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RAT RENAL AND ERYTHROCYTE CARBONIC ANHYDRASES

PURIFICATION AND PROPERTIES

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Summary

Rat renal and erythrocyte carbonic anhydrases (carbonate hydro-lyase, EC 4.2.1.1) were isolated by affinity chromatography. The erythrocytes contain two major forms of the enzyme. One of the forms has a specific activity (towards CO_2) 30 times higher than the other and constitutes the major part of the total cellular carbonic anhydrase. The amino acid compositions of this high-activity type and of the low-activity type are similar to the compositions reported for these types in other species.

The kidney appears to have only one high-activity form of carbonic anhydrase which is very similar to and probably identical with the erythrocyte high-activity form.

Introduction

There is still controversy [1] about whether the kidney uses only high-activity forms (towards CO_2) of carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) or whether this organ also has a cytoplasmic low-activity form like the erythrocytes [2,3]. These carbonic anhydrases not only differ in substrate activity [4] but are also inhibited differently by sulfonamides [5]. It is of obvious importance to know whether both forms have to be taken into account when attempts are made to assess the role of carbonic anhydrase in renal acidification and electrolyte excretion.

In renal physiology and pharmacology, particularly in micropuncture studies, the rat is the most frequently used experimental animal. Its renal car-

Abbreviations: enzyme HCA-B and enzyme HCA-C are isoenzymes of carbonic anhydrase from human erythrocytes. Enzyme RBC-B and enzyme RBC-C are isoenzymes of carbonic anhydrase from rat erythrocytes. HEPES: *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonate.

bonic anhydrase has not yet been purified to an extent which would allow of a detailed characterization. The same is true of rat erythrocyte enzymes. It was therefore considered appropriate to use the rat for a detailed study of these renal and erythrocyte carbonic anhydrases.

In the present paper the rat renal soluble enzyme was purified by an affinity chromatographic technique. Its properties were compared with those of the enzymic forms from the erythrocytes. Only one soluble renal form was found similar to the high-activity erythrocyte form and different from the low-activity erythrocyte form. In this respect the rat is therefore similar to man.

Materials and Methods

Materials

Sepharose 6B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Carrier ampholytes, Ampholine®, were purchased from LKB-Produkter (Bromma, Sweden). Acrylamide and bisacrylamide were purchased from Eastman, Kodak (Rochester, Ill., U.S.A.). Sulfanilamide (Sigma, St. Louis, Mo., U.S.A.) was used without further purification. All other chemicals employed were reagent grade.

Preparation of enzymes

Adult female Wistar strain rats of 200—250 g were anesthetized with 40 mg/kg of Mebumal® intravenously. The kidneys were perfused in situ with 0.9% NaCl solution via the abdominal aorta at constant pressure until the effluent from the veins was colorless. The kidneys were sliced, and only sices which were visibly free from blood were taken. They were minced and homogenized at 0° C in 5 vol. of 0.3 M sucrose buffered with 0.02 M sodium phosphate, pH 7.3. A glass homogenizer with a motor-driven Teflon® pestle was used. The homogenate was centrifuged at 75 000 × *g* for 12 h in an MSE high-speed centrifuge. The supernatant was dialyzed against 0.05 M Tris · H_2SO_4, pH 8.0, containing 200 μg of EDTA (Na salt). It was loaded directly on to the affinity-chromatography column. Its concentration of hemoglobin was measured by a cyanmethaemoglobin method [6]. The protein of the supernatant and of the chromatographic fractions was assayed by the method of Lowry et al. [7].

Blood from the same animals was collected in heparinized tubes. It was centrifuged and the erythrocytes were washed twice in 0.9% NaCl. They were lysed in 2 vols. of water. The hemolysate was centrifuged and filtered in order to remove the stroma. Solid Tris was added to 0.05 M and adjusted to pH 8 with H_2SO_4. The stroma-free hemolysate was diluted 1/10 in 0.05 M Tris · H_2SO_4 buffer, pH 8, and then loaded on to the affinity-chromatographic column as described above for the kidney supernatant.

Affinity chromatography of carbonic anhydrase on sulfanilamide-agarose

Sulfanilamide coupled to agarose via oxirane groups is a stable, effective and selective adsorbant for both low- and high-activity forms of carbonic anhydrase [8]. The unspecific adsorption is low and the enzymes eluted are highly purified [1,8]. The activated matrix is formed by reaction of Sepharose 6 B with 1,4-bis(2,3-epoxypropoxy-)butane (EGA Chemie, Steinheim, G.F.R.). Sulfanila-

mide is coupled to the long hydrophilic spacer by an alkylamine bond. The amount of coupled sulfanilamide was 200 μmol per ml gel, by sulphur and nitrogen analysis. After addition of the tissue extract to the column the proteins which were nonspecifically retained by the gel were first desorbed by lowering the pH and increasing the ionic strength of the buffer. The low-activity forms of carbonic anhydrase bound to the gel were then eluted by adding chloride, a specific displacer of these forms [1]. The high-activity forms were eluted with perchlorate, an inhibitor of both forms of the enzyme [1]. Finally sulfanilamide was added to desorb all retained enzyme proteins.

Enzyme assays

Fractions were pooled, concentrated by the pressure-dialysis technique [9], and finally dialyzed against 10 mM HEPES buffer of pH 8.3 containing 200 μM EDTA, in order to remove inhibitory anions before assaying the enzyme activities.

The enzyme activities in these pooled fractions and in the tissue material were assayed by the pH-changing method of Philpot and Philpot [10]. One enzyme unit (U) is defined as the amount of enzyme that doubles the rate of hydration of CO_2.

Enzyme concentrations

The concentrations of the purified enzymes were calculated from the absorbance, $A_{280}^{1\%}$ (cm^{-1}) using values of 18 and 17, as reported [11], for the B and C types of erythrocyte enzymes RBC-B and RBC-C) respectively. The kidney enzyme was assumed to have a value similar to that of enzyme RBC-C. The molecular weights from analytical ultracentrifugation (Table III) were used in the calculation of kinetic parameters. The enzymes were kept at −20° C until analyzed.

Isoelectric focusing

This was done in tubes according to a procedure similar to that of Conway-Jacobs and Lewin [12]. A pH gradient between 3.5 and 9.5 was obtained by mixing carrier ampholytes (Ampholine®) in polyacrylamide gels. Focusing was performed by a stepwise increase in the voltage to give a power per tube not exceeding 200 mW. Equilibrium was achieved after 4 h. The gels were stained with Coomassie Brilliant Blue (Svenska ICI AB, Gothenburg, Sweden) and destained according to the procedure described by LKB-Produkter (Bromma, Sweden). The human erythrocyte carbonic anhydrases HCA-C and HCA-B with known isoelectric points of 7.3 and 6.6 respectively [13] were used as standard proteins.

Amino acid analysis

This analysis was performed by ion-exchange chromatography according to the method of Moore and Stein [14] on a Beckman 121 M automatic amino analyzer. Protein samples were hydrolyzed in 6 M HCl at 110° C for 14 and 72 h.

Analytical ultracentrifugation

Sedimentation velocity experiments were performed in a Spinco Model E

analytical ultracentrifuge, equipped with an ultraviolet scanning optical system. The rotor speed was 40 000 rev./min for every sedimentation run. Exposures were made every 4 min for 3 h. The sedimentation constants were calculated and corrected to give values in water at 20° C ($s_{20,w}$) according to the procedure of Svedberg and Pedersen [15].

Sedimentation equilibrium ultracentrifugation was done by means of Chervenka's long-column meniscus-depletion technique [16]. The proteins had absorbance values A_{280} (1.2 cm^{-1}) of between 0.18 and 0.6. A 12-mm 4° capillary-type synthetic boundary double-sector cell was used at 20° C. The centrifuge was run at 28 000 rev./min for about 20 h. The molecular weights were calculated from the slope of ln C versus r^2. The partial specific volumes were obtained by the method of Cohn and Edsall (cf. ref. 13) from the amino acid composition in Table I.

Kinetic methods

The kinetic parameters were investigated by assaying the effects of the enzymes on the rate of CO_2 hydration by use of an electrometric method [17]. The decrease in pH after the addition of CO_2 to the reaction mixture was measured with a glass electrode, as a function of time. The initial pH was 7.0 and the change in pH was less than 0.01 pH unit. The composition of the reaction

TABLE I

AMINO ACID COMPOSITION OF RAT KIDNEY AND ERYTHROCYTE CARBONIC ANHYDRASES

Data on mole fractions for the erythrocyte enzymes are the average of two hydrolyses, one at 24 h and the other at 72 h. Half-cystine and methionine were measured as cysteic acid and methionine sulfone respectively, after performic acid oxidation. Values for threonine and serine were obtained by extrapolation to zero-hour hydrolysis. Values for isoleucine and valine are from the 72 h-hydrolysis. Data on the kidney enzyme are from one 24-h hydrolysis, and, for the sake of comparison, they are given with the data of the 24-h hydrolysis for the high-activity erythrocyte form.

Amino acid	100 X mole fraction		100 X mole fraction (one 24-h hydrolysis)	
	Enzyme RBC-B	Enzyme RBC-C	Kidney enzyme	Enzyme RBC-C
Lysine	6.6	8.2	7.9	8.3
Histidine	4.5	5.1	4.6	5.3
Arginine	2.7	2.5	3.0	2.6
Aspartic acid	10.9	10.5	10.8	10.8
Threonine	4.0	4.5	4.5	4.4
Serine	12.8	8.9	8.2	8.6
Glutamic acid	7.8	10.6	10.8	10.7
Proline	7.9	6.5	6.5	6.2
Glycine	7.1	7.8	8.1	8.0
Alanine	8.5	6.4	7.1	6.6
Valine	7.3	5.1	5.1	4.8
Methionine	0.4	0.8	0.9	0.8
Isoleucine	3.3	4.5	4.2	4.1
Leucine	9.6	9.3	9.4	9.4
Tyrosine	3.1	2.9	2.9	2.8
Phenylalanine	3.2	5.0	4.6	4.9
Tryptophan	—	—	—	—
Cysteic acid	0.4	1.0	1.4	1.0

TABLE II

KINETIC PARAMETERS FOR RAT KIDNEY AND ERYTHROCYTE (RBC-B AND RBC-C) CARBONIC ANHYDRASE ISOENZYMES

Conditions of test: 10 mM sodium phosphate buffer with 25 mM Na_2SO_4, 500 μM EDTA, 12.5 mg/ml bovine plasma albumin, initial pH 7, 0°C ± 0.05. $[E]_0$ was 1.7, 0.8 and 43 nM for renal enzyme, enzyme RBC-C and enzyme RBC-B, respectively. The uncatalyzed rate constant, k_{CO_2} under these conditions was 0.0021 s^{-1}. The parameters are given with their 95% confidence limits in brackets.

Enzyme	Hydration		Inhibition by sulfanilamide
	K_m (mM)	$10^{-4} \times V/[E]_0$ (s^{-1})	K_i (μM)
Kidney	4.3 (2.9—5.8)	3.7 (2.6—4.8)	1.9 (1.6—2.1)
RBC-C	5.6 (2.3—9.0)	8.2 (6.5—9.9)	1.1 (1.0—1.3)
RBC-B	12.8 (8.8—16.9)	0.3 (0.2—0.4)	16 (11—21)

mixture (4.0 ml) is given in the legend to Table II. 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 assay medium with standard H_2SO_4.

Each set of Michaelis parameters was determined with one enzyme-buffer solution and 6 different substrate concentrations of between 2 and 30 mM CO_2. The Michaelis-Menten equation was fitted to the experimental data by an iterative method where a non-linear least squares curve-fitting program was used [18]. The program was executed on an IBM 370/155 digital computer. By these calculations the kinetic parameters and their standard errors were obtained. Inhibition of the enzymes by sulfanilamide was studied with 6 inhibitor concentrations and various substrate concentrations. The data resulted in straight lines and indicated a noncompetitive type of inhibition when plotted according to the graphic method of Dixon [19]. It was therefore felt justified to use the non-linear equation

$$v = \frac{V_0}{1 + [I]/K_i}$$

to calculate K_i and its standard error, where v and V_0 are the measured initial rates in the presence or absence of the inhibitor, I, respectively. This was done by fitting the equation to the experimental data by an iterative least squares curve-fitting program [18].

Results

Enzyme purification

Erythrocyte enzymes. Fig. 1 A shows the chromatography of the hemolysate. The amount of protein which passed through the column without being adsorbed was more than 99% of that applied. Increasing the ionic strength by adding sodium sulphate to the buffer (point I) did not desorb any detectable

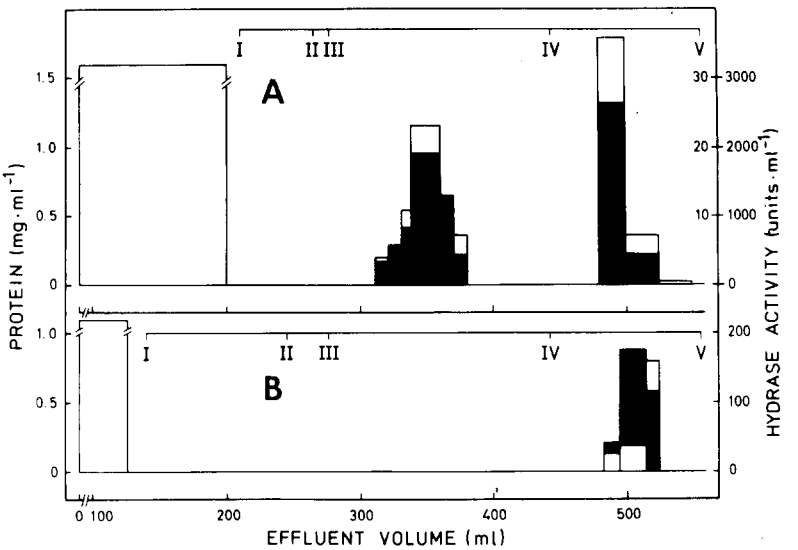

Fig. 1. Purification of rat carbonic anhydrases by affinity chromatography. The column (1.2 × 3 cm) was equilibrated with 0.05 M Tris · H_2SO_4 buffer, pH 8.0. At point I, nonspecifically bound proteins were displaced by changing the buffer to 0.2 M Na_2SO_4 in 0.1 M sodium phosphate buffer, pH 6.5. At point II, the column was washed with 0.1 M sodium phosphate buffer, pH 6.5. The carbonic anhydrases were desorbed by adding either 2 M sodium chloride, at point III, or 1 M sodium perchlorate at point IV to the buffer. Sulfanilamide, 40 μM, was added to the buffer at point V. All buffers contained 200 μM EDTA (Na salt). The course of the fractionation was evaluated by the absorbance at 280 nm of eluted fractions. Protein (open bars) and CO_2-hydrase activities (filled bars) were determined on fractions which had been pooled, concentrated and dialyzed against 10 mM HEPES buffer, pH 8.3. The flow was 10 ml/h and the fraction volume 3 ml. The temperature was 4°C. A. Isolation of erythrocyte carbonic anhydrases. The column was loaded with 200 ml of the hemolysate, corresponding to about 20 ml of packed red cells. The left-hand scale of hydrase activity is for enzyme RBC-B, eluted as the first peak. The right-hand scale is for enzyme RBC-C of the second peak. B. Isolation of renal carbonic anhydrase. The column was loaded with 0.45 g of renal soluble protein mixture in 38 ml of 0.05 M Tris · H_2SO_4 buffer, pH 8.0.

amounts of non-specifically bound proteins. When the specific elution started by adding chloride (point III) a protein peak appeared with a specific activity of approx. 20 U/mg of protein (range 11—24 U/mg). Changing to perchlorate (point IV) resulted in a second sharp protein peak with specific activities of between 1287 and 1167 U/mg. The final addition of sulfanilamide (point V) did not displace enzyme activities further. The total recovery of enzyme activity was 55%. This is lower than for methods using gel filtration and ion-exchange chromatography [13], and is probably explained by losses during the concentration and dialysis of the separate fractions. The effluent from the column must be dialyzed to remove the inhibitory anions chloride and perchlorate before assaying the hydrase activities.

Renal enzyme. The renal supernatant contained about 95% of the enzyme activity of the whole homogenate. The elution of enzyme from the chromatographic column is seen in Fig. 1 B. Like for the hemolysate, the nonretained proteins amounted to more than 99% of those applied. No hydrase activity was found among these fractions. The chloride elution (point III) did not displace any detectable enzyme activity or protein, whereas perchlorate (point IV) desorbed a sharp peak of protein (1.7 mg) at the same place as the high-activity

peak of the hemolysate. The specific activity of this protein was also high with approx. 1000 U/mg of protein throughout the peak. The activity eluted during these steps was about 50% of the one applied to the column.

The contamination of the renal tissues by blood carbonic anhydrase would have added non-detectable amounts of low-activity erythrocyte enzymes to the supernatant. This was calculated from the concentration of hemoglobin in the supernatant and the levels of enzyme in the erythrocyte, supposed to be 0.2 and 0.45 g/l packed cells of low- and high-activity forms, respectively.

The chromatographic fractions with the highest specific enzyme activities were taken for further analysis: see below.

Criteria of purity

The purity of the enzymes was tested by isoelectric focusing (Fig. 2) of the

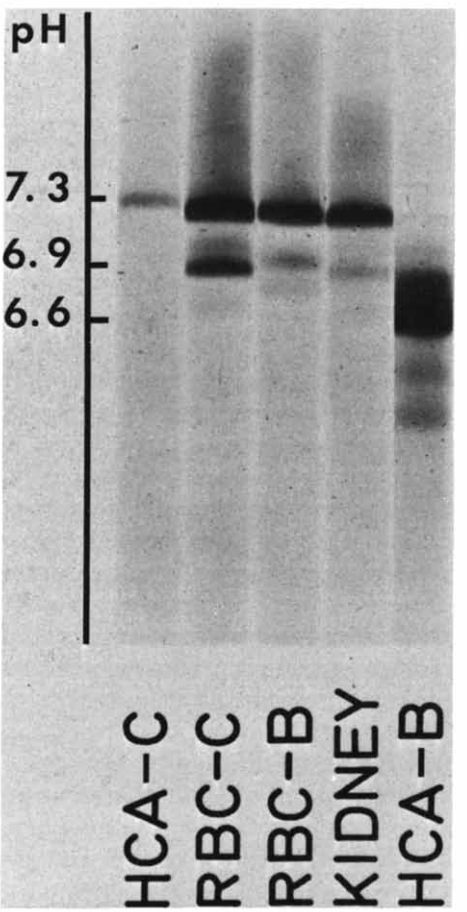

Fig. 2. Isoelectric focusing pattern of carbonic anhydrase isoenzymes separated by affinity chromatography. Erythrocyte fractions of the high (RBC-C) and low (RBC-B) activity enzymes (Fig. 1A) and of the renal enzyme (Fig. 1B) are compared with the human erythrocyte enzyme forms HCA-B and HCA-C, with known isoelectric points, of 7.3 and 6.6, respectively. The concentration of ampholytes in the gels was 2.1% (w/v) and acrylamide, T = 5.8% (w/v) and C = 4.4% (w/v) [28]. The amount of applied protein was about 20 μg.

fractions with the highest specific activities. Enzyme RBC-C exhibited distinct bands, at pH 7.2 and 6.9. The renal enzyme had two bands at the same locations, the only difference being a fainter band at pH 6.9. Enzyme RBC-B also exhibited two distinct bands, one at pH 7.2 and the other at pH 7.0. It is assumed here that both bands are carbonic anhydrases, since highly purified human enzymes, isolated by affinity chromatography exhibit the same focusing patterns [1].

These enzyme fractions were, however, homogeneous with respect to sedimentation in the ultracentrifuge. In the sedimentation velocity experiment the enzymes sedimented as a single symmetrical distribution curve throughout the run of 3 h. The molecular weight distribution curves also indicated a monodisperse system from the linear relation between ln C versus r^2.

Physical properties

The plotting of the sedimentation constant of the erythrocyte enzymes as a function of their protein concentration resulted in straight lines. Extrapolated values for $s^0_{20,w}$ for the high- and low-activity forms respectively, are given in Table III. These values are close to those observed for other mammalian carbonic anhydrases [13]. The results of the determination of the molecular weights of the enzyme are given in Table III. The molecular weights are the average values of runs at different initial protein concentrations. Enzyme RBC-B had a slightly lower weight as is usually found for the low-activity forms of erythrocytes of other species [13]. The centrifugation data gave values for the molecular weights of both enzymes which were slightly lower than those previously obtained [11] by gel filtration (Table III). The reason for this difference is not known.

Amino acid composition

The results of the amino acid analyses are seen from Table I. The renal enzyme and the erythrocyte enzyme RBC-C had identical compositions within the limits of the method. They had the same low content of serine in compari-

TABLE III

COMPARATIVE PROPERTIES OF THE CARBONIC ANHYDRASES FROM RAT KIDNEY AND ERYTHROCYTES

Property	Isoenzyme		
	kidney	RBC-C	RBC-B
Molecular weight (from gel filtration)		29 000 [11]	29 000 [11]
Molecular weight (from centrifugation)	25 700	26 000	24 900
$A^{1\%}_{280}$ (cm^{-1})		17 [11]	18 [11]
$S^0_{20,w}$ (Svedberg units)	2.7	2.9	2.3
Partial specific volume	0.734	0.734	0.734
Isoelectric point (pH), Fig. 2	7.2, 6.9	72., 6.9	7.2, 7.0
Amino acid composition (Table I)	same as RBC-C	same as kidney	different from kidney and RBC-C
Kinetic behaviour (Table II)	same as RBC-C	same as kidney	different from kidney and RBC-C

son with the low-activity erythrocyte enzyme as previously reported [13] for these enzymic forms of other species.

Kinetic properties

The enzymic reactions accurately obeyed the Michaelis-Menten equation. The inhibition of sulfanilamide was of the noncompetitive type for all enzymes. The data of Table II indicate the similarity between the high-activity renal and erythrocyte enzymes. They also show the great difference between these forms and the low-activity erythrocyte enzyme RBC-B with respect to both substrate and inhibitor kinetics. The data agree reasonably well with those previously reported for partially purified erythrocyte [5,11] and renal [4] enzymes.

Discussion

The presence of a pair of kinetically and chemically different isoenzymes in rat erythrocytes is typical of most mammalian species, except for ruminants [13]. The relative amounts and specific activities of these types vary somewhat among different species but the low activity enzymes are always responsible only for a minor part — in man about 15% [1] — of the total carbonic anhydrase activity of the erythrocyte. In the rat, enzyme RBC-B is even less important for the CO_2 activity of the cell, or less than 1% as calculated from the kinetic data of Table II, a CO_2 concentration of 1.2 mM in vivo, and assuming the molar concentrations of the low- and high-activity types to be 10 and 25 μM respectively in the cell water (0.6 of tissue wet weight). The physiological function of the high-activity type is presumably to catalyze the hydration and dehydration of carbon dioxide, whereas that of the low-activity type remains obscure [20].

Although the two forms of the enzymes were homogeneous with respect to sedimentation in the ultracentrifuge, isoelectric focusing revealed two bands for each isoenzyme. These acidic bands probably correspond to the minor erythrocyte forms reported by others [11,21] using different isolation techniques. They are probably subcomponents of the major types, with similar substrate and inhibitor kinetics [11,21]. The affinity chromatographic technique only separates enzymes with different affinities for sulfanilamide. Enzymes which are resistant to inhibition by sulfonamides such as those found in the liver of the male rat [5] are not adsorbed to the column and should therefore be found in the first fractions of the elution. However, no hydrase activity was found among these chromatographic fractions of the hemolyzate and those of the kidney supernatant. Only one form of the enzyme was isolated from the kidney.

Beside the chromatographic and kinetic similarities, the renal enzyme has the same amino acid composition (Table I) as the high-activity erythrocyte form. Its isoelectric focusing pattern is also the same. These high-activity forms of the kidney and erythrocytes are therefore probably identical, as previously found to be the case in man [1]. The rat kidney has been reported [21] to contain two isoenzymes, chromatographically and electrophoretically similar to the high- and low-activity forms, respectively, of the erythrocytes. However, we

believe that the low-activity form originates from blood contamination, since in our studies as well as in those of other investigators [4,22] only one form could be detected when kidney perfusion was done.

Carbonic anhydrase activity has been found histochemically along the whole nephron with the exception of the glomeruli [23]. Most of this activity probably originates from the cytoplasmic enzyme. However, about 5—10% of the total activity of the kidney homogenate is associated with the particulate fractions. Isolated glomeruli contain no activity [24], whereas isolated membranes of the microvilli and the basal infoldings of the proximal tubular cells have enzyme activity with a sensitivity to sulfonamides like the low activity erythrocyte form [29]. Nuclei [25], mitochondria [25,26] and microsomes [23,25, 27] have also been reported to contain enzyme activity. The function of these membrane-bound forms of carbonic anhydrase remains an outstanding problem.

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