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THE CARBONIC ANHYDRASE IN THE RUMEN EPITHELIAL TISSUE OF THE OX

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SUMMARY

Two carbonic anhydrases (carbonate hydro-lyase, EC 4.2.1.1) have been isolated from the rumen epithelium of the ox. In their amino acid compositions and in their kinetic properties, the enzymes are closely related to the "low activity" carbonic anhydrases that have been isolated from the bloods and large intestines of other species. The two enzymes are present in high concentrations in the rumen epithelium, and were isolated in nearly equal amounts. The "high activity"-type carbonic anhydrase, which occurs in blood and in non-ruminant stomachs, was not found.

The selective occurrence of the "low activity" enzyme in organs which harbour microorganisms may mean that the enzyme participates in some fashion in the absorption of substances peculiarly associated with those organs, such as NH_4^+ or volatile fatty acids.

INTRODUCTION

It was reported in 1960 that two chromatographically distinct forms of carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) had been isolated from bovine blood[1]. The two forms are similar both in their amino acid compositions and in their kinetic properties. Shortly thereafter, multiple forms of carbonic anhydrase were discovered in human blood[2]. In contrast to the bovine isoenzymes, the human isoenzymes are of two kinetically differing kinds. The CO_2-hydratase activity of one type of human blood isoenzyme exceeds that of the other type by an order of magnitude[3]. The two kinds of isoenzyme are also markedly different in their amino acid compositions[4,5].

The functional importance of the "high activity" and "low activity" carbonic anhydrases of blood is not known, but similar pairs of isoenzymes have been isolated from the bloods of several other mammalian species[6–9]. The "high activity"–"low activity" pattern appears to be the rule, and therefore, in lacking a "low activity" carbonic anhydrase[1,10], bovine blood is anomalous (see, however, refs. 2, 7).

Some attention has recently been given to the carbonic anhydrases of tissues

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other than blood. The dog kidney[11] and the rat dorsolateral prostate gland[8] are reported to contain only "high activity" isoenzymes, but no "low activity" isoenzymes. In a study of the blood and gastrointestinal tissues of the guinea pig, it was found that whereas the stomach contains only the "high activity" type of isoenzyme, the proximal colonic mucosa contains large amounts of both types of isoenzyme[9]. The concentration of "low activity" isoenzyme found in the caecal mucosa was greater than that found either in stomach or in proximal colonic mucosa[12,13]. Thus these studies show a clear difference between the tissue distributions of the two types of isoenzyme; and although the "low activity" isoenzyme compares unfavourably with the "high activity" isoenzyme in CO_2-hydratase activity, it evidently does possess a special importance. The selective distribution of the "low activity" isoenzyme in the gastrointestinal tract (there is very little in the small intestinal mucosa, and practically none in the stomach[12,13]) links it with the peculiar physiology of the large intestine.

The non-ruminant stomach contains large amounts of "high activity" carbonic anhydrase, but no "low activity" isoenzyme (see above). Although the rumen is a part of the stomach[13], it is functionally more akin to the large intestine[14]. In view of this physiological connexion, the rumen epithelium might be expected to contain a counterpart of the "low activity" isoenzyme that has been found in the large intestinal mucosa. This has proved to be so, and the present paper describes the purification and some properties of two "low activity" carbonic anhydrases from rumen epithelial tissue.

MATERIALS AND METHODS

Triethanolamine (reagent grade, British Drug Houses Ltd., Poole, Dorset, U.K.), Sephadex G-75 (Pharmacia (GB) Ltd.), DEAE-cellulose (H. Reeve Angel and Co. Ltd., London EC4, U.K.), Bovine plasma albumin and Folin and Ciocalteu's reagent (British Drug Houses Ltd., Poole, Dorset, U.K.), *N,N,N',N'*-tetraethylmethyl diamine (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.), acrylamide and bis-acrylamide (reagent grade, British Drug Houses Ltd., Poole, Dorset, U.K.), naphthalene black "12B" (British Drug Houses Ltd., Poole, Dorset, U.K.). Other reagents were analytical grade from British Drug Houses Ltd., Poole, Dorset, U.K., and water was deionized and glass distilled.

Homogenisation of rumen epithelial tissue

The tissue is very coarse and tough, and consequently the following procedure was used to homogenise it: Patches of epithelial tissue (about 3 cm^2) were frozen in liquid nitrogen, and crushed with solid CO_2 by using a mortar and pestle. The resulting mixture of solid CO_2 and frozen tissue was fed into a hammer-mill (Glen Creston, Stanmore, U.K.). The tissue emerged as a fine powder which was finally dispersed in about 400 ml of 0.3 M sucrose, 50 mM sodium phosphate buffer, pH 7.0, with a motor-driven homogeniser (Silverson Machines Ltd., London SE1, U.K.) (2–4°).

$(NH_4)_2SO_4$ fractionation

22.6 g $(NH_4)_2SO_4$ were added to each 100 ml of protein solution (initial volume),

pH 7.2, and the mixture stirred continuously for 2 h at 2–4°. Precipitated material was separated by centrifugation at about 50 000 × *g* for 20 min.

Gel filtration

Bead-form Sephadex G-75 was allowed to swell in a solution containing 0.1 M Na_2SO_4, 50 mM sodium phosphate buffer, pH 7.2, for 24 h. A column measuring 4 cm × 95 cm was used for fractionating crude samples of carbonic anhydrase (2–4°). Sample volume was 15 ml, and the above buffer was used for elution. The flow rate was 40–50 ml/h.

Ion-exchange chromatography

DEAE-cellulose (Whatman, microgranular pre-swollen) was equilibrated with 5 mM triethanolamine titrated to pH 8.2 with concentrated HCl. The column bed measured 1.6 cm × 25 cm. A 0.2–0.5-ml sample dialysed previously against the same buffer was applied to the column. Buffer was pumped through the column at 25–30 ml/h to elute a peak of enzymically active material ((a) in Fig. 2). 2 mM NaCl was added to the above buffer to elute a further peak of enzymically active material ((b) in Fig. 2). 2–5-ml fractions were collected.

Storage of isoenzymes

The enzyme solutions (<10 mg/ml) were dialysed against 10 mM sodium phosphate buffer, pH 7.2, and stored at 0–2°. Preparations retained their enzymic activity for several weeks.

Measurement of CO_2-hydratase activity

The activities of fractions collected during chromatography were measured with a pH-stat method[16]. The CO_2 concentration was 4 mM. The reaction mixture (5.0 ml) also contained 2 mM sodium phosphate buffer, 25 mg/l bovine plasma albumin, 1 mM EDTA (sodium salt). During a measurement, the reaction mixture was kept at pH 7.4 (0°) by continuous titration with 0.5 M NaOH. The rate of reaction was calculated from the rate of titration with alkali. The contribution of the spontaneous hydration rate was deducted from the total rate. A unit of CO_2-hydratase activity was defined as that amount of enzymic activity which raises the rate of hydration of CO_2 by 1 μmole/min, under the conditions described above.

To measure kinetic parameters, an electrometric method was used[17]. The rate of fall in pH of the reaction mixture was recorded as a function of time (0°). The total change in pH was about 0.05 pH unit, and the time taken for such a change in pH was usually not more than 10 sec. The reaction mixture (5.0 ml) contained 25 mM sodium phosphate buffer, 50 mM Na_2SO_4, 25 mg/l bovine plasma albumin, 1 mM EDTA (sodium salt), and 2–30 mM CO_2. The initial pH was 7.20. The rate of hydration was deduced from the rate of generation of hydrogen ions. The addition of H^+ to the reaction mixture was related to changes in pH by direct titration of the CO_2-free reaction mixture with standard H_2SO_4.

Reduction of large volumes of protein solutions

The "pressure dialysis" technique[18] was used.

Amino acid analysis

A 150–200 μg sample of enzyme was dried in a 1 cm × 12 cm test tube *in vacuo*. 0.2 ml of HCl (British Drug Houses "Aristar", diluted 1:1 with water) was added to the dried sample and the tube sealed as described by MOORE AND STEIN[19]. The tubes were incubated for 24–96 h at 110°. The tubes were dried in a vacuum, and the hydrolysate analysed by an amino acid analyser (Locarte Co., London SW7, U.K.).

Serine and threonine were estimated by extrapolating the measurements obtained to zero time. Cysteine was measured as cysteic acid after performic acid oxidation of the unhydrolysed sample[20]. The tryptophan:tyrosine ratio was determined by the methods of GOODWIN AND MORTON[21] and of BENCZE AND SCHMID[22], the results by the two methods being averaged. NH_3 was not determined.

Acrylamide gel electrophoresis

This was performed in the manner described by DAVIS[23], except that "spacer" and "sample" gels were omitted. The samples (10–100 μg) were applied in a strong solution of sucrose (<100 μl). The gel mixture was 7.5% (w/v) acrylamide. The voltage gradient was about 40 V/cm and the current through each gel was 2 mA. Electrophoresis was done in a cold room (2–4°) and was usually continued for 2 h. Proteins migrated in a zone of pH 9.5 (approximately)[23]. Gels were stained for protein with 1% naphthalene black in 5% (v/v) acetic acid, and de-stained electrophoretically.

Determination of protein concentration

The concentration of a protein mixture was determined by the method of LOWRY *et al.*[24], using bovine plasma albumin as standard.

The concentrations of enzyme solutions were determined from absorbance measurements at 280 nm (Zeiss spectrophotometer PMQ II) at room temperature. The medium was 10 mM sodium phosphate buffer, pH 6.0. The specific extinction coefficient of rumen enzyme (a) was determined from the quantities of amino acids recovered during amino acid analysis of enzyme taken from a stock solution of known extinction. The specific extinction coefficient was also calculated from the amino acid composition of enzyme (a) (Table III), using the data of WETLAUFER[25].

pH measurements

pH was measured, at the appropriate temperature with a glass combined electrode (Activion Glass Ltd., Fife, U.K.) and a Vibret pH meter Model 3920 (Electrical Instruments Ltd., Richmond, Surrey, U.K.).

RESULTS

A piece of the rumen wall, measuring about 20 cm × 20 cm, was taken from a freshly killed ox, and packed in ice during transport from the slaughterhouse (approximately 1 h). The mucosal surface was scrubbed and rinsed with 0.25 M sucrose, 50 mM sodium phosphate buffer, pH 7.0, and the epithelium stripped from the muscle layers. The wet weight of the epithelium was 80–100 g. The tissue was cut into patches about 2 cm^2, put into liquid nitrogen, and homogenised as described in

TABLE I

PURIFICATION OF BOVINE RUMEN EPITHELIAL CARBONIC ANHYDRASE

CO_2-hydratase activity was measured by the pH-stat method (see MATERIALS AND METHODS). Concentrations of protein mixtures were determined by the method of LOWRY *et al.*[24]. Concentrations of the purified enzymes were determined from absorbance measurements at 280 nm, taking $E_{1\ cm} = 17.0$ for both enzyme (a) and enzyme (b).

Purification step	*Total protein*	*Total enzyme activity (units × 10^{-4})*	*Specific activity (units/mg)*	*Yield (%)*
Homogenate	3.8 g	24	65	100
High speed supernatant	1.2 g	23	190	96
$(NH_9)_2SO_4$ precipitation	610 mg	19	310	79
Gel filtration	140 mg	18	1290	75
DEAE-cellulose				
Enzyme (a)	23 mg	4	1740*	
Enzyme (b)	22 mg	4	1820**	33

* Maximum value in peak.
** Average value in peak.

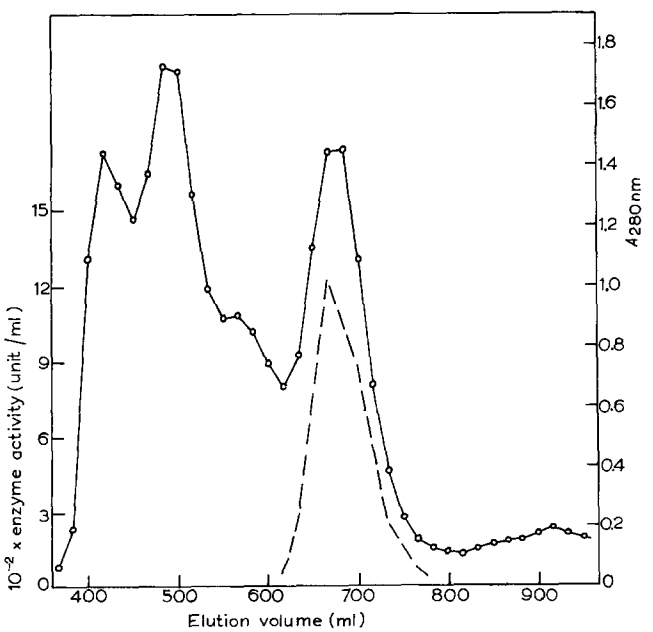


Fig. 1. Gel filtration, using Sephadex G-75, of crude carbonic anhydrase extracted from the rumen epithelial tissue of ox. For pre-treatment of the starting material, see text. The eluting buffer was 0.1 M sodium sulphate, 0.05 M sodium phosphate buffer, pH 7.2 (4°). ○—○, absorbance at 280 nm.; — —, CO_2-hydratase activity measured by the pH-stat method (see MATERIALS AND METHODS). Fractions were pooled so that at least 90% of the enzymic activity was recovered from this step.

MATERIALS AND METHODS. It is likely that a proportion of the enzyme activity of the tissue was destroyed during homogenisation. The specific activity of such a homogenate (Table I) was much less than that of ox whole blood (which was about 150 units/ml).

The homogenate (400 ml) was centrifuged at 75 000 × *g* for 60 min; more than 90% of the enzymic activity (pH-stat assay) was recovered in the supernatant. The volume of that supernatant was reduced to 15 ml, when it was fractionated by the addition of solid $(NH_4)_2SO_4$ (see MATERIALS AND METHODS). A somewhat variable proportion (70–90%) of the enzymic activity was recovered in the supernatant obtained from this step. The supernatant from the $(NH_4)_2SO_4$ fractionation step was fractionated further by gel filtration (see MATERIALS AND METHODS). Fig. 1 shows a typical elution pattern. Fractions were pooled in such a way that about 90% of the enzyme activity was recovered from this step.

The pooled fractions from the gel filtration step were reduced in volume to about 0.5 ml, and eluted from a column of DEAE-cellulose (Fig. 2), as described in MATERIALS AND METHODS.

The crude enzyme was resolved into two major peaks of enzyme activity (isoenzymes (a) and (b), Fig. 2). The specific activity of isoenzyme (a) was not uniform

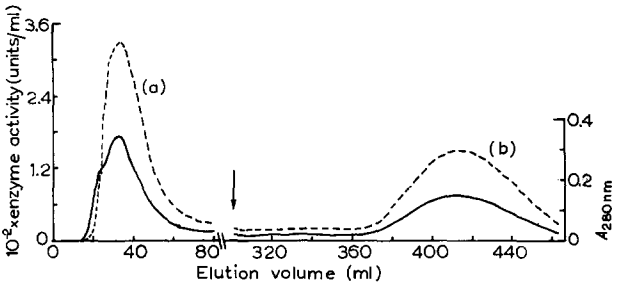


Fig. 2. Ion-exchange chromatography, using DEAE-cellulose, of crude carbonic anhydrase from ox rumen epithelial tissue. Starting material was obtained from the gel filtration step (see Fig. 1). The column was equilibrated, and elution started, with 5 mM triethanolamine titrated with HCl to pH 8.2 (4°). The ionic strength of the eluting buffer was subsequently raised (arrow) by the addition of 2 mM NaCl. ———, absorbance at 280 nm; — — —, CO_2-hydratase activity measured by the pH-stat method.

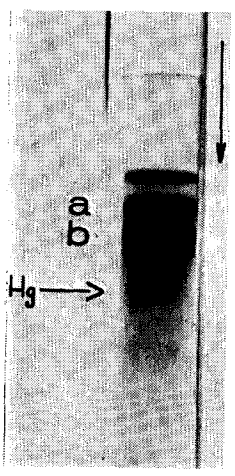
through the peak. Isoenzyme (b) appeared to be homogeneous. By re-chromatography with buffer of higher pH, the material in the first eluted peak was separated into a small peak of enzymically inactive material (eluted first) and an homogeneous peak of carbonic anhydrase (a). The specific activities of the rumen isoenzymes were both about 1800 units/mg (pH-stat assay), which should be compared with 3000 units/mg for the guinea pig "low activity" isoenzyme, and 130 000 units/mg for the guinea pig "high activity" isoenzyme[13].

Rumen isoenzyme (a) behaves in a manner very similar to the guinea pig "high activity" isoenzyme[13] during DEAE-cellulose chromatography. Accordingly, the rumen isoenzyme (a) is likely to be isoelectric at, or somewhat higher than, pH 7.4 (ref. 9). Likewise, rumen isoenzyme (b) was very similar to the ox blood isoenzyme "B", which is reported to be isoelectric at pH 5.9 (ref. 30).

LINDSKOG[1] did not describe a "low activity" carbonic anhydrase when reporting his discovery of multiple forms of carbonic anhydrase in ox blood. In the guinea pig it has been found that "low activity" isoenzymes from blood and gastrointestinal tissues are practically identical[9], and an attempt was therefore made to detect a "low activity" isoenzyme in ox blood. From the properties of the rumen isoenzymes described above, it was reckoned that a "low activity" carbonic anhydrase would be eluted from DEAE-cellulose before, or very close to, the "high activity" isoenzyme "B". A small amount of crude ox blood carbonic anhydrase was prepared by fractionating an haemolysate on Sephadex G-75. During elution of the crude enzyme from DEAE-cellulose, only two peaks of activity corresponding to LINDSKOG's isoenzymes "B" and "A" were observed. However since it was done on rather a small scale (10 ml packed erythrocytes) a "low activity" isoenzyme may have been overlooked if it were present in a very small amount.

Blood content of rumen epithelial tissue

An estimate of less than 5% blood in the rumen epithelial tissue (based on absorbance measurements for haemoglobin at 410 nm), probably overestimates quite substantially the true proportion of blood. Thus at such low levels of blood contamination, the contribution of pigments other than haemoglobin to the absorbance at 410 nm may be quite substantial. If the rumen epithelial tissue does indeed contain 5% blood, the blood "high activity" isoenzymes would contribute only about 10% to the CO_2-hydratase activity of the rumen epithelial tissue homogenates. There is reason to believe that this is also an over-estimate, for it is assumed that both rumen



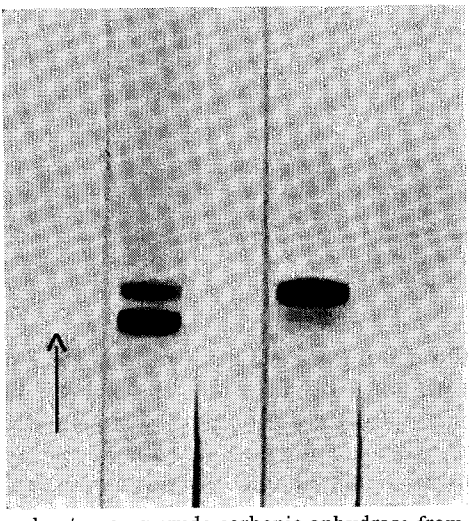


Fig. 3. Acrylamide gel electrophoresis. Sample was about 100 μg crude carbonic anhydrase from rumen epithelial tissue, obtained from gel filtration step of purification procedure (see text and Fig. 1). The 'low activity' isoenzymes (a) and (b) are marked. The arrow indicates the position of haemoglobin. Cathode at top of photograph; anode at bottom. Voltage gradient: 40 V/cm. Gels were run for 2 h (2–4°). Current through each gel was 2 mA. Gels were stained in 1% naphthalene black in 5% acetic acid, for 2 h.

Fig. 4. Acrylamide gel electrophoresis. Left, 30 μg rumen epithelium carbonic anhydrase (b); right, 30 μg rumen epithelium carbonic anhydrase (a). See legend to Fig. 3.

"low activity" and blood "high activity" iosenzymes are equally sensitive to inactivation during the harsh homogenisation procedure that was employed (see DISCUSSION).

Acrylamide gel electrophoresis

The enzymically active material obtained during gel filtration of the crude rumen carbonic anhydrase (Fig. 1) was analysed by acrylamide gel electrophoresis (Fig. 3). Carbonic anhydrases (a) and (b) were identified, after separation by ion-exchange chromatography (see above) as the two major protein bands in Fig. 3. The small band of material, of lower mobility than isoenzyme (a), was identified as the minor contaminant of isoenzyme (a) encountered during ion-exchange chromatography (see above). The minor band of material that migrated just ahead of isoenzyme (b) during electrophoresis was also identified during ion-exchange chromatography (not shown in Fig. 2); it was eluted after isoenzyme (b), and possessed no CO_2-hydratase activity.

Fig. 4 shows acrylamide gel analyses of preparations of carbonic anhydrases (a) and (b). Using the conditions described in the preceding section, it was not possible to obtain preparations of isoenzyme (a) completely free from isoenzyme (b). Densitometer measurements of such analyses indicated that the contamination of isoenzyme (a) with isoenzyme (b) was less than 5%. Preparations of isoenzyme (b) were practically homogeneous (Fig. 4) during electrophoresis.

Fig. 5 shows the pattern produced by gel electrophoresis of crude ox blood carbonic anhydrase (*cf.* ref. 10). The ox blood "high activity" isoenzyme "B" was very similar in mobility to the rumen "low activity" isoenzyme (b), whereas rumen isoenzyme (a) migrated much less rapidly than either of the erythrocyte "high activity" enzymes. No band of material corresponding to either rumen enzyme (a) or (b) was discernible in analyses of crude ox blood carbonic anhydrase.

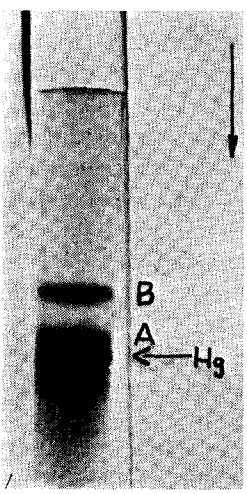


Fig. 5. Acrylamide gel electrophoresis. Sample was 30 μg ox blood crude carbonic anhydrase, obtained by gel filtration of an haemolysate of washed erythrocytes. The top band is the 'high activity' isoenzyme 'B', the band with slightly lower mobility than haemoglobin (arrowed) is the 'high activity' isoenzyme 'A'.

TABLE II

THE AMINO ACID COMPOSITIONS OF OX RUMEN 'LOW ACTIVITY' CARBONIC ANHYDRASES (a) AND (b)

The enzyme preparations were obtained as described in the text. Hydrolysis and analysis of enzyme samples was performed as described in MATERIALS AND METHODS. The data are the results of ten analyses of isoenzyme (a) (two preparations) and of five analyses of (b) (one preparation). Serine and threonine values were corrected for destruction during hydrolysis of the samples (see MATERIALS AND METHODS). Cysteine was measured as cysteic acid[20]. Tryptophan was deduced from the ratio tyrosine:tryptophan, as measured by the method of GOODWIN AND MORTON[21], and by that of BENCZE AND SCHMID[22]. It was assumed that each isoenzyme contains 22 alanine residues per molecule.

Amino acid	*Number of residues per molecule*	
	(a)	*(b)*
Aspartate	30.0	30.0
Threonine	9.8	10.0
Serine	25.6	25.8
Glutamate	22.0	21.3
Proline	20.3	20.3
Glycine	18.1	18.2
Alanine	22.0	22.0
Cysteine	0.8	—
Valine	15.6	15.3
Methionine	0.5	0.6
Isoleucine	9.8	9.5
Leucine	25.0	24.6
Tyrosine	6.8	6.6
Tryptophan	7.3	—
Phenylalanine	11.0	10.9
Histidine	10.1	10.5
Lysine	21.0	19.7
Arginine	7.8	7.2

Amino acid analyses

Table II shows the results of the amino acid analyses performed on rumen carbonic anhydrases (a) and (b). In calculating the results it was assumed that these isoenzymes, along with other mammalian carbonic anhydrases[6], have molecular weights of close to 30 000. The assumption is justified by observations made during gel filtration of the rumen crude carbonic anhydrase (Fig. 1). Thus the ox rumen isoenzymes were eluted from a column of Sephadex G-75 in the same volume and under identical conditions as were the guinea pig isoenzymes, which are already known to have molecular weights of about 30 000[9,13]. It was further assumed that each isoenzyme contains 1 zinc atom per molecule[6], and that the α-amino terminals are acetylated[26].

The differences between the analyses of the rumen isoenzymes (a) and (b) are probably within the limits of experimental error. Both enzymes show features that are common to all of the mammalian carbonic anhydrases so far studied, *viz.* a high proportion of aromatic residues (giving a relatively high specific extinction coefficient (see below)), a high proportion of proline residues, and only a few sulphur-containing residues. Table III compares the data for rumen enzyme (a) with the composition of bovine erythrocyte enzyme "B" (the figures have been rounded to give integral numbers of residues per molecule of enzyme). It was remarked earlier that the rumen

TABLE III

THE AMINO ACID COMPOSITIONS OF OX RUMEN 'LOW ACTIVITY' CARBONIC ANHYDRASE (a) AND OX BLOOD 'HIGH ACTIVITY' CARBONIC ANHYDRASE 'B'

The data for rumen enzyme (a) are from Table II, and those for the ox blood enzyme are from NYMAN AND LINDSKOG[4]. Values for each residue have been rounded to the nearest integer.

Amino acid	*Number of residues per molecule*	
	Rumen isoenzyme (a)	*Ox blood isoenzyme B*
Aspartate	30	32
Threonine	10	15
Serine	26	16
Glutamate	22	24
Proline	20	20
Glycine	18	20
Alanine	22	17
Cysteine	1	0
Valine	16	20
Methionine	1	3
Isoleucine	10	5
Leucine	25	26
Tyrosine	7	8
Tryptophan	7	7
Phenylalanine	11	11
Histidine	10	11
Lysine	21	19
Arginine	8	9
Total residues	265	263
Molecular weight	29 300	29 481

enzymes are related by their CO_2-hydratase activities to the "low activity" type of carbonic anhydrase. Bovine erythrocyte enzyme "B" is of the "high activity" kinetic type (see below).

Ultraviolet absorption

Using the amino acid analysis data of Table III, for rumen enzyme (a), the specific extinction coefficient was calculated[25] as 16.1. By measuring the quantities of amino acids recovered from the amino acid analyses, a figure of 17.2 $\pm$ 0.9 (S.E.) (four determinations) was obtained for rumen isoenzyme (a). In calculating molar enzyme concentrations, the latter figure was rounded to 17.0 and used both for isoenzyme (a) and for isoenzyme (b).

Kinetic measurements

Using CO_2 concentrations ranging from 2 to 30 mM, measurements of the catalysed rate of hydration were made at pH 7.2, 0°. Both rumen isoenzymes obeyed Michaelis–Menten kinetics (Figs. 6 and 7). K_m and $v_{max}/[E]$ values are given in Table IV, which also includes some values for ox blood carbonic anhydrase taken from ref. 27. Table IV shows that the rumen isoenzymes are very similar in their kinetic properties. They are clearly of the "low activity" type, for the $v_{max}/[E]$ values are

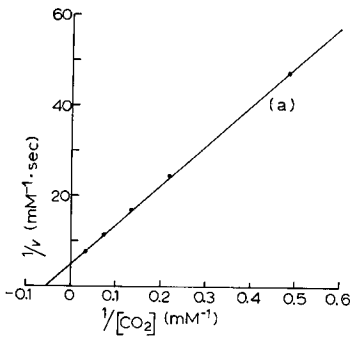


Fig. 6. Lineweaver–Burk plot for 'low activity' isoenzyme (a) of the bovine rumen epithelium, pH 7.2 (0°). Reaction mixture: 25 mM sodium phosphate buffer, 50 mM sodium sulphate, 1 mM EDTA (sodium salt); 25 mg/l bovine plasma albumin, 2–30 mM CO_2 dioxide, and 70 nM enzyme.

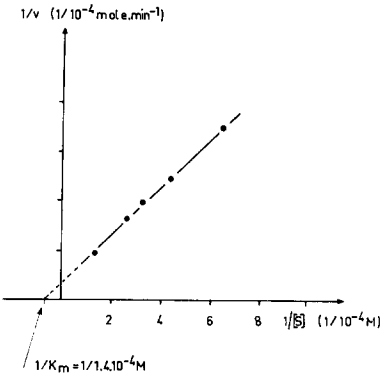


Fig. 7. Lineweaver–Burk plot for 'low activity' isoenzyme (b) of the bovine rumen epithelium, pH 7.2 (0°). See legend to Fig. 6 for details of reaction mixture. Enzyme concentration was 73 nM.

less than one-twentieth that of the ox-blood preparation studied by DEVOE AND KISTIAKOWSKY[27]. The K_m values indicate that the specific activities of blood and rumen isoenzymes are disparate at very low, as well as saturating, CO_2 concentrations (*cf.* ref. 9).

DISCUSSION

Chemically and kinetically differing types of carbonic anhydrase, "high activity" and "low activity", have been found in the erythrocytes of several mammalian species[6–9]. In addition, "high activity" isoenzymes have been isolated from stomach, large intestinal mucosal tissues[9,12,13], kidney[11], and prostate gland tissue[8]. "Low activity" carbonic anhydrases have been observed only in large intestinal mucosal

TABLE IV

KINETIC PARAMETERS OF SOME CARBONIC ANHYDRASES FROM BOVINE BLOOD AND BOVINE RUMEN EPITHELIAL TISSUE, MEASURED AT pH 7.2 (0°)

The data for rumen isoenzymes were obtained in the manner described in MATERIALS AND METHODS. The reaction mixture contained 25 mM sodium phosphate buffer, 50 mM sodium sulphate, 1 mM EDTA, 25 mg/l bovine plasma albumin, 2–30 mM CO_2, and 70–90 mM enzyme. Coefficients of variation for $v_{max}/[E]$ measurements were about 10%. Coefficients of variation for K_m measurements were about 15%. Data are from experiments with two enzyme preparations. The data for the blood preparation, which is probably a mixture of kinetically similar 'high activity' isoenzymes, are taken from DEVOE AND KISTIAKOWSKY[27].

Enzyme	K_m *(mM)*	$10^{-3} \times v_{max}/[E]$ *(sec⁻¹)*
Rumen isoenzyme (a)	19.8	3.4
Rumen isoenzyme (b)	22.5	3.2
Blood enzyme	9.1	82.1

tissues, notably the caecal mucosa, among tissues other than blood[9,12,13]. In this paper, two "low activity" enzymes from the rumen epithelial tissue of the ox have been described.

The fresh rumen epithelial tissue may contain one or several "high activity" isoenzymes (some due to blood contaminating the tissue) in addition to the "low activity" isoenzymes described here. Selective loss of the "high activity" isoenzymes through denaturation[28] during purification could account for the failure to recover any "high activity" isoenzymes. Thus a substantial loss of "high activity" enzyme is likely to have occurred in preparing the homogenate, as well as during some of the subsequent purification stages, *e.g.* $(NH_4)_2SO_4$ precipitation. The possible existence of "high activity" isoenzymes in the homogenate makes it difficult to estimate the real recovery, and thence the tissue concentration, of the "low activity" isoenzymes. If the enzymic activity of the homogenate were due solely to the "low activity" isoenzymes (a) and (b), then the homogenate would have contained 3–4 mg isoenzymes ((a) + (b)) per 100 mg homogenate protein. If losses of isoenzymes (a) and (b) during purification were small, the recovered quantities of the two isoenzymes allow an estimate of 1 mg isoenzymes ((a) + (b)) per 100 mg homogenate protein. In comparison, LINDSKOG's data indicate that whole ox blood contains about 0.15 mg "high activity" isoenzymes per 100 mg protein.

In spite of the fact that no "high activity" isoenzyme was observed during either ion-exchange chromatography or electrophoresis of the crude rumen carbonic anhydrase, it is conceivable that blood "high activity" isoenzyme was adsorbed by the rumen enzyme fractions to a significant degree. However, close agreement between the chemical and kinetic data (see Tables II and IV) for these fractions (together with the evidence for their kinetic homogeneity (Figs. 6 and 7)), makes it extremely unlikely that such contamination occurred.

The discrepancies between the amino acid analysis data for the two rumen isoenzymes concern only a small number of residues; indeed the isoenzymes may be identical in their contents of the residues measured. The physical dissimilarities may be due to differing degrees of amidination[31], or to differing tertiary structures[32]. Thus, in view of the harshness of the homogenisation method, it is possible that the fresh tissue contains but a single form of the "low activity" carbonic anhydrase. On the other hand, both of the erythrocyte "high activity" isoenzymes (which are in some ways analogous to the two rumen isoenzymes[1,4]) were observed in a crude preparation of ox blood enzyme made by gel filtration of an haemolysate, a very gentle method.

In their overall chemical compositions, "low activity" isoenzymes from various species resemble one another more closely than do "high activity" and "low activity" isoenzymes from the same species[29]. This is illustrated by a graphical comparison of the amino acid composition of ox rumen isoenzyme (a) with, firstly, the "high activity" isoenzyme from ox blood, and secondly, with the "low activity" isoenzyme from the blood of a distantly related species, man (Fig. 8). Two conspicuous differences between "high activity" and "low activity" isoenzymes from several species are shown in Table V. The difference in serine content is remarkably large and consistent ("low activity"/"high activity" approx. 1.6). The difference between "high activity" and "low activity" isoenzymes in their contents of basic residues holds good for four species ("low activity"/"high activity" approx. 0.8), but the rumen iso-

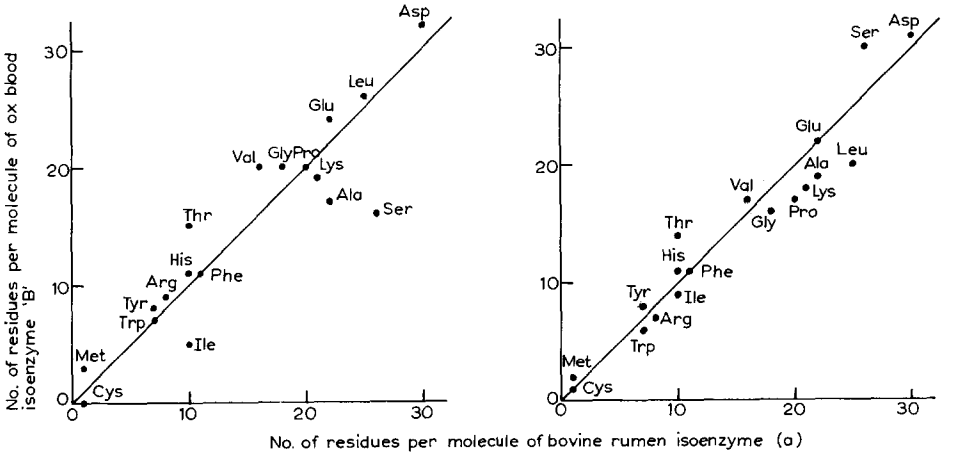


Fig. 8. The amino acid composition of the 'low activity' isoenzyme (a) of the bovine rumen epithelium compared with, left, the amino acid composition of the 'high activity' isoenzyme 'B' of bovine blood, and right, the amino acid composition of the 'low activity' isoenzyme 'B' of human blood. The figures compare the numbers of amino acid residues (nearest integer) per molecule of enzyme. For bovine isoenzymes see Table III; data for human isoenzyme 'B' are from ref. 5. Method of plotting is after NYMAN AND LINDSKOG[4].

enzyme (a) contains a larger number of basic residues than the other "low activity" isoenzymes studied.

There is evidence that the "high activity" and "low activity" carbonic anhydrases are under separate genetic control, and that the two forms arose through a gene duplication[4,7]. That the types of isoenzyme are governed by separate and independently-controlled genetic loci is shown by the wide variation in their tissue distributions (Table VI). It has been suggested that the "low activity" type of carbonic anhydrase appeared at a later stage in evolution than the "high activity" type, since the former shows greater phylogenetic variation than the latter. A "low activity" isoenzyme, for example, is lacking altogether in the bloods of some species[34,35], and in the blood of an human individual also[36]. But the absence of a "low activity"

TABLE V

COMPARISON OF THE 'HIGH ACTIVITY' AND 'LOW ACTIVITY' CARBONIC ANHYDRASES FROM DIFFERENT SPECIES, ACCORDING TO THEIR CONTENTS OF SERINE AND OF BASIC RESIDUES (ARGININE, LYSINE, HISTIDINE) (number of residues per enzyme molecule)

Species	*'Low activity'/'high activity'*	
	Serine residues	*Basic residues*
Human[5]	1.58	0.82
Monkey[29]	1.67	0.77
Horse[7]	1.56	0.85
Guinea pig[13]	1.50	0.81
Ox*	1.63	1.00

* From Table III.

TABLE VI

THE OCCURRENCE OF 'HIGH ACTIVITY' AND 'LOW ACTIVITY' CARBONIC ANHYDRASES IN MAMMALIAN TISSUE

+ signs show relative tissue concentrations of enzymes. — sign indicates that the enzyme has not been reported present, or that it occurs in a very low concentration, in a tissue. For references, see text.

Tissue	*'High activity' isoenzyme*	*'Low activity' isoenzyme*
Blood	+	+++
Stomach	+++	—
Small intestinal mucosa	—	—
Proximal colonic mucosa	++	+++
Distal colonic mucosa	—	++
Caecal mucosa	+	+++++
Kidney, carnivore	+	—
Kidney, herbivore	—	—
Prostate gland	+	—
Rumen epithelium	—	+++++

carbonic anhydrase from the blood does not mean that it is absent from tissues generally. Instead of searching for a "low activity" isoenzyme in the blood of non-mammalian species, it might indeed prove more fruitful to investigate large intestinal (or homologous) tissues and also, for example, shell gland tissue (avians) or gill tissue[12]. In mammals, the uterus and kidney need further study.

The high concentrations of "low activity" carbonic anhydrase in the large intestinal mucosal tissue suggests that the enzyme may be concerned with the absorption of substances produced by the action of microorganisms, for example, ammonium ions or volatile fatty acids[12,13]. The present study forges another link between "low activity" carbonic anhydrases and microbial actions, inasmuch as the rumen bears a close functional resemblance to the large intestine (see INTRODUCTION). From what is known about the tissue distributions of "low activity" and "high activity" carbonic anhydrases (Table VI), it appears that the respective roles of the two types of isoenzyme may not be closely allied. Thus the function of "low activity" carbonic anhydrase in the ox rumen epithelium may involve some activity beyond simply catalysing the reactions between CO_2, HCO_3^- and water[12,33].

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