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Biochem. J. (1964), **92**, 444

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

By E. R. HUEHNS

Department of Biochemistry, University College London, Gower Street, London, W.C. 1

G. H. BEAVEN AND B. L. STEVENS

Medical Research Council Laboratories, Holly Hill, London, N.W. 3

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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

volume of 0.2 ml. with the phosphate-cyanide buffer. The resulting mixtures were analysed immediately and after incubation at various temperatures by starch-gel electrophoresis either in phosphate buffer, pH 7.4 (Gammack, Huehns, Shooter & Gerald, 1960), or in a tris-citrate/borate discontinuous system (Poulik, 1957). The gels were analysed by transmission densitometry after being stained with Naphthalene Black and differentiated 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.

RESULTS

Reaction of haemoglobin α with haemoglobin β_4

Experiment 1. Fig. 1 shows the analyses of a mixture of Hb- α with Hb- β_4 . It can be seen that 'Hb-A' was immediately formed (Fig. 1a) and that incubation of the mixture caused all the Hb- α to combine with β -chains from Hb- β_4 to form 'Hb-A' (Fig. 1b). Alongside the starch-gel-electrophoretic analyses are shown the relevant densitometer tracings.

Experiments 2-9. The rates of formation of 'Hb-A' in mixtures of Hb- α and Hb- β_4 when

Fig. 1. Expt. 1: starch-gel-electrophoretic analyses in sodium phosphate buffer, pH 7.4, of a mixture of Hb- α and Hb- β_4 together with the corresponding densitometer tracings. (a) Analysis immediately after mixing; (b) analysis after several hours' incubation at room temperature and 12 hr. at 37°. Details are given in the text.

incubated at various temperatures are shown in Figs. 2 and 3. Expts. 2-5 (Fig. 2) were made with the same preparations of Hb- α and Hb- β_4 , whereas Expts. 6 and 7 (Fig. 3) were made with a further preparation of each haemoglobin. As an example the detailed results of the mixture of Hb- α /Hb- β_4 , which was incubated at 37°, are given in Table 1.

The times for conversion of 50 % of the total pigment into 'Hb-A' ($t_{1/2}$ 'Hb-A' formation) are given in Table 2. These results indicate that the formation of 'Hb-A' is approximately three times as fast at 37° as at 25°, and 20-30 times as fast as at 0°.

Fig. 2. Progressive formation of 'Hb-A' in mixtures of Hb- α with Hb- β_4 . ●, Incubated at 37° (Expt. 2); ■, incubated at 25° (Expt. 3); ○, mixture made and analysed at 25°, and incubated at 0° (Expt. 4); ▲, mixture made, incubated and analysed at 0° (Expt. 5). All experiments were made with the same preparation of Hb- α and Hb- β_4 . Details are given in the text.

Fig. 3. Progressive formation of 'Hb-A' in mixtures of Hb- α with Hb- β_4 . ■, Incubated at 25° (Expt. 6); ▲, mixture made, incubated and analysed at 0° (Expt. 7). These experiments were made with the same preparation of Hb- β_4 as those shown in Fig. 2, but a different preparation of Hb- α was used. Details are given in the text.

Table 3 compares the rate of formation of 'Hb-A' when Hb- α was mixed with various preparations of Hb- β_4 . It can be seen that the rate of 'Hb-A' formation is considerably slower in the stored samples. On electrophoretic analysis, the four preparations of Hb- β_4 all migrated at the same rate.

Reaction of haemoglobin α with haemoglobin γ_4

Experiment 10. Expt. 10 (Fig. 4) shows the analysis of a mixture of Hb- α with Hb- γ_4 . No 'Hb-F'

Table 1. *Reaction of haemoglobin α with haemoglobin β_4 at 37°*

Analyses were by quantitative starch-gel electrophoresis. Details are given in the text.

Time (hr.)	Hb- α (%)	'Hb-A' (%)	Hb- β_4 (%)
Immediate	34	23	43
1½	12	63	25
3½	7	72	21
6½	5	76	19
9½	7	83	10
24	Trace	90	10

Table 2. *Comparison of the time taken for 50% formation of 'Hb-A' ($t_{\frac{1}{2}}$ 'Hb-A' formation) in mixtures of haemoglobin α and haemoglobin β_4*

Expts. 2-5 were made with the same preparations of Hb- α and Hb- β_4 . Expts 6 and 7 were made with a different preparation of Hb- α but the same preparation of Hb- β_4 , the day after Expts. 2-5. Details are given in the text.

Expt. no.	Temperature of incubation	$t_{\frac{1}{2}}$ 'Hb-A' formation (hr.)
2	37°	1
3	25	2½
4	0 (analyses made at room temperature)	22
5	0 (analyses made at 4°)	27
6	25	6
7	0 (analyses made at 4°)	34

Table 3. *Comparison of 'haemoglobin A' formation in mixtures of haemoglobin α with haemoglobin β_4 to show the effect of storage of haemoglobin β_4*

The temperature of incubation was 25°. Details are given in the text.

Expt. no.	Time of storage of Hb- β_4 until use* (days)	Proportion of 'Hb-A' found on immediate analysis (%)	$t_{\frac{1}{2}}$ 'Hb-A' formation
8	1	73	10 min.
3	6	22	2½ hr.
6	7	18	6 hr.
9	21	16	16 hr.

* Measured from the time the blood was taken.

formation occurred immediately after mixing (Fig. 4a). After incubation at 37° 'Hb-F' formation gradually occurred (Fig. 4b). This experiment is directly comparable with Expt. 1 (Fig. 1), as the same preparation of Hb- α was used, the Hb- γ_4 was prepared from the same haemolysate and the incubation and starch-gel-electrophoretic analyses were made in parallel.

Experiments 11-13. The effect of temperature on the formation of Hb-F in mixtures of Hb- α with Hb- γ_4 is shown in Fig. 5 and Table 4. These experiments are directly comparable with Expts. 2-7. The same preparation of Hb- α was used in Expts. 2-5 as in Expts. 13 and 14, and the incubation and starch-gel-electrophoretic analyses of the two sets of experiments were made in parallel. In Expt. 15 the same Hb- α was used as in Expts. 6 and 7 and these were also carried out in parallel.

Experiment 14. The preparation of Hb- γ_4 made *in vitro* reacted with Hb- α at a rate comparable with that of natural Hb- γ_4 (Table 4). Expts. 10-14 indicate that in all cases the immediate analysis of Hb- α /Hb- γ_4 mixtures showed only traces of 'Hb-F'.

Fig. 4. Expt. 10: starch-gel-electrophoretic analyses in sodium phosphate buffer, pH 7.4, of a mixture of Hb- α and Hb- γ_4 together with the corresponding densitometer tracings. This experiment was made with the same preparation of Hb- α and with Hb- γ_4 isolated from the same haemolysate as in Expt. 1 shown in Fig. 1. The two incubations and analyses were made in parallel. (a) Analysis immediately after mixing; (b) analyses after several hours' incubation at room temperature and 12 hr. at 37°.

Comparison of values for 50 % conversion into 'Hb-A' and 'Hb-F' (Tables 2 and 4) indicate that the formation of 'Hb-A' is approximately ten times as fast as that of Hb-F.

Fig. 5. Progressive formation of 'Hb-F' in mixtures of Hb- α with Hb- γ_4 . ●, Incubated at 37° (Expt. 11); ■, incubated at 37° (Expt. 12); ▲, incubated at 25° (Expt. 13). Expts. 11 and 13 were made in parallel to and with the same preparation of Hb- α as Expts. 2-5. Expt. 12 was made in parallel to and with the same preparation of Hb- α as Expts. 6 and 7. Details are given in the text.

Table 4. Comparison of the time taken for 50 % formation of 'haemoglobin F' (t_1 'Hb-F' formation) in mixtures of haemoglobin α with haemoglobin γ_4

Expts. 11 and 13 were made in parallel to and with the same preparation of Hb- α as Expts. 2-5; Expt. 12 was made in parallel to and with the same preparation of Hb- α as Expts. 6 and 7. Details are given in the text.

(a) Hb- α with natural Hb- γ_4		
Expt. no.	Temperature of incubation	t_1 'Hb-F' formation (hr.)
11	37°	20
12	37	19
13	25	39
(b) Hb- α and Hb- γ_4 prepared <i>in vitro</i>		
14	37	30

Fig. 6. Expt. 15: starch-gel-electrophoretic analyses in tris-citrate/borate buffer system of a mixture of Hb- α with Hb- δ made immediately after mixing. Details are given in the text.

Reaction of haemoglobin α with haemoglobin δ

Experiment 15. The immediate analysis (Fig. 6) of this system showed 28 % of 'Hb-A₂' increasing to 42 % after 10 hr. at 37°. The rate of reaction of Hb- δ with Hb- α is therefore comparable with that of Hb- β_4 rather than with that of Hb- γ_4 .

DISCUSSION

It has already been demonstrated that Hb- α reacts with Hb- β_4 at neutral pH to form a haemoglobin species indistinguishable from Hb-A (Huehns & Shooter, 1962); it has now been shown that Hb- α reacts similarly with Hb- γ_4 and Hb- δ to form species that are electrophoretically indistinguishable from Hb-F and Hb-A₂ respectively. These three reactions have now been studied by quantitative starch-gel electrophoresis, and the rates of appearance of the new species have been followed at three different temperatures. Several practical factors limited the accuracy with which these measurements could be made.

On starch-gel electrophoresis in phosphate buffer, pH 7.4, the resolution of natural Hb- β_4 and Hb-A is complete, with no intermediate pigment, unless Hb- γ_4 or Hb- δ is also present. In incubated mixtures of Hb- α and Hb- β_4 , the formation of 'Hb-A' is frequently accompanied by the appearance of pigment which, on electrophoresis, migrates between Hb- β_4 and 'Hb-A', but is not clearly resolved from either. The nature of this material has not been studied. In the analyses, the proportion of this intermediate pigment was in no instance greater than 15 % of the total pigment and was allocated equally between the Hb- β_4 and 'Hb-A' zones.

Hb- α gradually precipitates during incubation for 24 hr. at 37° (Huehns *et al.* 1962), and a precipitate invariably formed in the present experiments when these were made at 37°. This factor only influenced the analyses in those experiments where the Hb- α was not rapidly utilized, i.e. Hb- α with Hb- γ_4 . In the other experiments no precipitate formed.

In spite of these technical difficulties, the results permit the following conclusions to be drawn.

- (i) Hb- α reacts rapidly with Hb- β_4 to form 'Hb-A' and this reaction is more rapid at 37° than at 0°.
- (ii) Storage of Hb- β_4 decreases the rate of formation of 'Hb-A' in mixtures of Hb- α with Hb- β_4 .
- (iii) The rate of formation of 'Hb-F' in mixtures of Hb- α with Hb- γ_4 is approximately tenfold slower than the formation of 'Hb-A' in mixtures of Hb- α with Hb- β_4 .
- (iv) The reaction of Hb- δ with Hb- α to form 'Hb-A₂' proceeds at approximately the same rate as the reaction of Hb- α with Hb- β_4 to form 'Hb-A'.

It may be envisaged that the difference in reactivity of α -chains with β -chains, as compared with γ -chains, applies not only *in vitro* but also during the synthesis of haemoglobin *in vivo*, as previously postulated to explain the relative proportions of the abnormal haemoglobin species found in Hb-H disease (Huehns *et al.* 1960). In those individuals with α -thalassaemia in whom the rate of α -chain synthesis is less than the combined rates of synthesis of β -, γ - and δ -chains, the latter compete for the available α -chains and analyses of haemolysates from such individuals indicate that Hb-A is formed in preference to Hb-F.

Two alternative consequences of this situation can be postulated, and both have been observed. In the first, the available α -chains exceed the available β -chains but are less than the total of β - and γ -chains. Almost all the available β -chains combine with α -chains to form Hb-A, the remaining α -chains combine with γ -chains to form Hb-F, and the excess of γ -chains form Hb- γ_4 . In this instance the haemolysate contains Hb-A, Hb-F and Hb- γ_4 without any detectable Hb- β_4 (Ager & Lehmann, 1958; Choremis, Zannos-Mariolea, Ager & Lehmann, 1959). In the alternative situation, the available α -chains are less than the available β -chains. Again Hb-A is formed in preference to Hb-F, the excess of β -chains and γ -chains forming Hb- β_4 and Hb- γ_4 respectively. In these individuals the haemolysate contains Hb-A, Hb- β_4 and Hb- γ_4 with only traces of Hb-F (Ramot, Sheba, Fisher, Ager & Lehmann, 1959).

The presence of Hb- δ has only been demonstrated in four individuals (Dance *et al.* 1963), but in each individual a higher proportion of Hb-A₂ than of Hb- δ was present. It would appear, therefore, that *in vivo* δ - and β -chains combine with α -chains at comparable rates, as was also observed in the experiments *in vitro* described above.

SUMMARY

1. The rate of reaction of haemoglobin- α^A (Hb- α) with Hb- β_4 , Hb- γ_4 and Hb- δ at neutral pH to form new haemoglobin species that are electrophoretically indistinguishable from Hb-A, Hb-F and Hb-A₂ respectively has been examined over the temperature range 0–37° by quantitative starch-gel electro-

phoresis. The rate of formation of 'Hb-A' in mixtures of Hb- α and Hb- β_4 was approximately three times as great at 37° as at 25°, and 20–30 times as great as at 0°. In mixtures of Hb- α with Hb- β_4 in which stored samples of Hb- β_4 were used the rate of formation of 'Hb-A' was significantly slower.

2. At 0° the rate of formation of 'Hb-F' from Hb- α and Hb- γ_4 was negligible, but was appreciable at 25° and approximately twice as fast at 37°. At corresponding temperatures the formation of 'Hb-F' in Hb- α /Hb- γ_4 mixtures was approximately tenfold slower than the formation of 'Hb-A' in Hb- α /Hb- β_4 mixtures. These findings *in vitro* are used to explain the situation *in vivo* in Hb-H disease, in which there appears to be a deficiency of α -chain synthesis and the observed compositions of haemolysates may be accounted for in terms of competition between β - and γ -chains for the available α -chains.

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