Studies of Haemoglobin Types in Barbary Sheep (*Ammotragus lervia*)

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(Received 17 October 1967)

1. Two haemoglobin types, haemoglobins Amm-C and Amm-B, were observed in five Barbary sheep (*Ammotragus lervia*). One animal was homozygous for haemoglobin Amm-C, a second was homozygous for haemoglobin Amm-B, and three were heterozygous for both. 2. Amino acid analyses of the globin from haemoglobin Amm-B showed that this type was related to, but not identical with, haemoglobin B of the domestic sheep. 3. The β -chain of haemoglobin Amm-C was found to be composed of 141 amino acid residues. Its amino acid composition differed from that of the β -chain of the anaemic domestic sheep in at least 14 residues. The Amm- β -chain contained one isoleucyl residue. 4. The amino acid compositions of tryptic peptides T-1, T-2, T-13 and T-14 of the Amm- β -chain were similar to those of the sheep β -chain. Peptides T-3, T-4, T-6, T-7, T-8, T-11 and T-15 were the same as the corresponding peptides of the sheep β^A - and β^C -chains. Peptide T-5 and to a smaller extent peptide T-9 resembled the corresponding peptides of the sheep β^A -chain, and peptide T-10 was identical with peptide γ T-10 of sheep haemoglobin F. Peptide T-12 was not recovered. 5. The results of these investigations were interpreted as being indicative that the structural Amm- β -gene is closely related to the β^C -gene of sheep, from which through domestication the present domestic sheep originated.

The primary structures of the β -chains of the two adult sheep haemoglobin types, designated as Hb-A* and Hb-B, have recently been determined (Beale, Lehmann, Drury & Tucker, 1966; Boyer *et al.* 1966, 1967; Huisman, Reynolds, Dozy & Wilson, 1965; Wilson, Edwards, McDaniel, Dobbs & Huisman, 1966). The two polypeptide chains are each composed of 145 residues; they differ by at least seven residues, which are located in positions 50, 58, 75, 76, 120, 129 and 144, counted from the N-terminal end (indicated as position 2). A third haemoglobin type, Hb-C, has been observed in sheep heterozygous or homozygous for Hb-A (Blunt & Evans, 1963; Braend, Efremov & Helle, 1964; van Vliet & Huisman, 1964). Hb-C becomes a major haemoglobin in animals severely anaemic from blood loss by replacing Hb-A but not Hb-B. The β -chain of Hb-C is composed of 141 residues and differs from the β^A -chain by at least 16 residues and from the β^B -chain by at least 21 residues (Beale *et al.* 1966; Boyer *et al.* 1966, 1967; Wilson *et al.* 1966). A similar phenomenon is also observed in the domestic goat (Huisman, Adams, Dimmock, Edwards & Wilson, 1967). The β -chain of Hb-C,

produced in goats during severe blood-loss anaemia, was found to be only slightly different from the sheep β^C -chain; only one structural variation has been observed so far.

Electrophoretic analyses of the haemoglobins of non-domestic sheep (Huisman, van Vliet & Sebens, 1958; Meyer, 1967) have indicated a marked variability in several species. It was thought that a comparison of the structures of some of these haemoglobin types with those of the three adult sheep haemoglobins might contribute to the elucidation of the position of the β^C -gene in the evolutionary scheme of the haemoglobin genes in sheep and goats. The present paper reports the results of such studies on the haemoglobins of Barbary sheep (*Ammotragus lervia*).

MATERIALS AND METHODS

Animals. Five adult Barbary sheep were available. Three, nos. 7, 8 and 108, were kept in the Zoological Garden Artis (Amsterdam, The Netherlands), and two, nos. 71 and 72, in the Blydorp Garden (Rotterdam, The Netherlands). Haematological studies were done in the Haematological Laboratory of the Department of Internal Medicine of the State University of Utrecht, The Netherlands (Dr K. Punt).

* Abbreviation: Hb, haemoglobin.

The erythrocytes were washed three times with 0.9% NaCl solution to which was added 50 units of penicillin and 250 μ g. of streptomycin/ml. The erythrocyte suspensions were mailed to the U.S.A. by air.

Electrophoretic and chromatographic studies. Starch-gel electrophoresis was performed as described by Huisman (1963). The haemoglobins were also separated by DEAE-Sephadex chromatography (Huisman & Dozy, 1965; Dozy, Kleihauer & Huisman, 1968). The isolated haemoglobin fractions were concentrated by vacuum dialysis at 4°, and their purity was checked by starch-gel electrophoresis. Only fractions without contamination by other haemoglobin and non-haemoglobin components were used for structural studies.

Determination of the amino acid compositions of acid hydrolysates. Isolated haemoglobin fractions from Barbary sheep nos. 8, 71 and 108 were converted into globin by the acid-acetone procedure of Anson & Mirsky (1930) and samples (2mg.) hydrolysed in 6N-HCl in sealed evacuated tubes at 110° for 24 hr. before analysis on a Spinco model 120B amino acid analyser (Spackman, Moore & Stein, 1958), modified to include a long-path photometer, which gave a tenfold increase in sensitivity (Jones & Weiss, 1964). The globin Amm-C from animal no. 8 was separated into α - and β -chains by a simplified countercurrent distribution procedure (Babin, Schroeder, Shelton, Shelton & Robberson, 1966) and the β -chain converted into its *S*-aminoethyl derivative (Raftery & Cole, 1963; Jones, 1964) before analysis of the amino acid composition.

Enzymic digestion and separation of the tryptic peptides. Tryptic digestion was performed on 280mg. of the *S*-aminoethyl- β -chain of the Hb-C from animal no. 8 by the technique described by Huisman *et al.* (1967). The initial separation of the tryptic peptides was made on a column (100 cm. $\times$ 1.0 cm.) of Dowex 50 (X2) and the separated zones were rechromatographed on a column of Dowex 1 (X2) according to the procedures of Schroeder, Jones, Cormick & McCalla (1962) and Schroeder & Robberson (1965).

Amino acid analyses of isolated tryptic peptides. Approx. 0.2–0.3 μ mole of each peptide was hydrolysed in 6N-HCl (2.0 ml. for each 0.1 μ mole) for 24 hr. at 110° in evacuated sealed tubes and analysed in a similar way to that described for total globin. Cysteine was determined as *S*-aminoethyl-cysteine on a 15 cm. column of the PA-35 resin, on which this derivative is eluted between lysine and histidine. Tryptophan was identified in two peptides by spot test on paper with Ehrlich's reagent (Baldrige & Lewis, 1953). The homology of the amino acid compositions of the various peptides from the Amm- β C-chain from Barbary sheep no. 8 with those of the non- α -chains of sheep Hb-A, Hb-C and Hb-F was the basis of the arrangement of the peptides corresponding to sequences starting at the *N*-terminal end (Boyer *et al.* 1966, 1967; Beale *et al.* 1966; Wilson *et al.* 1966).

RESULTS

Electrophoretic and chromatographic analyses of haemoglobins. Fig. 1 presents the electrophoretic patterns of the haemoglobins of three Barbary sheep and of some domestic sheep. Barbary sheep no. 8 (sample *e*, Fig. 1) showed one major haemoglobin type (Hb-Amm-C) with a mobility similar to that of sheep Hb-C. Two haemoglobin components

Fig. 1. Starch-gel electrophoresis of the haemoglobins of Barbary sheep. Sample (a): domestic sheep A; sample (b): lamb with the Hb-A, Hb-F, Hb-B and Hb-C; sample (c): Barbary sheep no. 7; sample (d): lamb with Hb-A, Hb-F and Hb-C; sample (e): Barbary sheep no. 8; sample (f): domestic sheep A; samples (g) and (h): Barbary sheep no. 108.

were observed in Barbary sheep nos. 7 (sample *c*, Fig. 1), 71 and 72; one was apparently similar to that of Barbary sheep no. 8, whereas the electrophoretic mobility of the second component (Hb-Amm-B) was similar to that of sheep Hb-B. Hb-Amm-B was the sole haemoglobin component in Barbary sheep no. 108 (samples *g* and *h*, Fig. 1).

The chromatographic separation of the haemoglobins of Barbary sheep nos. 8 and 71 are presented in Fig. 2. One major zone (Hb-Amm-C₀) was observed in the chromatogram of animal no. 8, and two major zones (Hb-Amm-C₀ and Hb-Amm-B₀) were observed in that of animal no. 71. Both isolated components behaved as single bands in starch-gel electrophoresis at pH 8.1. Minor haemoglobin components, usually observed in this type of chromatography (Huisman & Dozy, 1965), designated as Hb-Amm-C₁ and Hb-Amm-B₁ were also present and were not further characterized. Table 1 lists the percentages of the various haemoglobin components of the five Barbary sheep. The percentages of Hb-Amm-B in the heterozygous animals were distinctly higher than those of Hb-Amm-C. All animals were healthy with total haemoglobin concentrations in the range 14.1–16.0 g./100 ml. The mean cell volume was in the range 19.0–29.4 μ^3 , which is considerably lower than that observed for the domestic sheep and more comparable with that seen in the domestic goat (van Vliet & Huisman, 1964; Huisman *et al.* 1967). The values for the mean cell haemoglobin concentration and the amount of haemoglobin per cell were

in the ranges 26.2–27.9% and 7.1–10.7 μg . respectively.

Amino acid compositions of acid hydrolysates of total globins and of the Amm- β^{C} -chain. Table 2 presents the results of analyses of 24 hr. acid hydrolysates of the globins Amm-B and Amm-C of Barbary sheep nos. 108, 71 and 8. Some marked differences were noted, which included the presence of two isoleucyl residues and four methionyl residues in globin Amm-C (no isoleucyl residues and eight methionyl residues in globin Amm-B) and of a considerably lower number of threonyl residues, slightly lower numbers of valyl and histidiny

residues and slightly higher numbers of seryl and glutamyl residues in globin Amm-C as compared with globin Amm-B. Comparison of these data with those of sheep globin B (Huisman *et al.* 1965) indicates a close similarity between sheep globin B and globin Amm-B. The proteins, however, are probably not the same, since a marked difference in the numbers of aspartyl residues and possibly of leucyl, seryl and valyl residues was apparent.

Because of the close similarity of the amino acid composition of Amm-C globin with that of sheep globin C (Huisman *et al.* 1965) this globin type was further analysed. Small amounts (2 mg.) of the isolated β -chain were hydrolysed in 6 N-hydrochloric acid for 24 hr. and 72 hr. The results of the amino acid analyses are also presented in Table 2; the data include the average or extrapolated values and the integral values for each of the amino acids. In one instance (threonine) it was found impossible to state with certainty the exact number of residues; the possibility exists that contamination of the isolated β -chain with small amounts of the threonine-rich α -chain may be responsible for a slight excess of threonyl residues. The Amm- β^{C} -chain contains one isoleucyl, one methionyl and one cysteinyl residue, and probably two threonyl, two tyrosyl and two tryptophyl residues. When the total amino acid composition of the Amm- β^{C} -chain was compared with that of the sheep β^{C} -chain (Boyer *et al.* 1966, 1967; Huisman *et al.* 1965; Wilson *et al.* 1966) a close similarity was observed; both proteins seem to be composed of 141 amino acid residues. Several differences were also noted; the Amm- β^{C} -chain seems to contain one more residue each of lysine, aspartic acid and methionine, two more residues each of serine and alanine, one less residue each of glycine and valine, two less residues of glutamic acid and three less residues of threonine.

Amino acid compositions of tryptic peptides of the S-aminoethyl-Amm- β^{C} -chain. Thirteen major zones were obtained from the chromatography of the soluble peptides of a tryptic digest of the Amm- β^{C} -chain by Dowex 50 chromatography (Fig. 3) and were rechromatographed on Dowex 1 to obtain pure peptides. Zones numbered I–V, VII, X, XII and XIII

Fig. 2. Chromatographic separation of the haemoglobins of Barbary sheep no. 8 (a) and no. 71 (b).

Table 1. Haematological data on five Barbary sheep

Animal no.	Sex	Age (years)	Concn. of Hb (g./100 ml.)	Packed-cell volume (ml./100 ml.)	10 ⁻⁶ × Erythrocyte count (per mm. ³)	Proportions of each Hb type (%)			
						Amm-C ₀	Amm-C ₁	Amm-B ₀	Amm-B ₁
7	F	3	15.4	43	16.32	32.4		62.2	5.4
8	F	2	16.0	43	14.96	86.5	13.5		
71	M	2	15.7	42	22.13	45.4		47.7	6.9
72	F	4	14.1	37	15.88	42.8		46.5	10.7
108	F	3	n.d.	n.d.	n.d.			95.4	4.6

n.d., Not determined.

Table 2. Amino acid compositions of 24 hr. acid hydrolysates of the globin from Hb-Amm-B and Hb-Amm-C of three Barbary sheep, and of 24 hr. and 72 hr. acid hydrolysates of the β -chain isolated from Hb-Amm-C

The values given are averages of duplicate hydrolysates prepared in 6N-HCl in sealed evacuated tubes at 110°. n.d., Not determined.

Amino acid	No. of residues/mol. of globin (assumed mol. wt. 66000)				No. of residues/mol. of β -chain (assumed mol. wt. 16500)			
	Amm-B (no. 108)	Amm-B (no. 71)	Amm-C (no. 71)	Amm-C (no. 8)	Amm- β^C (no. 8)		Average or extrapolated values	Integral values
	24 hr.	24 hr.	24 hr.	24 hr.	24 hr.	72 hr.		
Lys	42.6	42.3	41.9	42.6	11.96	12.02	11.99	12
His	39.3	37.8	34.0	35.7	8.36	8.24	8.30	8
Arg	16.8	16.4	16.2	17.6	4.82	4.76	4.79	5
Asp	54.6	53.7	52.7	54.0	14.30	13.60	13.95	14
Thr	31.9	29.1	23.0	23.2	2.44	2.22	2.60*	2-3
Ser	36.7	36.7	39.0	39.1	7.84	6.70	8.35*	8
Glu	30.7	32.2	35.2	33.5	10.58	10.86	10.72	11
Pro	21.6	21.7	19.7	21.0	4.50	4.32	4.41	4
Gly	43.7	40.4	40.3	40.0	10.19	9.95	10.07	10
Ala	65.0	66.9	67.0	67.5	15.89	15.77	15.83	16
Cys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		(1)†
Val	56.1	56.2	53.3	52.0	13.14	15.63	15.63‡	16
Met	7.3	7.9	4.3	3.5	1.03	0.75	0.89	1
Ile	0	0	2.1	2.1	1.00	0.86	0.93	1
Leu	69.9	68.1	68.8	71.8	18.14	17.48	17.81	18
Tyr	10.4	9.9	11.0	10.6	2.30	2.10	2.20	2
Phe	33.9	33.8	36.4	34.9	9.62	9.58	9.60	10
Trp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		(2)†
Total								141-142

* Obtained by extrapolation to zero time.

† From results of analyses of the tryptic peptides (Table 3)

‡ Average of 72 hr. values only.

Fig. 3. Separation of the tryptic peptides from a 2 hr. digest of 280 mg. of the S-aminoethyl-Amm- β^C -chain by Dowex 50 (X2) chromatography. The values in parentheses represent the elution pH values of the various zones.

Table 3. Amino acid composition of the tryptic peptides from a 2 hr. digest of the S-aminoethyl-β-chain of Hb-Amm-C₀

Data are presented as residues per peptide relative to lysine, arginine or histidine. Amino acids present to less than 0.1 residue are omitted. Tryptophan was determined by colour reactions on paper (Balbridge & Lewis, 1953). No correction factors were applied to amino acids that were partially destroyed during acid hydrolysis. The arrangement of the peptides is based on the analogy between the peptides and corresponding sections in sheep and goat β-chains (Boyer *et al.* 1966, 1967; Huismann *et al.* 1967; Wilson *et al.* 1966). The zone numbers refer to those given in Fig. 3. The percentage yield of each peptide was calculated on the basis of the recovery after Dowex 50 and subsequent Dowex 1 chromatography, assuming 100% hydrolysis of the appropriate cleavage points during tryptic hydrolysis. AE-Cys, S-Aminoethylcysteine.

Peptide... Amino acid	No. of residues/peptide relative to lysine, arginine or histidine																		
	T-1	T-2	T-3	T-3 ^b	T-4	T-5	T-6	T-7	T-8	T-9 ^a	T-9 ^b	T-10	T-11	T-12 ^b	T-13	T-14	T-15		
Lys	1.00	1.00	0.82			1.00	1.00	1.00	1.00	1.00	1.00	1.00		1.00	1.00				
AE-Cys																			
His			1.00	1.00		0.80		0.80								0.93	1.00		
Arg	0.91		1.03	1.00		4.15				1.95	2.05	1.03	2.03		1.01				
Asp		0.90			0.91														
Thr	0.20	0.98			0.93	1.01	1.78			1.90		1.65	1.01		4.62	1.10			
Ser			1.98	2.00	0.88					0.20					1.00				
Glu	0.76																		
Pro										1.16									
Gly		1.08	1.88	1.89		1.02		0.94		0.25		0.94		1.13	1.01	1.00			
Ala	0.92	0.98	2.00	2.05		2.86		0.92		0.75		1.98				3.92			
Val			2.60	1.96	1.43*	1.02	1.13						0.94			2.22*			
Met						0.97													
Ile		0.97																	
Leu		0.94	1.00	1.02	1.88	1.12				1.77	2.01	1.94	1.01		1.03	1.04			0.90
Tyr					0.65														
Phe		0.86				2.71				1.04		0.98	0.99	0.97	1.80				
Trp		+			+														
Total	3	9	13	11	10	19	2	4	1	10	6	13	9	4	12	12	2		
Positions	6-8	9-17	18-30	20-30	31-40	41-59	60-61	62-65	66	67-76	77-82	83-95	96-104	117-120	121-132	133-144	145-146		
Zone	VI	XII	V	I	XI	VI	VIII	XI	IV	III	VII	X	IX	XIII	II	VIII	IX		
Yield (%)	39	21	33	42	12	34	123	48	51	42	44	14	31	28	51	25	37		

* Low recovery due to the presence of Val-Val bond.

each yielded one major peptide, and two major peptides were isolated from each of the remaining zones. The amino acid composition and the recovery of each of these peptides are given in Table 3. The peptides are arranged in order of their assumed location in the total polypeptide chain, starting from the *N*-terminal end. All tryptic peptides were isolated from the digest except peptide T-12 (positions 105–116), which is probably insoluble in the buffer system used in the development of the chromatogram (Huisman *et al.* 1967). The *N*-terminal peptide T-1, (Pro,Asn,Lys), was isolated in a relatively high yield of 39%. The one isoleucyl residue was present in peptide T-2. Peptide T-3 (positions 18–30) contained 13 residues. The same residues were also observed in peptide T-3^b (positions 20–30), which is peptide T-3 minus the first two residues. This dipeptide, Val-Lys, was eluted in zone VIII. The high recovery of this peptide (123%) is explained by the fact that the same sequence is found in positions 60 and 61 (peptide T-6). The one methionyl residue was present in peptide T-5 (zone VI). Peptide T-9 was recovered as two peptides, the first ten residues (peptide T-9^a) from zone III and the last six residues (peptide T-9^b) from zone VII. Zone VII also contained a considerable amount of the tripeptide Thr-Gln-Arg, which was derived from peptide T-4 as a result of chymotryptic activity. Tryptophan was identified in peptide T-2, isolated from zone XII, and in peptide T-4, isolated in low yields from zone XI.

DISCUSSION

Two distinct haemoglobin types have been observed in the non-anaemic Barbary sheep that occurred either alone or in combination with each other, indicating an apparent homozygosity or heterozygosity for these proteins. The electrophoretic and chromatographic characteristics of Hb-Amm-C were similar to those of Hb-C found in the anaemic domestic sheep, whereas slight differences were observed between Hb-Amm-B and Hb-B of domestic sheep. The quantity of Hb-Amm-B in the heterozygous animal was slightly greater than that of Hb-Amm-C. None of the five Barbary sheep was anaemic.

The limited structural studies of Hb-Amm-B suggested a close resemblance of this haemoglobin type to that of Hb-B of the domestic sheep. Analyses of the total amino acid compositions of 24 hr. acid hydrolysates, however, indicated some marked differences, and more extensive studies are needed for a closer comparison.

The structural studies of the β -chain of Hb-Amm-C, which is present as a major haemoglobin type in non-anaemic Barbary sheep, indicated a marked

resemblance to the β -chain of Hb-C of the anaemic sheep. The Amm- β^C -chain was found to be composed of 141 amino acids; additional characteristics included the presence of one methionyl and one isoleucyl residue and of perhaps only two threonyl residues. When compared with the sheep β^C -chain, at least 14 differences in the total numbers of amino acid residues per chain were noted. All these differences could be accounted for from the analyses of the total amino acid composition of the tryptic peptides of the Amm- β^C -chain with the exception of the presence of one less aspartyl residue and of one more alanyl residue. It may be that peptide T-12 (positions 105–116), which in the sheep β^C -chain has the composition (Asp,Gly,Ala,Val₄,Leu₄,Arg) (Boyer *et al.* 1966, 1967), has the slightly different composition (Gly,Ala₂,Val₄,Leu₄,Arg).

In comparing the amino acid composition of the tryptic peptides of the Amm- β^C -chain with those of corresponding peptides of the non- α -chains of the sheep Hb-A, Hb-C and Hb-F the following observations can be made. (a) Peptides T-3, T-4, T-6, T-7, T-8, T-11 and T-15 of the β -chains of Hb-Amm-C, sheep Hb-A and Hb-C have identical amino acid compositions. (b) Peptides T-1, T-2, T-13 (except for the aspartyl residue in the assumed position 123) and T-14 of the Amm- β^C -chain are identical with the corresponding peptides of the sheep β^C -chain. (c) Peptide T-5 of the Amm- β^C -chain was the same as that of the sheep β^A -chain, and peptide T-9 of the Amm- β^C -chain resembled that of the sheep β^A -chain (positions 73 and 77) as well as that of the sheep γ -chain (positions 75 and 76). (d) The amino acid composition of peptide T-10 of the Amm- β^C -chain was identical with that of peptide T-10 of the sheep γ -chain. This comparison therefore indicates that the *N*-terminal end (positions 2–49 at least) and the *C*-terminal end (positions 117–146) of the Amm- β^C -chain are probably the same as the corresponding structures of the sheep β^C -chain, and a relationship with the β^A -chain is apparent for positions 50–77 or thereabouts, and with the γ -chain for positions 75–87. It seems that Hb-Amm-C is more closely related to Hb-C of the anaemic domestic sheep than to any other haemoglobin type of this species.

The evolution of the three β -genes (β^A , β^B , β^C) in the domestic sheep has been explained by a divergence of the β^A - and β^B -genes long after the creation of an ancestral β^C -gene, with a vigorous selection in favour of animals with β^B -gene mutations (Boyer *et al.* 1966). Ancestors of the domestic sheep may lack such a polymorphism and may be homozygous for a non-silent β^C -gene. The Barbary sheep, of the genus *Ammotragus*, order Artiodactyla, family Bovidae, which is native to Northern Africa, is not believed to be the direct ancestor of our domestic sheep, since the earliest domestication of

sheep probably took place in southwestern Asia (*Encyclopedia Britannica*, 1965; *Colliers Encyclopedia*, 1967). *Ammotragus lervia*, is, moreover, quite distinct from all other wild sheep; a mixture with other wild sheep is considered to be limited. It therefore seems improbable that the β^C -gene of the Barbary sheep is the direct ancestral gene from which diverged the silent β^C -gene of the domestic sheep. However, the close similarity of the structures of the Amm- β^C -chain and the sheep β^C -chain suggests a close relationship of *Ammotragus* species with the direct ancestors of the domestic sheep and supports the hypothesis of an ancestral structural β^C -gene from which through mechanisms of duplication and mutation the β^A - and β^B -genes emerged, resulting in the A-B-C polymorphism of the domestic sheep.

This study was supported by U.S. Public Health Service Grant HE-05168. The authors are indebted to Dr Jacobs (Artis, Amsterdam) and Dr Van Bommel (Blydorp, Rotterdam) for their help in collecting the blood samples, and to Dr K. Punt and Miss J. Boss (University of Utrecht, The Netherlands) for haematological analyses.

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