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Purification and Properties of Carbonic Anhydrase from Sheep Erythrocytes*

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ABSTRACT: The single major component and three minor components of sheep red cell carbonic anhydrase were purified using a combination of column chromatography and isoelectrofocusing. The major form behaved as a single component in sedimentation velocity experiments ($s^0_{20,w}$ = 3.0 S; observed mol wt 30,000) and during starch gel electrophoresis. Hydrase and esterase activities in conjunction with amino acid composition data suggest that the single major component is homologous with the high-activity form known to occur in other mammals. Comparison of the amino acid composition data of the sheep major and minor forms indicates these structures are also apparently conformational isomers of the same protein. Cyanogen bromide treatment of sheep enzyme releases two soluble fragments. Composition and sequence data indicate the C-terminal fragment is identical with the corresponding fragment seen in bovine carbonic anhydrase except for a Gly–Val substitution four residues from the C terminus. We conclude that sheep red cells contain a single high-activity carbonic anhydrase in contrast to most other mammalian red cell systems where both high- and low-activity forms are known to occur.

The erythrocytes of most mammalian species studied to date (*cf.*, *e.g.*, Tashian *et al.*, 1972) contain two isozymes of carbonic anhydrase which differ markedly in specific activity. In addition, genetic studies (*cf.* Tashian *et al.*, 1971) and comparative sequence analyses of the two human isozymes (Henderson *et al.*, 1971) clearly indicate that the two enzymes are the products of two different autosomal genes. Additional isozymes, seen at low levels in many mammalian carbonic anhydrase systems, appear not to be products of additional genetic loci, but rather represent secondarily altered forms of the major isozymes (*cf.* Funakoshi and

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Deutsch, 1968; Reynaud *et al.*, 1970; Headings and Tashian, 1971).

Because only one form of carbonic anhydrase, which appears to be homologous to the high-activity (CO_2 hydrase) form seen in other mammals, is present in bovine red cells (Nyman *et al.*, 1968), it was of interest to examine another ruminant species such as sheep in order to determine whether their red cells also contained only one form of the enzyme. Preliminary results (Tanis *et al.*, 1970) in this laboratory strongly suggested that the red cells of the domestic sheep (*Ovis aries*) contain only a single major form of carbonic anhydrase. Thus, the present study was undertaken to confirm this finding, and to determine whether the enzyme is more homologous to the high or to the low-activity isozyme seen in other nammals. Furthermore, this study gives us an opportunity to compare physical and chemical properties of the carbonic anhydrases of two species (domestic cattle and sheep) both of which characteristically possess a single major form of carbonic anhydrase in their red cells.

Materials and Methods

Purification. Sheep red blood cells were isolated from citrated whole blood obtained from Colorado Serum Co., Denver, Colo. The blood used in this study was pooled from several donor animals of the Hampshire, Rambouillet, and Suffolk breeds of sheep. The cells were washed and hemolyzed as previously described (Tanis *et al.*, 1970). Extraction of the hemolysate with chloroform and ethanol selectively removed the hemoglobin (Tashian *et al.*, 1966). The concentrated enzyme extract was dialyzed extensively and applied directly to a DEAE-Sephadex column equilibrated with 0.05 M Tris-HCl buffer (pH 8.7). This same buffer was also employed as the initial eluting buffer of the column. Subsequently, a 0.2 M NaCl gradient in Tris-HCl buffer was applied to the column to selectively remove the enzyme. Protein concentration of the column effluent was monitored using a Zeiss spectrophotometer.

Electrofocusing. Electrofocusing experiments were carried out at 9° using the LKB apparatus and carrier ampholytes (Tanis *et al.*, 1970). The time for a typical separation, using the pH 5–8 ampholyte, was 72 hr. All subsequent experiments involving the major and minor forms of the enzyme were carried out using material prepared in this fashion.

Protein Concentration. The method of Babul and Stillwagon (1968) was used to determine protein concentration. The refractive increment (fringes per milligram per milliliter was obtained using techniques previously described (Tanis *et al.*, 1970).

Starch Gel Electrophoresis. Starch gel electrophoresis was carried out using techniques previously described (Tashian, 1969). Gels were made up in 0.02 M borate–NaOH buffer (pH 8.6) and the bridge buffer consisted of 0.3 M borate–NaOH (pH 8.0), containing 0.03 M NaCl. A gradient potential of 8.3 V/cm was applied for 18 hr at 4°. The gels were subsequently stained for both protein and esterase activity.

Sedimentation Runs. Ultracentrifuge runs to determine sedimentation and diffusion coefficients were made using a Spinco Model E ultracentrifuge equipped with an RTIC temperature control. A valve-type synthetic boundry cell was used at a rotor speed of 52,640 rpm. Suitable temperature and buffer corrections were made to obtain the observed $s_{20,w}$ values. Apparent diffusion coefficients were determined by integration of the area under a series of peaks obtained during the early portion of the sedimentation run (Elias, 1970).

Amino Acid Composition. Amino acid analysis was carried out using hydrolysis times of 24, 48, and 72 hr to permit corrections for the destruction or slow release of certain amino acids (Moore and Stein, 1963). Duplicate samples (0.5–0.7 mg of protein) were analyzed for each of the time intervals. Cysteine content was determined using the dimethyl sulfoxide procedure (Spencer and Wold, 1969) and by the technique of treating with 2-bromoacetamido-4-nitrophenol (Tanis *et al.*, 1970). Tryptophan determinations of whole protein were made by the method of Edelhoch (1967), while the tryptophan content of the cyanogen bromide fragment was quantitated using molar extinction coefficients at 287.5 and 278 mμ of 4.6×10^3 and 5.5×10^3 M^{-1} cm^{-1}, respectively (Beaven and Holiday, 1967). The partial specific volume calculation of the sheep carbonic anhydrase was derived from the amino acid composition (Cohn and Edsall 1943).

Cyanogen Bromide Cleavage. Cyanogen bromide cleavage was carried out in 70% trifluoroacetic acid at a protein concentration of 18 mg/ml. A 30- to 40-fold excess of cyanogen bromide (Eastman) was added and the reaction allowed to proceed at room temperature for 8–20 hr. The reaction was terminated by diluting the reaction mixture with water to a final trifluoroacetic acid concentration of 5%. At this point, much of the material became insoluble and was removed by centrifugation. The supernatant, containing the soluble fragments, was lyophilized and resuspended in 0.17 M pyridine–acetate (pH 4.7). This material was applied directly to a 0.5 × 12 cm SE-Sephadex column equilibrated with 0.17 M pyridine–acetate (pH 4.7). The column was eluted with a 100-ml linear gradient (0.17–2 M pyridine–acetate). Each fraction was checked for ninhydrin-positive material and the individual pooled fractions were recycled through similar columns to ensure adequate purification.

Carboxypeptidase A (Worthington) crystals were prepared according to the method of Ambler (1967). Digestion times used were 20 min, 1 hr, and 2 hr.

Enzyme Activities. The CO_2 hydrase and esterase activity measurements of the sheep carbonic anhydrase were made using techniques previously described (Tashian 1969; Nyman, 1963). Esterase activities were determined using *p*-nitrophenyl acetate, 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone, and β-naphthyl acetate as substrates. In all cases, the values reported represent an averaging of three or more separate determinations.

Sequence Determinations. A Beckman Model 890 automatic protein–peptide sequencer was employed to determine the partial amino acid sequence of the C-terminal fragments. Both the standard peptide program (Beckman 080570) and the C-terminal program (092370) were used without modification. Beckman sequencer grade reagents were used without further purification or alteration. The phenylthiohyantoylamino acid derivatives were identified using a Beckman Model GC-4 equipped with a 2 mm × 4 ft DC-560 column (Pisano and Bronzert, 1969). The temperature program consisted of 2-min isothermal at 167° followed by a 16-min linear rise to 277° and was terminated 5 min later. Standard phenylthiohydantoylamino acid derivatives, used for identification and quantitation purposes, were obtained from the Pierce Chemical Co. Arginine, isolated from the water layer by treatment with 1 M Na_2HPO_4, was detected by spotting on paper and treating with phenanthrenequinone in alcoholic NaOH (Yamada and Itano, 1966). The thiazolinone derivatives were converted to corresponding phenylthiohydantoin derivatives by heating at 80° in the presence of 1 N HCl for 10 min (Edman and Begg, 1967).

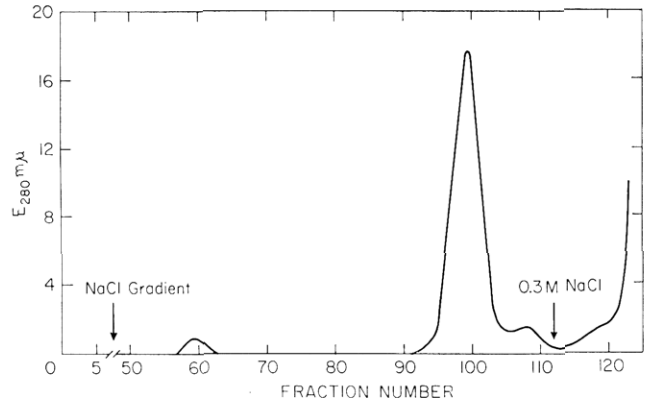

FIGURE 1: Elution pattern of sheep carbonic anhydrase from a column (1.5 × 100 cm) of DEAE-Sephadex equilibrated with 0.05 M Tris-HCl buffer (pH 8.7). A linear 0.2 M NaCl gradient (200 ml) was used to selectively remove the enzyme. Fraction volumes were 3 ml at all stages of development. Fractions 95–101 were pooled and represented the bulk of the major component; fractions 102–107 were also pooled and contained primarily minor component material.

Results

Enzyme Purifications. By utilizing methods previously found effective for other sources of the enzyme, it was possible to purify carbonic anhydrase from sheep red blood cells. A liter of whole blood, after chloroform–ethanol treatment, produced approximately 800 mg of protein, the bulk of which was carbonic anhydrase. This material, after extensive dialysis against 0.05 M Tris-HCl (pH 8.4), was concentrated and applied directly to a 1.5 × 100 cm DEAE-Sephadex column equilibrated with the above Tris buffer. To develop properly the DEAE-Sephadex column, a linear NaCl gradient was found necessary. A typical elution pattern is shown in Figure 1. Only a single carbonic anhydrase peak is observed, centered at fraction 98, even when the column is stripped with 0.3 M NaCl. The minor peak at fraction 60, the shoulder from fractions 105 to 111, and the large residual hemoglobin peak starting to emerge at fraction 118 were all shown, after concentration, to contain no carbonic anhydrase activity.

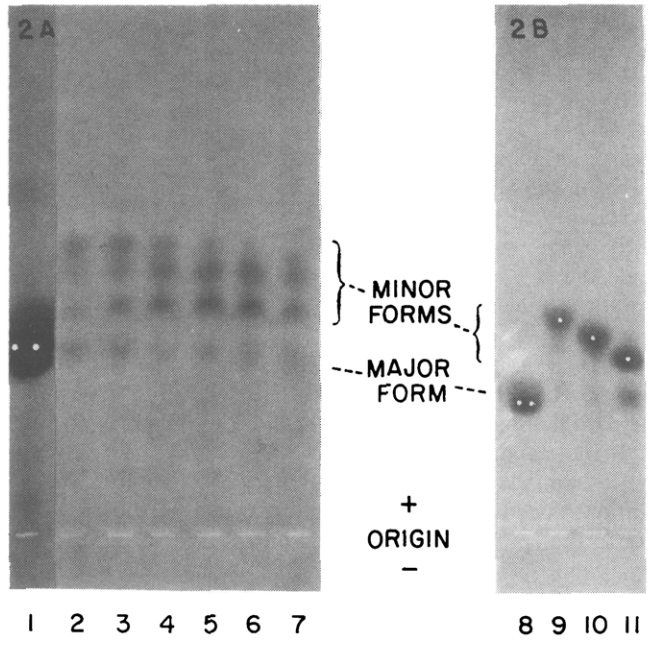

FIGURE 2: Starch gel electrophoresis of sheep carbonic anhydrases. Bands marked with double white dots are considered major forms; single white dots indicate minor components. (A) Well 1, contains the single major component with represents the bulk of the DEAE-Sephadex peak centered at fraction 98 in Figure 1. Wells 2–7, contain the unpooled trailing edge of the DEAE-Sephadex peak. This shows the distribution of the three minor components before separation. (B) Well 8, contains major component after electrofocusing. No contamination could be shown. Wells 9–11, contain the three minor components, representing pools C, B, and A, respectively (see Figure 3), after electrofocusing. The gel electrophoresis run was made at 4° for 18 hr using sodium borate buffer (pH 8.6).

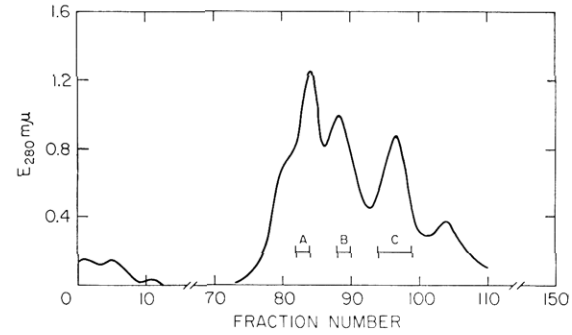

FIGURE 3: Isoelectric focusing of the pooled minor components (see text). The pH gradient, using the LKB ampholine, was approximately 5–8 with the cathode at the alkaline region. Pools A, B, and C were made to minimize cross contamination. No carbonic anhydrase activity could be shown for the peak centered at fraction 104.

Starch Gel Electrophoresis. When the single major enzyme from the DEAE-Sephadex column peak was subjected to starch gel electrophoresis, it was shown to be composed of a single major component and three partially resolved minor components. This is clearly seen on the starch gel patterns in Figure 2A. Well 1, representing the pooled leading edge of the enzyme peak, contains the bulk of the major component plus a small amount of nonenzymatic protein. The unpooled column fractions containing the trailing edge of the peak are shown in wells 2–7. Primarily, these latter fractions contain differing amounts of the minor components of sheep carbonic anhydrase.

Electrofocusing. To purify further both the major and minor forms of the enzyme previously employed (Tanis *et al.*, 1970) electrofocusing techniques were utilized. By using

TABLE I: Comparative Physical Properties of the C-Type Carbonic Anhydrase from Several Ungulate Species.

	Sheep	Horse CA C[a]	Bovine[b]	Porcine CA C[c]
$s^0_{20,w} \times 10^{-13}$ (S)	3.0	2.7[d]	2.9	3.2
Partial specific volume[e]	0.73	0.73	0.73	0.73
Molecular weight $\times 10^{-4}$	2.9	2.8	3.0	2.9
$A^{1\%}_{280}$	16.1	13.4	19.0	15.2

[a] Furth (1968). [b] Nyman and Lindskog (1964). [c] Tanis *et al.* (1970). [d] Reported as $s^{app}_{20,w}$. [e] Based on amino acid composition.

TABLE II: Amino Acid Composition of Sheep (Major and Minor Components), Bovine, Porcine C, and Equine C Carbonic Anhydrases.[e]

	Sheep						
	Major		Minor				
	Obsd	Nearest Integer	Obsd	Nearest Integer	Bovine[a]	Porcine CA C[b]	Equine CA C[c]
Lys	18.0	18	17.8	18	19	20	19
His	11.1	11	11.3	11	11	13	12
Arg	9.5	10	9.1	9	9	9	9
Asp	33.0	33	32.4	32	32	27	27
Glu	24.8	25	24.1	24	24	28	26
Thr	11.4	11	8.5	9	15	13	12
Ser	18.1	18	14.7	15	16	17	18
Pro	20.2	20	23.2	23	20	17	16
Gly	17.9	18	18.0	18	20	22	23
Ala	19.5	20	19.5	20	17	13	17
Cys[d]	0.0	0			0	0	1
Val	22.0	22	21.7	22	20	13	19
Met	3.0	3	3.0	3	3	3	1
Ile	3.1	3	3.0	3	5	10	7
Leu	26.4	26	26.2	26	26	23	22
Tyr	6.8	7	6.4	6	8	7	7
Phe	12.2	12	12.0	12	11	12	11
Try[d]	5.9	6			7	6	5

[a] Data taken from Nyman and Lindskog (1964). [b] Data taken from Tanis *et al.* (1970). [c] Data taken from Furth (1968). [d] Determined by procedures detailed in the Materials and Methods section; no data for the minor component. [e] Hydrolysis periods of 24, 48, and 72 hr were used to permit corrections for slow rate of release and amino acid destruction. The composition data for both forms of sheep are based on an assumed methionine content of three residues per molecule.

a pH 5–8 gradient of 2% ampholine, it was possible to cleanly separate the major sheep component from the slight protein contamination seen after DEAE-Sephadex separation. Material purified in this manner appeared homogeneous by several criteria including starch gel elctrophoresis (Figure 2B) and analytical ultracentrifugation. The minor components were also partially separated and purified using the preparative LKB electrofocusing system. After pooling the DEAE-Sephadex fractions containing the minor components, isoelectrofocusing was carried out using a pH 5–8 gradient at a concentration of 1%. The resultant protein profile is shown in Figure 3. The leading shoulder, centered at fraction 80, represents residual major component while the small peak at fraction 104 is nonenzymatic material. Portions of each of the three minor component peaks were pooled in such a way as to minimize cross contamination, and analyzed by starch gel electrophoresis. The results, seen in Figure 2B (wells 9–11), show that it is possible to separate the three sheep minor components even though a slight amount of contamination still remains. Since the intermediate minor component appeared to be almost completely pure, amino acid composition data were obtained (*vide infra*). No attempt, using denaturing agents, was made to try and reconvert these minor components to the major form; however, it appears they will not undergo such an alteration spontaneously in solution.

Physical Properties. Sedimentation velocity determinations made with the analytical ultracentrifuge indicated a homogeneous component over the protein concentration range of 5–12 mg/ml. As shown in Table I, sheep carbonic anhydrase has an $s^0_{20,w}$ of 3.0 S. The corresponding $D^0_{20,w}$, obtained from schlerin peak area, was 9.2×10^{-7}. These values, in conjunction with the partial specific volume calculated from amino acid composition, indicate a molecular weight of 29,500. This value agrees well with the assumption that sheep carbonic anhydrase is a single polypeptide chain composed of about 260 amino acid residues.

Amino Acid Composition. Table II lists the amino acid composition of the major component and one (intermediate) of the three minor components of sheep carbonic anhydrase. The average values of the major form were obtained from duplicate runs made after 24-, 48-, or 72-hr hydrolysis. In the case of the minor form, duplicate samples hydrolyzed for 32 hr were analyzed and no corrections for destruction or slow release were applied. Because of its purity, the intermediate minor component was chosen for composition studies. In both cases the average values reported here were made on the assumption that both forms contained three methionine residues. Such an assumption agrees well with the molecular weight data calculated from the ultracentrifuge. As indicated under Materials and Methods both cysteine and tryptophan were determined spectrophotometrically; in addition, cysteine was also determined using the dimethyl sulfoxide procedure. No cysteine could be detected in either case. Cysteine and tryptophan determinations were not made on the minor component due to the low amount of material available.

Although there are some notable exceptions (*e.g.*, proline

Residue No.	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
Human CA B	Glx-	His-	Asn	- Asn-	Arg	-Pro	- Thr-	Gln	-Pro-	Leu	-Lys	- Gly -	Arg	- Thr -	Val-	Arg-	Ala-	Ser-	Phe-	COOH
Human CA C	Val-	Asp-	Asn	- Trp	- Arg	-Pro	- Ala	- Gln	-Pro-	Leu	-Lys	- Asn	- Arg	- Gln	-Ile-	Lys-	Ala-	Ser-	Phe-	Lys -COOH
Bovine CA C	Leu-	Ala-	Asn	- Trp	- Arg	-Pro	- Ala	- Gln	-Pro-	Leu	-Lys	- Asn	- Arg	- Gln	-Val-	Arg-	Gly-	Phe-	Pro-	Lys -COOH
Sheep CA C	Leu-	Ala-	Asn	- Trp	- Arg	-Pro	- Ala	- Gln	-Pro-	Leu	-Lys	- Asn	- Arg	- Gln	-Val-	Arg-	Val-	Phe-	Pro-	Lys -COOH

FIGURE 4: Amino acid sequence of the carboxyl-terminal portions of the various carbonic anhydrases. The data for the human and bovine enzymes are taken from Nyman *et al.* (1968) and reflect a residue numbering scheme starting at the C-terminal end of the peptides. The boxes show the locations of agreement between the sequences. See text for details of sheep sequence.

Residue No.	38	37	36	35	34	33	32	31	30	29	28	27	26	25	24	23	22	21
Human CA B	Ala-	Gln-	Phe	-Arg	-Ser-	Leu	- Leu-	Ser	-Asn-	Val-	Glu	- Asp-	Asn-	Gly -	Ala -	Val -	Pro -	Met
Human CA C	Val-	Lys	- Phe	-Arg	-Lys-	Leu	- Asn-	Phe	-Asp-	Gly-	Glu	- Gly-	Glu-	(Pro	),Glu,	Glu)-	Leu	- Met
Sheep CA C	Leu-	Lys	- Phe	-Arg	-Ser-	Leu	- Asn-	Phe	-Asn-	Ala-	Glu	- Gly-	Glu-	Pro	-Leu-	Glu-	Leu	- Met

FIGURE 5: Amino acid sequence of the penultimate CNBr fragment of sheep carbonic anhydrase and the corresponding human isozyme sequences. The boxes show the location of agreement between the sequences. Arrows to the right (→) indicate sheep sequence determined with the automatic sequencer; arrows to the left (←) indicate carboxypeptidase A sequence data.

and serine), it appears that amino acid composition of the minor component is very similar to that of the major form. This finding is in agreement with the observation of Laurent *et al.* (1966) that the minor form of human carbonic anhydrase (CA A) has an amino acid composition identical with the major component (CA B).

C-Terminal CNBr Fragment. As in the case of bovine (Nyman and Lindskog, 1964) and pig (Tanis *et al.*, 1970) carbonic anhydrases, the sheep molecule appears to contain three residues of methionine. Thus, it was relevant to determine if one of these corresponded to the methionine located 21 residues from the C-terminal end of the bovine enzyme. Cyanogen bromide treatment and isolation of the peptide were carried out using techniques previously found successful (Tanis *et al.*, 1970). Advantage was taken of the observation that the C-terminal fragment was soluble in water, while the bulk of the CNBr fragments were not. To separate the fragment from another soluble peptide of very different amino acid eomposition, SE-Sephadex columns were employed. Following this separation, the amino acid composition indicated the C-terminal peptide was highly purified and suitable for sequencing in the automatic sequencer. The amino acid composition data are presented in Table III. It was assumed that the fragment contained 1 mole of phenylalanine/mole of peptide. These composition data indicate that bovine and sheep appear to have homologous C-terminal fragments except for a single Gly–Val substitution. To confirm this homology, automatic sequencing of the fragment was undertaken using the volatile buffer peptide program. The following sheep sequence was confirmed by repeated sequencer runs: Leu-Ala-Asn-Trp-Arg-Pro-Ala-Gln-Pro-Leu-Lys-Asn-Arg-Gln-Val-Arg-Val-Phe-Pro-Lys. Aside from the Gly–Val substitution this sequence is identical with that reported for the bovine (Nyman *et al.*, 1968) C-terminal fragment (Figure 4).

TABLE III: Amino Acid Composition of the Sheep C-Terminal CNBr Fragments.[c]

	Terminal Fragment		Penultimate Fragment	
Amino Acid	Obsd (μm)	Nearest Residue Integer	Obsd (μm)	Nearest Residue Integer
Asp	0.18	2	0.14	2
Ser			0.07	1
Glu	0.22	2	0.21	3
Pro	0.31	3	0.08	1
Gly			0.06	1
Ala	0.17	2	0.08	1
Val	0.19	2		
Leu	0.22	2	0.26	4
Phe	0.10	1	0.13	2
Lys	0.20	2	0.08	1
Arg	0.33	3	0.07	1
Trp[a]	0.10	1		
Met[b]			+	1

[a] Based on observed OD_{278}. [b] Based on observed homoserine. [c] The terminal fragment was assumed to contain one residue of phenylalanine while the penultimate fragment was assumed to contain two residues of this amino acid.

Penultimate CNBr Fragment. An additional CNBr fragment, produced during cyanogen bromide treatment of the whole molecule, was purified and sequenced. Chromatographic isolation of the peptide, using a SE-Sephadex column developed with a pyridine acetate gradient, was necessary before amino acid and sequence data could be obtained. The composition data shown in Table III indicate that it is an 18-residue peptide terminating with homoserine. Using the automatic sequencer it was possible to determine the sequence of the first 14 residues of this peptide without equivocation. These data are presented in Figure 5 as residues 38–25. To complete the sequence carboxypeptidase A treatment of 0.04 μM of the CNBr fragment was undertaken. Using the amino acid analyzer the following data were observed: 20 min, 1.0 residue homoserine and 0.9 residue of leucine; 1 hr, 1.0 residue homoserine and 1.0 residue of leucine. Since the affinity of the enzyme for glutamic acid is very low (Ambler,

TABLE IV: Hydrase and Esterase Activities of Sheep, Porcine, Bovine, and Human Carbonic Anhydrase.

Carbonic Anhydrase Type	Esterase Activities[a]			CO_2 Hydrase Act., ΔOD_{278} (mg/sec)
	β-Naphthyl Acetate, $v/[E_0]$, min^{-1}	*p*-Nitrophenyl Acetate, $v/[E_0]$, min^{-1}	Sultone[b] 10^{-4} k_{enz} ($1\ mole^{-1}\ min^{-1}$)	
Human B	1.47	32.0	5.1	7.9
Porcine B	6.40	11.8	1.0	4.8
Human C	1.00	166.7	7.6	27.2
Porcine C	0.90	35.0	11.3	32.9
Sheep C	0.89	56.1	11.3	42.5
Bovine C	0.89	61.8	14.6	38.0

[a] Data for human and porcine from Tanis *et al.* (1970). [b] 2-Hydroxy-5-nitro-α-toluenesulfonic acid sultone.

1967), this indicates that the C-terminal sequence of this fragment is Leu-Glu-Leu-Met.

Enzyme Activity. Previous reports of mammalian red cell carbonic anhydrase (*cf.* Tashian *et al.*, 1972) generally have shown two isozymes which differed significantly in their specific CO_2 hydrase and esterase activities. Such differences have been useful in grouping the low-activity forms into the CA B isozyme category and the high specific activity forms into the CA C isozyme category. In the case of sheep, we observed only a single major component of carbonic anhydrase. The observed hydrase and esterase activities are reported in Table IV; for comparison purposes, previously reported values for human and porcine carbonic anhydrase have been included. The markedly high specific CO_2 hydrase activity value reported here for the sheep enzyme clearly indicates that it is probably homologous to the high activity CA C forms seen in other mammalian species. This is analogous to the observation that bovine red cells also contain a single high-activity enzyme.

Discussion

The amino acid composition and ultracentrifuge data indicate that sheep red cell carbonic anhydrase is a single polypeptide chain of about 260 amino acid residues. Typically, the protein is low in sulfur-containing amino acids and contains no cysteine residues. It also appears to be unique in having a low isoleucine content of only three residues per molecule of enzyme. On the basis of its low serine content and comparative enzyme activity data, the single sheep red cell carbonic anhydrase appears to correspond most closely to the high-activity C isozyme seen in those mammalian systems which contain the two isozymes. Therefore, in line with previous nomenclature suggestions (Furth, 1968), the sheep major component would be designated as sheep CA C. The correctness of this assumption is further strengthened by the observed sequence similarities in the C-terminal portions of sheep, bovine, and human C enzyme (see below).

When the sheep and bovine red cell carbonic anhydrase systems are compared, additional similarities are observed. Both are single component systems containing a high-activity (CO_2 hydrase) enzyme. Presumably, other ruminants also show the single high-activity enzyme, suggesting that the suppression in red blood cells of the gene which determines the low-activity enzyme occurred early in the divergence of the Artiodactyla line which led to the present day ruminant species. However, on the basis of the recent finding by Carter (1971), that bovine rumen epithelium contains only high levels of the low-activity B isozyme of carbonic anhydrase, it can be concluded that the B enzyme of certain ruminant red cells has been suppressed, rather than genetically lost, in these species. Other features shared by the sheep and bovine enzymes are their almost identical starch gel migration behavior and similar amino acid compositions.

The three enzymatically active minor bands which migrate ahead of the major component during starch gel electrophoresis were always observed to occur at low concentration levels. Each of the three had, on the basis of zymogram staining, equal levels of hydrase or esterase activity. They appear not to reflect a polymorphic system which has been reported from Columbia, Targhee, and Merino breeds of sheep (Tucker *et al.*, 1967) since all three components were repeatably present in red cells obtained from many individual donor animals. Furthermore, the amino acid composition of the intermediate minor component is almost identical with the major component suggesting that these minor components are not the products of additional genetic loci, but rather reflect some conformational change or side-chain modification. Each of the three minor components appeared stable to long-term storage in solution and showed no tendency to generate the major component or other minor components.

The sheep cyanogen bromide fragment reported to represent the C terminus of the molecule did not appear to contain homoserine lactone and demonstrated a striking sequence similarity to the bovine C-terminal fragment. Under the conditions employed for generation and purification of the C-terminal CNBr fragment there appeared to be no major deamidation reactions occurring as has been previously reported for other sources (Nyman *et al.*, 1968). Inspection of Figure 4 reveals there are 8 identical residues in all 4 fragments while there are 13 identities if the low-activity form, human CA B, is excluded. What is noteworthy is that 12 of these 13 identities are grouped as a single contiguous run. Although this would suggest some importance to the maintenance of the high-activity form, it is obviously not possible as yet to assign a functional role to these residues. Nyman *et al.* (1968) have discussed this region in some detail and the reader is referred to their work for suggestions concerning possible functional implications. The same high degree of identical homology is also seen in the penultimate fragment (Figure 5). Additional sequence data (Tashian *et al.*, 1972) of the N-terminal 84 residues of the sheep enzyme reflects a higher degree of homology between the human (Henderson *et al.*, 1972) and sheep carbonic anhydrases than does

the C-terminal data presented here. Corresponding bovine data are also available and indicate it too is highly homologous to the human C isozyme.

Acknowledgment

We thank Mrs. Ya-Shiou L. Yu for her highly skilled research assistance.

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Hepatic Epoxide Hydrase. Structure–Activity Relationships for Substrates and Inhibitors*

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ABSTRACT: An epoxide hydrase preparation solubilized and partially purified from guinea pig liver microsomes catalyzes the hydration of a variety of epoxides to corresponding glycols. The activity of various epoxides as substrates or as inhibitors of this enzyme is dependent on the nature and stereochemistry of substituents on the oxirane ring. Oxiranes with a 1-aryl substituent (styrene oxides) or with certain 1-alkyl substituents (1-octene oxide, phenyl 2,3-epoxypropyl ethers) are among the best substrates for the enzyme and are *competitive* inhibitors for hydration of styrene-*t* oxide. 1,1-Disubstituted and cis-1,2-disubstituted oxiranes are less active as substrates and inhibitors. Trans-1,2-disubstituted, trisubstituted, and tetrasubstituted oxiranes are virtually inactive as substrates or inhibitors. Certain alicyclic oxiranes such as 1,2-epoxy-1,2,3,4-tetrahydronaphthalene and cyclohexene oxide are relatively inactive as substrates but very effective as inhibitors. Inhibition by these compounds appears to be *noncompetitive* with respect to substrate. 1,1,1-Trichloropropene 2,3-oxide is a very potent *uncompetitive* inhibitor of epoxide hydrase. Various analogs of epoxides such as azaridines, thiiranes, and oxaziridines, either do not inhibit the enzyme or are relatively weak inhibitors. Alcohols and certain ketones, such as metyrapone, activate the enzyme. Structure–activity correlations with the epoxide hydrase are distinctly different from those of squalene oxidocyclase.

Hepatic epoxide hydrase is an important enzyme in the metabolism of olefinic and aromatic substrates, since it controls one of two major enzymatic pathways for the further bioalteration of intermediate epoxides and arene oxides (Jerina *et al.*, 1968, 1970a; Leibman and Ortiz, 1968); the conversion of these compounds to glycols and dihydrodiols, respectively.

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