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CATALYTIC ACTIVITY AND INHIBITION OF CARBONIC ANHYDRASE OF RAT TISSUES*

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Abstract—Carbonic anhydrase activity was studied in stomach and kidney homogenates, and isoenzymes were purified from erythrocytes and livers of male and female rats. Two liver isoenzymes of male and female rats and one erythrocyte isoenzyme had low CO_2 hydration activity. The enzymes of stomach and kidney, one isoenzyme of erythrocytes and one of female rat liver had high CO_2 hydration activity. The esterase activity toward *p*-nitrophenyl acetate paralleled the CO_2 hydration activity of all the isoenzymes. However, the esterase activity toward β-naphthyl acetate was either absent or did not show any correlation with the CO_2 hydration activity of isoenzymes. Male rat liver carbonic anhydrases were 1000 times less sensitive to sulfonamides than female rat liver carbonic anhydrases for the inhibition both of CO_2 hydration and esterase activity. Male rat liver carbonic anhydrases were as sensitive to inhibition by monovalent anions as were the female rat liver carbonic anhydrases. It is concluded that the active site of carbonic anhydrases from male rat liver is more hydrophilic than the active site of carbonic anhydrase from female rat liver or other tissues of the rat.

CARBONIC anhydrase is present in erythrocytes and the organs of secretion and excretion.[1] Human erythrocytes contain two major isoenzymes designated as B (low catalytic activity) and C (high catalytic activity), while the erythrocytes of certain other species, i.e. dog, ox and dolphin, have only one isoenzyme (high catalytic activity). Isoenzymes of carbonic anhydrase occur in other organs; their properties and distribution have been reviewed by Carter.[2]

Because of our recent findings of very low catalytic activity and susceptibility to inhibition of liver carbonic anhydrase of the male rat,[3,4] it was of interest to study catalytic activity and inhibition of carbonic anhydrase in other tissues of this species: stomach, kidney and erythrocytes. The results of this study are reported in the present paper.

METHODS

Preparation of the supernatant fractions of the rat tissues. The method used for the preparation of the supernatant fractions from stomach, kidney and liver of the rat was the same as described for rat liver.[4] It involved perfusion of each tissue with 0·9% NaCl, homogenization, and centrifugation at 100,000 *g*. The supernatant fractions were used for inhibition studies and for enzyme purification.

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Isolation of liver and erythrocyte carbonic anhydrase. The method used for purification of liver and erythrocyte carbonic anhydrase was the same as described previously.[4] It involved ammonium sulfate precipitation, gel filtration and ion exchange chromatography.

Determination of carbon dioxide hydration activity and its inhibition by sulfonamides. The fractions obtained during purification of liver and erythrocyte carbonic anhydrases were assayed for carbonic anhydrase activity by the method of Maren *et al.*[5] The basic measurement was time required for carbon dioxide to yield H^+ and titrate 1 ml carbonate buffer from pH 10 to 7·4. The end point was change in color of phenol red. The enzyme activity was expressed as enzyme units which are defined as:

$$\text{enzyme unit (e.u.)} = \frac{\text{uncatalyzed time} - \text{catalyzed time}}{\text{catalyzed time}}.$$

The relationship between the time required for catalyzed reaction and the product formed per unit time has been described in detail by Maren *et al.*[6] Under the conditions of the experiment using 60 mM CO_2 at 0°, 1 e.u. of carbonic anhydrase produces 800 μmoles/l./sec of H^+ in the carbonate buffer system. However, for kinetic work, 2 ml of an equimolar mixture of 0·025 M barbital and sodium barbital was substituted for 1 ml carbonate buffer. The advantage of the barbital system over the carbonate system is that the change in pH in the former case is only 0·5 pH units (from pH 7·9 to pH 7·4,) as compared to the latter where the change in pH during the reaction is 2·6 pH units (from pH 10 to pH 7·4). Under the conditions of the experiment, neutralization of 2 ml barbital buffer from pH 7·9 to pH 7·4 required 38 μmoles standard acid. The rates of CO_2 hydration by the the enzyme were calculated as H^+ formed per unit time as described by Maren *et al.*[6] The variability of the method is about 10 per cent. The variation in samples from different animals was about 50 per cent. However, in the context of comparison of more than 10-fold differences in catalytic activity of low and high activity enzyme, this variability is not very significant.

The enzyme concentration in the 100,000 *g* supernatant fraction of stomach and kidney homogenates was determined by its titration with ethoxzolamide as described by Maren *et al.*[6] Ethoxzolamide is reversible, noncompetitive and a very potent inhibitor having a K_i of 10^{-9} M for carbonic anhydrase so that I_0 reacts with E_0 without the necessity of excess I_0 being present. One molecule of carbonic anhydrase combines with one molecule of ethoxzolamide. The enzyme concentration was calculated from the Easson and Stedman[7] equation:

$$\frac{I}{i} = \frac{1}{1-i} \cdot K_i + E_0$$

where I is the inhibitor, i is the fractional inhibition of the enzyme and E_0 is the total concentration of the enzyme.

The solutions of purified enzymes were prepared by dissolving a known quantity of freeze-dried powders in a known volume of Tris buffer. The molar concentration was calculated using a molecular weight of 29,000.[3,4]

For inhibition studies, various concentrations of the inhibitor were added in the presence of a fixed quantity (approximately 2 e.u.) of enzyme and the residual activity of carbonic anhydrase was determined.

Determination of esterase activity of purified isoenzymes of carbonic anhydrase. The method has been described by Thorslund and Lindskog.[8] *p*-Nitrophenyl acetate and *β*-naphthyl acetate were used as substrates. A 3-mM stock solution of *p*-nitrophenyl acetate was prepared by dissolving 27·2 mg *p*-nitrophenyl acetate in 1 ml acetone and diluting this rapidly with distilled water to 50 ml. The reaction solution contained 1·0 ml of the stock solution of *p*-nitrophenyl acetate and 2·0 ml of enzyme solution and 10 mM Tris buffer, pH 8·0. The increase in the E_{348} (the isosbestic point of *p*-nitrophenol and the *p*-nitrophenolate ion) of the reaction mixture was measured in a Beckman DB spectrophotometer at 25°. The reference solution contained all the reactants except the enzyme. The molar absorption of *p*-nitrophenol at 348 nm is $5{\cdot}4 \times 10^3\ M^{-1}\ cm^{-1}$. For inhibition studies, various concentrations of acetazolamide were added to the reaction mixtures.

A stock solution of *β*-naphthyl acetate was prepared by dissolving 18·6 mg *β*-naphthyl acetate in 5·0 ml acetone. The reaction mixture was prepared by mixing in a 1-ml cuvette 0·1 ml of 100 mM Tris, pH 8·0, 50 *μ*l of stock solution of *β*-naphthyl acetate and a suitable volume of enzyme solution and water to give a total volume of 1·0 ml. An increase in the E_{330} was measured at 25°. *β*-Naphthyl acetate has a molar absorption at 330 nm of $1{\cdot}7 \times 10^3\ M^{-1}\ cm^{-1}$.

RESULTS

Relationship between enzyme concentration and enzyme unit. Figure 1A shows the results of inhibition of carbonic anhydrase of rat stomach by ethoxzolamide in the barbital buffer system. The ordinate intercept (E_0) is $5{\cdot}4 \times 10^{-9}$ M. This yields $2{\cdot}7 \times 10^{-9}$ M for 1 enzyme unit in the barbital buffer system. Figure 1(B) shows similar data

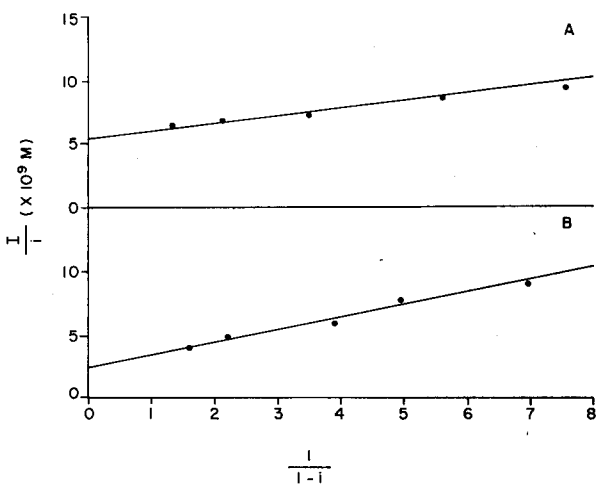

FIG. 1. Inhibition of carbonic anhydrase (in 100,000 *g* supernatant fraction) by ethoxzolamide in barbital buffer. Plot according to equation:

$$\frac{I}{i} = K_i \cdot \frac{1}{1-i} + E_0 \text{ (see Methods).}$$

I = molar concentration of ethoxzolamide; i = fractional inhibition of the enzyme. (A) Rat stomach carbonic anhydrase. Intercept = $E_0 = 5{\cdot}4 \times 10^{-9}$ M. Since enzyme was 2·0 e.u., therefore, E_0/e.u. = $2{\cdot}7 \times 10^{-9}$ M. Slope = $K_i = 0{\cdot}6 \times 10^{-9}$ M. (B) Rat kidney carbonic anhydrase. Intercept = $E_0 = 2{\cdot}4 \times 10^{-9}$ M. Since enzyme was 1 e.u., therefore, E_0/e.u. = $2{\cdot}4 \times 10^{-9}$ M. Slope = $K_i = 1 \times 10^{-9}$ M.

for carbonic anhydrase of rat kidney, which yields $2{\cdot}4 \times 10^{-9}$ M for 1 enzyme unit in this system. Under similar conditions, carbonic anhydrase from dog erythrocyte gave 1×10^{-9} M for 1 enzyme unit in the barbital system (see also Maren *et al.*[6]).

CO_2 hydration activity of rat tissue carbonic anhydrases. The catalytic activities of carbonic anhydrases from rat tissues are compared as V/E_0 for 60 mM CO_2, as an approach to the turnover numbers. Theoretically:

$$\text{Turnover number} = \frac{V_{max}}{E} = \frac{V \times (K_m + S)}{E \times S}.$$

If S is large compared to K_m, then:

$$\text{Turnover number} = \frac{V}{E}.$$

In the present experiments, S was 60 mM and the K_m was not known. The K_m of human erythrocyte carbonic anhydrases is less than 10 mM for CO_2; the K_m of carbonic anhydrases of rat tissues can reasonably be expected to be less than 60 mM; therefore, the possible variation of V/E_0 from the real turnover number will not exceed 2-fold and is probably much less.

TABLE 1. CO_2 HYDRATION ACTIVITY OF RAT TISSUE CARBONIC ANHYDRASES IN BARBITAL BUFFER AT 0°

Tissue	V (μM/sec)	E_0 (nM)	V/E_0 (10^{-3} sec^{-1})
Stomach	373	5·4	70
Kidney	183	2·4	76
Erythrocyte			
Isoenzyme 1	253	200	1·3
Isoenzyme 2	167	2·4	64
Male rat liver			
Isoenzyme 1	312	260	1·2
Isoenzyme 2	130	50	2·6
Female rat liver			
Isoenzyme 1	136	64	2·1
Isoenzyme 2	298	64	4·6
Isoenzyme 3	107	1·4	76
Dog erythrocyte*	342	2·0	171
Human erythrocyte C*	900	5·0	180
Human erythrocyte B*	420	28·0	15

* Studied under same conditions as used for rat tissues and included for comparison.

Carter[2] has shown that the guinea-pig stomach contains only one type of carbonic anhydrase. Dog kidneys are reported to contain only one type of carbonic anhydrase.[9] A preliminary analysis of carbonic anhydrase in supernatant fractions of tissue homogenates on gel electrophoresis showed that there was only one type of carbonic anhydrase in the stomach and kidneys of rats. Therefore, carbonic anhydrase from stomach and kidney was studied for CO_2 hydration activity in blood-free homogenates. The purified enzymes from erythrocytes and liver are designated by numbers corresponding to their order of elution from DEAE Sephadex® column. Table 1 shows

TABLE 2. ESTERASE ACTIVITY OF RAT TISSUE CARBONIC ANHYDRASE ISOENZYMES AT pH 8·0 AND 25°

Isoenzyme	V/E_0 ($\times 10^2$ sec^{-1})* *p*-Nitrophenyl acetate	*β*-Naphthyl acetate
Erythrocytes		
Isoenzyme 1	15·0	2·0
Isoenzyme 2	48·0	0·2
Male rat liver		
Isoenzyme 1	2·0	0
Isoenzyme 2	2·0	0
Female rat liver		
Isoenzyme 1	16·0	0·2
Isoenzyme 2	10·0	0
Isoenzyme 3	49·0	0
Human erythrocyte C†	100·0	4·0
Human erythrocyte B†	30·0	4·0

* With 1 mM substrate.

† Studied under same conditions as used for rat tissues and included for comparison.

that carbonic anhydrase from stomach and kidney and isoenzyme 2 of erythrocytes and isoenzyme 3 of liver of the female rat have high CO_2 hydration activity. Human erythrocyte C isoenzyme had V/E_0 of 180×10^3 sec^{-1} under these conditions. Dog erythrocytes, which have only high activity carbonic anhydrase, had V/E_0 of 171×10^3 sec^{-1} under similar experimental conditions. The high activity carbonic anhydrases of rat tissues have about half the CO_2 hydration activity of human carbonic anhydrase C. Table 1 also shows that isoenzyme 1 of rat erythrocytes, isoenzymes 1 and 2 of male rat liver and isoenzymes 1 and 2 of female rat liver have low CO_2 hydration activity which is about 40 times less than the human erythrocyte carbonic anhydrase C and about 4 times less than human erythrocyte carbonic anhydrase B.

TABLE 3. INHIBITION OF RAT TISSUE CARBONIC ANHYDRASES BY ETHOXZOLAMIDE

Tissue isoenzyme	$K_i \times 10^9$ M
Stomach	1·1
Kidney	0·9
Erythrocytes	
Isoenzyme 1	55·0
Isoenzyme 2	2·3
Female rat liver	
Isoenzyme 1	15·0
Isoenzyme 2	7·0
Isoenzyme 3	5·0
Male rat liver	
Isoenzyme 1	150,000
Isoenzyme 2	143,000
Dog erythrocyte*	1·4
Human erythrocyte C*	1·4
Human erythrocyte B*	1·9

* Studied under same conditions as used for rat tissues and included for comparison.

TABLE 4. INHIBITION OF RAT TISSUE CARBONIC ANHYDRASES BY MONOVALENT ANIONS

Tissue isoenzyme	$K_i \times 10^6$ M CN^-	HS^-
Stomach	5·0	1·0
Kidney	6·0	2·0
Erythrocytes		
Isoenzyme 1	15·0	20·0
Isoenzyme 2	14·5	6·0
Female rat liver		
Isoenzyme 1	15·0	8·0
Isoenzyme 2	20·0	6·0
Isoenzyme 3	10·0	1·5
Male rat liver		
Isoenzyme 1	25·0	20·0
Isoenzyme 2	23·0	22·0
Dog erythrocyte*	21·0	11·5
Human erythrocyte C*	19·0	1·9
Human erythrocyte B*	24·0	15·0

* Studied under same conditions as used for rat tissues and included for comparison.

Esterase activity. Table 2 shows that the esterase activity of purified carbonic anhydrases from erythrocytes and livers of male and female rats toward *p*-nitrophenyl acetate almost parallels their CO_2 hydration activity (shown in Table 1). In contrast to their esterase activity toward *p*-nitrophenyl acetate, isoenzyme 1 had higher esterase activity toward β-naphthyl acetate than isoenzyme 2 of erythrocytes (Table 2). Except for isoenzyme 1 of the female rat liver, none of the liver carbonic anhydrase had any esterase activity toward β-naphthyl acetate.

Inhibition of CO_2 hydration activity. Table 3 shows that the K_i of ethoxzolamide for inhibition of carbonic anhydrases from stomach, kidney, erythrocytes and female rat liver was within the 60-fold range. On the other hand, both isoenzymes of the male rat liver had a K_i 1000-fold higher than those of other tissues.

TABLE 5. INHIBITION OF ESTERASE ACTIVITY OF RAT ERYTHROCYTE AND LIVER CARBONIC ANHYDRASES FOR *p*-NITROPHENYL ACETATE BY ACETOZOLAMIDE

Isoenzymes	$I_{50} \times 10^7$ M
Erythrocytes	
Isoenzyme 1	50·0
Isoenzyme 2	1·0
Female rat liver	
Isoenzyme 1	1·5
Isoenzyme 2	2·0
Isoenzyme 3	1·0
Male rat liver	
Isoenzyme 1	5,000
Isoenzyme 2	10,000
Human erythrocyte C*	6·6
Human erythrocyte B*	37·0

* Studied under same conditions as used for rat tissues and included for comparison.

TABLE 6. IONIZATION CONSTANT (pK_a) AND INHIBITION CONSTANT (K_i) OF SULFONAMIDES FOR INHIBITION OF LIVER CARBONIC ANHYDRASES OF MALE AND FEMALE RATS

Sulfonamide	pK_a*	K_i for liver carbonic anhydrase (μM) Male rat isoenzyme 1	Female rat isoenzyme 1
Sulfanilamide	10·4	1000	5·0
Ethoxzolamide	8·1	150	0·008
Methazolamide	7·2	80	0·04
Acetazolamide	7·4, 9·1	100	0·03
Benzolamide	3·2, 9·0	4	0·007

* Taken from Ref. 1.

Table 4 shows that the K_i of monovalent anions for carbonic anhydrases of all tissues (including male rat liver) was within the 30-fold range.

Inhibition of esterase activity. Table 5 shows that the I_{50} of acetazolamide for inhibition of esterase activity toward *p*-nitrophenyl acetate of carbonic anhydrases from erythrocytes and female rat liver was within the 50-fold range. However, the I_{50} of acetazolamide for liver isoenzymes of the male rat was at least 1000-fold higher than that of female rat liver.

Relationship between K_i *and* pK_a *of sulfonamides.* Table 6 shows the K_i of some sulfonamides for inhibition of carbonic anhydrase isoenzymes of male and female rat livers. Sulfanilamide, ethoxzolamide and methazolamide have the unambiguous pK_a of the $—SO_2NH_2$ group. With the decrease in pK_a of these three sulfonamides, there was a decrease in the K_i for isoenzyme 1 of male rat liver but not for that of female rat liver. Figure 2 shows that there is a linear relationship between the pK_a of these three sulfonamides and the pK_i for isoenzyme 1 of male rat liver. The pK_a of the $—SO_2NH_2$ group in acetazolamide is probably 7·4, but the pK_a of benzolamide is 9·0. However, if the pK_i for isoenzyme 1 of male rat liver is plotted against the pK_a values 7·4 for acetazolamide and 3·2 for benzolamide, the points fall on the line

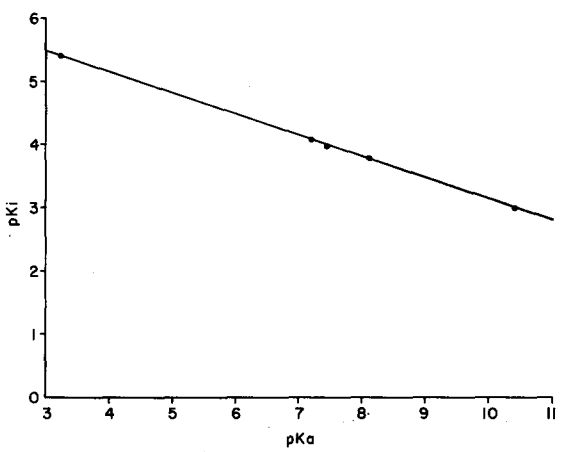

FIG. 2. Plot of pK_i (negative log of K_i for inhibition of carbonic anhydrase isoenzyme 1 of male rat liver) against pK_a of five sulfonamides: sulfanilamide, ethoxzolamide, acetazolamide, methazolamide and benzolamide.

drawn for the other three sulfonamides with the unambiguous pK_a (Fig. 2). The results suggest that ionic forces play a major role in the binding of sulfonamides with the male rat liver carbonic anhydrase (see Discussion).

DISCUSSION

Human erythrocytes have two major isoenzymes of carbonic anhydrase designated as B, a low catalytic activity type, and C, a high catalytic activity type. The low activity type isoenzyme is missing in erythrocytes of many mammalian species. Except in the bovine rumen and the large intestine of the guinea-pig,[2] all the tissues investigated for carbonic anhydrase isoenzymes have high activity type enzymes. In the present study, it has been shown that both major isoenzymes of male rat liver and two isoenzymes of female rat liver are low activity isoenzymes.

Inhibition of carbonic anhydrase isoenzymes by sulfonamides showed a marked difference between the susceptibility to inhibition of male rat liver isoenzymes and all the other tissues' isoenzymes. Male rat liver carbonic anhydrases were very resistant to sulfonamides, but this resistance was not of the same order of magnitude for each of the sulfonamides tested. This suggests that the active site of male rat liver carbonic anhydrase is different than the active site of carbonic anhydrases of other tissues of the rat. Sulfonamides obtain their binding energy from: (a) coordination with metal at the active site, and (b) hydrophobic interactions with the amino acids at the active site. The linear relationship between pK_a and pK_i of some sulfonamides suggests that ionic forces play a major role for binding of sulfonamides at the active site of the enzyme of male rat liver. This is also supported by the fact that: (1) monovalent anions like CN^- and HS^- are as active against male rat liver carbonic anhydrase as against other tissue carbonic anhydrases, and (2) even the most potent sulfonamide is about as potent ($K_i = 1 \times 10^{-6}$ M) as the potent anions for inhibition of male rat liver carbonic anhydrase. On the other hand, some of the sulfonamides are about 1000-fold more potent than the potent anions for inhibition of other tissue carbonic anhydrases. However, further work is required to see if this relationship exists in the case of large numbers of other unsubstituted sulfonamides.

The difference in susceptibility between male and female rat liver carbonic anhydrase to inhibition by sulfonamides is probably due to the difference in the structure of the enzymes at the active site. It has been shown[4] that castration of the male rat produces female rat liver type enzyme (susceptible to inhibition by sulfonamides) in male rat liver. Administration of testosterone to female rats produces the male rat liver type enzyme (refractory to inhibition by sulfonamides) in female rats. This change occurs only in liver enzyme and not in carbonic anhydrase of the other tissues of the rat. It has been suggested that major carbonic anhydrase isoenzymes, corresponding to human enzymes B and C, arose through a process of gene duplication and subsequent independent evolution.[10] However, additional polymorphism may occur due to the presence of modified forms of each of the two isoenzymes. These modifications may arise through mutational changes or may result from secondary modifications occurring *in vivo* or *in vitro*. Tashian *et al.*[10,11] reported that B isoenzyme shows greater variability than C isoenzyme. It seems that, in the case of rat liver carbonic anhydrase, the gene for production of low activity isoenzyme is influenced directly or indirectly by testosterone. In the presence of testosterone, the rat

liver synthesizes an isoenzyme which not only has a low catalytic activity but is also refractory to inhibition by sulfonamides. In the absence of testosterone, as in the female rat, the liver produces normal low and high activity type isoenzymes which are sensitive to inhibition by sulfonamides.

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