A Study of Hepatic Carbonic Anhydrase

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SUMMARY

Dog and rat liver contain carbonic anhydrase in supernatant and particulate fractions. Hepatic bile of both species has higher HCO_3^- and lower Cl^- concentration than plasma. The enzyme in all fractions of dog liver was inhibited by unsubstituted aromatic sulfonamides at concentrations of 10^{-6} to 10^{-9} M. *In vivo*, administration of these drugs at doses which yield > 99% enzyme inhibition lowered biliary HCO_3^- and raised biliary Cl^- concentrations. Certain of the drugs were secreted by liver and concentrated in the bile of both species. Rat liver supernatant carbonic anhydrase was nearly refractory to sulfonamide inhibition *in vitro;* this is the first example of such lowered response in the animal kingdom. Biliary electrolytes in the rat were accordingly not altered by high doses of these sulfonamides.

INTRODUCTION

In the course of experiments designed to yield information about the hepatic transfer of certain sulfonamide carbonic anhydrase inhibitors in the rat, it was noted that the drugs had no effect upon bile flow or electrolytes. Since this was contrary to the report of Wheeler and Ramos (1) for the dog, it was decided to study the hepatic enzyme in both species and to document further the electrolyte pattern in these animals.

Previous work on this subject is relatively sparse. Although hepatic carbonic anhydrase has been recognized for some time (8), and recently found in both supernatant and particulate fractions in rat (2, 3), it is present in rather low concentrations (about 10% that in kidney); only three studies have appeared on the effects of the specific inhibitor acetazolamide on bile. Wheeler and Ramos (1) used trained unanesthetized, splenectomized, and cholecystectomized dogs, infused with sodium taurocholate. Acetazolamide, 65 mg/kg i.v., gave an increased flow, an increased chloride concentration, and decrease in bicarbonate concentration in the bile. Bizard *et al.* (4) saw no change in bile flow in the rat after 200 mg/kg acetazolamide. Fink (5) gave 500 mg of acetazolamide, followed by 250 mg every 6 hours, orally to a patient with a chronic biliary fistula. He attributed the small rise in Cl^- and decline in HCO_3^- concentration in bile, to the urinary loss of HCO_3^-, which follows the typical renal effect of carbonic anhydrase inhibition. However, the data show that 3 hours after initial drug administration bile chloride rose 9 mM, but plasma chloride had not risen. There have been no reports on the *in vitro* sensitivity of the hepatic enzyme to any of the sulfonamides.[1]

In spite of these discrepancies, the over-

[1] After completion of the present work, O. Pihar reported that 39% of the catalytic action of rat liver carbonic anhydrase could not be inhibited with 21 μM acetazolamide. In parallel experiments, all red cell enzyme activity was abolished. (*Biochim. Biophys. Acta* **104**, 608 (1965)).

all chemical composition of the bile in mammalian species (6) suggests strongly that carbonic anhydrase might be involved in its formation. Like pancreatic juice, bicarbonate concentration is higher, and the chloride concentration lower, than plasma. In both organs, metabolic or circulating CO_2 may be converted to HCO_3^-, which is thereby concentrated in the secretion.

METHODS AND MATERIALS

Holtzman rats weighing 300–450 g and mongrel dogs weighing approximately 10 kg were used; they were fasted for 12 hr prior to testing. The rats were anesthetized with ether during cannulation of the common bile duct. Following this procedure, some animals remained under anesthesia for the experiment, others were allowed to recover in Bollman-type wire restraining cages. In certain of the rats sodium taurocholate was intubated into the duodenum. The dogs were anesthetized with sodium pentobarbital during the entire experiment. The femoral vein was used for the intravenous infusion of 0.5% sodium taurocholate in saline (Sigma) at about 1 ml/min which was started 20 min before operation. Prior to cannulation of the common bile duct, the cystic duct was ligated. The hepatic bile flowed into graduated centrifuge tubes containing mineral oil. Breathing was spontaneous with the exception of the hyperventilation experiments. Venous samples were obtained at 30-min intervals in the middle or end of each collection period.

Respiratory alkalosis was accomplished by hyperventilation with a Harvard respiration pump model 607, via a tracheal tube. The pump was set for 50 cycles per minute.

The drugs were supplied by the Lederle Division of American Cyanamid Company. Their structure and chemical and physical properties are given in references (9) and (10). They were administered intravenously as their sodium salts.

Drug analyses and inhibition assays were done by an adaptation of the changing pH method for carbonic anhydrase. The variability of the analytical method, which is about 10%, has been elsewhere discussed (7). Chloride was determined by mercuric nitrate titration, CO_2 by manometry in the Kopp-Natelson microgasometer. pH was determined in the Metrohm meter, and sodium and potassium in the Eppendorf flame photometer. The terms HCO_3^- and total CO_2 are used interchangeably, since at the pH of bile (about 7.6) 97% of CO_2 is HCO_3^- ion. Carbonic anhydrase assays (7) were done on thoroughly perfused livers. Perfusion was done through the hepatic artery with cold isotonic saline until the organ attained a whitish tan aspect and the effluent was colorless.

Fractionation of liver was done as follows: Animals were killed by pentobarbital, and the liver was removed and perfused with cold 0.25 M sucrose through the hepatic artery as described above. The tissue was cooled at 4°, weighed, minced and homogenized in a motor-driven glass pestle. Particulate cellular components were isolated following the differential centrifugation procedure of Schneider and Hogeboom (12). In some cases particulate matter was washed once, and in others 3 times. Protein was determined by the method of Lowry *et al.* (13).

RESULTS

Enzymic

Distribution of carbonic anhydrase in liver. Both dog and rat liver contain carbonic anhydrase activity. Using the assay method of this laboratory (7), the values ranged from 7 to 26 enzyme units per gram wet perfused tissue for dog and rat. By comparison with other organs in the same units: dog kidney cortex = 546; dog red cell = 1400; dog pancreas = 20; rat kidney cortex = 250; rat red cells = 2600; rabbit ciliary process = 23; cat choroid plexus = 250.

Separation of perfused dog and rat liver into the various fractions by ultracentrifugation yields the data of Table 1. There is no essential difference between the two species with respect to distribution of the enzyme among the different cell structures. In terms of total cell volume or wet weight,

TABLE 1
Carbonic anhydrase in cell fractions of dog and rat liver[a]

Fraction	Enzyme units/g whole wet tissue		Mg protein/g whole wet tissue		Enzyme units/mg protein	
	Rat	Dog	Rat	Dog	Rat	Dog
			A. One wash			
Whole liver	22	20	122	164	0.18	0.12
Supernatant[b]	16	16	43	48	0.27	0.33
Nuclei	2.4	2.2	27	27	0.09	0.08
Mitochondria	1.9	2.4	24	25	0.08	0.09
Microsomes	1.4	2.6	12	20	0.12	0.14
			B. Three washes			
Whole liver	24	11	95	185	0.25	0.06
Supernatant[b]	21	7	30	37	0.7	0.19
Nuclei	2	1	26	34	0.08	0.03
Mitochondria	0.7	0.5	11	12	0.06	0.04
Microsomes	0.5	0.4	2	2	0.25	0.2

[a] These values after 2 min equilibration of enzyme and CO_2 in the test system. If no equilibration is done, activity is about half. This is the case only for rat liver, not for any other carbonic anhydrase we have studied.
[b] Represents supernatant after separation of all the other fractions.

the supernatant has most of the enzyme. Based on protein weight, the concentration of enzyme in the microsomal and supernatant fractions are not greatly different, and nuclei and mitochondria contain about one-third as much.

Inhibition in vitro. Table 2 shows the effect of five representative carbonic anhydrase inhibitors on dog and rat liver and the subcellular fractions *in vitro*. Each datum is a mean of 2–5 experiments, in which an individual point is the result of 3–10 runs in the vicinity of the I_{50} or I_{90} (7, 10). The concentration of drug to inhibit 50% (I_{50}) and 90% (I_{90}) of enzyme is given, along with comparative data, for red cell enzyme. It is evident that each of the 5 drugs has a characteristic and reasonably consistent activity against the various fractions of dog liver, dog blood, rat subcellular fractions, and rat blood. There is some suggestion that the microsomes of dog liver require 2 to 3-fold more drug than the other fractions for the same degree of inhibition, indicating some difference in enzyme structure. Such differences, however, appear too small to have a pharmacologic implication.

Figure 1 shows a plot, originally developed by Easson and Stedman and used in this laboratory for the carbonic anhydrase problem (10) of dog liver against the very powerful inhibitor, CL 13,580, 2-*o*-chlorphenyl - 1,3,4 - thiadiazole 5-sulfonamide. This particular drug has the important attributes of high activity and instantaneous equilibrium with enzyme. In the type plot of Fig. 1, the ordinal intercept yields the molar concentration (E_0) of enzyme. E_0 divided by the activity term, enzyme units (e.u.) yields a measure of the activity of the enzyme. The value 5×10^{-9} M obtained for E_0/e.u. in Fig. 1 is in the range found for dog blood (10) and kidney, and the human red cell pure fraction C (11).

Table 2 shows that whole rat liver and supernatant yield an entirely different result. Among the five drugs, activity based on I_{50} data is 0.16–0.014 times that found against dog liver and dog and rat red cells, as well as dog and rat kidney (Maren and Ellison, manuscript in preparation). At the I_{90} level, differences are even greater; all the drugs are less than 0.001 time as active in the whole rat liver and supernatant. Because of the lack of relation between the I_{50} and the I_{90} in whole rat liver and supernatant, it seems reasonable to assume that

TABLE 2

Inhibition of liver and red cell carbonic anhydrase in the rat and dog, by sulfonamides ($\times 10^{7}$ M)

Inhibitor		Dog liver					Rat liver					Red cells	
		Whole	Sup.[a]	Nuc.	Mit.	Mic.	Whole	Sup.	Nuc.	Mit.	Mic.	Dog	Rat
Sulfanilamide	I_{50}	33	32	30	22	52	208	208	32	18	30	28	40
	I_{90}	66	68	—	—	—	>8000	>8000	—	—	—	200	245
Methazolamide	I_{50}	0.18	0.18	0.20	0.32	0.58	75	90	0.18	0.20	0.39	0.24	0.4
	I_{90}	0.48	0.72	2.0	1.8	2.7	~3000	~2400	115[b]	56[b]	60[b]	0.91	2.0
Acetazolamide	I_{50}	0.45	0.27	—	—	—	643	623	—	—	—	0.2	0.2
	I_{90}	1.1	1.9	—	—	—	>10000	>10000	—	—	—	1.6	1.8
Cl 11,366	I_{50}	0.03	0.08	—	—	—	0.43	0.43	—	—	—	0.03	0.07
	I_{90}	0.13	0.3	—	—	—	—	2200	—	—	—	0.12	—
CL 13,580	I_{50}	0.04	0.03	—	—	—	26	26	0.04	0.03	0.04	0.02	0.03
	I_{90}	—	0.16	—	—	—	2600	2700	6[b]	6[b]	6[b]	0.15	—

[a] Abbreviations: *Sup.*, supernatant; *Nuc.*, nuclei; *Mit.*, mitochondria; *Mic.*, microsomes.

[b] These values, which are higher than theoretical based on the I_{50}, are explicable if about 15% of enzyme in particulate is similar to or contaminated with that of the supernatant. It may be readily shown that this would have a negligible effect on the I_{50}, but would greatly increase the observed I_{90}.

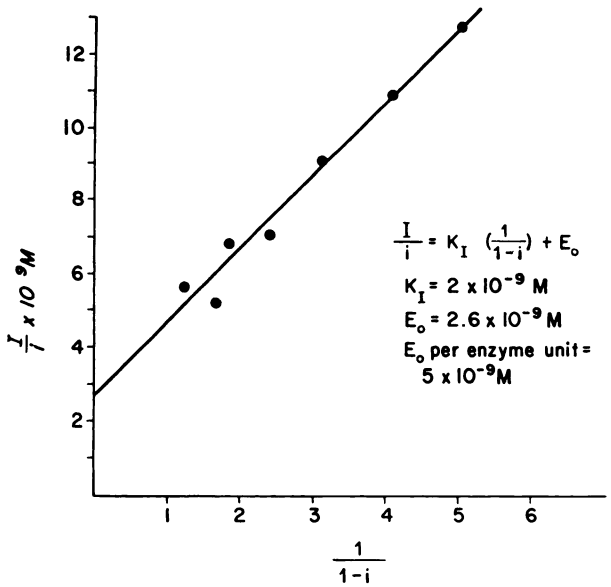

FIG. 1. *Interaction of CL 13, 580 [2(o-chlorphenyl)-1, 3, 4-thiadiazole-5-sulfonamide] with 0.52 enzyme units of whole dog liver carbonic anhydrase in vitro*

The data are plotted according to the equation given, in which i = fractional inhibition; I = drug added to system; E_o = ordinal intercept yields molar concentration of enzyme in system; K_I = slope, dissociation constant of the enzyme–inhibitor complex.

several enzymes are present. All the major components of activity are relatively insensitive to inhibition, but some component(s), amounting to at least 20%, must be exceptionally resistant to the drugs, so that the approach to complete inhibition is impossible except for the very strongest inhibitors; these require some 10,000 times as much drug as needed for the corresponding experiment with dog liver. Whole liver seems to reflect the supernatant fraction in resistance to the sulfonamides; surprisingly the enzyme in the particulate fractions shows sensitivity akin to other organs and species.

A survey of several other species showed that the guinea pig liver and subcellular fractions reacted identically to those of the dog against methazolamide, acetazolamide, and sulfanilamide. Mouse and rabbit livers and fractions tested against these three drugs appeared to resemble those of the rat.

Physiological Disposition of Drugs

Figures 2 and 3 show plasma and bile concentrations of the drugs following their intravenous injection into the rat and dog. Acetazolamide shows higher concentration in bile than in plasma, for both species. The ratio of concentration in bile:unbound drug in plasma is 3–10. CL 11,366 shows a higher ratio for the dog, about 50 (Fig. 3). In the rat, a single experiment with CL 11,366 at 20 mg/kg i.v. yielded at 1 hr a plasma concentration of 9 μg/ml and bile concentration of 5 μg/ml (not shown). Since plasma binding is about 90% (14), this also yields a high ratio of bile:plasma unbound. Both these drugs also show higher concentrations in the livers of the rat than simultaneous concentration in plasma unbound (14). Methazolamide shows relatively little concentration in bile (Fig. 3) or in liver (14), over that in plasma. All three drugs, however, appear in both liver parenchyma and bile in at least the concentrations in plasma water, and thus are suitable for the functional studies which follow.

Biliary Electrolytes in the Dog

Table 3 shows the fundamental change in dog liver bile elicited by carbonic an-

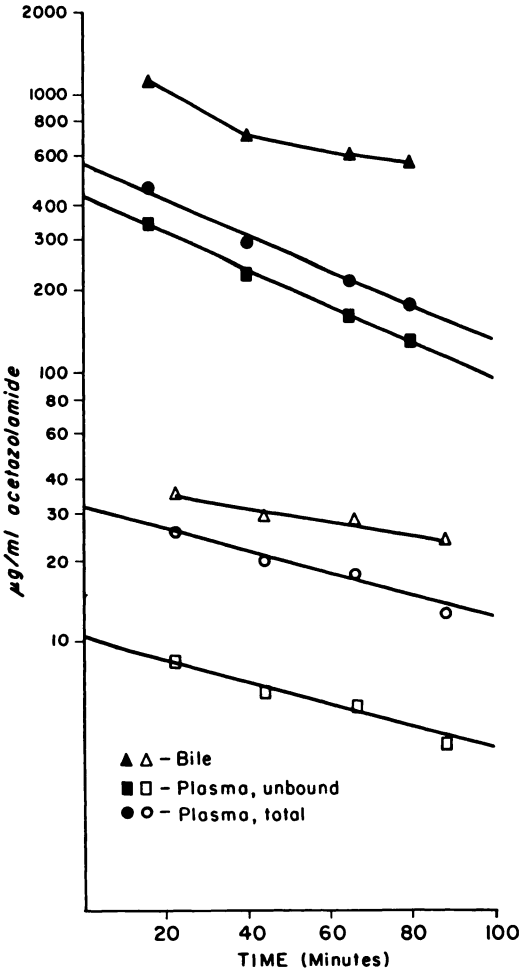

FIG. 2. *Concentrations of acetazolamide in bile and plasma of the rat, after intravenous injection*

Filled symbols, 100 mg/kg: open symbols, 10 mg/kg.

hydrase inhibition. The dose and drug chosen (3 mg/kg of CL 11,366) elicit no respiratory effect (14) and thus are particularly suitable for this purpose. Bile Cl^- concentration rises while HCO_3^- falls; Na^+ concentration rises, reflecting the fact that Cl^- rise exceeds HCO_3^- fall. K^+ concentration is unchanged. Flow was unaffected.

Figure 4 shows the time course of changes in biliary electrolytes following 20 mg/kg of acetazolamide (A) and methazolamide (B). Both experiments show a rise in Cl^- of about 25 mM. A second dose of acetazolamide, given when bile Cl^- returned to normal, reproduced the initial effect (A); a second dose of methazolamide, given when bile Cl^- was falling slowly toward normal, also restored the initial elevation (B). Acetazolamide produced a 7 mM fall in HCO_3^- (A) but methazolamide (B) did not. Methazolamide appears to be longer acting than acetazolamide, reflecting its longer half-life in both bile and plasma (Fig. 3).

Additional experiments at different doses with all three drugs were done to find the limits of activity, and to inquire why the HCO_3^- effect was not seen, for example in Fig. 4B, where Cl^- effect and the dose appear maximal. Table 4 summarizes the results of 22 experiments in the dog, using identical procedures. Several significant points emerge which were not entirely clear from individual experiments. Overall, the Cl^- rise is greater in magnitude and more reliable than the HCO_3^- fall. In all 18 experiments (those on the inactive dose of 1 mg/kg being excepted) there was a rise in biliary Cl^- concentration, while in 5 of these experiments no HCO_3^- fall (<1 mM) was observed. It appears that the maximal effect is a rise in the range 11–23 mM of Cl^-, with a decline of 6–9 mM of HCO_3^-. This is elicited with 20 mg/kg acetazolamide or methazolamide, and 6 mg/kg CL 11,366. The lower doses of 5 mg/kg acetazolamide and methazolamide, and 3 mg/kg CL 11,366 appear to elicit about half maximal Cl^- rise; the HCO_3^- fall is too small to quantify according to dose. If all experiments over 1 mg/kg are combined ($n = 18$; mM $\pm$ SE_{mean}) the Cl^- rise is 13.3 ± 1.6, and the HCO_3^- fall is 4.5 ± 0.8. Combining all experiments with no drug and 1 mg/kg ($n = 6$), Cl^- rise was 2.3 ± 0.8 and HCO_3^- fall 0.2 ± 0.9. For the drug effect on Cl^- rise $P = 0.005$, and for the HCO_3^- fall $P = 0.02$. Analyses of these experiments shows no consistent effect on bile flow (cf. Table 3 and Fig. 4). In the 18 drug experiments just cited there was a slight average increase of flow, from 171 μl/min before drug to 207 μl/min after drug. In the six controls flow was initially 163 μl/min and ultimately 161 μl/min.

Table 5 shows the effect of hyperventilation on biliary electrolytes in the dog. This

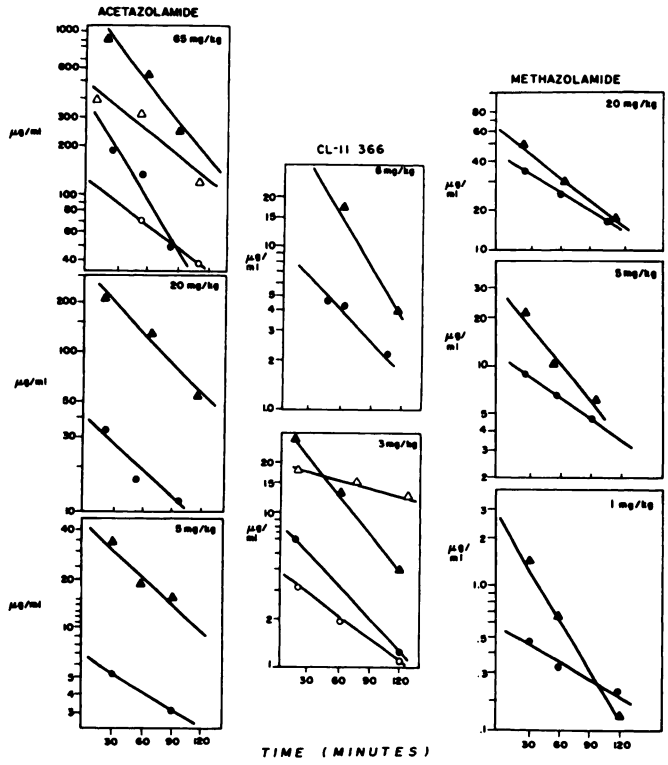

FIG. 3. *Decay of sulfonamides from plasma and bile of dog*

All drugs given intravenously at 0 time. ▲ and △ (replicate experiments) show drug concentration in bile. ● and ○ show total drug concentration in plasma. Plasma binding (%) of these three drugs in the dog:

μg/ml	Acetazolamide	CL 11,366	Methazolamide
1–10	60–40	93–92	55
10–100	33	—	—

clearly mimics that of carbonic anhydrase inhibition, qualitatively and quantitatively, with the reservation that the HCO_3^- fall appears to exceed that following the sulfonamides. In the experiments of Table 5, carbonic anhydrase inhibition produces no further change, following the hyperventilation effect.

Biliary Electrolytes in the Rat

Table 6 shows representative experiments with acetazolamide and CL 11,366, in which there was no change in the biliary output of chloride or bicarbonate. Table 7 summarizes all experiments with both drugs, and again shows that biliary output of these ions after carbonic anhydrase inhibition in this species does not differ from those of controls.

DISCUSSION

The data obtained in the dog agree in the main with those of Wheeler and Ramos (1). It is worth noting that there were some differences in procedure. Their dogs were trained, splenectomized, and used without anesthesia; these factors do not appear to be significant in the present context since their results and ours are similar. In several experiments (not shown) it

TABLE 3

The effect of carbonic anhydrase inhibition (CL 11,366) on biliary electrolytes in the dog

	A. No drug[a]				B. 3 mg/kg i.v. of CL 11,366 at 60 min[b]							
		Conc. in bile (mM)		Plasma		Concentration in bile (mM)				Plasma		
Time (min)	Flow (μl/min)	CO_2	Cl^-	CO_2 (mM)	Flow (μl/min)	CO_2	Cl^-	Na^+	K^+	CO_2 (mM)	pH	pCO_2 (mm Hg)
15	112	28	76	—	210	37	88	168	5.3	—	—	—
30	100	27	78	21	210	41	88	—	5.3	—	—	—
45	100	27	76	—	220	39	88	168	5.4	—	—	—
60	100	28	78	—	240	40	88	168	5.3	21	7.36	36
75	75	29	78	23	240[c]	40	100	178	5.5	21	7.33	38
90	87	29	78	—	240	38	90	174	5.6	—	—	—
105	87	29	80	—	230	35	100	178	5.7	—	—	—
120	100	29	80	23	220	35	102	182	5.7	21	—	—
135	—	—	—	—	230	34	104	184	5.7	—	—	—
150	100	31	—	—	220	34	102	—	5.7	21	—	—
240	50	31	84	—	—	—	—	—	—	—	—	—

[a] Experiment 9, weight 10.2 kg.

[b] Experiment 7, weight 13.2 kg.

[c] Drug given at the beginning of this period.

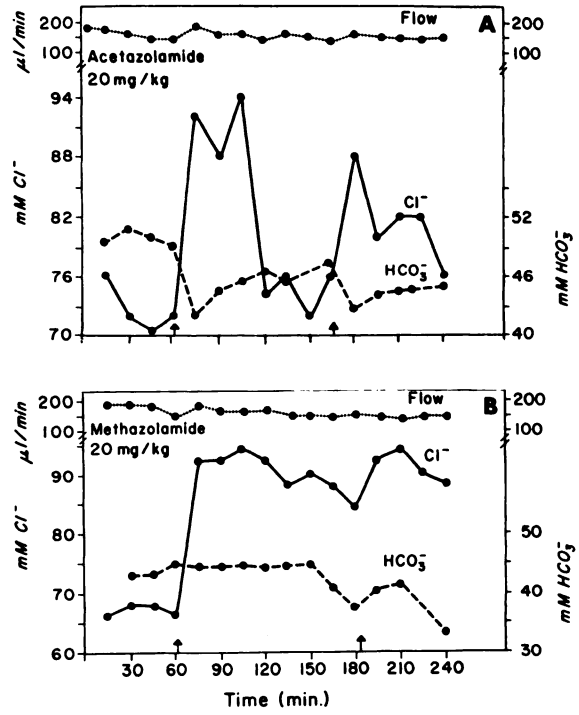

FIG. 4. *Cl^- concentration (•——•) and HCO_3^- concentration (•–•–•) and flow (•...•...•) in dog bile following 20 mg/kg i.v. of acetazolamide (A) or methazolamide (B) given at arrows.*

TABLE 4
Dose response of biliary anions to carbonic anhydrase inhibitors in the dog

Drug and number (n) of experiments at each dose	Mean of 4 periods (15 min) after—before drug (mM)									
	1 mg/kg		3 mg/kg		5 mg/kg		20 mg/kg		65 mg/kg	
	Cl^-	HCO_3^-	Cl^-	HCO_3^-	Cl^-	HCO_3^-	Cl^-	HCO_3^-	Cl^-	HCO_3^-
Methazolamide (2)	−0.5	0	—	—	+7	−9	+23	−3.5	+16	−4
Acetazolamide (2)	0	0	—	—	+9	−7	+11	−6	+18	−2
CL 11,366 $\pm SE_{mean}$	—	—	+8±4 (n = 5)	−3±5	+17 (1)[a]	−3	—	—	—	—

[a] 6 mg/kg.

TABLE 5
The effect of hyperventilation on biliary electrolytes in the dog: addition of carbonic anhydrase inhibition to the respiratory alkalosis

Time of sample	Experiment 4[a]				Experiment 5[b]					
	Flow (μl/min)	Conc. in bile (mM)		Plasma CO_2 (mM)	Flow (μl/min)	Conc. in bile (mM)		Plasma		
		CO_2	Cl^-			CO_2	Cl^-	CO_2 (mM)	pH	pCO_2 (mm Hg)
15	230	36	86	—	157	41	76	—	—	—
30	220	35	88	—	166	—	78	—	—	—
45	220	34	90	—	160	—	76	24	7.36	41
60	240	31	86	21	160	36	78	—	—	—
				Hyperventilation started						
75	166	17	118	—	166	31	88	17	7.53	20
90	140	—	116	19	170	—	98	—	—	—
105	140	17	116	—	166	26	98	—	—	—
120	150	18	120	12	166	25	102	17	7.63	17
135	150	19	120	—	200[d]	24	102	14	7.48	19
150	150	—	—	—	186	24	102	—	—	—
165	150	—	116	—	173	—	—	16	7.44	23
180	145	18	116	12	173	—	102	—	—	—
195[c]	133	16	108	14	—	—	—	—	—	—
210	133	17	104	—	—	—	—	—	—	—
225	145	20	108	—	—	—	—	—	—	—
240	150	—	108	14	—	—	—	—	—	—
255	157	19	—	—	—	—	—	—	—	—

[a] Weight 11.2 kg.
[b] Weight 13.2 kg.
[c] 3 mg/kg CL 11,366 i.v. at beginning of this period, i.e., 180 min.
[d] 3 mg/kg CL 11,366 i.v. at beginning of this period, i.e., 120 min.

was observed that without taurocholate infusion, but with flows in the range found with taurocholate, acetazolamide had somewhat less effect in increasing chloride and reducing bicarbonate output. Whether this is significant or not must be reserved for a separate study. Taurocholate alone has no immediate effect of raising Cl^- or lowering HCO_3^- concentrations. Table 3 (control) shows a trivial rise in Cl^- and HCO_3^-

TABLE 6

Effect of carbonic anhydrase inhibition on biliary anions in the rat. Representative types of protocol

Experiment[a]	Collection time (min)	Total CO_2 (mM)	Cl^- (mM)	Flow (ml/10 min)
Control, ether anesthesia, 300-g rat	0–28	27.7	80.8	0.071
	28–50	29.4	86.0	0.086
	50–75	33.9	85.5	0.070
Control, no anesthesia, 440-g rat.	0–20	32.3	86.0	0.100
	20–35	31.1	89.3	0.147
	35–45	31.6	93.0	0.143
Acetazolamide, 20 mg/kg, ether anesthesia, 410-g rat.	0–12	30.8	84.8	0.250
	12*–25	30.6	87.3	0.154
	25–38	31.5	90.2	0.166
	38–60	31.7	74.0	
11,366, 20 mg/kg, no anesthesia, 340-g rat.	0–13	26.7	86.2	0.150
	13*–33	27.6	90.5	0.150
	33–51	29.7	95.3	0.120
	51–73	30.2	95.6	0.170
Acetazolamide, 20 mg/kg, no anesthesia, 400-g rat.	0–18	25.5	87.0	0.167
	18*–32	31.1	85.8	0.164
	32–47	37.2	88.2	0.133
	47–60	38.1	91.0	0.154

[a] Representative experiments from each of the groups. The first collection period is a control; subsequent periods are after administration of drug as indicated by asterisk.

TABLE 7

Summary of experiments on biliary anions in the rat. Means of data for all groups

Time (min)	Bile CO_2 (mM)				Bile Cl^-, mM			
	Acetazolamide			CL 11,366	Acetazolamide			CL 11,366
	0[a]	20	75	20	0	20	75	20
15	—	30.3 ± 1.1[b] (12)	—	—	—	84.6 ± 1.7 (12)	—	—
30	30.6 ± 1.7 (6)	29.2 ± 1.5 (4)	—	27.6 (1)	87 ± 1.7 (6)	91 ± 2.2 (5)	—	90 (1)
45	33.0 ± 1.7 (6)	32.1 ± 2.2 (5)	30.5 (1)	29.7 (1)	90 ± 1.8 (5)	92 ± 2.7 (5)	92 (1)	95 (1)
60	33.8 (2)	34.1 ± 3.0 (4)	32.3 (1)	—	87 (2)	89 ± 4 (4)	—	96 (1)
75	—	32.0 (2)	35.6 (1)	30.2 (1)	—	95 (2)	96 (1)	—

[a] Drugs were given intravenously, in the doses (mg/kg) indicated, at the end of the first (15 min) period. Plasma concentrations in the rat are about 22 mM for CO_2 and 110 mM for Cl^-.

[b] Means and SE_{means} are given. Number of replicates indicated in parentheses.

with time. In experiments without taurocholate, a slight rise in chloride was also observed.

Biliary secretion is essentially like that of the pancreas with respect to anions, being high in bicarbonate and low in chloride, relative to plasma. Both liver and pancreas contain carbonic anhydrase, and inhibition of the enzyme lowers bicarbonate and increases chloride output in the secreted fluid (17). There are certain differences of interest: enzyme inhibition decreases flow of pancreatic juice, but there is usually no change and occasionally an increase in the flow of bile. Pursuit of these points are beyond the present scope; it appears to us that the fundamental chemistry of the two fluids is similar with respect to electrolyte formation. A significant parallel is the response to hyperventilation; in both pancreas (17) and liver (Table 5) chloride output rises and bicarbonate output falls, just as in carbonic anhydrase inhibition. Thus, reduction of substrate (CO_2) has the same effect as reduction of enzyme.

But on closer analysis it seems implausible that reduction of pCO_2 per se should lower the observed rate in pancreas or liver. For the observed *in vivo* rate is only a small fraction of the enzymic rate; the true enzymic rate, based on E_0 in the organ is so great that it should function at any known pCO_2 (discussion for pancreas in ref. 17 and for kidney in ref. 16). Further evidence in this direction is given in the discussion to follow, which will show that physiological inhibition of biliary electrolytes is not apparent until enzyme inhibition exceeds 99%.

How, then, does hyperventilation produce such profound effects, which precisely mimic carbonic anhydrase inhibition, in kidney, pancreas, and liver? The most reasonable explanation appears to be in the large pH rise which is engendered in the secretory cell by sudden lowering of pCO_2. In this view, the role of the enzyme normally is to maintain a constant pH by buffering of OH^- with CO_2, in cells which secrete H^+ or OH^-, or have secretory functions which in some way are dependent on separation of H^+ and OH^- within the cell. Hydroxyl ion may rise *either* from insufficiency (inhibition) of enzyme to equilibrate $CO_2 \rightleftharpoons H_2CO_3$ at the proper rate *or* from loss of CO_2 in the presence of enzyme, which will instantly lower H_2CO_3.

Data are available from which the degree of enzyme inhibition during the physiological response may be measured. This may be done, as previously described (15), using the Easson-Stedman rearrangement of the Michaelis equation:

$$I_0 = K_I\left(\frac{i}{1-i}\right) + iE_0$$

where I_0 is the concentration of inhibitor in tissue, available to react with enzyme; K_I is the dissociation constant of the enzyme inhibitor complex; i is fractional inhibition; and E_0 is the concentration of enzyme in liver of untreated dogs.

The numerical values are estimated as follows: For acetazolamide, drug concentration in liver (14) and bile (Fig. 3) are higher than the unbound concentration in plasma. However, this latter value will be taken as the minimal one which will be in equilibrium with enzyme in the liver. I_0 at 30 min after 20 mg/kg, which gives a maximal response (cf. Table 4), is taken from the unbound concentration in plasma. Figure 3 shows the total concentration in plasma to be 30 μg/ml at this time, whence the unbound concentration is 15 μg/ml or, $I_0 = 67$ μM. K_I for acetazolamide against dog liver enzyme (Table 2) is 0.45×10^{-7} M (16) and since in general the K_I at 37° is higher than that at 0°, we will take 1×10^{-7} M as the figure for dog liver. E_0, the concentration of enzyme, is derived from the activity, approximately 11 units/gram (Table 1), times the factor which we have used to convert activity units into micromolar terms. This factor, for dog blood, was 0.017; it was based on the fact that 1 enzyme unit/7 ml in the *in vitro* carbonate buffer system was equal to 2.4×10^{-9} M (10). As shown above, the corresponding value for dog liver is 5×10^{-9} M, whence the conversion factor is 0.035. E_0 is then 11 units/gram $\times$ 0.035 = 0.39 μM. In the above equation the term

$K_I\left(\frac{i}{1-i}\right) \gg i\ E_0$ for values of i above 0.9, so that the second term on the right may be dropped yielding:

$$i = \frac{I_0}{I_0 + K_I}$$

This is equivalent to stating that $I_0 \cong I_{free}$, for the case where $I_0 \gg E_0$ (16). Solving, in μM:

$$i = \frac{67}{67 + 0.1} = 0.9984$$

Thus, for acetazolamide, 99.8% of dog liver enzyme must be inhibited to give a full response, since lower doses than 20 mg/kg are not fully effective. For the totally inactive dose of 1 mg/kg, inhibition in the liver may be calculated in similar fashion as 97%.

It is of interest that methazolamide, which does not appear to be actively secreted or accumulated by the dog liver, gives a similar physiological response to that of acetazolamide and CL 11,366. This is similar to the situation in the kidney (15), and suggests that a drug may arrive at the enzymic site by either diffusion or active transport. It is also significant that CL 11,366 produces its hepatic effect at doses (3–6 mg/kg) below that necessary for inhibition of red cell carbonic anhydrase (14).

Carbonic anhydrase in whole dog liver and the fractions respond *in vitro* to the inhibitors tested in much the same way as dog blood and rat blood (Table 2). In general, this is the quantitative pattern observed for human red cell carbonic anhydrase C (11). The finding of enzyme in particulate fractions agrees with the data of Karler and Woodbury (3) and Datta and Shepard (2), although the latter authors attributed this to contamination by the very high concentration in the supernatant.[2] The value of E_0/e.u. obtained for dog liver carbonic anhydrase (Fig. 1) is close enough to those for blood and kidney of various species, and that of human red cell C (11) that we may tentatively take its turnover number—the fundamental measure of enzyme activity—to be close to those of the other, more commonly studied, enzymes. Thus, the dog liver enzyme conforms, in its *in vitro* and *in vivo* activities, to many of the other carbonic anhydrases.

Results in the rat were completely at variance with those in the dog. From the control data, it appeared that the two species were alike, based on the anion composition of bile, the presence of hepatic carbonic anhydrase, and the distribution of enzyme in the various cell fractions. The pharmacology of the drugs in the two species, with respect to appearance in the bile, also appears similar. However, inhibition of whole rat liver and supernatant *in vitro* required enormous concentration, particularly when $i \geqq 0.90$ was sought, and there was no *in vivo* effect following acetazolamide or CL 11,366 in large doses. This situation was entirely unexpected. It is the first example of a vertebrate carbonic anhydrase whose sensitivity to the sulfonamides is (depending on the criterion used) 10–3000 times less than that seen in the red cell or kidney preparations, crude or purified, which are generally studied. It is also the first example of such a striking species difference in the *in vivo* response.

A review of the literature shows that while physiological responses have been obtained with carbonic anhydrase inhibitors in a variety of vertebrate species, only rarely has the sensitivity of the organ *in vitro* been studied. Indeed, this has never been an issue, since responses to these drugs have been quite uniform. In mammals, for example, there is no known case where the renal response, or the ocular response, cannot be elicited. Admittedly, a wide range has not been studied; renal work includes man, dog, rat, rabbit, and mouse; ocular work includes man, dog, rabbit, and guinea

[2] These authors (2, 3) found a similar distribution for kidney carbonic anhydrase. All fractions of rat and dog kidney carbonic anhydrase are sensitive to sulfonamides, to the degree shown in Table 1 for blood (T. H. Maren and A. C. Ellison, manuscript in preparation). Rat and dog gastric carbonic anhydrase is also sensitive to the sulfonamides (T. F. Muther, personal communication).

pig. Frogs and alligators show a renal response to acetazolamide. Failure of renal response in the marine elasmobranch and teleost is due to absence of enzyme in secretory tissue. Red cell enzyme in fish is sensitive to the sulfonamides, both *in vivo* and *in vitro* (11). The only carbonic anhydrase previously found unresponsive to the sulfonamides *in vitro* is the zinc-free enzyme of the plant (18). The present finding provides a stimulus to the systematic examination of some vertebrate carbonic anhydrases, with respect to their kinetic properties and susceptibility to inhibition. Isolation and structural studies of rat liver enzyme would be exceedingly difficult because of the very low concentration involved—about 0.003 that in red cells.

Based on the I_{50} data for CL 11,366 and rat liver, one might expect an *in vivo* response, since this figure (0.43×10^{-7} M) while considerably higher than I_{50} for this drug against other carbonic anhydrases (Table 1) is about the same as the I_{50} for acetazolamide against dog liver. However, the I_{90} for CL 11,366 is 1000 times greater than the I_{50}. All the drugs tested showed an increment of about this magnitude for rat liver, which is completely at variance with data for the other carbonic anhydrases and with the theoretical relation, which states that $I_{90}:I_{50}$ is approximately 10, when $I > E$, or less than 10 when I approaches E. There is no final explanation at hand for this anomalous finding, although it does suggest that a mixture of enzymes is present, with radically differing responses to the drugs. But since *in vivo* response to carbonic anhydrase inhibition (ref. 15 and above) has been invariably associated with $i > 0.99$, it is clear that the *in vitro* data are entirely satisfactory in explaining the lack of *in vivo* response in the rat.

It is of further striking interest that carbonic anhydrase in the particulate fractions of rat liver is normally responsive to the sulfonamides. Obviously, enzymes in supernatant and particulate are very different, and the physiologic effect is due to the supernatant enzyme.

It is significant to note that the I_{50} and I_{90} data for dog liver among the different drugs agree well with those for dog and rat blood. On the other hand the I_{50} data for rat liver show acetazolamide to be somewhat less active than sulfanilamide, which has never been observed in any other carbonic anhydrase system (11).

The present work explains the apparent discrepancy in the literature between the results of Ramos and Wheeler (1), who used the dog, and Bizard *et al.* (4), who used the rat. The question of the response of human liver carbonic anhydrase remains open, although the single study by Fink (5) suggests that man may respond like the dog. It is evident that further chemical and physiological work in this field remains to be done, but encouraging that the *in vivo* response is a faithful consequence of enzyme inhibition as it is revealed *in vitro*.

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