Changes in the Haemoglobin Types of Sheep as a Response to Anaemia

By G. VAN VLIET*
Baarn, The Netherlands

AND T. H. J. HUISMAN
Department of Biochemistry, Medical College of Georgia, Augusta, Ga., U.S.A.

(Received 2 March 1964)

The existence of two genetic variants of haemoglobin in sheep of different breeds has been demonstrated (e.g. Harris & Warren, 1955; Cabannes & Serain, 1955; Evans, King, Cohen, Harris & Warren, 1956; Evans, Harris & Warren, 1957; van der Helm, van Vliet & Huisman, 1957; Huisman, van Vliet & Sebens, 1958*a*). The haemoglobin type moving more rapidly towards the anode in paper electrophoresis at pH 8.6 was called Hb-A or Hb-II, and the slow-moving haemoglobin has been designated as Hb-B or Hb-I. Further experiments have shown that the two haemoglobin types differ in chromatographic behaviour on the cation exchangers CM-cellulose and Amberlite IRC-50, in rate of denaturation by alkaline reagents, and in solubility in concentrated salt solutions; analyses of the amino acid compositions of the two variants have shown that Hb-A contains more threonine, serine and glutamic acid and Hb-B more glycine, alanine and aspartic acid (van der Helm *et al.* 1957; Huisman, van de Brande & Meyering, 1960). The hybridization behaviour of the haemoglobins was studied by Shreffler & Vinograd (1962), who demonstrated that Hb-B more readily dissociates into its sub-units than does Hb-A at both acid and alkaline pH. Studies of the equilibria of the haemoglobin variants with oxygen revealed a higher affinity of the Hb-A type as compared with the Hb-B type (Huisman *et al.* 1958*a*).

It has been shown (Huisman *et al.* 1958*a*; Huisman, van der Helm, Visser & van Vliet, 1958*b*; van Vliet, 1960) that quantitative changes in the amounts of Hb-A and Hb-B may occur in a sheep heterozygous for both variants (AB sheep) when such an animal is submitted to the stress of severe anaemia. According to the observations of Blunt & Evans (1963) this response to anaemia may be due to either an increased production of foetal haemoglobin or the formation of a new haemoglobin, electrophoretically similar to Hb-B and different from foetal haemoglobin (Hb-F). The present paper describes additional studies of the changes in the haemoglobin in AB sheep and in sheep homozygous for Hb-B (B sheep) before, during and

after experimental anaemia. Results suggesting the formation of a new haemoglobin type (Hb-C) in the AB sheep, but not in the B sheep, are presented and some characteristic properties of the Hb-C are described.

METHODS

Material. Adult sheep of the Texel breed were studied. Two AB ewes (no. 2357 and no. 9087, weighing 93 and 81 kg. respectively) and one male (no. 289, body wt. 70 kg.) were made anaemic by repeated bleeding according to the scheme presented below. Blood samples from these animals were collected at regular intervals and analysed. In addition, blood samples from two lambs heterozygous for Hb-A and Hb-B and two lambs homozygous for Hb-B were studied at periodic intervals after birth. All animals, who were studied while in the animal quarters of the Laboratory, were kept free of parasites by the administration of antihelminthics. It may therefore be assumed that no additional blood loss due to intestinal parasites had occurred.

Haematological studies and the preparation of erythrocyte haemolysates. The blood samples were collected in heparin and centrifuged to remove the plasma. The erythrocytes were washed three times with 0.9% NaCl solution to which were added 50 units of penicillin and 250 µg. of streptomycin/ml. The packed erythrocytes were mailed immediately from The Netherlands to the U.S.A. by air. No deterioration of the samples was noted under these conditions. Haematological studies were performed in the haematological laboratory of the Department of Internal Medicine of the State University of Utrecht, The Netherlands (Dr K. Punt), by the conventional laboratory procedures (Wintrobe, 1962). The erythrocytes of sheep 2357 were also separated by centrifugation into young and old erythrocytes by the method of Borum, Figueroa & Perry (1957).

Electrophoretic and chromatographic studies. Starch-gel electrophoresis was carried out by a slight modification (Huisman, 1963) of the procedure developed by Smithies (1955). Chromatographic separation of Hb-A, Hb-B, Hb-C and Hb-F was obtained with DEAE-cellulose (Huisman & Dozy, 1962). The same procedure, but on a larger scale, was used for the isolation of greater quantities of the different haemoglobin types (Huisman & Dozy, 1962).

Characterization of the isolated haemoglobin components. Absorption-spectral measurements were performed with a Beckman DK-2 spectrophotometer and quartz cuvettes of 1.0 cm. light-path. The solubility of the three isolated haemoglobin variants A, B and C in concentrated salt solutions was determined by the method of Derrien (1952):

* Present address: Research Laboratories, Philips-Duphar Inc., Weesp, The Netherlands.

the final haemoglobin concentration was 1.0 g./100 ml. Gross structural differences between the polypeptide chains of the haemoglobins were studied by hybridization experiments and by starch-gel electrophoresis in sodium formate-formic acid buffer, pH 1.9 (Muller, 1960; Huisman, 1963). The hybridization procedure was similar to that described for human haemoglobin types (Gammack, Huehns, Lehmann & Shooter, 1961; Huisman, 1963). A small volume (0.5 ml.) of a particular haemoglobin solution (5 g./100 ml.) was mixed with an equal quantity of canine haemoglobin solution of approximately similar concentration and the mixture separated into two equal portions. One portion was dialysed for 7 hr. against 0.1 M-sodium acetate-acetic acid buffer, pH 4.7, at 4° followed by dialysis overnight against the tris-EDTA-borate buffer used in starch-gel electrophoresis (Huisman, 1963). The control mixture was treated similarly except that the dialysis was against water rather than sodium acetate-acetic acid buffer. The formation of hybrid haemoglobins was studied by the starch-gel-electrophoretic procedure mentioned above.

Oxygen equilibrium experiments. Construction of oxygen dissociation curves at 37° was performed by the procedure described by Brinkman & Dirken (1940). Total erythrocyte haemolysates containing 5 g. of haemoglobin/100 ml. and isolated haemoglobin components in solutions of 0.8–1.0 g. of haemoglobin/100 ml. were studied. The solutions, which had been dialysed for 24 hr. against a large volume of a 0.1 M-potassium phosphate buffer, pH 7.4, at 4° were each equilibrated with oxygen at a different pressure (10–80 mm.Hg) in 150 ml. tonometers at 40 mm. $p\text{CO}_2$ and 37°. The determination of the percentage oxygen saturation was made with a Zeiss spectrophotometer (model PMQ-II), by the procedure described by Jonxis & Boeve (1956) for the concentrated haemoglobin solutions, and by that described by Meyering, Israels, Sebens & Huisman (1960) for the more dilute haemoglobin solutions. The pH measurements were carried out on a Radiometer model 4 pH-meter at 25°; each value obtained was corrected to 37° by using the factor given by Rosenthal (1948). The $\log P_{50}$ value, which is the value of the $p\text{O}_2$ at which 50% saturation of the haemoglobin with oxygen was observed, and the factor n , a measure of the haem-haem interaction, were calculated from the linear relationships attained by plotting the logarithms of the $p\text{O}_2$ against the logarithms of $y/(100-y)$, y representing the percentage oxygen saturation of the haemoglobin.

RESULTS

Electrophoretic and chromatographic characteristics of sheep haemoglobins. An example of the separation of Hb-A and Hb-B by starch-gel electrophoresis is presented in Fig. 1. The haemoglobin sample obtained from the AB sheep 2357 under normal conditions consisted of two major fractions, the fast-moving Hb-A and the slower-moving Hb-B, which possesses an electrophoretic mobility identical with that of human Hb-A. A minute amount of a slower-moving fraction was also detectable. This component, designated Hb-C, was present in much larger quantities during the experimental anaemia and ultimately replaced Hb-A entirely (Fig. 1).

In the separation of the Hb-A, Hb-B and Hb-C, starch-gel electrophoresis was found to be superior to any other electrophoretic procedure, paper electrophoresis particularly. The degree to which the three haemoglobins were separated by DEAE-cellulose chromatography is shown in Fig. 2. As expected from the electrophoretic mobilities, the Hb-C was eluted first, followed successively by Hb-B and Hb-A. Since the separation of the haemoglobin variants was almost complete, calculation of the proportion of each haemoglobin type in mixtures was possible. Fig. 2 also shows a chromatogram of the haemoglobin of a newborn lamb (no. 11) from a blood sample collected 15 days after birth. Four haemoglobin fractions were detectable. The major haemoglobin fraction, which was identified as Hb-F, was eluted between the Hb-B and the Hb-A. A small but distinct proportion of Hb-C was also present. The greater part of each haemoglobin component, eluted from these columns, was combined and concentrated by CM-cellulose chromatography (Huisman & Meyering, 1960). Some idea of the degree of purity of these isolated components can be obtained by reference to their mobilities in starch-gel electrophoresis (Fig. 3). Mutual contamination of the haemoglobin variants was minimal or absent. Fig. 3 also shows that the small proportion of Hb-C, which was demonstrated in the blood of the newborn lamb by DEAE-cellulose chromatography, was detectable

Fig. 1. Separation of the haemoglobin types A, B and C by starch-gel electrophoresis. Experimental details are given in the text. Total Hb concentrations: (a) 11.2 g./100 ml.; (b) 7.8 g./100 ml.; (c) 6.1 g./100 ml.; (d) 4.3 g./100 ml. All blood samples were taken from sheep 2357.

by starch-gel electrophoresis. The percentages of the different haemoglobin components in blood samples from newborn lambs, which were collected at different time-intervals after birth and were analysed by the DEAE-cellulose chromatographic procedure, are shown in Table 1. Two lambs, no. 10 and no. 11, were heterozygous for Hb-A and Hb-B, and two lambs, no. 12 and no. 13, homozygous for Hb-B. The results demonstrate the fast disappearance of Hb-F after birth and its replacement by either Hb-A and Hb-B or Hb-B alone, and also suggest the presence of small but distinct amounts of Hb-C in newborn AB sheep and its absence in newborn B sheep.

Response of an AB sheep to extreme blood loss. Sheep 2357 was submitted to experimental anaemia. The quantities of blood removed two or three times each week varied from 250 to 750 ml. and are plotted in the upper section of Fig. 4. As a result of this treatment a very severe anaemia developed (Table 2 and Fig. 4), which was microcytic and slightly hypochromic with a moderate reticulocytosis. The total haemoglobin concentration decreased from 12.2 g./100 ml. to a final value of 2.8 g./100 ml. Electrophoretic and chromato-

graphic analyses of the haemoglobin in the blood samples collected at various intervals revealed a continuous decrease in the percentage of Hb-A and a corresponding increase in the percentage of Hb-C, whereas no significant change in the percentage of Hb-B was observed. No Hb-A was detectable during the final 2 weeks of the anaemic period, whereas the total proportion of Hb-C increased to such an extent that it was only slightly less than that of Hb-B (Fig. 4). Significant differences in the proportions of Hb-A and of Hb-C were found for young and old erythrocytes. There was a marked rise in the percentage of Hb-C in the young erythrocytes as compared with that of old erythrocytes during the course of the anaemia, and the concentration of Hb-A was lower in the young erythrocytes than in the older cells. No significant difference in the percentage of Hb-B in the two cell layers was observed.

Response of an AB sheep and a B sheep to moderate blood loss and the recovery from the anaemia. A similar experiment was carried out with the AB

Fig. 2. Separation of the different haemoglobin types by DEAE-cellulose chromatography. Experimental details are given in the text. (a) Blood sample of sheep 2357 (total Hb concn.: 7.8 g./100 ml.); (b) blood sample of lamb 11, collected 15 days after birth.

Fig. 3. Starch-gel-electrophoretic patterns of haemoglobin types, isolated by DEAE-cellulose chromatography. Experimental details are given in the text. (b) Hb-B; (c) Hb-F; (d) Hb-A. For the purpose of comparison the separations of the haemoglobins present in a blood sample of sheep 2357 (total Hb concn.: 4.3 g./100 ml.) and in a blood sample of lamb 11 (15 days after birth) are also shown; these samples are labelled (a) and (e) respectively.

Table 1. Percentages of the different haemoglobin fractions in the blood of lambs at various ages

Experimental details are given in the text.

Lamb no.	Age (days)	Hb-A (%)	Hb-B (%)	Hb-C (%)	Hb-F (%)
10	15	27.3	19.3	1.6	51.8
	35	51.4	34.8	0.9	12.9
11	15	24.6	19.0	2.0	54.4
	35	47.8	36.0	1.4	14.8
	210	57.2	42.8	0	0
12	12	0	31.6	0	68.4
	32	0	76.6	0	23.4
13	3	0	7.5	0	92.5
	25	0	72.6	0	27.4

Fig. 4. Changes in the concentrations of Hb-A (○), Hb-B (△) and Hb-C (●) in the blood of sheep 2357 before and during severe experimental anaemia. Experimental details are given in the text.

sheep 9087 and with the B sheep 289. The quantities of blood removed each week were slightly less than those removed from sheep 2357. This treatment resulted in a rather severe anaemia with severely hypochromic and microcytic erythrocytes and a moderate reticulocytosis (Table 3 and Fig. 5). The animals were maintained at a total haemoglobin concentration of approx. 5 g./100 ml. for 3 weeks, whereupon the bleeding was discontinued. A rapid recovery from the anaemia resulted with a persistence of the microcytosis and hypochromia as judged from the values of the mean corpuscular volume and mean corpuscular haemoglobin. The changes in the haemoglobin types as a response of

the AB sheep 9087 to this treatment were comparable with those seen in sheep 2357; the Hb-A was partially replaced by the Hb-C, but the concentration of Hb-B was not affected. For example, the proportions of Hb-A, Hb-B and Hb-C in the blood samples collected 165 days after the start of the experiments were 24.9, 45.5 and 29.6 % respectively, whereas 57 % of Hb-A, 43 % of Hb-B and 0 % of Hb-C were found in the blood sample collected before the induction of the anaemia. A considerable formation of Hb-C was maintained in the post-anaemic period. Although the percentage of Hb-C decreased rapidly with a corresponding rise in the percentage of Hb-A, the total concentration of Hb-C in the blood remained fairly constant (Fig. 5). The haematological changes observed for the B sheep 289 submitted to a similar experimental anaemia were comparable with those found for the AB sheep 9087. No differences, however, were observed in the type of haemoglobin produced. The electrophoretic and chromatographic mobilities of the single haemoglobin type present in this animal before, during and after the anaemic state were identical and no formation of an additional haemoglobin component was observed.

Physicochemical properties of the haemoglobins A, B, C and F. The visible-light-absorption spectra of the oxy, deoxy, carbonmonoxy, met and cyanmet derivatives of the four haemoglobin types, isolated by DEAE-cellulose chromatography, were identical. Also, no striking differences were observed between the ultraviolet-absorption spectra of the four haemoglobin types. The solubilities of Hb-A, Hb-B and Hb-C in phosphate solutions of different molarities, as determined by the technique of Derrien (1952), were markedly different. The relatively low solubility of Hb-A compared with that of Hb-B (van der Helm *et al.* 1957) was confirmed; the solubility of Hb-C was intermediate between the solubilities of Hb-A and Hb-B.

The existence of possible gross structural differences between Hb-A, Hb-B and Hb-C was studied by starch-gel electrophoresis of the corresponding

Table 2. *Haematological data on sheep 2357 before and during extreme blood loss*
Experimental details are given in the text.

Time after start of experiment (weeks)	Vol. of blood removed in preceding week (l.)	Total vol. of blood removed (l.)	Concn. of Hb (g./100 ml.)	Packed cell volume (ml./100 ml.)	10 ⁻⁶ × Erythrocyte count (per mm. ³)	Mean cell volume (μ ³)	Mean cell Hb (μg.)	Mean cell Hb concn. (g./100 ml.)	Reticulocyte count (per 1000 erythrocytes)
0	0	0	12.2	36	7.81	46	15.7	34	0
1	0	0	12.2	36	7.33	49	16.7	34	0
7	1.85	6.3	8.9	26.5	5.31	50	16.8	33.5	6
8	1.8	8.1	7.8	24	5.20	46	15.0	32.5	14
14	2.2	18.7	4.8	16	2.90	55	16.5	30	16
16	2.1	23.2	4.3	13	2.65	47	16.2	33	43
17	1.3	24.5	2.8	9.5	2.13	45	13.2	29.5	38

Table 3. *Haematological data on sheep 9087 and 289 before, during and after experimental anaemia*
Experimental details are given in the text.

Sheep no. and Hb type	Time after start of experiment (weeks)	Vol. of blood removed in preceding week (l.)	Total vol. of blood removed (l.)	Concn. of Hb (g./100 ml.)	Packed cell volume (ml./100 ml.)	10 ⁻⁶ × Erythrocyte count (per mm. ³)	Mean cell volume (μ ³)	Mean cell Hb (μg.)	Mean cell Hb concn. (g./100 ml.)	Reticulocyte count (per 1000 erythrocytes)
9087 (AB)	0	0	0	12.5	33	5.38	61.5	23.2	38	0
	1	0.8	0.8	10.5	28	5.28	53	20.0	37.5	0
	8	2.0	5.0	8.3	24.5	5.52	44.5	15.1	34	2
	21	1.5	24.0	5.5	18	5.63	32	9.7	30.5	23
	24	2.4	29.4	4.9	17	5.23	32.5	9.4	29	115
	27	0	29.4	8.8	28.5	8.50	33.5	10.3	31	1
	30	0	29.4	11.1	32	8.55	37.5	13.0	34.5	0
289 (BB)	33	0	29.4	13.3	37	9.50	39.0	14.0	36	0
	0	0	0	11.2	31.5	6.43	49	17.4	35.5	0
	1	0.8	0.8	10.5	26	4.61	56.5	22.8	40.5	2
	15	1.3	7.5	7.2	19	4.17	45.5	17.3	38	0
	26	1.2	19.8	5.2	17	4.62	37	11.2	30.5	12
	29	2.1	25.6	4.2	14	4.02	34.5	10.4	30	45
	32	0	25.6	9.2	27	7.83	34.5	11.7	34	0
	37	0	25.6	9.7	27.5	7.92	35	12.4	35.5	0
	40	0	25.6	10.9	26.5	7.96	33.5	13.7	41	0

globins in sodium formate-formic acid buffer, pH 1.9 (Muller, 1960; Huisman, 1963), since with this technique a complete separation of the two basic polypeptide chains of these proteins can be obtained. Examples of the results obtained are shown in Fig. 6. The three globin types share one polypeptide chain, which has been identified as the α -polypeptide chain (Muller, 1961). The non- α -polypeptide chains possessed different electrophoretic mobilities, indicating differences in structural composition. The non- α -chain (i.e. the γ -chain) of the foetal haemoglobin showed an electrophoretic mobility that was slightly greater than that of the corresponding chain of Hb-C under these experimental conditions.

The presence of identical α -polypeptide chains and different non- α -polypeptide chains in Hb-A, Hb-B and Hb-C was confirmed by the formation of hybrid haemoglobins from mixtures of these haemoglobin types with canine haemoglobin. Results of such hybridization experiments are shown in Fig. 7. The starch-gel-electrophoretic patterns of the hybrid mixtures each show the

presence of two newly formed haemoglobin fractions. The fraction that showed the slowest electrophoretic mobility and that is composed of the α -chains of sheep haemoglobin and the β -chains of canine haemoglobin, $\alpha_2^{\text{sheep}}\beta_2^{\text{can}}$ (Huisman, 1963), was present in each of the three hybrid mixtures, indicating the presence of identical α -polypeptide chains in the sheep haemoglobin types A, B and C. The other hybrid haemoglobins produced by recombining the sub-units of canine haemoglobin and the three sheep haemoglobins behaved differently in starch-gel electrophoresis. The mobility of that produced from mixtures of canine haemoglobin and Hb-A was greater than that of Hb-A. The component formed in mixtures of canine haemoglobin and Hb-B moved ahead of the Hb-B and was slightly slower than Hb-A. The hybrid haemoglobin formed in mixtures of canine haemoglobin and Hb-C possessed an electrophoretic mobility that was slightly greater than that of Hb-B.

Fig. 5. Changes in the concentrations of Hb-A (○), Hb-B (△) and Hb-C (●) in the blood of sheep 9087 before, during and after moderate experimental anaemia. Experimental details are given in the text.

Fig. 6. Separation of the polypeptide chains of Hb-C (a), Hb-B (b) and Hb-A (c) by starch-gel electrophoresis in sodium formate-formic acid buffer, pH 1.9. Experimental details are given in the text.

Physiological properties of haemoglobins A, B and C. Fig. 8 shows examples of the oxygen dissociation curves of Hb-A, Hb-B and Hb-C, which had been isolated by DEAE-cellulose chromatography. The curves were constructed from data obtained for solutions containing approx. 1 g. of haemoglobin/100 ml., which were dialysed against 0.1 M-potassium phosphate buffer, pH 7.4. The oxygen

Fig. 7. Formation of hybrid haemoglobins from mixtures of sheep haemoglobins with canine haemoglobin as demonstrated by starch-gel electrophoresis. Experimental details are given in the text. (b) Mixture of Hb-C and canine haemoglobin; (c) mixture of Hb-B and canine haemoglobin; (d) mixture of Hb-A and canine haemoglobin. C, Control; H, hybridization experiment. The haemoglobins in the total erythrocyte haemolysates of sheep 2357 (total Hb concn.: 6.1 g./100 ml.) and of the dog are also shown; these samples are labelled (a) and (e) respectively.

Fig. 8. Oxygen dissociation curves of isolated Hb-A (○), Hb-B (△) and Hb-C (●). Experimental details are given in the text.

saturation values were plotted versus the corrected logarithms of the pO_2 at pH 7.0 (at 37°). The difference in oxygen affinity between the Hb-A and Hb-B, which has been described by Huisman *et al.* (1958a), was confirmed. The affinity of Hb-C for molecular oxygen was identical with that of Hb-A; the $\log P_{50}$ values for both haemoglobin types were 1.240, whereas the $\log P_{50}$ value for Hb-B was 1.440. Similar experiments were carried out with more concentrated total haemolysates of the red blood cells of the AB sheep 9087 and the B sheep 289, which were collected at various times before, during and after the experimental anaemia. The total haemoglobin concentrations were adjusted to approx. 5 g./100 ml. before the construction of the oxygen affinity curves. The results of these studies are expressed as the $\log P_{50}$ values at pH 7.0 (at 37°) and are shown in Fig. 9. The proportions of the three haemoglobin types of the different red-cell haemolysates of sheep 9087 and the total haemoglobin concentrations at the time of collection are also presented in Fig. 9. No significant change in the $\log P_{50}$ values was observed; any difference noted fell within the limit of accuracy of the method, namely 0.040, which is two standard deviations of a mean value of 1.400. Similar results were obtained on analysing the various haemolysates of the erythrocytes of the B sheep 289. The

Fig. 9. Changes in the percentages of Hb-A (○), Hb-B (△) and Hb-C (●), and the logarithms of the P_{50} values of the total haemoglobins (□) of sheep 9087 before, during and after moderate experimental anaemia. The logarithms of the P_{50} values of the total haemolysates (△) of the B sheep 289, being collected under identical conditions, are also given. Experimental details are given in the text.

mean value observed for $\log P_{50}$ was 1.495, with a similar value of 0.040 as two standard deviations of the mean. The mean values of the exponent n in Hill's equation, representing the physiological interaction of the haem groups, were 2.0 ± 0.25 for the haemolysates of sheep 9087 and 1.95 ± 0.2 for those of sheep 289.

DISCUSSION

The simultaneous use of more advanced electrophoretic and chromatographic techniques has provided additional information on the changes in the quantities of the haemoglobin types of sheep during the stress of severe anaemia. As a result of this the interpretation of the observations of the changes in the proportions of the two haemoglobin types in AB sheep during severe blood loss, made by Huisman *et al.* (1958*a*), is no longer tenable. The present findings strongly suggest that under such circumstances a new haemoglobin type with distinct physicochemical properties is produced. This haemoglobin component, designated Hb-C, replaces one of the two adult haemoglobin types, namely Hb-A, whereas the formation of the second haemoglobin, Hb-B, seems not to be affected. The oxygen affinity of Hb-C was found to be identical with that of Hb-A. The new haemoglobin type was often detectable in small amounts in the blood of heterozygous sheep that were suffering from a mild hypochromic microcytic anaemia.

The results of the hybridization experiments and starch-gel-electrophoretic studies at low pH leave little doubt that one of the two polypeptide chains of Hb-C differs from the corresponding chains of the other haemoglobin types, whereas the same second polypeptide chain, the α -chain, is present in all four haemoglobin variants. Preliminary results of experiments, which are not described in detail in the present paper, indicate the existence of more than one difference in the amino acid composition of Hb-C as compared with those of Hb-A, Hb-B and Hb-F. Particularly striking were the differences in the number of methionine and isoleucine residues. No isoleucine was present in either Hb-A or Hb-B, whereas 2 and 4 residues of this amino acid were found in Hb-C and Hb-F respectively. The number of methionine residues were 6, 8, 2 and 4 for Hb-A, Hb-B, Hb-C and Hb-F respectively. Because of these differences and of other deviations in the amino acid compositions of Hb-A and Hb-B (van der Helm *et al.* 1957), it seems of basic importance to investigate the primary structure of the different sheep haemoglobin variants. Such studies are at present in progress.

There is apparently a specific replacement of Hb-A and not of Hb-B by another haemoglobin type in experimental anaemia. Although the

present study failed to demonstrate any change in the Hb-B component, the possibility of the formation of an altered haemoglobin type with identical electrophoretic and chromatographic properties cannot be excluded. Application of additional techniques, including structural studies of the 'Hb-B' types isolated before and during severe anaemia, may prove of further value. The physiological characteristics of Hb-B, as determined by its oxygen equilibrium, did not change during the period of severe anaemia.

The continuous presence of rather large amounts of Hb-C in the post-anaemic period is also noteworthy. During the 70 days after the last bleeding of the AB sheep 9087 the total concentration of Hb-C present in the blood had remained constant at approx. 1.5 g./100 ml. (Fig. 5), whereas during the recovery phase a continuous rise in the concentrations of both Hb-A and Hb-B was observed. One might therefore postulate that during this period distinct erythrocyte types containing specific (mixtures of) haemoglobin variants are produced. The rate of production of erythrocytes containing mainly Hb-C and probably Hb-B (or an unknown variant with similar properties) seems to balance their destruction rate, whereas the formation of erythrocytes with mainly Hb-A and probably Hb-B greatly exceeds the rate of breakdown of these cells.

The nature of the genetic control of the sheep haemoglobins A and B is of fundamental interest because of the fact that the non- α -polypeptide chains of these haemoglobin types appear to differ by perhaps 10–20 amino acid residues, though the evidence both from pedigrees and population data for single-gene control of these polypeptide chains seems to be convincing (van der Helm *et al.* 1957; Huisman *et al.* 1958*a*). Different mechanisms of mode of inheritance, including one postulating single-gene control of the two different polypeptide chains by a pair of alleles at a regular locus, have been discussed (Shreffler & Vinograd, 1962). The problem has become more complicated by the present evidence for the existence of a regulatory mechanism that is active in anaemic states and results in the increased production of a third (and possibly a fourth) type of haemoglobin in heterozygous AB sheep. It seems, at present, not possible to develop a genetic scheme of mechanisms that will adequately explain all the observed facts. Any final conclusion must be based on the results of detailed analyses of the primary structures of the haemoglobin types.

SUMMARY

1. The response of sheep heterozygous for the two haemoglobin variants Hb-A and Hb-B and

sheep homozygous for Hb-B to the stress of severe experimental anaemia has been reinvestigated.

2. In animals subjected to extreme blood loss the Hb-A was replaced entirely by a new haemoglobin variant (Hb-C), whereas the production of Hb-B apparently was not affected. Under conditions of moderate blood loss, the replacement of Hb-A by Hb-C was partial. Hb-C was detectable in fairly constant concentrations in the blood of heterozygous sheep during the recovery phase of the anaemia.

3. Hb-C differs from Hb-A and from Hb-B in electrophoretic mobility and in its behaviour in DEAE-cellulose chromatography. Haemoglobins A, B, C and foetal haemoglobin seem to share the same α -polypeptide chain, but the non- α -chains of each of the four haemoglobin types are distinctly different.

4. The affinity of Hb-C for molecular oxygen was identical with that of Hb-A and therefore different from that of Hb-B. No changes in oxygen affinities of total erythrocyte haemolysates of a heterozygous AB sheep and a homozygous B sheep were observed during the experimental anaemia.

We acknowledge our gratitude to Dr K. Punt and Miss J. Bos (Utrecht, The Netherlands) for their help in the haematological studies, and to Miss A. M. Dozy, Mrs C. A. Reynolds, Mrs M. Sheley and Miss J. M. Schillhorn van Veen for skilful technical assistance. The study was in part supported by U.S. Public Health Service Grants no. H-5168 and no. H-6982.

REFERENCES

- Blunt, M. H. & Evans, J. V. (1963). *Nature, Lond.*, **200**, 1215.
 Borum, E. R., Figueroa, W. G. & Perry, S. M. (1957). *J. clin. Invest.* **36**, 676.

- Brinkman, R. & Dirken, M. N. J. (1940). *Acta brev. neerl. Physiol.* **10**, 228.
 Cabannes, R. & Serain, C. (1955). *C.R. Soc. Biol., Paris*, **149**, 1193.
 Derrien, Y. (1952). *Biochim. biophys. Acta*, **8**, 631.
 Evans, J. V., Harris, H. & Warren, F. L. (1957). *Biochem. J.* **65**, 42F.
 Evans, J. V., King, J. W. B., Cohen, B. L., Harris, H. & Warren, F. L. (1956). *Nature, Lond.*, **178**, 849.
 Gammack, D. B., Huehns, E. R., Lehmann, H. & Shooter, E. M. (1961). *Acta genet.* **11**, 1.
 Harris, H. & Warren, F. L. (1955). *Biochem. J.* **60**, xxix.
 Huisman, T. H. J. (1963). *Advanc. clin. Chem.* **6**, 231.
 Huisman, T. H. J. & Dozy, A. M. (1962). *J. Chromat.* **7**, 180.
 Huisman, T. H. J. & Meyering, C. A. (1960). *Clin. chim. Acta*, **5**, 103.
 Huisman, T. H. J., van de Brande, J. & Meyering, C. A. (1960). *Clin. chim. Acta*, **5**, 375.
 Huisman, T. H. J., van der Helm, H. J., Visser, H. K. G. & van Vliet, G. (1958b). In *Abnormal Haemoglobins*, p. 181. Ed. by Jonxis, J. H. P. & Delafresnaye, J. F. Oxford: Blackwell Scientific Publications Ltd.
 Huisman, T. H. J., van Vliet, G. & Sebens, T. (1958a). *Nature, Lond.*, **182**, 171.
 Jonxis, J. H. P. & Boeve, H. W. (1956). *Acta med. scand.* **115**, 157.
 Meyering, C. A., Israels, A. L. M., Sebens, T. & Huisman, T. H. J. (1960). *Clin. chim. Acta*, **5**, 208.
 Muller, C. J. (1960). *Nature, Lond.*, **186**, 643.
 Muller, C. J. (1961). Doctoral Thesis. Assen, The Netherlands: Van Gorcum and Co.
 Rosenthal, T. B. (1948). *J. biol. Chem.* **187**, 25.
 Shreffler, D. C. & Vinograd, J. (1962). *Biochim. biophys. Acta*, **65**, 101.
 Smithies, O. (1955). *Biochem. J.* **61**, 629.
 van der Helm, H. T., van Vliet, G. & Huisman, T. H. J. (1957). *Arch. Biochem. Biophys.* **72**, 331.
 van Vliet, G. (1960). Doctoral Thesis. Groningen, The Netherlands: van Genderen.
 Wintrobe, M. M. (1962). *Clinical Hematology*. Philadelphia: Lea and Febiger.

Biochem. J. (1964), **93**, 409

Effect of Cofactors, Oestrogens and Magnesium Ions on the Activity and Stability of Human Glutamate Dehydrogenase

By J. C. WARREN, D. O. CARR AND S. GRISOLIA

Department of Biochemistry, University of Kansas School of Medicine, Kansas City 3, Kans., U.S.A.

(Received 27 February 1964)

It is well known that glutamate dehydrogenase [L-glutamate-NAD(P) oxidoreductase, EC 1.4.1.3] undergoes aggregation and deaggregation under a variety of conditions, including changes in concentration (Olson & Anfinsen, 1952). It has been claimed (Tomkins, Yielding & Curran, 1961) that

with aggregation there are reciprocal changes in the rates of oxidation of glutamate and other amino acids (such as alanine) that are substrates for this enzyme. Other investigators believe that in the range of 0.1–1.0 mg./ml. (e.g. completely deaggregated to essentially fully aggregated enzyme)