbiturate buffer and isolating the zone nearest the cathode after starch-block electrophoresis in the same buffer. This sub-unit, like the isolated human Hb- α^A -chain sub-unit, is largely monomeric at neutral and acid pH (D. Labie, M. Jacobs, E. R. Huehns & E. M. Shooter, unpublished work).

The species Hb- $\alpha_2\beta_2^{\text{can}}$ was made by recombining Hb- G_{Bristol} with Hb-canine (Huehns, Shooter & Beaven, 1962a) and isolating the hybrid species by starch-block electrophoresis at pH 8.6. Sperm-whale myoglobin was obtained from Seravac Laboratories Ltd. (Maidenhead, Berks.). Starch-gel electrophoresis was carried out as described by Gammack, Huehns, Shooter & Gerald (1960) with a tris-EDTA-borate buffer, pH 8.3 (69.9 g. of tris, 5.84 g. of disodium EDTA and 30.9 g. of boric acid/l.), diluted 1:20 for making the gel and 1:7 for the buffer vessels.

Transmission densitometry. The relative proportions of pigment in the various zones were determined by transmission densitometry after staining with Naphthalene Black and differentiating in a glycerol-water-acetic acid solvent mixture (Gratzer & Beaven, 1960; G. H. Beaven & B. L. Stevens, unpublished work). The densitometer records were evaluated by planimetry.

Recombination experiments followed the procedure outlined by Gammack *et al.* (1960) with 0.1M-sodium acetate-0.01M-KCN buffer, pH 4.7, and dialysing against developer 4 for the adjustment from acid to neutral pH. The time-course experiment followed the procedure of Huehns, Shooter & Beaven (1964a).

Gel-filtration experiments. These were carried out on Sephadex G-100 (Pharmacia, Uppsala, Sweden) in 150 cm. $\times$ 1.1 cm. columns with 0.1M-sodium acetate-0.01M-KCN buffer, pH 4.7, and flow rates of 6–10 ml./hr. The appropriate haemoglobin or myoglobin solution was dialysed against the pH 4.7 buffer for 6 hr. with stirring and then adjusted to a concentration of 10 mg./ml. with the same buffer. To 0.2 ml. of this solution was added 10 mg. of bovine serum albumin (fraction V, crystalline; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex), 10 mg. of ovalbumin, 20 mg. of ribonuclease (Seravac Laboratories Ltd.) and Blue Dextran 2000 (Pharmacia) to mark the void volume. Apparent molecular weights were calculated by the procedure of Whitaker (1963).

Sedimentation coefficients were determined in a Spinco model E ultracentrifuge operated at 59 780 rev./min. and 5°.

Spectrophotometric procedures. These were as described by Dance *et al.* (1963).

Determination of radioactivity of the haemoglobin species. The ^{59}Fe -containing haemoglobins were measured with a well-type scintillation counter. The ^{14}C -labelled haemoglobins were measured in an automatic liquid-scintillation counter. The protein was first precipitated with trichloroacetic acid (5 g./100 ml.), then washed twice with acid acetone and finally dried with ether. The dried protein was transferred to the counting vials and dissolved in 1 ml. of 2M-Hyaminate hydroxide at 60°. After cooling, 15 ml. of scintillation fluid was added [4 g. of 2,5-diphenyloxazole and 0.05 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 l. of toluene]. The efficiency for ^{14}C was approx. 40% (Vaughan, Steinberg & Logan, 1957).

RESULTS

Dissociation of Hb-F_I at acid pH. The apparent molecular weight of Hb-F_I at acid pH is compared with those of Hb-F_{II} and Hb-A in Table 1. At the same concentration but at neutral pH all three haemoglobins have elution volumes corresponding to apparent molecular weights of approx. 45 000. The effluent volume at neutral pH increases with increasing dilution (Guidotti, 1964), and both this and the anomalous elution volumes themselves are indications of the mobile equilibrium at neutral pH. Ackers & Thompson (1965) have used such results to calculate the equilibrium constant for the dissociation. Hb-F_I is retarded in the columns at acid pH to the same extent as Hb-F_{II} and Hb-A (Fig. 1). The sedimentation coefficients of the three haemoglobins at acid pH are also within the same range and significantly lower than the corresponding values at neutral pH (Table 2).

Table 1. Gel filtration of Hb-F_I, Hb-F_{II}, Hb-A and myoglobin on Sephadex G-100 with 0.10M-sodium acetate-0.01M-potassium cyanide buffer, pH 4.7

Serum albumin (68000), ovalbumin (45000) and ribonuclease (13000) were used as molecular-weight markers. The initial concentration of haemoglobin or myoglobin was 1.0g./100ml.

Hb species	<i>M</i> (app.)
F _I	30200
F _{II}	30600
A	30900
Myoglobin	17400

Fig. 1. Gel filtration of Hb-F_I on Sephadex G-100. A 0.2ml. volume of Hb-F_I solution (1.0g./100ml.) in 0.10M-sodium acetate-0.01M-KCN buffer, pH 4.7, containing also 10mg. of bovine serum albumin, 10mg. of ovalbumin, 20mg. of ribonuclease and Blue Dextran 2000 was eluted from the column with the pH 4.7 buffer. The temperature was 2° and the flow rate 7ml./hr. —, *E*₂₈₀; ····, *E*₄₂₀.

Both sets of measurements indicate that Hb-F_I dissociates at acid pH. The *S*_{20,w} values for both Hb-F_I and Hb-F_{II} at acid pH are higher than that for Hb-A. Thus, at the concentrations used in the sedimentation analysis, neither Hb-F_I nor Hb-F_{II} dissociates to the same extent as Hb-A. At the lower concentrations reached in the gel-filtration experiments, where the haemoglobin is finally eluted at about 0.05g./100ml., these differences are not so marked.

Recombination experiments with Hb-F_I. (a) Recombination of Hb-F_I with canine haemoglobin. The time-course of the recombination of Hb-F_I with canine haemoglobin is shown in Table 3, which shows that equilibrium is reached in approx. 45hr. Under the same conditions, equilibrium is reached in the exchange of sub-units between Hb-A and canine haemoglobin in approx. 24hr. (Huehns *et al.* 1964a). Since, in a symmetrical dissociation, the exchange takes place at the level of individual monomer chain sub-units, these results suggest that the dissociation of the αγ sub-units under these conditions is less than that of the αβ sub-unit. Taken together with the sedimentation analyses, it follows that, though Hb-F_I is clearly in equilibrium with monomer and dimer sub-units at acid pH, the equilibrium is displaced, in comparison with Hb-A, towards the tetramer.

(b) Recombination with Hb-α₂^Gβ₂^{Can}. Since the acid dissociation of Hb-F_I at concentrations above 0.5g./100ml. is less complete than that of Hb-A, it could be argued that the stability of Hb-F_I itself at neutral pH is a result of the equilibrium between tetramer and αγ sub-units being displaced in favour of the tetramer at these pH values. However, if this were so, the hybrid of Hb-F_I with unlike α-chains, e.g. Hb-α^Gα^γγ^{AcF}, should also be stable and resolvable by starch-gel electrophoresis. Fig. 2 shows the results of the recombination of Hb-F_I and Hb-F_{II} with the species α₂^Gβ₂^{Can}. This species

Table 2. Sedimentation coefficients of Hb-F_I, Hb-F_{II} and Hb-A at neutral and acid pH

A Spinco model E ultracentrifuge operated at 59780 rev./min. and 5° was used. The solvent at neutral pH was 0.05M-phosphate-0.10M-NaCl-0.01M-KCN buffer, pH 7.0, and that at acid pH was 0.10M-sodium acetate-0.01M-KCN buffer, pH 4.7. The haemoglobin concentrations were 0.62g./100ml. at neutral pH and 0.57g./100ml. at acid pH.

Hb species	<i>S</i> _{20,w} (s)	
	Neutral pH	Acid pH
F _I	4.27	3.41
F _{II}	4.21	3.30
A	4.25	2.98

Table 3. *Dissociation and recombination of Hb-F_I with canine haemoglobin*

Equal volumes of Hb-F_I solution (1.0g./100ml.) and canine haemoglobin solution (1.0g./100ml.) were mixed and dialysed with stirring against 0.10M-sodium acetate-0.01M-KCN buffer, pH4.7, for various times and then against developer 4 (Allen *et al.* 1958) for 16hr. The resultant haemoglobin solutions were analysed by starch-gel electrophoresis as described in the Materials and Methods section.

Exposure to acid pH (hr.)	Proportion of total Hb (%)			
	$\alpha_2^A\beta_2^{\text{Can}}$	Hb-F _I + Hb-Can	$\alpha_2^{\text{Can}}\gamma^F\gamma^{\text{AcF}}$	Hb- γ_4
0		100		
6	9	83	8	Trace
21	17	65	18	2
30	19	56	22	3
45	24	48	25	3

Fig. 2. Dissociation and recombination of $\alpha_2^G\beta_2^{\text{Can}}$ with Hb-F_I and Hb-F_{II}. Equal volumes of $\alpha_2^G\beta_2^{\text{Can}}$ solution (1.0g./100ml.) and Hb-F_I solution (1.0g./100ml.) or Hb-F_{II} solution (1.0g./100ml.) were mixed, dialysed against 0.10M-sodium acetate-0.01M-KCN buffer, pH4.7, for 50hr. and then against developer 4 for 16hr. Starch-gel analysis was carried out in the tris-citrate/borate discontinuous buffer system, pH8.6 (Poulik, 1957); the migration time was 4hr. The bands were stained with *o*-dianisidine. (a) $\alpha_2^G\beta_2^{\text{Can}}$ + Hb-F_{II} control mixture; (b) $\alpha_2^G\beta_2^{\text{Can}}$ + Hb-F_I dissociated and recombined; (c) $\alpha_2^G\beta_2^{\text{Can}}$ + Hb-F_I control mixture; (d) $\alpha_2^G\beta_2^{\text{Can}}$ + Hb-F_I dissociated and recombined.

was chosen because of its relatively large charge difference from Hb-F_I and Hb-F_{II} and because the new hybrid species would be resolved both from each other and from the parent species. The compositions of the usual type of hybrid species are given in the following equations:

The identity of the new haemoglobin zones in the recombination with Hb-F_{II} follows from the known properties of the mixed human-canine haemoglobin hybrids (Huehns *et al.* 1962a; Huehns, Shooter, Beaven & Gratzner, 1962b). The two new

Scheme 1. Dissociation equilibria of Hb-F_I.

species formed in the recombination with Hb-F_I can be identified as $\alpha_2^A\beta_2^{\text{Can}}$ and $\alpha_2^G\gamma^F\gamma^{\text{AcF}}$. The species $\alpha_2^A\alpha^G\gamma^F\gamma^{\text{AcF}}$, which would migrate between $\alpha_2^G\gamma^F\gamma^{\text{AcF}}$ and Hb-F_I, was not observed. An analogous result was obtained in the recombination of Hb-A with $\alpha_2^G\beta_2^{\text{Can}}$. Thus the properties of Hb-F_I at acid pH and its behaviour in recombination experiments do not markedly differentiate it from Hb-A or Hb-F_{II} and cannot therefore be used to argue against a mobile equilibrium for Hb-F_I. Its postulated stability is therefore determined by other factors.

Self-hybridization of Hb-F_I. Allowing that Hb-F_I is a mobile equilibrium system at neutral pH of the type suggested by Guidotti *et al.* (1963), it follows that it shares a common $\alpha\gamma^F$ sub-unit with Hb-F_{II} (Scheme 1). The other $\alpha^A\gamma^{\text{AcF}}$ sub-unit is potentially in equilibrium with the theoretical species $\alpha_2^A\gamma_2^{\text{AcF}}$. Hb-F_I dissociates at acid pH into its $\alpha\gamma$ sub-units, and self-hybridization experiments permit a test of which of the three potential tetramers can form. Isolated Hb-F_I was dialysed against the usual acid buffer for various times and then neutralized by dialysis against developer 4. The resulting haemoglobin species were isolated by chromatography on Amberlite IRC-50 (Fig. 3), and the properties of the various haemoglobins found in these solutions are listed in Table 4. Some precipitation of haemoglobin occurred in these experiments, the amount increasing with lengthening exposure to acid pH. As well as some Hb-F_{II} and Hb- γ_4 , some Hb- α^A was also formed in these experiments, but this amount was too small to be detected by the

chromatographic system used. Starch-gel electrophoresis of the whole acid-treated solution showed that this was less than 1% of total haemoglobin. To confirm the identity of the various haemoglobin fractions eluted from the Amberlite IRC-50 column, each fraction was concentrated and examined by starch-gel electrophoresis (Fig. 4). Hb- $\gamma_4^{F_1}$ migrated as a single zone ahead of Hb-F_I (Huehns, Dance, Jacobs, Beaven & Shooter, 1965). The fraction labelled 'X' appears in aged samples of Hb-F_I as well as on exposure to acid pH (e.g. Table 4). It

Fig. 3. Self-hybridization of Hb-F_I, analysed by chromatography after concentration on a 1 cm. $\times$ 35 cm. column of Amberlite IRC-50 with developer 4 as elution buffer. The column was brought to 25° for elution of Hb-F_{II}. (a) Untreated control solution of Hb-F_I; (b) solution of Hb-F_I (0.5 g./100 ml.) dialysed to pH 4.7 for 43 hr. and then for 16 hr. against developer 4.

formed two zones on the starch gel, one migrating like Hb-F_I and the other, more diffuse, migrating between Hb-F_I and Hb- $\gamma_4^{F_1}$. This haemoglobin zone has not been further identified. Hb-F_I migrated at the same rate as untreated Hb-F_I and the presumed Hb-F_{II} at the same rate as natural Hb-F_{II}.

Hb- $\gamma_4^{F_1}$ was further identified by ultraviolet spectroscopy. The tryptophan fine-structure band was well resolved at approx. 2900 Å, with a fractional resolution $R = 4.3 \times 10^{-2}$ at a spectral band-width of approx. 8 Å, which is close to that obtained for natural Hb- γ_4^F by Dance *et al.* (1963), namely $R = 3.9 \times 10^{-2}$ at a spectral band-width of 5 Å. This clearly distinguishes it from Hb-F_{II}, in which $R = \text{approx. } 2 \times 10^{-2}$ (Dance *et al.* 1963), and also Hb-F_I, where $R = 2.3 \times 10^{-2}$.

Table 4 shows that the major species that is re-formed in these self-hybridization experiments is Hb-F_I itself. Exposure to acid pH produces

Fig. 4. Self-hybridization of Hb-F_I: identification of haemoglobin fractions eluted from Amberlite IRC-50. Starch-gel analysis was carried out in the tris-EDTA/borate buffer, pH 8.3; the migration time was 4 hr. The bands were stained with *o*-dianisidine. (a) Fraction 'X'; (b) Hb-F_I fraction; (c) Hb-F_{II} fraction; (d) natural Hb-F_{II} (no acid treatment); (e) natural Hb-F_I (no acid treatment). The analysis of fraction 'X' shows the presence of trace amounts of Hb- γ_4 as well as some Hb-F_I.

Table 4. Self-hybridization of Hb-F_I

A 0.35 ml. volume of Hb-F_I solution (5.0 g./100 ml.) in developer 4 plus 3.15 ml. of 0.10 M-sodium acetate-0.01 M-KCN buffer, pH 4.7, was dialysed for various times against the same acetate buffer and then against developer 4 for 16 hr. The resultant solutions were analysed by chromatography on a 1 cm. $\times$ 35 cm. column of Amberlite IRC-50 with developer 4 as elution buffer. The column was brought to 25° for elution of Hb-F_{II}.

Dialysis time at pH 4.7 (hr.)	Composition of Hb solution (% of total Hb)			
	Hb- $\gamma_4^{F_1}$	'X'	Hb-F _I	Hb-F _{II}
0	0	0	100	0
2	1	4	93.5	1.5
22	3	3	92.5	1.5
43	6	13	76.5	4.5
96	5	10	78.5	6.5
Control, 168 hr. in developer 4	0	4	96	<0.5

trace amounts of Hb- α^A and Hb- γ^F , as was noted by Huehns *et al.* (1965), and increasing quantities of the unidentified fraction 'X'. Small amounts of Hb-F_{II} appear after short exposure to acid pH, and the proportion increases with increasing time at acid pH. Therefore, even when Hb-F_I has been exposed to conditions where it is known to be largely dissociated into $\alpha^A\gamma^F$ and $\alpha^A\gamma^{AcF}$ sub-units, only one tetramer, namely Hb-F_I, is re-formed to any extent in the subsequent association process. Huehns & Shooter (1964) have argued that such a result would be expected if one of the other two tetramer molecules in the overall equilibrium (Scheme 1) is unstable. Since Hb-F_{II} obviously does not fall into this category, they suggested that the species in which the *N*-terminal amino acids of both γ^F -chains are acetylated, $\alpha_2\gamma_2^{AcF}$, was not a viable haemoglobin molecule.

Hb-F_{II} was also formed during the reconversion of $\alpha\gamma$ globin prepared from Hb-F_I (Winterhalter & Huehns, 1964) into haemoglobin. The losses of globin during dialysis to neutral pH in this experiment were, however, so large that no quantitative results were obtained. In this instance, Hb-F_{II} was isolated in small yield by chromatography on Amberlite IRC-50 and its identity confirmed by starch-gel electrophoresis. The Hb-F_{II} formed in these experiments presumably arises from the production of $\alpha\gamma^F$ sub-units during either the acid treatment of haemoglobin or the formation of globin by the action of acid acetone.

Sub-unit exchange between Hb-F_I and radioactive labelled Hb-F_{II}. (a) Exchange at neutral pH or after brief exposure to acid pH. The suggestion that Hb-F_I is stable because the species $\alpha_2\gamma_2^{AcF}$ cannot form can be tested directly by using radioactive Hb-F_{II}. Because Hb-F_{II} and Hb-F_I share a common $\alpha^A\gamma^F$ sub-unit (Scheme 1) radioactive label should be transferred rapidly from Hb-F_{II} to Hb-F_I at neutral pH. ⁵⁹Fe-labelled Hb-F_{II} was mixed with an equal amount of non-radioactive Hb-F_I and kept for 1 hr. at neutral pH and 0°; the two haemoglobins were subsequently separated by

chromatography on Amberlite IRC-50. The results (Table 5) show that virtually no exchange of radioactivity occurred, only 2.4% of the label appearing in Hb-F_I and 2.1% in fraction 'X'. In a second experiment, no radioactivity was detected in the isolated Hb-F_I.

There are two possible explanations for this failure to exchange radioactive label from Hb-F_{II} to Hb-F_I. The first, that Hb-F_I is not in equilibrium with its sub-units at neutral pH, has already been discounted on the grounds that an F_I-type haemoglobin with unlike α -chains was not produced in an appropriate recombination experiment. The alternative explanation is that the two foetal haemoglobins do not share a common $\alpha^A\gamma^F$ sub-unit and that Hb-F_I does not contain any γ^F -chains. Such a conclusion can be tested by allowing sub-unit exchange between the two haemoglobins to occur after dissociation to acid pH. In the first experiment, Hb-F_I and ⁵⁹Fe-labelled Hb-F_{II} were separately dissociated to their $\alpha\gamma$ sub-units by dialysis to acid pH, mixed and then dialysed immediately to neutral pH (Table 5). Again, only a very small amount of label was found in Hb-F_I. In subsequent experiments, the sub-unit exchange at acid pH was allowed to proceed to equilibrium.

(b) Exchange at equilibrium. ⁵⁹Fe-labelled Hb-F_{II} could not be used for experiments in which haemoglobin mixtures were kept at acid pH for any length of time because of the exchange of haem groups. In control experiments, between 25 and 80% of theoretical haem exchange occurred during 80 hr. at acid pH.

Therefore these experiments were carried out with [¹⁴C]leucine- and [¹⁴C]isoleucine-labelled Hb-F_{II}. In both instances mixtures of equal, or nearly equal, amounts of Hb-F_I and labelled Hb-F_{II} were dialysed against the pH 4.7 buffer for 40 hr. Haemoglobin concentrations were chosen (cf. Table 4) so that the systems would be near equilibrium after this period of time. The solutions were then dialysed to neutrality against developer 4 and analysed in the usual manner on Amberlite

Table 5. *Exchange of sub-units between Hb-F_I and ⁵⁹Fe-labelled Hb-F_{II}*

Equal volumes (0.2 ml.) of Hb-F_I solution (2.0 g./100 ml.) and ⁵⁹Fe-labelled Hb-F_{II} solution (2.0 g./100 ml.) in developer 4 were (a) mixed and kept at 0° for 1 hr. or (b) dialysed separately for 16 hr. against 0.10 M-sodium acetate-0.01 M-KCN buffer, pH 4.7, then mixed and immediately dialysed against developer 4 for 16 hr. The resultant solutions were analysed on a 1 cm. x 35 cm. column of Amberlite IRC-50 with developer 4 as elution buffer.

Hb species	(a) Neutral pH		(b) Acid pH	
	(counts/min.)	(% of total radioactivity)	(counts/min.)	(% of total radioactivity)
'X'	610	2.1	320	2.7
F _I	690	2.4	310	2.6
F _{II}	27500	95.5	11310	94.7

IRC-50. Each haemoglobin fraction was concentrated and a sample taken for determination of radioactivity. The results are given in Table 6 for the [^{14}C]leucine-labelled Hb-F_{II} and in Table 9 for the [^{14}C]isoleucine-labelled Hb-F_{II}.

In contrast with the experiments described in the preceding section, considerable transfer of label to Hb-F_I occurred with [^{14}C]leucine-labelled Hb-F_{II}, approx. 25% of the label now being found in Hb-F_I. Hb- γ_4 produced during the incubation in acid also contained radioactive label (Table 6). The recovery of haemoglobin in these experiments was high, the difference between the recoveries for Hb-F_I and Hb-F_{II} being due mainly to the known conversion of Hb-F_I into Hb-F_{II} under these conditions (see the section on self-hybridization).

The specific activity of the recovered Hb-F_I was less than one-half that of Hb-F_{II}, implying that equilibrium was not attained or that not all the sub-units in Hb-F_I exchange with those in Hb-F_{II}. Assuming equilibrium is reached in the exchange of sub-units, it is possible to calculate the theoretical distribution of the recovered label between the two haemoglobins, and these calculations are given in

Table 7 for two different models. The experimental results agree closely with the calculated values if it is assumed that only the α^A -chains in Hb-F_I exchange. The actual distribution of label in Hb-F_I was determined by separating the α^A - and γ -chains by hybridization with canine haemoglobin, i.e.:

Samples of the recovered Hb-F_I from this experiment were dissociated and recombined with an excess of canine haemoglobin to achieve as much transfer of the labelled chains to their complementary canine sub-unit partners as possible. The resulting haemoglobin species were isolated by starch-block electrophoresis and counted as described in the Materials and Methods section. The results outlined in Table 8 indicate that the α^A -chains of Hb-F_I have 92% of the label, whereas the γ -chains have only 8%, the latter value being

Table 6. *Exchange of sub-units between Hb-F_I and [^{14}C]leucine-labelled Hb-F_{II}*

Hb-F_I (20 mg.) and [^{14}C]leucine-labelled Hb-F_{II} (20 mg.) in a volume of 6.0 ml. were dialysed against 0.10 M-sodium acetate-0.01 M-KCN buffer, pH 4.7, for 40 hr. and then against developer 4 for 16 hr. The resultant haemoglobin solution was analysed by chromatography on a 1 cm. $\times$ 35 cm. column of Amberlite IRC-50 with developer 4 as elution buffer.

Hb species	Hb recovered (mg.)	Total radioactivity (disintegrations/min.)	Specific activity (disintegrations/min./mg. of Hb)
γ_4	1.64	25 800	15 700
F _I	16.4	133 700	8 200
F _{II}	20.3	490 000	24 000

Table 8. *Distribution of isotopic label in Hb-F_I after incubation with [^{14}C]leucine-labelled Hb-F_{II}*

Samples of Hb-F_I from the acid incubation with [^{14}C]leucine-labelled Hb-F_{II} (Table 6) and of [^{14}C]leucine-labelled Hb-F_{II} were dissociated and recombined with a sixfold excess of canine haemoglobin at a total concentration of 0.5 g. of haemoglobin/100 ml. The solutions were dialysed against 0.10 M-sodium acetate-0.01 M-KCN buffer, pH 4.7, for 40 hr. and then under pressure dialysis (to concentrate the haemoglobin solutions) against 0.05 M-sodium diethyl barbiturate buffer, pH 8.6. Haemoglobin species in the mixture were separated by starch-grain electrophoresis in the same buffer. Haemoglobin zones were eluted with the pH 8.6 buffer.

Original Hb species	Hb species isolated	Total radioactivity (disintegrations/min.)	% of total radioactivity
F _I + canine	$\alpha_2^A \beta_2^{\text{Can}}$	34 400	92.0
	$\alpha_2^{\text{Can}} \gamma_2^{\text{F AcF}}$	2 860	8.0
F _{II} + canine	$\alpha_2^A \beta_2^{\text{Can}}$	51 900	46.0
	$\alpha_2^{\text{Can}} \gamma_2^{\text{F}}$	60 700	54.0

Table 7. *Exchange of sub-units between Hb-F_I and [^{14}C]leucine-labelled Hb-F_{II}: a comparison of the experimental values with the distributions of isotope calculated by using two models*

Hb species	Calculated					
	Experimental		Assuming exchange of α -chains only		Assuming exchange of α -chains and one γ -chain	
	Radioactivity (disintegrations/min.)	Specific activity (disintegrations/min./mg. of Hb)	Radioactivity (disintegrations/min.)	Specific activity (disintegrations/min./mg. of Hb)	Radioactivity (disintegrations/min.)	Specific activity (disintegrations/min./mg. of Hb)
F _I	133 700	8 200	139 300	8 500	229 000	14 000
F _{II}	490 000	24 000	484 300	23 900	394 600	19 400

Table 9. *Exchange of sub-units between Hb-F_I and [¹⁴C]isoleucine-labelled Hb-F_{II}*

Hb-F_I (10 mg.) and [¹⁴C]isoleucine-labelled Hb-F_{II} (9 mg.) in a volume of 50 ml. were dialysed against 0.10 M-sodium acetate-0.01 M-KCN buffer, pH 4.7, for 40 hr. and then against developer 4 for 16 hr. The resultant haemoglobin solution was analysed on a 1 cm. × 35 cm. column of Amberlite IRC-50 with developer 4 as elution buffer.

Hb species	Hb recovered (mg.)	Total radioactivity (disintegrations/min.)	% of total radioactivity	Specific activity (disintegrations/min./mg. of Hb)
γ ^A	1.3	21000	12	16200
F _I	6.3	3500	2	560
F _{II}	9.7	145000	86	14900

probably within the range of cross-contamination found in this type of experiment.

A control experiment under the same conditions with uniformly labelled Hb-F_{II} gave nearly equal distribution of label between the α^A- and γ^F-chains (Table 8).

The finding that almost all the radioactivity originally transferred from Hb-F_{II} to Hb-F_I is in the α^A-chains of Hb-F_I confirms that the system had reached equilibrium and that the comparisons in Table 7 are valid.

The only human haemoglobin polypeptide chain that contains isoleucine is the γ-chain, and by using this amino acid the γ-chains of Hb-F_{II} can be specifically labelled. By using [¹⁴C]isoleucine-labelled Hb-F_{II} in a similar experiment to the previous one the results outlined in Table 9 were obtained; in this experiment fraction 'X' was discarded. Virtually no label was exchanged between the non-α-chains of Hb-F_{II} and Hb-F_I, the little radioactivity found in Hb-F_I probably arising from a residual contamination of labelled Hb-F_I in the original Hb-F_{II} fraction.

DISCUSSION

Although Hb-F_I does not dissociate as readily as Hb-A at acid pH, as judged by both its sedimentation behaviour and the time required to reach equilibrium in recombination experiments, it gives rise to only the usual type of hybrid haemoglobins in the latter experiments. The absence of, for example, an Hb-F_I species with two unlike α-chains in the analysis of the appropriate recombination mixture leads to the conclusion that Hb-F_I, like Hb-A and Hb-F_{II}, is in mobile equilibrium with its sub-units at all pH values. The behaviour of Hb-F_I on self-hybridization, previously interpreted as an indication that the species α₂γ₂^{AcF} is unstable (Huehns & Shooter, 1964), can now be re-evaluated in the light of the results of the exchange experiments with isotopically labelled Hb-F_{II}. The latter show (a) that dimer sub-units cannot be exchanged

between Hb-F_I and Hb-F_{II} at neutral pH and (b) that non-α^A-chains do not exchange under conditions where the α^A-chains equilibrate completely between the two species. Thus there can be no γ^F-chains in Hb-F_I. The equilibria depicted in Scheme 1 are not therefore the true equilibria for Hb-F_I, since it does not share a common α^Aγ^F sub-unit with Hb-F_{II}. There is thus no *a priori* reason for Hb-F_I to form Hb-F_{II} on self-hybridization. However, the fact that small amounts of Hb-F_{II} appear in these experiments and, moreover, increase with increasing exposure to acid pH, does have some relevance to the structure of Hb-F_I.

In attempting to reconcile the results of the exchange experiments with a structure for Hb-F_I a number of apparently conflicting observations have to be taken into account. The chemical studies showed clearly that Hb-F_I has 1 acetyl group/unit of 66000 molecular weight (Schroeder *et al.* 1962). These authors also found that Hb-F_I gave rise to 0.4–0.6 residue of DNP-glycine (the N-terminal residue of the γ^F-chain)/unit of 66000 molecular weight. On the other hand, the tryptic digests showed some peptide γTp(1+2) (the N-terminal tryptic dipeptide of the γ^F-chain), but none of the expected acetylated derivative of this peptide. Finally, the exchange experiments reported above indicate that Hb-F_I does not contain γ^F-chains.

If two haemoglobins, Hb-α₂γ₂^X and Hb-α₂γ₂^Z, are indistinguishable from each other by electrophoresis and chromatography, the analysis of a mixture of equal amounts of these two species would lead to the conclusion that this was a haemoglobin with the structure α₂γ^Xγ^Z, and it is possible that this is the situation with Hb-F_I. On this basis, Hb-F_I would comprise the species α₂γ₂^{AcF} and α₂γ₂^{XF} (and presumably α₂γ^{AcF}γ^{XF}). Although one can only speculate on the nature of the postulated γ^{XF}-chain, its charge relative to the γ^F-chain must be exactly the same as that of the γ^{AcF}-chain in phosphate buffer at neutral pH and at pH 8.6, and

the simplest explanation would be that the *N*-terminal amino group of this chain is also blocked, this time by an unidentified substituent. The formation of Hb-F_{II}, i.e. of γ^F -chains on self-hybridization, the appearance of DNP-glycine in the end-group reaction and of peptide $\gamma\text{Tp}(1+2)$ in the peptide analysis all require that either the acetyl group or the unknown substituent or both are relatively labile under certain conditions.

Evidence that this may be so comes from data on the adult haemoglobin analogous to Hb-F_I, namely Hb-A_{Ic}, $\alpha_2\beta^A\beta^{xA}$ (Holmquist & Schroeder, 1964). In this haemoglobin, the nature of the substituent on the terminal amino acid of one β^A -chain has not yet been determined. However, both *N*-terminal peptide $\beta\text{Tp}1$ and its derivative $\beta^x\text{Tp}1$ have been isolated, and Holmquist & Schroeder (1964) found that the substituent on $\beta^x\text{Tp}1$ could be removed during subsequent chromatography on Dowex 1. Self-hybridization experiments were also made with Hb-A_{Ic}, and it was reported that, although between 25 and 75% of the haemoglobin was precipitated in the experiment, some 25% of Hb-A_{Ic} was converted into Hb-A and a second component, presumed to be $\alpha_2\beta_2^{xA}$.

From the present results it would appear that Hb-F_I is not an exception to the general rule that haemoglobins are in mobile equilibrium with their sub-units, but rather that it has a more complex structure than that originally proposed by Schroeder *et al.* (1962). They also help to explain the observation (Huehns *et al.* 1965) that Hb- γ_4^{FII} is not formed when the sub-units of Hb-F_I are separated by exposing the haemoglobin to acid pH. The adult haemoglobin Hb-A_{Ic} thus remains the one exception to the equilibrium rule. If, however, the structure of Hb-A_{Ic} is also of the type postulated above for Hb-F_I, then all haemoglobins would presumably be in equilibrium with their sub-units.

Thanks are due to Dr G. H. Beaven for the densitometry results given in Table 3. This research was supported by Grant NB-04270 from the National Institutes of Health. E. R. H. is a Senior Beit Memorial Fellow.

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