Recombination Studies on Haemoglobins at Neutral pH

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It is well known that haemoglobins dissociate into half and quarter molecules at both high and low pH values, and that these are re-formed into tetramers when the pH is readjusted to neutrality (for references see Schroeder, 1959). Exposure of mixtures of haemoglobin species to dissociating conditions, followed by readjustment to neutrality, can result in the formation of new haemoglobin species, in accordance with the overall reaction:

When the relative charges of the two reacting species and the new species are different, the resulting four species can be resolved by electrophoresis (Itano, Singer & Robinson, 1959; Vinograd & Hutchinson, 1960; Gammack, Huehns, Shooter & Gerald, 1960).

It has previously been shown that the natural haemoglobin species containing only a single type of chain, namely Hb- β_4^A (Hb-H), Hb- γ_4^F (Hb-Bart's) and Hb- δ^A , can combine with Hb- α^A at neutral pH to form Hb-A, Hb-F and Hb-A₂ respectively (Huehns & Shooter, 1962; Dance, Huehns & Beaven, 1963). These reactions proceed more rapidly at 37° than at lower temperatures (Huehns, Beaven & Stevens, 1964*b*). When neutral mixtures of haemoglobins, which are known to give rise to electrophoretically detectable new species during dissociation-recombination experiments, are stored at 4° no new species are formed over a period of at least 2 weeks.

In the present work, the effect of incubating mixtures of various haemoglobin species at neutral pH and 37° has been studied, and it is shown that exchange of chains can occur under these conditions.

MATERIALS AND METHODS

All the haemoglobin species were used in the methaemoglobin cyanide (HiCN) form, prepared as described by Huehns, Dance, Shooter, Beaven & Gratzner (1962*a*), and were dialysed before use against a buffer containing Na₂HPO₄ (0.01 M) and KCN (0.01 M) adjusted to pH 7.0 with conc. H₃PO₄. The various haemoglobin species were obtained as follows.

Hb- α^A was prepared from Hb-A as described by Huehns, Dance, Shooter & Beaven (1962*c*).

Natural Hb- β_4^A and Hb- γ_4^F were isolated and purified from haemolysates of patients with Hb-H disease (Dance *et al.* 1963).

Hb- γ_4^F was also prepared from normal Hb-F ($\alpha_2^A\gamma_2^F$) as described by Dance & Huehns (1962).

Other haemoglobin variants, namely Hb-J ($\alpha_2^A\beta_2^J$; Gammack, Huehns, Shooter & Lehmann, 1961), Hb-I ($\alpha_2^A\beta_2^I$; Murayama, 1960) and Hb-C ($\alpha_2^A\beta_2^C$; Ingram, 1959; Itano *et al.* 1959), were isolated from appropriate haemolysates by starch-block electrophoresis (Kunkel, 1954).

For canine (Hb-Can; $\alpha_2^{\text{Can}}\beta_2^{\text{Can}}$) and human adult (Hb-A; $\alpha_2^A\beta_2^A$) haemoglobins the appropriate haemolysates were used without further purification.

Dissociation-recombination experiments were carried out as described by Huehns & Shooter (1961), overnight dialysis against acetate buffer, pH 4.7 and 1.0-1, being used for the dissociation step.

The proportion of alkali-resistant haemoglobin was estimated as described by Huehns *et al.* (1962*a*).

Recombination experiments at neutral pH. These were made with 1.0 mg. portions of each haemoglobin, mixed in a final volume of 0.2 ml. of the phosphate-cyanide buffer, pH 7.0 (see above). This was divided into two equal portions, one of which was kept at 4° while the other was incubated at 37° for approximately 40 hr. The separate portions were then submitted to starch-gel electrophoresis, usually in phosphate buffer, pH 7.4 (Gammack *et al.* 1960), or in a tris-citrate/borate discontinuous buffer system (Poulik, 1957). 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 (Gratzner & Beaven, 1960; G. H. Beaven & B. L. Stevens, unpublished work). The densitometer records were evaluated by planimetry.

RESULTS

The following systems were examined: (a) Hb- α^A with Hb-I; (b) Hb- β_4^A with Hb-C; (c) Hb- β_4^A with Hb-J; (d) Hb- β_4^A with Hb-I; (e) Hb- β_4^A with Hb-Can; (f) Hb- γ_4^F with Hb-Can; (g) Hb- β_4^A with Hb-F; (h) Hb-I with Hb-C; (i) Hb-A with Hb-Can; (j) Hb-C with Hb-J.

The results of the analyses of seven of these systems after storage at 4° or incubation at 37° are listed in Table 1, together with equations showing the anticipated exchanges of chains. Examples of typical starch-gel-electrophoretic patterns and transmission-densitometry records are shown in Figs. 1 and 2 respectively.

In system (a) some precipitate formed in the incubated portion. This presumably consisted of Hb- α^A and Hb- α^I , which are known to be unstable at 37° (Huehns *et al.* 1962c). In system (b) no pre-

cipitate was seen and any Hb- β_2^C formed presumably migrated with Hb-A (cf. Hb-A₂ and Hb- δ ; Dance *et al.* 1963). In system (c) Hb- β_4^I was presumably not resolved from Hb- β_4^A and this would account for the proportion of Hb- β_4^A not falling in this experiment. In system (d), where Hb- β_4^A was incubated with Hb-I ($\alpha_2^I\beta_2^I$), exchange of the β^A -chains cannot lead to the formation of any new species, and no changes in composition were observed in the experiments at either 4° or 37°. In system (e) the species Hb- β_4^{Can} probably migrates with Hb-Can (Huehns, Shooter & Beaven, 1962b), and this explanation is supported by the disproportionately small decrease in the proportion of the Hb-Can compared with that found for the Hb- β_4^A component; the species β_4^{Can} component was also not seen in the analyses of system (f).

In system (g) exchange of chains between Hb- β_4^A and Hb-F would result in the formation of Hb- γ_4^F and Hb-A. Since Hb-A was not adequately resolved from Hb-F by starch-gel electrophoresis, this system was examined on a slightly larger scale by the following procedure, and instead of pure Hb-F a normal cord-blood haemolysate containing 75 % of Hb-F was used. The reaction mixtures (0.25 ml.) were analysed by starch-block electrophoresis, with phosphate buffer, pH 7.0, 0.08M with respect to Na⁺ ions. Under these conditions Hb- β_4^A is resolved from both Hb- γ_4^F and the mixed Hb-(A + F) zone, as shown by Dance *et al.* (1963). No Hb- γ_4^F was found in either reaction mixture. The Hb-(A + F) zones were cut out, eluted and concentrated. The proportion of Hb-A in each was estimated by the alkaline-denaturation method, and found to be 26 % in the experiment at 4° and 25 % in the 37° experiment. Thus no detectable exchange of chains had occurred in this experiment.

In system (h), as the species $\alpha_2^I\beta_2^C$ has the same net charge as Hb-A (Itano *et al.* 1959), the 'Hb-A' zone presumably also contains this species. In system (j), in which both components contain α^A -chains, no new species would be expected to form and no change in composition was found after incubation at 37°.

Table 1. Recombination experiments at neutral pH

The values are the proportions of the various species in each system, expressed as percentages of the total haemoglobin. Where two sets of numbers are given these represent the results of duplicate analyses.

System (a) $2\alpha^A + \alpha_2^I\beta_2^A \rightleftharpoons 2\alpha^I + \alpha_2^A\beta_2^A$			
	Hb- α^A	Hb-A	Hb-I
4°	43	0	57
37°	Trace	28	72
System (b) $2\alpha_2^A\beta_2^C + \beta_4^A \rightleftharpoons 2\alpha_2^A\beta_2^A + \beta_4^C$			
	Hb-C	Hb-A	Hb- β_4^A
4°	48, 48	0, 0	52, 52
37°	47, 42	13, 11	40, 47
System (c) $2\alpha_2^A\beta_2^I + \beta_4^A \rightleftharpoons 2\alpha_2^A\beta_2^A + \beta_4^I$			
	Hb-A	Hb-J	Hb- β_4^A
4°	0, 0	66, 66	34, 34
37°	6, 4	56, 58	38, 38
System (e) $2\alpha_2^{Can}\beta_2^{Can} + \beta_4^A \rightleftharpoons 2\alpha_2^{Can}\beta_2^A + \beta_4^{Can}$			
	Hb-Can	$\alpha_2^{Can}\beta_2^A$	Hb- β_4^A
4°	50	0	50
37°	46	33	21
Acid-dissociated and recombined	40	39	21
System (f) $2\alpha_2^{Can}\beta_2^{Can} + \gamma_4^F \rightleftharpoons 2\alpha_2^{Can}\gamma_2^F + \beta_4^{Can}$			
(i) Natural Hb- γ_4^F			
	Hb-Can	$\alpha_2^{Can}\gamma_2^F$	Hb- γ_4^F
4°	52	0	48
37°	52	Trace	48
Acid-dissociated and recombined	42	16	42
(ii) 'in vitro' Hb- γ_4^F			
4°	39	0	61
37°	40	5	55
Acid-dissociated and recombined	38	21	41
System (h) $\alpha_2^A\beta_2^C + \alpha_2^I\beta_2^A \rightleftharpoons \alpha_2^A\beta_2^A + \alpha_2^I\beta_2^C$			
	Hb-C	Hb-A + $\alpha_2^I\beta_2^C$	Hb-I
4°	44	0	56
37°	35	16	49
System (i) $\alpha_2^{Can}\beta_2^{Can} + \alpha_2^A\beta_2^A \rightleftharpoons \alpha_2^A\beta_2^{Can} + \alpha_2^{Can}\beta_2^A$			
(i) Analysis in discontinuous buffer system:			
	$\alpha_2^A\beta_2^{Can}$	Hb-Can	Hb-A
4°	Trace	49	51
Acid-dissociated and recombined	25	23	25
(ii) Analysis in phosphate buffer system: (Hb-A + Hb-Can)			
4°	Trace	100	0
37°	8	85	7
Acid-dissociated and recombined	20	52	28

Fig. 1. Starch-gel electrophoresis in phosphate buffer, pH 7.4, of system (a): (i) neutral mixture of Hb- α^A with Hb-I kept at 4°; (ii) same mixture as in (i) incubated at 37° for 40 hr.; (iii) Hb-A marker. Details are given in the text.

In the other systems examined, for which results are listed in Table 1, the identities assigned to the new components, in accordance with the expected equilibria, are consistent with their known electrophoretic mobilities relative to the initial components under the conditions of starch-gel electrophoresis used, i.e. Hb-A in systems (a), (b), (c) and (h), Hb- $\alpha_2^{\text{Can}}\beta_2^{\text{A}}$ in system (e), Hb- $\alpha_2^{\text{Can}}\gamma_2^{\text{F}}$ in system (f), Hb-A ($+\alpha_2^{\text{I}}\beta_2^{\text{C}}$) in system (h) and Hb- $\alpha_2^{\text{A}}\beta_2^{\text{Can}}$ and Hb- $\alpha_2^{\text{Can}}\beta_2^{\text{A}}$ in system (i).

Fig. 2. Densitometry tracing of starch-gel analyses of system (a) shown in Fig. 1: (a) mixture of Hb- α^{A} with Hb-I kept at 4°; (b) same mixture incubated at 37° for 40 hr. Details are given in the text.

Control experiments. All the individual haemoglobin species were incubated separately at 37° and then examined as described above. No new haemoglobin zones were detected in these analyses. The pigment in the Hb- α^{A} control was precipitated completely during the incubation period (cf. Huehns *et al.* 1962c).

DISCUSSION

Previous work has shown that the naturally occurring haemoglobins containing a single type of polypeptide chain will react with Hb- α^{A} at neutral pH to form the corresponding natural species (Huehns & Shooter, 1962). The present work indicates that exchange of chains can also occur at neutral pH between other haemoglobins when these are incubated at 37°. These reactions fall into four categories:

(i) Hb- α^{A} will exchange with the α -chains of haemoglobin variants with aberrant α -chains, e.g. Hb-I ($\alpha_2^{\text{I}}\beta_2^{\text{A}}$; Expt. a).

(ii) Hb- β_4^{A} will exchange with the β -chains of haemoglobin variants with aberrant β -chains, e.g. Hb-C ($\alpha_2^{\text{A}}\beta_2^{\text{C}}$; Expt. b) and Hb-J ($\alpha_2^{\text{A}}\beta_2^{\text{J}}$; Expt. c). In a similar experiment (Expt. g) with Hb-F ($\alpha_2^{\text{A}}\beta_2^{\text{F}}$) the expected exchange of chains could not be demonstrated.

(iii) Hb- β_4^{A} and Hb- γ_4^{F} will also exchange chains with Hb-Can to form the species $\alpha_2^{\text{Can}}\beta_2^{\text{A}}$ and $\alpha_2^{\text{Can}}\gamma_2^{\text{F}}$ respectively (Expts. e and f).

(iv) Exchange of chains also occurs between haemoglobin species which contain both α - and β -chains, e.g. Hb-C with Hb-I (Expt. h) and Hb-A with Hb-Can (Expt. i).

The proportion of new species formed varies widely, although these experiments were carried out under comparable conditions with respect to the haemoglobin derivative used (HiCN), pigment concentration, buffer ion concentration, temperature and time. Loss of material by precipitation occurred only in the experiment in which Hb- α^{A} was used. The formation of new species proceeded to the greatest extent (approx. 30 %) in the Can/ β_4^{A} and α^{A} /I systems. Significantly lower formation of new species (approx. 10 %) occurred in the C/ β_4^{A} , J/ β_4^{A} , Can/ γ_4^{F} , C/I and A/Can systems. No detectable exchange occurred in the F/ β_4^{A} system.

Exchange of sub-units between rat and canine haemoglobins at neutral pH has been reported by Drabkin & Wise (1962). The HbO₂ derivatives were used, the canine pigment having been labelled *in vitro* with [2-¹⁴C]glycine. A hybrid species was isolated in low yield by crystallization and the ratio of the specific activities of its haem and globin moieties compared with the ratios for the parent Hb-Can and its separated α - and β -chains. The results suggested that considerable incorporation

of labelled canine sub-units (probably β -chains) into the hybrid had occurred after 3–6 hr. at 37°. Because of differences in technique and interpretation, however, direct comparison of this result with those of the present experiments is not possible.

These results imply that there is significant dissociation of many haemoglobin species at neutral pH. Vinograd & Hutchinson (1960) suggested that at high pH haemoglobins dissociate symmetrically and that a proportion of the $\alpha\beta$ -sub-units thus formed dissociate further to give single α - and β -chains, with exchange of chains taking place at the level of single chains. A similar suggestion has been made (Huehns, Shooter & Beaven, 1964*a*) to explain the time-dependence of the appearance of new species in dissociation–recombination experiments at acid pH. It is conceivable, therefore, that the dissociation process is identical over a wide range of pH values:

The main species present is known to depend on the pH; between approx. pH 6.0 and 10.5 the tetramer species is predominant, whereas below pH 6.0 the degree of dissociation increases progressively until at pH 4.7 the dimer is the main species. Similarly at pH 11.0 the dimer species again predominates whereas at pH 11.5 mainly single chains are present (cf. Schroeder, 1959). Outside the approximate pH range 3.5–11.5 haemoglobins are denatured.

The failure of Antonini, Wyman, Bucci, Fronticelli & Rossi-Fanelli (1962) to detect new species in suitable mixtures of haemoglobins dissociated at neutral pH in buffers of high ionic strength was interpreted as indicating that the dimer formed under these conditions had the composition $\alpha\beta$, and that a negligible proportion of dimer dissociated to form monomers.

The proportion of new species formed is significantly lower with haemoglobins containing γ^F -chains, in comparison with haemoglobins containing β^A -chains. This difference is found at both neutral and acid pH, as shown by the following examples:

(i) The rate of formation of Hb-F in mixtures of Hb- α^A and Hb- γ_4^F at neutral pH is slower than the rate of formation of Hb-A in mixtures of Hb- α^A and Hb- β_3^A (Huehns *et al.* 1964*b*).

(ii) The proportion of species $\alpha_2^{\text{Can}}\gamma_2^F$ formed is less than that of the species $\alpha_2^{\text{Can}}\beta_2^A$ in mixtures of Hb-Can with Hb- γ_4^F and Hb- β_4^A respectively, in both neutral and acid pH recombination experiments (Expts. *e* and *f*).

(iii) No exchange of chains could be demonstrated in the mixture Hb- β_4^A /Hb-F (Expt. *g*). In contrast, exchange occurred in the mixtures Hb- β_4^A /Hb-C (Expt. *b*) and Hb- β_4^A /Hb-J (Expt. *c*).

(iv) Acid dissociation and recombination of Hb-Can/Hb-(F+A) mixture (Huehns *et al.* 1962*a*) indicated a greater degree of exchange involving the β^A -chains than the γ^F -chains.

The lower reactivity in these dissociation–recombination experiments of haemoglobins containing γ^F -chains could be explained on the basis of a generally lower rate of turnover through the monomeric state, as compared with haemoglobin species not containing γ^F -chains under the same conditions. (The rate of recombination of monomeric units, which appears to be the essential step for the formation of hybrid species, may well, for steric reasons, be much slower than their rate of formation, as only collisions involving complementary regions of the α - and β -monomers are likely to be effective.)

The markedly different rates of alkaline denaturation of haemoglobin species containing γ^F -chains as compared with species containing β -chains (Huehns *et al.* 1962*a*) may also be a consequence of differences in dissociation behaviour at high pH. This hypothesis implies that all monomeric species formed at high pH are rapidly denatured, whereas dimer species are alkali-resistant. Then, if $\alpha\gamma$ -dimer sub-units dissociate to form α - and γ -monomers more slowly than do $\alpha\beta$ -dimer sub-units to form α - and β -monomers, Hb-F would be expected to be denatured more slowly than Hb-A and other tetramer species that do not contain γ -chains. On this view the rate of formation of monomer species is the rate-determining step of the overall alkaline-denaturation process. A similar explanation has been proposed by Baglioni (1963).

The demonstration that exchange of chains can occur at neutral pH at 37° may well be relevant to the interpretation of studies on the specificity of haemoglobin biosynthesis in 'mixed' cell-free systems (Lamfrom, 1961; Kruh, Rosa, Dreyfus & Shapira, 1961; Bishop, Favelukes, Schweet & Russell, 1961).

SUMMARY

1. The exchange of chains in mixtures of haemoglobins at neutral pH and 37° has been demonstrated by detection of the appropriate hybrid species by using quantitative starch-gel electrophoresis. Exchange occurs not only when one of the haemoglobin species used contains a single type of chain, but also between haemoglobins containing both α - and β -chains.

2. With haemoglobins containing γ -chains, the proportion of new species formed is less than with haemoglobins containing β -chains, both in experiments at neutral pH and when acid pH is used to increase the extent of dissociation.

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Reaction of Haemoglobin α^A with Haemoglobins β_4^A , γ_4^F and δ^{A_2}

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The natural occurrence of haemoglobin species consisting solely of β^A -chains (Hb- β_4 or Hb-H; Jones, Schroeder, Balog & Vinograd, 1959), γ^F -chains (Hb- γ_4 or Hb-Bart's; Hunt & Lehmann, 1959; Kekwick & Lehmann, 1960) or δ^{A_2} -chains (Hb- δ ; Dance & Huehns, 1962*a*) is well established, and these three species have been shown to occur simultaneously in several individuals with Hb-H disease (Dance, Huehns & Beaven, 1963). The relative proportions of Hb- β_4 and Hb- γ_4 found in such individuals suggest that *in vivo* α -chains combine more rapidly with β -chains to form Hb-A than with γ -chains to form Hb-F (Huehns, Flynn, Butler & Shooter, 1960).

It has been shown (Huehns & Shooter, 1962) that haemoglobin α^A (Hb- α) reacts rapidly with natural Hb- β_4 at neutral pH to form Hb-A. In the present paper it is shown that Hb- α combines with Hb- γ_4 to form 'Hb-F' and with Hb- δ to form 'Hb-A₂', and that the rate of formation of 'Hb-F' is significantly slower than the rate of formation of 'Hb-A' or 'Hb-A₂'.

MATERIALS AND METHODS

Since all the haemoglobin species used in the present work contain normal adult or foetal chains, the superscripts A, F and A₂ have been omitted, from this point onwards.

Hb- α was prepared as the methaemoglobin cyanide (HiCN) derivative, as described by Huehns, Dance, Shooter & Beaven (1962), from normal adult haemolysates, normal cord-blood haemolysates and the Hb-A isolated from individuals with Hb-H disease.

Hb- β_4 was isolated as the HiCN derivative from the erythrocytes of two individuals with Hb-H disease either by starch-block electrophoresis in a 0.04M-sodium phosphate buffer, pH 7.0, or as described by Dance *et al.* (1963).

Hb- γ_4 and Hb- δ were isolated in the HiCN form from the erythrocytes of two individuals with Hb-H disease as described by Dance *et al.* (1963). Hb- γ_4 was also prepared from Hb-F in the HiCN form as described by Dance & Huehns (1962*b*).

The haemoglobins were finally dialysed against a buffer containing NaH₂PO₄ (0.01M) and KCN (0.01M), adjusted to pH 7.0 with conc. H₃PO₄.

In each experiment, 0.6 mg. of each of the two haemoglobin species were mixed at 25° or 0° and made up to a