MOLECULAR PHARMACOLOGY, 10, 335-343

The Isolation and Partial Characterization of Sulfonamide-Resistant Carbonic Anhydrases from the Liver of the Male Rat

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(Received August 15, 1973)

SUMMARY

KING, RODNEY W., GARG, LAL C., HUCKSON, JUDITH, AND MAREN, THOMAS H.: The isolation and partial characterization of sulfonamide-resistant carbonic anhydrases from the liver of the male rat. *Mol. Pharmacol.* **10**, 335-343 (1974).

The liver of the adult male rat contains carbonic anhydrase activity which is 10,000 times less sensitive to inhibition by acetazolamide than the carbonic anhydrase present in female rat liver or canine liver from either sex, or in rat kidney or erythrocytes from either sex. By contrast, inhibition of the carbonic anhydrase activity from the livers of male rats by cyanide or bisulfide anions occurs with I_{50} values similar to those of rat erythrocyte carbonic anhydrase. Using standard biochemical techniques, we have shown that the activity exists as four isoenzymatic forms, consisting of two major and two minor species. The two major species have been isolated, and run as single bands on polyacrylamide gel electrophoresis. They both have a molecular weight of approximately 29,000, as determined by molecular sieve chromatography, and probably contain 1 atom of zinc per molecule. The enzymes retain their resistance to inhibition by acetazolamide throughout the isolation procedure. The turnover number is about 3300 sec^{-1}, roughly 1% of that of human red cell carbonic anhydrase C and most tissue carbonic anhydrases.

INTRODUCTION

In 1966 Maren *et al.* (1) reported that the carbonic anhydrase activity of rat liver homogenates, and also of the supernatant solution from centrifuged homogenates, exhibited an abnormal lack of sensitivity to inhibition by acetazolamide. The acetazolamide concentration required for 50% inhibition of rat liver carbonic anhydrase activity was 62.3 μM, whereas the red cell carbonic anhydrase required only 20 nM. The concentration required for 90% inhibition showed an even more startling disparity, being more than 1 mM for rat liver carbonic anhydrase and only 180 nM for red cell carbonic anhydrase. Pihar (2) independently found that 39% of rat liver carbonic anhydrase activity could not be inhibited by 21 μM acetazolamide.

Maren *et al.* (1) did not attempt to purify the enzyme, since its concentration in rat liver tissue was estimated to be very low, about 0.003 that in red cells. It was clear, however, that studies of resistant enzyme could add significantly to the present knowledge of the forces governing the binding of sulfonamides to the sensitive enzyme.

This work was supported by National Institutes of Health Grant GM 16934.

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This paper describes the purification and partial characterization of the resistant rat liver carbonic anhydrase.

METHODS

A standard buffer solution, 50 mM in Tris adjusted to pH 9.3 with H_2SO_4, was used for most of the work. The compositions of other buffer solutions are given where appropriate. All reagents were of analytical grade. Carbonic anhydrase activity was measured by the methods of Maren and coworkers (3, 4). Inhibition assays were carried out after incubating enzyme and inhibitor for 1 min in the reaction vessel prior to starting the measurement. Inhibition is expressed as $100 \times (EU - EU_i)/EU$, where EU is the concentration of enzyme (in units in the bath) with no inhibitor present, and EU_i is the concentration of active enzyme in the presence of inhibitor. The variability of the method is about 10% (4). The variation in samples from different animals was greater, differences in I_{50} ranging from 1- to 2-fold. However, in the context of comparison with sensitive enzyme, where I_{50} values are 300–10,000 times smaller, this variability is insignificant.

Protein concentrations were determined by the method of Lowry *et al.* (5), using bovine serum albumin as standard. Optical densities were measured on a Gilford model 2400 ultraviolet spectrophotometer.

Preparation and treatment of liver tissue. The animal was anesthetized with ether and the hepatic portal vein was cannulated. Cold 0.9% NaCl solution was then perfused through the organ until it began to swell, whereupon the chest cavity was opened and the vena cava was cannulated. The perfusion rate was increased until the liver became pale tan. The liver was then removed, and perfusion was continued until the lobes appeared free from blood and the perfusate was colorless. After thorough washing in cold NaCl, the liver was cooled, weighed, and used immediately.

For most inhibition experiments the liver tissue was minced, suspended in 2 volumes of standard buffer, and homogenized for 2 min at approximately 4°, using a Polytron submersible-probe homogenizer. The suspension was then centrifuged at 100,000 × *g* and 4° for 90 min, and the supernatant solution was used as a crude enzyme preparation. Variations in the techniques used in the isolation procedure are described under RESULTS.

Column chromatography. All chromatographic procedures were carried out at 4°. Column materials were prepared and poured by standard methods. Ionic strength gradients used with DEAE-Sephadex A-50 columns were monitored using an Industrial Instruments model RC 16B2 conductivity bridge.

Molecular weight determinations, using a 1.1-cm-diameter × 195-cm Sephadex G-100 column, were carried out exactly according to Whitaker (6). Samples of 1 ml, containing approximately 5 mg of protein, were applied consecutively, spaced by 20 ml of buffer solution. The void volume was determined using 1 ml of a solution of blue dextran containing 1 mg/ml. The column effluent was monitored spectrophotometrically at 280 nm. Standard proteins were obtained from commercial sources.

Isoelectric focusing. Isoelectric focusing experiments were performed using an LKB model 8102 preparative isoelectric focusing column circulated with ethylene glycol–water at 0.4°. Ampholines were used at a concentration of 1 g/100 ml, and the sample was mixed with the "light" gradient solution. Equilibrium was reached after 60 hr, as judged by the decrease in current.

The preferred orientation of anode at the top could not be used, since in our first experiment considerable precipitation of protein occurred at the anode. This precipitate fell slowly down the column and interfered with the stability of the sucrose gradient. In subsequent experiments we used the anode at the bottom.

After equilibration the column was eluted into a fraction collector at 80% of the maximum recommended rate; 5-ml fractions were collected.

Polyacrylamide gel electrophoresis. Gel electrophoresis was carried out by modifications of the method of Davis (7). The systems used were: pH 8.3, Tris-HCl buffer solutions, using 7% gels; pH 3.5, formic acid–sodium formate buffer solutions, using 15%

gels containing 8 M urea. All experiments were done at room temperature.

For the experiments at high pH the samples were mixed with an equal volume of 40% sucrose solution containing bromphenol blue as tracking dye, and layered directly on top of the gels. Experiments at low pH required a prolonged preliminary period of electrophoresis to remove excess persulfate. In this case samples were mixed with an equal volume of solution containing 30% sucrose, 8 M urea, and methyl green as tracking dye.

After electrophoresis the gels were stained with 0.1% Amido black dissolved in 7% acetic acid, then electrophoretically destained and photographed.

RESULTS

In our initial work we made the entirely unexpected finding that female Wistar or Holtzman rats differed from the males, in that female liver supernatant fractions gave I_{50} (35 nM) and I_{90} (400 nM) data for acetazolamide similar to those for erythrocyte carbonic anhydrases of rats and other species (Fig. 1A). Male rats gave I_{50} data similar to those reported by Maren *et al.* (1), who had not specified the sex of their rats. The I_{50} value of our male liver preparations for acetazolamide was increased significantly, from 62.3 to 100–200 μM, by exhaustive perfusion of the liver prior to homogenization, and indeed the abnormal I_{50}:I_{90} ratio found by Maren *et al.* (1) could be virtually abolished by this treatment. A typical inhibition curve is shown in Fig. 1B. The liver of a 6-month ovariectomized rat was also tested for carbonic anhydrase inhibition, but was not significantly different from the normal female (Fig. 1A).

Table 1, columns *a*, shows that five other sulfonamides of widely differing structural type and activity resemble acetazolamide in their strikingly lower activity against male liver carbonic anhydrase than against the red cell enzyme. Columns *b* compare previous data on relatively crude supernatant fractions; notice that the effect of purification is to raise the I_{50}, an expected finding if the impure preparation contained some sensitive enzyme from blood.

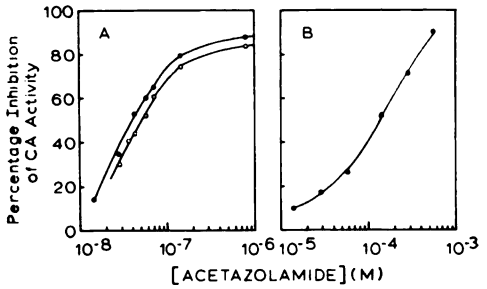

FIG. 1. *Typical liver carbonic anhydrase (CA) inhibition curves using supernatant solutions*

A. ●, female rat; O, ovariectomized rat. B. Male rat; note change in abscissa scale.

TABLE 1

Inhibition of liver and red cell carbonic anhydrases from male rats

Inhibitor	Liver I_{50}		Liver I_{90}		Red cells[a]	
	b	*a*	*b*	*a*	I_{50}	I_{90}
	M	*M*	*M*	*M*	*M*	*M*
Sulfanilamide	$>10^{-3}$	2.1×10^{-5}	$>10^{-3}$	$>8 \times 10^{-4}$	4×10^{-6}	2.5×10^{-5}
Methazolamide	1.5×10^{-5}	9×10^{-6}	4×10^{-4}	2.4×10^{-4}	4×10^{-8}	2.0×10^{-7}
Acetazolamide	$1\text{–}2 \times 10^{-4}$	6.2×10^{-5}	$1\text{–}2 \times 10^{-3}$	$>10^{-3}$	2×10^{-8}	1.8×10^{-7}
Benzolamide	4×10^{-6}	4.3×10^{-8}	1×10^{-4}	2.2×10^{-4}	7×10^{-9}	
CL 13,580	1×10^{-6}	2.6×10^{-6}	2×10^{-5}	2.7×10^{-4}	3×10^{-9}	
Ethoxzolamide	1.5×10^{-4}		$>5 \times 10^{-4}$		7×10^{-9}[b]	5×10^{-8}[b]
CN^-	2×10^{-5}		3×10^{-4}		1.5×10^{-5}[b]	1.5×10^{-4}[b]
SH^-	7×10^{-6}		7×10^{-5}		3×10^{-5}[b]	3×10^{-4}[b]

[a] Determined by Maren *et al.* (1).

[b] Determined by the present authors, using the supernatant solution as a crude enzyme preparation, as described in METHODS. The chemistry of these inhibitors has been described (8).

Table 1 also shows that, unlike the six sulfonamides, CN^- and SH^- have the same inhibitory activity against liver as against red cell carbonic anhydrase.

Preparation of crude enzyme fraction. Fifteen exhaustively perfused livers were cut into small pieces, suspended in 2 volumes of standard buffer solution, and homogenized. The pH of the suspension fell to 8.7 during homogenization and was returned to 9.3 by adding solid Tris. After centrifugation for 90 min at 4° and 100,000 × *g*, the tubes contained three distinct layers: a fat layer containing 6.8 enzyme units/ml, an aqueous layer containing 4.8 enzyme units/ml, and packed debris containing approximately one-fifth of the total activity of the preparation. The fat layer was removed with some of the aqueous layer and emulsified with 10 volumes of ice-cold chloroform, then centrifuged at low speed. The enzyme activity was located in the aqueous layer and was pooled with the aqueous supernatant fluid from the initial centrifugation. The pellets were resuspended in 2 volumes of standard buffer, homogenized, and centrifuged a second time. The activity remaining in the pellet was now less than 10% of the total and was discarded. The total combined activity from both sets of supernatant fractions was 3050 units, 90% of the activity of the original homogenate.

Ammonium sulfate precipitation. Ammonium sulfate was added to the combined reddish brown supernatant fractions slowly with stirring at 4° to a final concentration of 40% of saturation. Under these conditions 98% of the enzyme activity remained in solution while 75% of the total protein [as determined by the method of Lowry *et al.* (5)] was precipitated. The precipitate was removed by low-speed centrifugation, and the supernatant fraction was dialyzed against standard Tris buffer for 2 days with several changes of buffer solution.

Chromatography on Sephadex G-75. The pale pink solution obtained from the previous dialysis was concentrated to 90 ml by ultrafiltration, using an Amicon UM-10 membrane. Of this solution, 30 ml were applied to a column of Sephadex G-75 (80 × 2.5 cm) equilibrated with standard buffer, using reverse flow. The effluent was monitored by ultraviolet absorption at 280 nm and also by carbonic anhydrase activity. Figure 2 shows a typical elution profile. The ultraviolet-absorbing material was eluted as six peaks, only one of which was associated with carbonic anhydrase activity. Recovery of activity from the column was 70%, and was exactly reproducible for each of the three samples applied from this preparation. No other peak of carbonic anhydrase activity was found. The fractions containing carbonic anhydrase activity were pooled and concentrated to a small volume, giving a pale yellow solution.

Isoelectric focusing. Following chromatog-

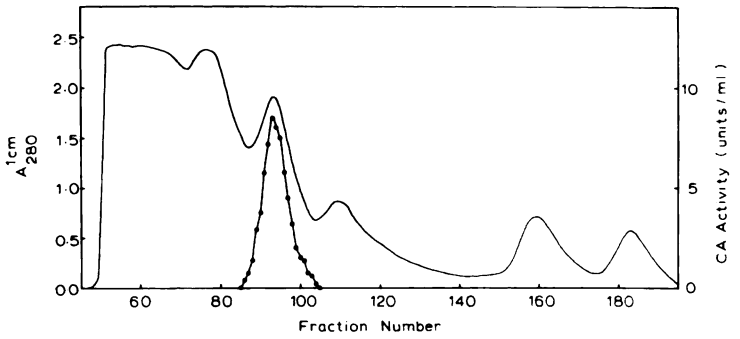

FIG. 2. *Elution profile from Sephadex G-75 column*

After treatment of the supernatant fraction from the homogenized livers with 40% ammonium sulfate and removal of the precipitate, the solution was thoroughly dialyzed and concentrated, and one-third of it was applied to a 5.0 × 80 cm column of Sephadex G-75 (medium) equilibrated with standard buffer. Elution of 10-ml fractions was performed with the same buffer. ——, optical density; ●——●, carbonic anhydrase (CA) activity.

raphy on Sephadex G-75 attempts were made to purify the rat liver carbonic anhydrase further by preparative isoelectric focusing. Using a pH gradient from 5 to 8, two large and two small peaks of carbonic anhydrase activity were detected, and six peaks of optical density at 280 nm (Fig. 3). It was impossible to obtain experimental values for uncatalyzed times, because of variation in the buffering power of the solution along the length of the column. Therefore we used an estimated uncatalyzed time of 100 sec and expressed activity as $100/t - 1$, where t is the catalyzed time. After the active fractions had been thoroughly dialyzed, activity measurements showed that over-all recovery from the isoelectric focusing column was only 25 %. The isoelectric pH of each of the fractions is given in Table 2.

Chromatography on DEAE-Sephadex A-50. Attempts were made to separate the enzymes from each other and from interfering material by ion-exchange chromatography. Using the standard pH 9.3 Tris buffer solution previously used for the Sephadex G-75 column, the enzymes were firmly attached to the ion-exchange column and could not be eluted under equilibrium conditions. A linear gradient increase of the buffer concentration from 0.05 to 0.10 M and from 0.10 to 0.20 M was also unsuccessful. A NaCl gradient from 0.0 to 0.2 M in the 0.2 M buffer eluted all the protein, but with no separation of the carbonic anhydrases.

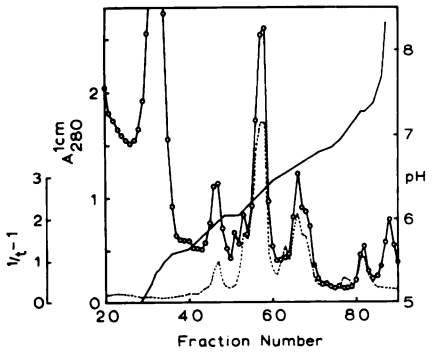

FIG. 3. *Isoelectric focusing of active fractions from Sephadex G-75*

The active fractions from the Sephadex G-75 column were pooled and concentrated, and a sample was applied to a preparative isoelectric focusing column. After equilibration 5-ml samples were collected. ——, pH; O——O, optical density; -----, carbonic anhydrase activity.

TABLE 2

Isoelectric points of male rat liver carbonic anhydrases

Fractions are numbered as in Fig. 4, assuming that the slopes of the titration curves of the enzymes are similar, and that the order of elution from DEAE-Sephadex A-50 is in decreasing order of their isoelectric points.

Fraction	Isoelectric pH
1	7.25
3	6.60
4	6.28
5	5.97

The column then was equilibrated with 0.05 M Tris buffer, pH 8.5, and another sample was applied. In this case there was very little retention of the carbonic anhydrases and only very poor separation of the isoenzymes.

Raising the alkalinity of the column to pH 8.7 gave good separation of two of the isoenzymes (peaks 4 and 5 in Fig. 4) and removed much of the non-carbonic anhydrase protein as a large peak when 0.2 M NaCl in 0.2 M Tris buffer was used for elution. Of the other activity peaks, peak 1 still contained non-carbonic anhydrase protein, and because of the very small amount available no further attempt was made to purify it. Fractions 2 contained nonrefractory enzyme, probably arising from erythrocytes not washed out of the liver during the initial perfusion, while fractions 3 contained mostly refractory enzyme.

The over-all recovery of activity from this column was 75 %, which compared favorably with the 25 % obtained from isoelectric focusing.

After chromatography the active fractions were dialyzed against 10 mM ammonium carbonate solution and freeze-dried.

Inhibition of isolated fractions by acetazolamide. Table 3 contains the results obtained from inhibition experiments using the pooled samples. Fractions 1, 3, 4, and 5 were relatively refractory to inhibition, and within the limitations of the method each of these fractions showed similar inhibition at both

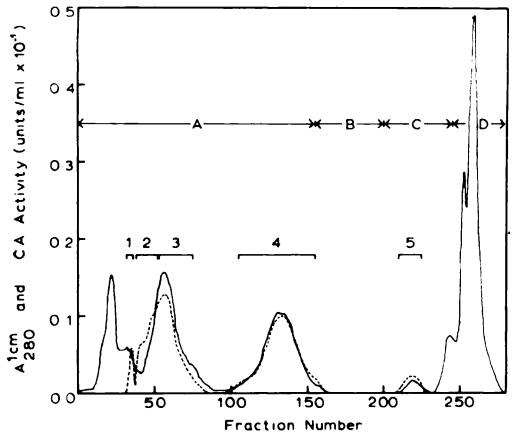

FIG. 4. *Elution profile from DEAE-Sephadex A-50 column*

A sample of the active fractions from the Sephadex G-75 column was dialyzed against Tris-sulfate buffer, pH 8.7, 50 mM in Tris, and applied to the column (2.5 × 45 cm), which had been equilibrated with the same buffer solution. Elution was continued with starting buffer (*A*), then linear gradients (*B*, 50–100 mM in Tris; *C*, 100–200 mM in Tris; *D*, 200 mM in Tris–200 mM in Tris plus 200 mM NaCl). Samples 1–5 were pooled as shown. ——, optical density; - - -, carbonic anhydrase (CA) activity. Samples of 10 ml were collected.

TABLE 3

Inhibition by acetazolamide of active fractions from DEAE-Sephadex A-50 column

Fraction	I_{50}	I_{90}
	M	*M*
1	7×10^{-5}	2×10^{-4}
2	3×10^{-8}	1×10^{-4}
3	1.5×10^{-4}	6×10^{-4}
4	1×10^{-4}	6×10^{-4}
5	1×10^{-4}	5×10^{-4}

the I_{50} and I_{90} levels. Fraction 2 was a mixture of refractory and nonrefractory enzyme, evidenced by the value of I_{50}, 30 nM, about the same as rat erythrocyte carbonic anydrase, and that of I_{90}, 100 μM. The values of I_{50} and I_{90} in a mixture of refractory and nonrefractory enzymes were dependent on the relative proportion of the enzymes in the mixture. At least half the activity in fraction 2 appeared to be due to nonrefractory enzyme.

Electrophoresis on polyacrylamide gels. Figure 5 shows the results obtained from electrophoresis of the purified proteins eluted from the ion-exchange column. Using the Tris-borate buffer system at pH 8.3, fraction 4 produced only one band, and fraction 3 produced one strong band and one very weak band, indicating a high degree of purity in both cases. As expected, fractions 1 and 2 both contained several bands, at least one band in fraction 2 having the same mobility as one of the rat erythrocyte carbonic anhydrases. Fraction 5 also contained a multiplicity of bands and was clearly very impure.

Electrophoresis in the formate buffer system at pH 3.5 using 15% gels containing 8 M urea revealed a similar pattern. Al-

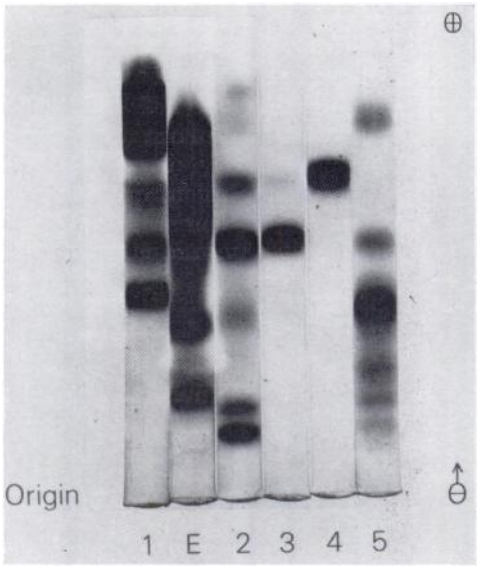

FIG. 5. *Electrophoresis of samples eluted from DEAE-Sephadex A-50*

Freeze-dried material obtained from the pooled fractions from DEAE-Sephadex A-50 were dissolved in water and subjected to electrophoresis on 7% polyacrylamide gels at pH 8.3. The gels are numbered with the sample number in Fig. 4. The gel marked *E* is a crude preparation of rat erythrocyte carbonic anhydrase. The very heavily stained band in *E* is hemoglobin. The two heavily stained bands nearer the origin are carbonic anhydrase, identified by comparison with an identical gel soaked in a 0.1 mM solution of *N*,*N*-dimethylaminonaphthalenesulfonamide and viewed under ultraviolet light.

though the mobilities of the proteins were altered, the same numbers of bands were seen.

Molecular weight determination. Figure 6 contains the results of the molecular weight determination for fractions 3 and 4 from the ion-exchange column. Both fractions were eluted at the same volume, and from the graph of V/V_0 against $\log_{10}$ molecular weight, a molecular weight of 29,000 ± 1000 was obtained. The error in the determination is higher than expected from Whitaker's results (6), and is due to (a) considerable broadening of the effluent peak and (b) the use of only five standards. However, the measurement is sufficiently accurate to demonstrate the close similarity in molecular weight between the refractory rat liver carbonic anhydrases and the sensitive carbonic anhydrases from other mammalian species.

Zinc content. The zinc content of freeze-dried samples of fractions 3 and 4 was determined by atomic absorption spectrometry. Based on a protein molecular weight of 29,000, fraction 3 contained 0.3 mole of zinc per mole of protein and fraction 4 contained 0.5 mole/mole. These values are considerably lower than the anticipated value of unity, and may be explained by the partial loss of activity of the preparations when dialyzed against 10 mM ammonium carbonate solution prior to freeze-drying and by residual water and salt in the preparation. Alternatively, the preparation may not have been completely pure, although this seems unlikely in view of the results from electrophoresis at high and low pH.

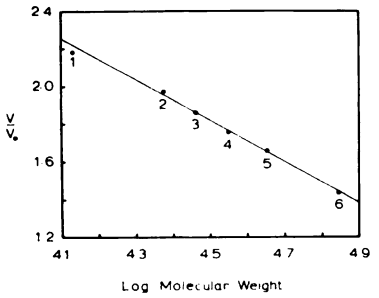

FIG. 6. *Molecular weight determination by analytical gel filtration on Sephadex G-100*

Rat liver carbonic anhydrase samples and standards were applied consecutively in 1 ml of solution containing approximately 5 mg, with 20-ml buffer spacers. V_0, the void volume, was determined using blue dextran. Samples of 1 ml were collected. V, the elution volume, was taken as the center of the eluted peak. Numbered points are: *1*, ribonuclease; *2*, trypsin; *3*, rat liver carbonic anhydrases 3 and 4; *4*, pepsin; *5*, ovalbumin; *6*, bovine serum albumin.

The protein concentration of the solutions used for atomic absorption spectrometry may also be estimated from the absorbance at 280 nm, as long as the molar extinction coefficient is known. Since we do not have a value for the molar extinction coefficient of the rat enzymes, we used the value 5.7×10^4 M^{-1} cm^{-1} for the bovine enzyme, which should not be grossly different from the values for the rat enzymes. The estimates of the zinc content obtained by this method are 0.72 mole/mole of protein for fraction 3 and 1.7 moles/mole of protein for fraction 4.

Clearly the enzymes do contain zinc, though further work will be necessary to obtain accurate estimates of the molar ratio.

Turnover number. This was estimated by calculation of the molar concentration of liver enzyme (E_0) per enzyme unit (EU) compared to that of pure red cell human carbonic anhydrase C or other tissue enzymes with known E_0/EU ratios (3). Under the assay conditions used (substrate concentration, 60 mM; temperature, 1°; buffer concentration, approximately 300 mM), the value of E_0/EU for most mammalian carbonic anhydrases is 2 nM/unit. Since the K_m value is much less than the substrate concentration, this is equivalent to a turnover number for CO_2 hydration (V_{max}/E_0) of about 5×10^5 sec^{-1} (Tables 2 and 3 of ref. 8).

Addition of known amounts of purified fractions 3 and 4 (estimated from the above data, including the zinc content) to the reaction which measures CO_2 hydration (4) yielded the much higher value of about 300 nM/unit. Thus the enzyme from male rat liver has an apparent turnover number somewhat less than 1% of that of human red cell carbonic anhydrase C, or about 3300 sec^{-1}. This apparently low turnover number may be modified to some extent when the true K_m value is determined, although it is

unlikely to change the value by more than a few times.

DISCUSSION

The presence in male rat liver of carbonic anhydrase resistant to sulfonamides appears so far to be unique to rodent species. We have also examined the effect of acetazolamide inhibition on the hepatic carbonic anhydrase from rabbits and mice of both sexes. In these species the carbonic anhydrase was similarly resistant to inhibition, showing no sex differentiation. However, in other organs, such as kidney and red cell of these rodents, the carbonic anhydrase was normally susceptible to the sulfonamide (1) (results above). Dog and guinea pig livers appear susceptible, and from a review of physiological data, human liver is also susceptible to acetazolamide (1).

From the point of view of the biochemist the resistant enzymes are particularly interesting as tools for elucidating the mechanism of inhibition of normal carbonic anhydrases by sulfonamides. A further point of interest is the very low turnover number of the rat liver enzyme compared to other animal carbonic anhydrases. It should be indicated, however, that turnover numbers of sulfonamide-sensitive hepatic enzymes have not yet been studied, and such work is under way in our laboratory.

The role of carbonic anhydrase in hepatic physiology has only been subject to a few studies, and no new data have appeared since the matter was reviewed in 1967 (8). Physiological inhibition could not be achieved with the doses used (although it is certainly likely that with doses some hundred times greater it could be done) in the male rat, but from the data in the dog a reasonable scheme can be proposed. There is no reason to believe that the "refractory" and low-activity enzymes in the male rat have a different function from the normally susceptible enzyme, although this point is certainly worth exploring. The data from the dog, following administration of the sulfonamides, suggest that the chief role of carbonic anhydrase in hepatic cells is the reabsorption of chloride from the hepatic duct, in exchange for bicarbonate.

The results obtained using purified enzymes demonstrate that carbonic anhydrase activity resistant to inhibition by sulfonamides is not an artifact caused by using crude homogenate. We carefully monitored the inhibition of activity at each stage of the purification and, apart from the variability expected from the assay method (see below), found no change in the I_{50} value for acetazolamide. For this reason it is probable that the results obtained with the other inhibitors, using an unpurified homogenate, closely reflect the properties of the purified enzymes.

Strong inhibitors of carbonic anhydrase fall into two main classes, aromatic or heterocyclic sulfonamides, unsubstituted in the sulfonamide group, and certain anions. The considerable body of evidence available (9) suggests that the anions act by binding directly to the metal at the active site, while the sulfonamides obtain their binding energy from coordination at the active site and from hydrophobic interaction with the active site wall. The results presented in Table 1 show that the inhibition of rat liver carbonic anhydrase by anions is not significantly different from that of erythrocyte carbonic anhydrase. This strongly suggests that the metal in the rat liver carbonic anhydrase is not in an abnormal environment. On the other hand, the results with sulfonamides show that their binding energy is considerably decreased relative to erythrocyte carbonic anhydrase. This decrease may well be explained by amino acid replacements either directly at the active site, causing a change in the hydrophobic binding energy, or at some other point in the peptide chain, causing some distortion at the active site. Our results indicate that, in general, weak inhibitors remain the weakest and strong inhibitors remain the strongest, but this simple picture is not totally consistent; viz. ethoxzolamide. Table 4 shows the ratio of I_{50} of the liver extract to I_{50} of a crude erythrocyte carbonic anhydrase preparation compared with the ether/water partition coefficient. There is no correlation between the two values, indicating that changes in the forces governing the combination of the inhibitor and enzyme are not wholly due to simple changes in hydrophobic interactions.

TABLE 4

Comparison of 50% inhibitory ratio of liver and erythrocyte carbonic anhydrases with sulfonamide partition coefficient

Sulfonamide	I_{50} liver enzyme /I_{50} erythrocyte enzyme	Partition coefficient, ether/water[a]
Benzolamide	280	0.001
CL 13,580	333	79
Methazolamide	400	0.62
Acetazolamide	8,000	0.14
Ethoxzolamide	20,000	140
Sulfanilamide	>250	0.15

[a] Data taken from Sanders and Maren (10).

Turning now to the purification of the enzymes, the total yield of freeze-dried fractions 3 and 4 was approximately 0.01% of the wet weight of liver tissue taken. This represents at most 70% of the total carbonic anhydrase protein present in the original supernatant fraction.

The major fractions, 3 and 4, appear to be reasonably pure by the criteria we have used. First, they were eluted as single peaks from the analytical gel filtration column; second, they ran as single bands (except for a very small amount of a second band in fraction 3) during electrophoresis at both high and low pH.

The molecular weight, isoelectric points, susceptibility to CN^- and SH^-, and zinc content of the purified male rat liver enzymes suggest that, apart from their resistance to sulfonamide inhibition and low turnover number, they closely resemble other mammalian carbonic anhydrases (8). Since only one peak of activity was eluted from the preparative Sephadex G-75 column and the molecular weight determination gave the same result for both fractions 3 and 4, it is likely that the minor unpurified species 1 and 5 also have a molecular weight near 29,000. In this context the presence of four isoenzymatic forms may be misleading. Further work will be required to isolate the minor species and demonstrate whether or not they arise from minor chemical modification of the major species.

ACKNOWLEDGMENT

We wish to thank Dr. Joseph E. Coleman of Yale University for carrying out the zinc determinations.

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