A Study of Renal Carbonic Anhydrase

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SUMMARY

Renal carbonic anhydrase from the dog and rat is found chiefly in the supernatant fraction. Based on molar activity and inhibition kinetics, the cytoplasmic enzyme has properties closely akin to the human red cell fraction C, which may be taken as a prototype for most vertebrate tissue and red cell enzymes. The data show that acetazolamide and allied drugs can readily inhibit >99% of cytoplasmic enzyme *in vivo* at low (5–20 mg/kg) doses. A smaller but definite concentration of enzyme is found in microsomes; on the basis of present criteria this enzyme is different from that of the supernatant. Although somewhat higher concentrations of drugs are necessary for inhibition of microsomal enzyme, these are still within the range attainable *in vivo*. Physiological manifestations of the system are discussed.

INTRODUCTION

The kinetic characteristics and intracellular localization of renal carbonic anhydrase are of great physiological importance, in view of the role of this enzyme in kidney function (1). Nothing has heretofore been published on the kinetics; preliminary inhibition data from this laboratory show that the activities of acetazolamide and sulfanilamide against dog renal carbonic anhydrase are close to those against crude dog red cell enzyme and the pure human red cell enzyme designated C (1, 2). The question of intracellular localization resulted in divergent reports, one study showing the enzyme from rat kidney in mitochondria as well as in the soluble fraction (3) and another claiming that the particulate enzyme was artifactual (4). In another study, small amounts of enzyme were also found in nucleus and microsomes, as well as mitochondria, but whether this was native to the structures was not determined (5). The present paper, together with earlier data from this laboratory on the separation (2) and immunology (6) of canine renal carbonic anhydrase provide a characterization of the renal enzyme from dog and rat in terms of its activity and cellular distribution.

METHODS AND MATERIALS

The kidneys were removed from adult male Sprague Dawley rats and mongrel dogs and thoroughly perfused with 0.25 M sucrose through the renal artery until the kidney was a light tan in color and the effluent colorless. The tissue was then placed in ice and the following manipulations were carried out at 0–4°. This tissue was weighed, thoroughly minced, and homogenized using a Potter-Elvehjem glass homogenizer with a motor-driven glass pestle. Particulate cellular components were isolated following the differential centrifugation procedure of Schneider and Hogeboom (7). All precipitates were washed in 0.25 M sucrose three times or until the washes were devoid of enzymic activity.

Contamination due to blood in case of incomplete perfusion, or the presence of whole cells due to incomplete homogenization, is likely to occur. Thus, the effect of

blood contamination was evaluated by comparing perfused (as above) with unperfused kidneys (Table 1). As a test for adsorption of supernatant upon microsomal elements, partially purified canine renal CA, as prepared by Byvoet and Gotti (2), and kindly supplied by Dr. P. Byvoet, was added to microsomes in the same concentration as that normally present in the cytoplasmic fraction. Incubation of this mixture was carried out for 24 hours in the cold and then centrifuged and reanalyzed (Table 2).

Enzyme assays and drug analyses using bicarbonate buffer were done by a modification of the changing pH method for carbonic anhydrase (8). The system as used for kinetic analysis is described in (9). The drugs were supplied by the Lederle Division of American Cyanamid Company. Their structure, physical and chemical properties are given in (1).

Protein concentrations were determined by the method of Lowry *et al.* (10).

RESULTS

Enzymic Distribution

The distribution of carbonic anhydrase in the various cell fractions of dog and rat kidney is shown in Table 1. It is seen that 87% of the total activity is found in the supernatant portion of the cell. The supernatant fraction also contains the largest enzyme concentration, 3.1–5.5 enzyme units (e.u.) per milligram of protein. The microsomal fraction contains one-half to one-third this concentration. The amount of enzyme in the mitochondria is small and might occur as a result of contamination with the supernatant. The data on unperfused rat kidney show that contamination by red cell enzyme appears to affect largely the nuclear fraction and does not alter the other relations observed.

TABLE 2

Incubation of pure renal carbonic anhydrase with the microsomal fraction

The microsomal fraction was derived from 3.7 g of wet canine kidney. For B–E, 10 ml of solution was used, and the microsomal pellet was separated and analyzed.

Sample	Enzyme units in particulate
A. Original microsomal fraction	32
B. A + 1 mg pure enzyme (2)	72
C. B after 24 hours	47
D. C after 2 washes	30
E. C after 6 washes	30

Table 2 shows a separate set of experiments, in which pure dog kidney enzyme was added to the microsomal fraction. Data indicate clearly that added enzyme was readily washed out, suggesting that the carbonic anhydrase originally found in

TABLE 1

Carbonic anhydrase in various fractions of dog and rat kidney

Rat data are means of 2 animals which agreed within 5%. Dog data are from one animal and are representative of 3 experiments, which agreed within 5% for particulate fractions and about 20% for soluble fraction.

Fraction	Enzyme units/gram whole tissue			Mg protein/gram whole tissue			Enzyme units/mg protein		
	Rat	Rat[a]	Dog	Rat	Rat[a]	Dog	Rat	Rat[a]	Dog
Homogenate	248	381	402	138	154	130	1.8	2.5	3.1
Nucleus (3 washes)	14	55	12	16	11	13	0.86	5.0	0.92
Mitochondria (3 washes)	12	10	12	17	19	18	0.65	0.52	0.65
Microsomes (3 washes)	13	18	12	9	11	8	1.6	1.6	1.9
Supernatant	215	328	350	90	93	93	3.1	3.7	3.8

[a] Unperfused.

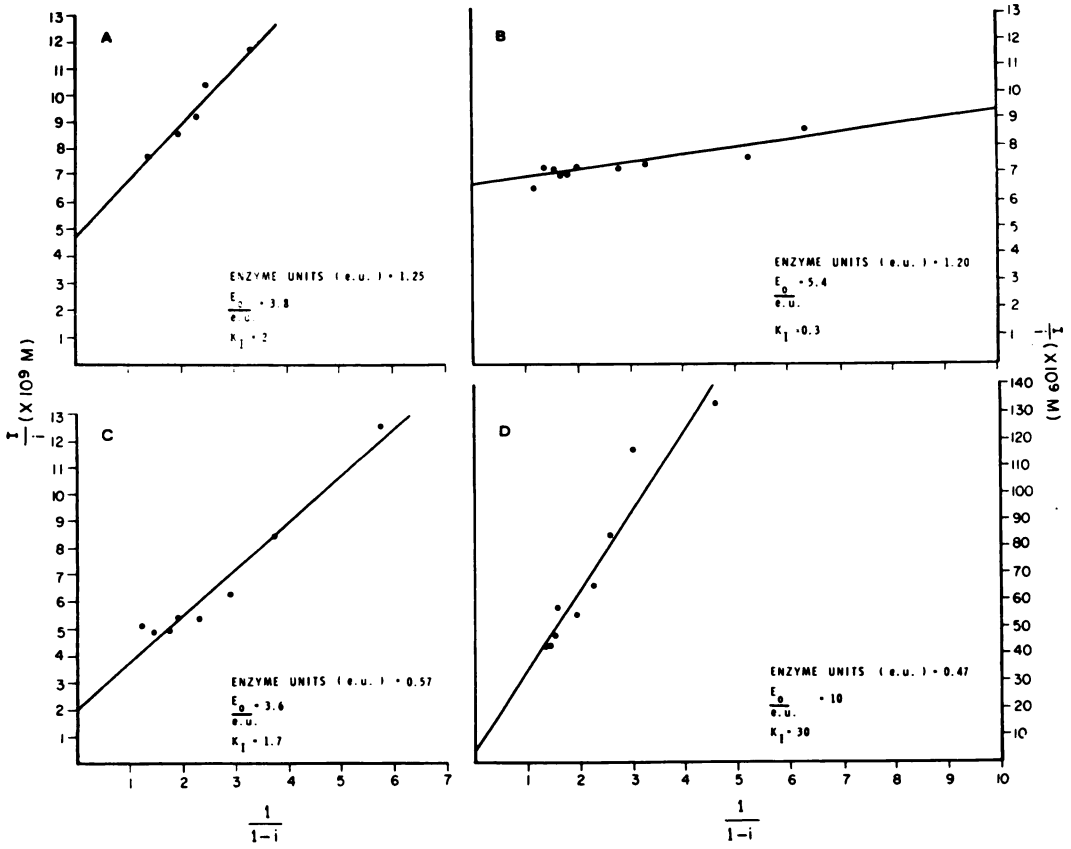

FIG. 1. *Renal carbonic anhydrase inhibition and enzyme activity*

The equation for these lines is

$$\frac{I}{i} = K_I\left(\frac{1}{1-i}\right) + E_0$$

Experimental details are according to Maren *et al.* (11). E_0/e.u. and K_I values given in the inset are $\times 10^9$ M. The drug used is CL 13,580, 2-*o*-chlorphenyl-1,3,4-thiadiazole-5-sulfonamide. Dog renal enzyme used as follows: (A) Whole cortex; (B) purified (2); (C) supernatant; (D) microsomes.

microsomes (and which could not be washed out) was not the result of adsorption from the cytoplasm.

The real nature of the microsomal enzyme fraction is further established by the fact that on further homogenization of the microsomal fraction in distilled water no enzymic activity was observed in the supernatant. Thus, it is concluded that the microsomal enzyme is an entity and is intrinsically bound to the membrane.

From Table 1 the homogenates of rat (248 e.u./g tissue) and dog (402 e.u./g tissue) kidney may be compared to lysates of red cells, which for rat contains 2600 e.u./ml and for dog 1600 e.u./ml using the same method of assay.

Specific Activity and Inhibition

Figure 1 shows plots, originally developed by Easson and Stedman (11) of various fractions of dog kidney enzyme (2) against a very powerful inhibitor, CL 13,580 (2-*o*-chlorphenyl-1,3,4-thiadiazole-5-sulfonamide). The ordinal intercept yields the molar concentration of enzyme

(E_0) in the procedure, and the value E_0/enzyme unit (e.u.) is a measure of the specific activity of the enzyme. The slope yields the K_I or, dissociation constant of (EI), the enzyme inhibitor complex (9).

Figures 1A, 1B, and 1C show E_0/e.u. and K_I values in the same range as those previously determined for dog blood (9), dog liver (12) and pure human carbonic anhydrase HCA-C (1). E_0/e.u. is about $2\text{–}5 \times 10^{-9}$ M and K_I about 10^{-9} M. It is thus clear that the supernatant fraction (Fig. 1C) which contains the largest amount and concentration of enzyme (Table 1) is closely related to the other vertebrate enzymes of which HCA-C is the kinetic prototype. Figure 1D shows that the microsomal enzyme is different in having higher K_I and lower specific activity (see discussion for relation between E_0/e.u. and turnover number).

Table 3 shows inhibition data for three other drugs; these fit with the findings of Fig. 1. For each of the compounds, the I_{50}'s and I_{90}'s (molar concentration to inhibit 50% and 90% of enzyme) are similar among the various renal fractions, and red cells, except for renal microsomes. All drugs tested were less active against the microsomes than any of the other preparations. The relation between the I_{50} and the I_{90} for all fractions is within the range predicted by theory; for weak inhibitors where $E_0 < I_{50}$, I_{90}/I_{50} is theoretically 9, and where $E_0 \simeq I_{50}$, I_{90}/I_{50} is ~ 2.

TABLE 3

Inhibition of carbonic anhydrase in kidney fractions of dog (M $\times 10^7$)

Fraction	Acetazolamide		Sulfanilamide		Methazolamide		CL 13,580	
	I_{50}	I_{90}	I_{50}	I_{90}	I_{50}	I_{90}	I_{50}	I_{90}
Homogenate	0.33	—	37	—	0.39	—	0.04	—
Nuclear	0.48	—	37	—	0.39	—	0.03	—
Mitochondria	0.47	—	34	—	0.52	—	0.03	—
Microsomes	1.2	6.3	60	653	0.89	—	0.32	2.6
Supernatant	0.28	1.2	34	128	0.29	—	0.03	0.15
Dog RBC	0.30	1.6	28	200	0.24	0.91	0.02	0.15
Rat RBC	0.20	1.8	40	245	0.40	2.0	0.03	—
HCA-C	0.30	1.9	24	—	0.20	—	0.04	0.10

DISCUSSION

The data are of intrinsic biochemical interest, and also are useful in the interpretation of physiologic and pharmacologic events. We shall discuss localization of the enzyme and kinetic properties.

Localization. The present data appear to resolve the issue as to whether renal carbonic anhydrase is truly present in particulate matter (3) or whether it is an artifact (4). Based on weight of whole tissue, about 85% enzyme is found in the supernatant fraction in both rat and dog. However, the small amount in microsomes (3–5%) has been shown not to be artifactual, as had been claimed (4). On the basis of weight of protein, the microsomes contain one-half to one-third as much enzyme as the supernatant fraction. The mitochondria contain lesser amounts on this basis, and it is not certain whether this may result from contamination by the soluble fraction. We have reported a similar distribution of carbonic anhydrase in the liver (12).

The idea of two sites for carbonic anhydrase in the proximal tubule was put forth by Rector *et al.* (13) on the basis of their finding that enzyme inhibition increased [H^+] of proximal lumen fluid. Thus while inhibition of (presumably) the supernatant enzyme results in a lowered acidification rate, the H_2CO_3 formed in the lumen is not rapidly dehydrated, because

of inhibition of a (particulate) enzyme at the luminal border. A model for this situation is given in (1). Histochemical localization of the enzyme at the luminal border has been claimed (14), but we do not feel that such methods are necessarily specific (15).

Kinetic properties. A review of the kinetic properties of the various vertebrate carbonic anhydrases reveals that the prototype enzyme may be considered to be the isoenzyme from human (or monkey) red cells designated HCA-C. Two principal criteria are considered: turnover number or specific activity, and *in vitro* I_{50} against two representative drugs, acetazolamide and sulfanilamide.

Since turnover number has not been determined for crude tissue enzymes, the specific activity, molar concentration of enzyme per enzyme unit (E_0/e.u.), may be used, as in Fig. 1 and earlier studies cited. All the vertebrate enzymes heretofore examined (except HCA-B which is much less active, and rat liver enzyme (14) which is not inhibited) have E_0/e.u. values in the range 2 to 5×10^{-9} M. In the present study all the renal fractions examined except the microsomes fit this pattern. Thus the use of the turnover number established for HCA-C or dog red cells (about 40×10^6 min^{-1}) is provisionally used for kidney supernatant fraction.

This relation between turnover number (V_{max}/E_0) and E_0/e.u. holds strictly only at V_{max}. In the present experiments we are working at high substrate concentration and reasonably close to V_{max} (16). We claim only that for a given pattern of E_0/e.u. and K_I, it is reasonable that a corresponding turnover number will be found. Ultimately a kinetic analysis of the different fractions should include K_m, to solidify these points. With the present techniques, however, there is more variability in determining K_m than in K_I (16).

The I_{50}'s of the several drugs against HCA-C are noted in Table 3. It will be seen that rat and dog red cells, and dog renal supernatant, give almost exactly the same figure. Microsomal enzyme is clearly different, requiring some 2–15 times more drug for 50% inhibition. While this is an interesting point with respect to the chemical individuality of particulate and supernatant enzyme, the activity is still high enough so that inhibition *in vivo* can be readily achieved; acetazolamide, for example, achieves renal concentration of free drug of about 50 μM following 5 mg/kg, whence fractional inhibition of supernatant and microsomal fractions are both >99%, and the renal effect is near maximal (1).

Other information on the renal enzyme will be briefly reviewed. Using the cytoplasmic fraction there appears to be but one main peak on DEAE chromatography. Using gel electrophoresis, the mobility of dog kidney supernatant enzyme (like that of dog red cells) corresponds to that of HCA-B (2). Canine kidney homogenate gave precipitation reactions with rabbit antisera prepared from HCA-B and HCA-C. In this study it was also reported that Tween 80 increased the activity of kidney homogenates (6), presumably by liberating membrane-bound enzyme; kinetically this must be of the HCA-C type (2). This technique will be of value in further investigation.

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