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THE ACTIVITY OF SULFONAMIDES AND ANIONS AGAINST THE CARBONIC ANHYDRASES OF ANIMALS, PLANTS, AND BACTERIA

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INTRODUCTION

For nearly thirty years after the discovery of carbonic anhydrase in red cells and tissues of the animal kingdom (1), susceptibility to inhibition by sulfonamides was found to be about the same, whatever the enzyme source. Although no systematic studies were done, tissues as different as human red cells and clam mantle showed similar susceptibility to sulfonamide inhibition (reviewed in 2); at the time isozymes were not in the forefront, and there seemed to be no special problem at hand. It was found that the plant enzyme was different, requiring some 10^3-fold greater concentration of acetazolamide, for example, than the 10^{-8} M needed for 50% inhibition of the animal enzymes thus far studied (3).

The first example of very different behavior in animal tissues was discovered accidentally in this laboratory. We found that rats did not respond to acetazolamide in terms of the alterations in hepatic electrolyte excretion known for dog and man (4). After an initial rude dismissal of the possibility that the rat liver enzyme might be different (in all other carbonic anhydrase systems rats behaved normally), we found that this was indeed the case. In male rat liver the K_I for acetazolamide was 10^4 times higher than the "standard value." The universe had been disturbed (5), at least a microcosm of it.

Meanwhile, work was being done on the differing properties of the two isozymes in primate red cells (and those of certain other species) (6), now designated carbonic anhydrases I and II, the latter having nearly 10-fold

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higher CO_2 hydrase activity. The role of isozyme I in respiration is negligible (7), but in view of its high concentration in the red cells and its close structural relation to II (which is also the secretory enzyme in tissues), it has been studied as an example of gene duplication (8) and as a possible catalyst for substrates other than CO_2 (9).

Sulfonamide inhibition required some 10-fold higher concentration for isoenzyme I when sulfanilamide or acetazolamide were used, but there was less difference, and often none, for other compounds (2). Of far greater magnitude, and probably of basic significance, was the discovery that human carbonic anhydrase I was about 100 times *more* susceptible to inhibition by inorganic anions than enzyme II (10).

The most recent chapter in this story has been the finding of a hitherto undetected, very low-activity carbonic anhydrase, now designated isozyme III, in skeletal muscle. It is resistant to sulfonamides, and its susceptibility to anions resembles that of isozyme I; astonishingly, it appears to be the same protein as the enzyme from male rat liver (11).

Because of the complexity of these developments, their theoretical implications, and the practical application of using inhibitors in studying the isozymes, we decided to compile the available data on the reversible inhibition of these carbonic anhydrases by certain sulfonamides and anions. We do not discuss irreversible inhibitors that form covalent bonds with active site histidines. For interest and completeness, we include data on crude enzyme preparations through the animal and plant kingdoms, and on bacteria. We give data for inhibition of CO_2 hydrase activity. These kinetic K_I's will be strictly equal to the dissociation constants of the E-I complexes only when inhibition is purely noncompetitive with CO_2. As will be evident from the next section, the nature of anion inhibition is largely noncompetitive with CO_2 at physiological pH, so that the measured I_{50} is independent of the concentration of substrate used (12, 13). For sulfonamides, the exact nature of inhibition still eludes us, but we treat this issue as a special case of anion inhibition with some justification, as described below. Thus, in general, $K_I = I_{50}$, as the concentration of enzyme used in the assay is usually much less than I_{50}. Where this is not the case, either because for very active inhibitors I_{50} is small, or for low-activity enzyme E is high, the relation $K_I = I_{50} - 0.5E$ is used.

THE CHEMICAL AND KINETIC NATURE OF INHIBITION

The amazing chemical specificity of the unsubstituted aromatic sulfonamides for carbonic anhydrase remains unchallenged since it was discovered 43 years ago (14). The matter has recently been reviewed (15). All chemicals

of this class are inhibitory, with dissociation constants ranging from 10^{-4} M to 10^{-9} M; no other class of organic compounds has notable activity ($K_I < 10^{-5}$ M), except certain mercaptans (16).

Anions were discovered to be inhibitory when the enzyme was first found, and experiments with CN^- were decisive in revealing catalytic activity (1). Further work over the years showed that there was an orderly progression of activity against the bovine red cell enzyme (chemically analogous to human red cell enzyme II) ranging from the K_I of chloride (about 10^{-1} M) to that of CNO^- (10^{-5} M). More recently, as noted above, we have found that these values are 100-fold less for human red cell enzyme I (10). The progression of activity among the halides conforms to a Hofmeister series and agrees with their activity in certain other biological systems (17).

The mechanism of inhibition is a complex topic, treated here in outline. There is ample experimental evidence to suggest that for binding of anions the protonated form of the catalytic group (metal-bound water) is required (6). The inhibition by anions weakens as the pH is raised due to competition with OH^-. X-ray diffraction work suggests that anions occupy the fourth coordination site of zinc (18). The strongest anionic inhibitors, such as cyanide, react with the metal in the enzyme in a complexometric fashion. This was shown by a spectroscopic study using Co (II) substituted human enzyme I (19). At low pH, HCN (pKa = 9.3) reacts with $E\text{-}H_2O^+$ forming E-CN, with concomitant release of H^+ from HCN and H_2O from the enzyme. At high pH, CN^- displaces OH^- from E-OH. These findings are supported by recent kinetic data (20). The effects of weaker inhibitors depend on their size, charge, and ability to affect the water structure in the active site (6). Thus, the order of inhibitory activities of the halides follows the Hofmeister series, which runs opposite to the order of the stability of zinc-halide complexes (21).

The kinetic mechanism of anion inhibition has been successfully studied, as anion binding is not usually complicated by the problem of slow dissociation that prevails for many sulfonamides. When HCO_3^- is the substrate, anion inhibition appears classically competitive (12). Kinetic competition of anions with OH^- has also been shown (12). With CO_2 as substrate, in the neutral pH range, anion inhibition has been found to be noncompetitive (13) or mixed with a large noncompetitive component (12). At higher pH, uncompetitive inhibition has been reported for bovine red cell carbonic anhydrase (22). Independently, two other laboratories have made this observation for human red cell isozyme II [(23); Conroy and Maren, unpublished observations]. Pure uncompetitive inhibition is rarely encountered, especially for a single substrate-single product interconversion. One observes an increase in inhibitory activity with increasing substrate concentration, an effect that is qualitatively opposite that demanded by competitive

inhibition. Two different interpretations have been proposed for carbonic anhydrase II. 1. The binding of CO_2 to the basic form of the enzyme (E-OH) relieves the negative charge density constraint, allowing binding of anion as a fifth ligand (22). A more plausible interpretation (23), consistent with the proton-transfer mechanism of carbonic anhydrase catalysis (24, 25), is the following: 2. A high CO_2 concentration favors a rapid conversion of CO_2 to HCO_3^-, and simultaneously of E-OH to E-H_2O^+ where the catalytic group is protonated. In the overall catalytic cycle the rate-limiting step, when external buffer (B) is present at 10mM or higher, is the transfer of proton from the catalytic group (zinc-water) to an acceptor group (most likely His-64 in isozyme II) in the active-site, i.e. E-H_2O^+ ⇌ H^+E-OH. Since this is the slowest step, E-H_2O^+ accumulates in the steady state when CO_2 concentration is high, leading to an increase in the affinity of anions. This scheme is described in Figure 1. An important finding is that human carbonic anhydrase I does not show uncompetitive inhibition (C. Conroy and T. H. Maren, unpublished observations), which suggests that it has a different active site mechanism than isozyme II.

X-ray diffraction work suggests that the sulfonamide group of aromatic sulfonamides occupies the fourth coordination site of the metal, linked to the nitrogen atom, replacing water (26). In addition, one of the sulfonamide oxygens is in the fifth ligand site (26). Based on the pH-dependence of the rate constants for the formation of carbonic anhydrase-sulfonamide complexes, a direct association of the ionized sulfonamide group (R-SO_2NH^-) with the protonated form of the active site was proposed (27). More recently, evidence for coordination between the metal and the sulfonamide nitrogen has been found by nuclear magnetic resonance experiments on ^{113}Cd-substituted carbonic anhydrase (28). The SO_2NH_2 groups in all known

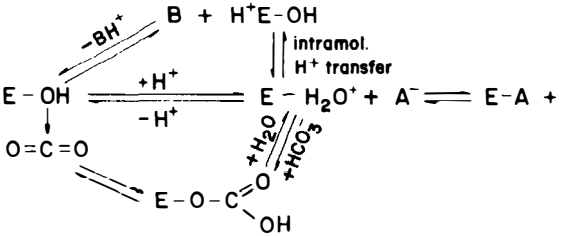

Figure 1 A schematic representation of one catalytic cycle of carbonic anhydrase and anion binding to the enzyme. E-OH and E-H_2O^+ are the basic and the protonated forms of the catalytic group. E-OH is active in CO_2 to HCO_3^- conversion; E-H_2O^+ catalyzes the reverse reaction. H^+E-OH is the form where the proton on the catalytic group has been transferred to an acceptor group. B and BH^+ are the basic and the acidic forms of the external buffer. Most anions (A^-) bind only to E-H_2O^+, and not to E-OH. For simplicity we consider only the most likely mode of binding for univalent anions, direct metal coordination by displacing H_2O.

mides are weak acids with pKa's ranging from 6.6 to 10.4 (29). In addition to this ionization, the lipophilicity of the compound is also important. The aromatic part of the molecule is in contact with the hydrophobic region of the enzyme active site. Thus sulfonamides have been found to bind to apocarbonic anhydrase (30), although association is some 10^4 times weaker than that with the holoenzyme. The binding to holoenzyme is thus a two-stage process: (*a*) hydrophobic association also demonstrable with apoenzyme; (*b*) pH-dependent coordination of $R\text{-}SO_2NH^-$ with the metal (30). The overall or final K_I is a product of these two interactions. For inorganic anions, interaction (*a*) does not occur, which accounts for their weaker activity against the enzyme compared to aromatic sulfonamides. The dissociation constants for apoenzyme-sulfonamide complexes are 10^{-3} to 10^{-4} M. This is of the same order of magnitude as the difference in activity (against isozyme type II) between the most potent of the anions and the most potent sulfonamide; this then is an independent measure of the hydrophobic stage (*a*) of sulfonamide binding.

The kinetic mechanism of inhibition by sulfonamides is not clearly known. Competitive inhibition patterns have been found that use HCO_3^- as substrate (31). Reports of noncompetitive inhibition with respect to CO_2 have appeared (32, 33), but these did not take into account the slow rates of dissociation of the enzyme-sulfonamide complexes. If these rates are significantly slower than the rates of decomposition of the enzyme-substrate complex, then noncompetition with the substrate will be seen regardless of the true mechanism (34). Inspection of the plots for sulfanilamide, acetazolamide, and ethoxzolamide in(35) suggests a mixture of noncompetitive and competitive kinetics. This is borne out by the finding that, for these and many other drugs tested, the I_{50}'s or K_i's increase in the presence of CO_2. For the binding of sulfanilamide to Co(II) substituted bovine carbonic anhydrase, biphasic kinetics was observed, the latter phase of which was competitive with CO_2 (36).

Worth brief mention although outside the scope of this review is the fact that the individual rate constants (k_{on} and k_{off}) have been measured for eight of the sulfonamides, against the two human red cell enzymes (30a).

PROPERTIES OF INHIBITORS

In this essay, data are given on the equilibrium inhibition (dissociation) constants of four sulfonamides and three inorganic anions against many of the isozymes. The sulfonamides cover a wide range of inhibitory activity, and do not discriminate in the same way against the different enzymes. Among the anions, we show a high- and a low-activity halide, and the most

active of the group, a nonhalide. Most investigators, of course, did not use all seven compounds, so to compare different studies it is necessary to have several reference points. Specific reasons for using these compounds and brief references to their properties are given below. The characteristics of the four sulfonamides have been described (2).

SULFANILAMIDE This was the chief compound used between 1940 and 1952, when acetazolamide was discovered. However, sulfanilamide is still of great importance. It is quite water-soluble and highly diffusible. Its pKa is 10.4. Like acetazolamide, it discriminates between enzymes I and II. It is, however, a much weaker inhibitor, lacking a thiadiazole structure. The relation between off and on rates is such that equilibrium with enzyme is reached within about 10 seconds.

ACETAZOLAMIDE This has become the standard drug in the field since it was the first to get into widespread clinical and physiological use. Virtually every study used this compound, but it is a mistake to generalize from it. Activity is greater against enzyme II than enzyme I. It is only moderately diffusible. Its first pK is 7.4; the second (macroscopic) is 8.8, but microscopic is 7.4 (27). Full equilibration with enzyme requires two minutes in presence of CO_2 at 0°C.

METHAZOLAMIDE Although rather closely related to acetazolamide and usually with the same activity, it has some very different properties. It is both more water- and lipid-soluble and more diffusible. Its pKa is 7.4. It does not discriminate between I and II. It is better suited for physiological work than acetazolamide, and many consider that it is also more appropriate for use in glaucoma. Equilibration time is 30 seconds or less.

ETHOXZOLAMIDE This is a very high-activity drug, so it can be used to "titrate" many of the isozymes and develop Easson-Stedman or Straus-Goldstein plots, yielding the molar concentration of enzyme. It is very lipid-soluble; pKa is 8.0. Ethoxzolamide does not discriminate between I and II. Equilibration with enzyme takes about 5 minutes at 0° when CO_2 is present.

ANIONS Cl^- is used because of obvious physiological importance. I^- parallels Cl^-, and eliminates the problem of ionic strength as it is active at lower concentration. CNO^- is used because it is not a halide, and because of its very high activity against some of the isozymes, which may make it suitable for physiological work.

INHIBITION DATA

Activity Against Pure Red Cell Isozymes

The pattern of inhibition shown in Table 1 for human red cell carbonic anhydrase II and associated enzymes of high turnover number is considered as a base. As will be seen, this is the common pattern for the red cells of most species, and importantly, for the enzyme(s) in secretory tissues of vertebrates, many invertebrates, and even bacteria. For sulfonamides, the features of this pattern are the K_I ranging from about 10^{-6} to 10^{-9}M; for anions from 0.2M to 2×10^{-5}M. In the case of sulfonamides, weaker inhibitors are known (2), but stronger ones are not detectable because the enzyme concentration in the assays used cannot be much lower than 10^{-9} M. Among the anions, weaker inhibitors than Cl^- cannot readily be detected because of ionic strength effects. Under ordinary conditions F^- and SO_4^{2-} are without effect at 0.5 M. The most potent anions are CNO^- (10), CN^- (23), and HS^- (6).

Red cell enzyme I departs from the pattern seen with II in two respects. The first and less important is that certain compounds (e. g. acetazolamide and sulfanilamide) are about 20-fold less inhibitory. This is not a fundamental property of enzyme I, as it is not found with methazolamide or ethoxzolamide. The discrimination appears limited to those compounds that have a dissociable proton in the molecule, other than in the $-SO_2NH_2$ group. The second and more significant finding is that enzyme I is 30–100 times more sensitive to anions than is II. This includes CN^- (23), which is not listed in the original paper (10). The only known exception is perchlorate (10). The data in Table 1 are from the human enzyme I; red cells from other primates (37), as well as those from rat (38), mouse (39), and turtle (40), also have what appears to be the same isozyme. It will clearly be important to test the anion sensitivity from these sources.

As shown below, other forms of low-activity carbonic anhydrases share the anion sensitivity of isozyme I. The reason for this is entirely unknown; conceivably these proteins have a role in Cl^- or I^- transport that remains to be discovered.

Table 1 also shows that the purified enzyme from red cells of a primitive vertebrate, the hagfish, *Myxine glutinosa,* has the same susceptibility to acetazolamide and the anions as human II. A complete study on the single enzyme from red cells of a typical bony fish, *Archosargus probatocephalus,* shows again a susceptibility for all seven compounds tested, akin to enzyme II. As shown in Table 1, the turnover number of this teleost enzyme is intermediate between those of human I and II. These data, along with structural studies of the fish enzyme (41), strongly suggest that the main features of carbonic anhydrase II were laid down some 500 million years ago.

Table 1 Inhibition by sulfonamides and anions of purified carbonic anhydrase isozymes from vertebrate red cells[a]

Enzyme source[b]	$k_{cat} \times 10^{-4}$ (sec^{-1})[c]	K_I							
		Sulfanilamide (μM)	Acetazolamide (μM)	Methazolamide (μM)	Ethoxzolamide (μM)	CNO^- (μM)	I^- (mM)	Cl^- (mM)	References
Man I	2.9	50	0.2	0.01	0.002	0.7	0.3	6	12, 43, 44
Man II	23	2	0.01	0.01	0.002	20	26	200	12, 43, 44
Ox	9.3	—	0.004	—	—	—	8	94	13, 45, 46
Rabbit I	~1[d]	—	0.2	—	—	—	—	—	47, 48
Rabbit II	14	—	0.02	—	—	—	—	—	47, 48
Toad	—	—	0.01	—	—	—	—	—	48
Teleost	7.7	5	0.03	0.03	0.002	7	9	200	41
Hagfish	—	—	0.009	—	—	3	—	300	49

[a] All data are for CO_2 hydration. For sulfonamides, temperature was 0° in all cases except for the ox enzyme, in which it was 25°. For anion inhibition, temperature was 0° for teleost and hagfish enzymes, but 25° for man and ox. Recent unpublished data of C. Conroy, in our laboratory, show that the increase in K_i with temperature from 0° to 25° is at most 3-fold.

[b] I and II refer to low- and high-activity isozymes respectively. Ox red cells do not contain isozyme I. Toad, teleost, and hagfish do not have kinetically distinct isozymes.

[c] k_{cat} values (turnover numbers) are for 0° and pH 7.0–7.5.

[d] Recalculated from data in (47) to correct for chloride inhibition.

Kinetic and inhibition data on pure carbonic anhydrases from the several vertebrate classes are available (12, 13, 41–49). It will be observed that there is a high-activity type enzyme in the blood of each class studied.

Activity Against Crude Red Cell Hemolysates

All of the red cells tested, except those from the elasmobranch, *S. acanthias,* showed about the same susceptibility to acetazolamide as human red cell II (50). The same was true for sulfanilamide. None of the red cell preparations showed high susceptibility to the anions. This strongly suggests that in no case is carbonic anhydrase I the dominant enzyme in red cells. When there is a mixture of I and II, as in human (6), dog (51), turtle (40), guinea pig (52), or rat (38) red cells, the catalytic properties and apparent susceptibility to inhibition of II dominate because of higher turnover number. The concentration of drug that reduces the rate by 50% reflects its attack on the "faster" enzyme, II, or its equivalent.

Although all vertebrate classes have some species with red cell carbonic anhydrases functionally akin to II, chemical differences from human II are seen for example in chicken (53) and frog (54). Until more work is done in *Agantha,* it will not be clear whether the pattern arose in that subclass (c.f. *Myxine,* Table 1) or in the bony fish, in which it seems well-developed. The elasmobranchs appear to represent a curious divergence from the main pattern; in these red cells sulfonamide inhibition is at least one order of magnitude less than for the standard II, but anion susceptibility is not marked and is probably like II. Chemical work also suggests that the

Table 2 Inhibition by sulfonamides and anions of carbonic anhydrase activity in hemolyzed red cells of vertebrates

Enzyme source	Kinetic isozyme type[e]	K_I Sulfanilamide (μM)	Acetazolamide (μM)	Methazolamide (μM)	Ethoxzolamide (μM)	CNO^{-}[b] (μM)	Cl^{-}[b] (mM)
Human[a]	I + II	4	0.03	0.05	0.005	6	50
Dog[a]	I + II	5	0.01	0.01	0.002	32	250
G. pig[b]	I + II	15	0.01	—	0.002	4.5	—
Rat[c]	I + II	4	0.02	0.04	—	—	—
Chicken[b]	II	22	0.03	—	0.002	9	50
Frog[b]	II	6	0.04	—	0.002	6	150
Turtle[b]	I + II	17	0.02	—	0.002	10	530
Dogfish[d]	I	90	0.2	0.1	0.01	30	70

[a] Reference (2).
[b] Data of C. Conroy in this laboratory, obtained at 0°.
[c] Reference (4).
[d] Reference (50).
[e] Except for human enzyme, the designation I and II is not chemically precise, but means low- and high-activity respectively. See text for references. For guinea pig, rat, and turtle, there are no adequate inhibition studies on the pure isozymes.

elasmobranch red cell enzyme has special characteristics, including some 70 additional amino acids and disulfide bridges (55).

Activity Against Cytoplasmic Enzymes in Tissues

From the point of view of mammalian physiology and pharmacology, the first row (A) of Table 3 shows critical data. The enzyme from cytosol of all the secretory tissues yields an inhibition pattern indistinguishable from enzyme II. This agrees with kinetic and structural studies on some of these tissues showing that the enzyme is indeed of high activity and closely akin to II.

Row B of Table 3 gives data for the intestinal enzyme of the guinea pig taken from the caecum. This enzyme is supposed to be of the I type with respect to low turnover number, and there have been speculations that its function might be unrelated to CO_2 (52). However, sequence studies have not been reported, so its relations to enzymes I and II are not firmly known. The present data suggest that the intestinal enzyme is not I, as its K_I against Cl^- is 10 times higher, and against acetazolamide 10 times lower, than human I. However, bovine intestinal enzyme has been sequenced and appears analogous to isozyme I from human red cells (69). Further work is surely necessary.

Row C gives data for the cytosolic enzyme of male rat liver, and row D for striated muscle of cat (66, 67). Two rather astonishing facts emerge: For the first time we see a vertebrate carbonic anhydrase resistant to the sulfonamides—four orders of magnitude less sensitive than for enzyme II. In addition, the inhibition patterns of the enzymes from these two very different sources are alike, and indeed, the amino acid sequence of the cyanogen bromide fragments is indistinguishable (11). They are now designated carbonic anhydrase III. Additionally we note that these enzymes have high susceptibility to anions—the K_I values are the same as for enzyme I. We now see a pattern emerging in which low activity of the enzymes is associated with sensitivity to anions.

It should be emphasized that the male rat liver enzyme is a decided anomaly, as the enzyme in the female rat is sensitive to the sulfonamides, like the liver enzymes from both sexes in dog, man, and rabbit (4, 66). However, the muscle enzyme from both sexes and all species thus far studied in our laboratory (ox, rabbit, dog) appears to have the characteristics shown in Table 3, Row D.

These enzymes show for the first time a reversal of the relative activities of sulfonamides and anions. Now cyanate is some 200–600-fold more active than actazolamide; importantly, cyanate can inhibit muscle enzyme and leave enzyme II relatively untouched. This discrimination should become an important pharmacological tool. When the structural basis at the active

Table 3 Inhibition by sulfonamides and anions of cytoplasmic carbonic anhydrases of vertebrate tissues

Tissues	Kinetic isozyme type	K_I Sulfanilamide (μM)	Acetazolamide (μM)	Methazolamide (μM)	Ethoxzolamide (μM)	CNO^- (μM)	I^- (mM)	Cl^- (mM)
A. Mammalian secretory tissues[a]	II	3–7	0.004–0.04	0.01–0.05	0.001–0.008	10	9–80	100–700
B. Intestine g. pig caecum[b]	I	13	0.008	0.01	0.002	2.5	—	50
C. Male rat liver (66, 67)	III	>1000	150	15	150	0.7	1.0	11
D. Skeletal muscle (67)	III	>5000	306	96	54	0.5	1.1	6
E. Rectal gland of dogfish (50, 68)	II	—	—	0.2	0.01	30	390	>1000
F. Chicken shell gland[c]	?	25	0.04	—	—	10	—	140

[a] Includes the following mammalian tissues which have been shown to be of high activity like isozyme II: kidney, both cortex and medulla (44, 56, 57); liver except male rat (4, 58); stomach (2, 58); ciliary process (56, 59); lens (60, 61); and *Stria vascularis* (62). Presumptive evidence (not proved for type II) from inhibition data and/or in vivo responses exist for pancreas (63); choroid plexus (64); and parotid gland (65). Not all the sulfonamides or anions have been tested against all the tissues. The individual references must be consulted.

[b] Reference (52), and unpublished data of C. Conroy from this laboratory.

[c] Data of C. Conroy from this laboratory.

site for the vast fall-off in both activity and sulfonamide affinity is revealed, it will be a major advance in the understanding of how this enzyme works.

Rows E and F show several enzyme sources from secretory tissues in fish and birds. All show the usual sensitivity to acetazolamide. The shell gland of the chicken shows the anionic pattern of response of enzyme II. Interestingly, the rectal or salt gland of the elasmobranch, which produces a secretion of 0.5 M NaCl, is totally resistant to chloride inhibition. Note that the red cells of this same species, *S. acanthias,* is inhibited at 0.07 M chloride (Table 2).

In addition to the data of Table 3, there are some important unpublished responses to acetazolamide alone from the field of comparative physiology. The gill enzyme of teleost is inhibited (K_I) at $\sim 10^{-8}$M; that from elasmobranchs at $\sim 10^{-7}$ M; the nasal salt gland of the herring gull, *L. argentatus,* at 4×10^{-8} M; and the toad bladder enzyme at $\sim 10^{-8}$ M. Carbonic anhydrase from turtle bladder has a K_I of 4×10^{-8}M (70).

Activity Against Particulate Cell Fractions

The first row of Table 4 shows that the membranes of kidney have a different inhibition pattern from that of the cytosol, being less affected by acetazolamide (and other drugs; see Table 3) as well as by Cl^- and CNO^-. It will be essential to continue this work with other tissues.[1] These findings are of great interest; fortunately they do not change the basic idea that a very high degree of inhibition is necessary for physiological effects. Since the K_I of acetazolamide for kidney microsomes is 10-fold greater than previously thought (on the basis of data from II or from cytosol), fractional inhibition at full pharmacological doses for renal HCO_3^- excretion is not, for example, 0.9995 but 0.995. It is also significant that the renal membrane enzyme is entirely refractory to inhibition by Cl^- (44), very likely reflecting the fact that the ion is in high concentration at the membrane site. These considerable differences between cytosolic and membrane-bound enzymes reflect important structural differences, now being explored in several laboratories. Membranes from basolateral and apical surfaces show the same inhibition pattern (44, 73). The membrane enzyme may have a molecular weight of about 66,000 and is antigenically different from the cytoplasmic enzyme (71, 72). On the other hand, isolation of the membrane-bound form from brain has shown that the enzyme has the same molecular weight and isoelectric point as the cytoplasmic enzyme (73a). Although not complete, data from these papers suggest that the membrane-bound enzyme is of the high-turnover type.

[1]Note added in proof. Lung membrane enzyme, presumably from capillary endothelium, has been found to conform to properties described here for other membrane carbonic anhydrases: acetazolamide K_I 3X higher than for cytosolic enzyme, activity half as great, antigenically different, M.W. 52,000 (70a).

Table 4 Inhibition by sulfonamides and anions of carbonic anhydrases in particulate fractions of vertebrate tissues

Tissue	K_I						
	Sulfanil-amide (μM)	Acetazol-amide (μM)	Methazol-amide (μM)	Ethoxzol-amide (μM)	CNO^- (μM)	Cl^- (M)	References
A. Kidney microsomes	21	0.12	0.10	0.04	42	>1	44, 73
B. Kidney mitochondria	3.4	0.05	0.05	—	—	—	74
C. Liver microsomes (rat, dog)	4	—	0.05	—	—	—	4
D. Liver mitochondria	2.2	—	0.03	—	—	—	4
E. Muscle mitochondria[a]	—	—	0.2	—	25	>0.1	
F. Brain myelin	—	0.003	—	—	17	—	75
G. Chick chorioallantoic membrane	25	15	—	—	—	—	76

[a] Unpublished observations by the authors.

The microsomal enzyme from male rat liver is entirely different from the soluble enzyme, as is evident from comparison of Table 3 (Row C) with Table 4 (Row C); the microsomal fraction is fully susceptible to the sulfonamides.

Table 4 shows that mitochondrial enzyme(s) from liver (4), kidney (74), and muscle (unpublished data from this laboratory) are sensitive to the sulfonamides. Note that the pattern of inhibition of muscle mitochondrial enzyme by methazolamide and anions (Row E) is totally unlike that for muscle cytosol (Table 3, Row D). Brain myelin shows the inhibition pattern of isozyme II (Row F) (75). The relative refractiveness of the chorioallantoic membrane to acetazolamide (Row G) (76) appears anomalous; there is no other example of this compound and sulfanilamide having nearly the same activity.

Activity Against Carbonic Anhydrases from Invertebrates, Bacteria, and Plants

Table 5 shows inhibition by three sulfonamides and two anions against invertebrate, bacterial, and plant enzymes. It was surprising to find that the inhibition pattern against the mantle enzyme from the Mollusc *S. officinalis* was the same as that for human red cell II (77). So far as now known, the same is true for acetazolamide and chloride inhibition of oyster (78) and crab (78a). Notably, the enzyme in invertebrates is largely in particulate fractions of cells and in serum. Purification and kinetic work is not complete on the enzyme from these tissues, but to date it appears that the turnover number is high and that (at least in oyster serum) the molecular weight might be as high as 500,000, suggesting a polymeric form of the enzyme (79). In this phylum, the role of the enzyme is largely, if not entirely, in shell formation. The same is true for Arthropoda and Coelenterata; examples are the barnacle (80) and coral (81). Fragmentary work suggests that carbonic anhydrases in these species are also fully sensitive to the sulfonamides, but quantitative data are lacking. In Lepidoptera, the enzyme in the larval midgut epithelium subserves K^+ secretion; again it appears fully sensitive to acetazolamide (82–84). Acetazolamide is also a strong inhibitor of the plasma enzyme of Annelida, for example *Arenicola marina* (85). A recent general review of carbonic anhydrase (86) includes invertebrates, and points to interesting unsolved problems. In the present context, it should be noted that in many physiological studies using the inhibitors, their sensitivity against the enzyme itself was not tested.

It is not surprising that bacterial carbonic anhydrase is sensitive to all the sulfonamides in roughly the same pattern as found against the standard human red cell II (87, 88). In our hands (87) inhibition was some 10 times less than for type II. It is clear that bacterial carbonic anhydrase is asso-

Table 5 Inhibition of carbonic anhydrases of invertebrates and plants

Species	K_I Sulfanilamide (μM)	Acetazolamide (μM)	Ethoxzolamide (μM)	Cl^- (mM)	CN^- (mM)	References
Sepia officianlis mantle muscle	7	0.0025	0.001	370	0.1	77
Crassotrea virginica serum	—	0.015	—	150	—	78, 79
Arenicola marina plasma	—	0.23	—	—	—	—
Lepidoptera midgut	—	0.03	—	—	—	83, 84
Phototropic bacteria	—	0.01	—	—	0.1–1	89
Neisseria perflava, strain E	17	0.29	0.16	—	—	87
Neisseria sicca, strain 6021	3	0.05	0.01	—	—	88
Green algae	13	0.008	—	56	—	90
Blue-green algae	10	0.8	—	50	—	91
Plant leaves	>10000	20–30	0.5	10	—	3, 92–96

ciated with a high requirement for CO_2, as in *Neisseria* species. Presumably HCO_3^- is utilized, and the interconversion of the two chemical species is important. We do not know the anion sensitivity of the enzyme from *Neisseria.* A single report (89) suggests that phototropic (purple) bacterial enzyme is sensitive to acetazolamide, but relatively refractory to CN^-. Green algae show the same inhibition pattern as bacteria (90); this enzyme (in *Chlamydomonas reinhardii*) appears to be a hexamer of the usual vertebrate type. Blue-green algae are also sulfonamide sensitive (91).

All plant carbonic anhydrases studied to date (3, 92–96) are relatively refractory to the sulfonamides, roughly like the male rat liver or the muscle cytosol enzyme. The turnover numbers for parsley (92) and spinach (93) enzymes are high. The activity of ethoxzolamide against spinach enzyme ($K_I = 5 \times 10^{-7}$ M) was still great enough that inhibition studies could be carried out and an Easson-Stedman plot constructed, yielding the specific activity of the enzyme in molar terms (94). Table 5 shows that both ethoxzolamide (94) and acetazolamide [(3); also gives other sulfonamides] are 10^3 times less active than against type II. The plant enzyme is resistant to 0.01 M sulfanilamide (96). However, the plant enzyme shows anion sensitivity like isozyme III or I (3). The spinach chloroplast enzyme, like all carbonic anhydrases, contains zinc and appears to be a hexamer of the vertebrate type. Its role is the storage of CO_2 in the cell (as HCO_3^-) and subsequent release as CO_2 to enter the first stage of the Calvin cycle. An excellent general review is available (97).

CONCLUSION

In broad outline, we may say that nearly all carbonic anhydrases in the animal kingdom are sulfonamide-sensitive by the criteria of K_I for acetazolamide or methazolamide of 10^{-7}–10^{-8} M, a higher order of magnitude for sulfanilamide, and a lower order for ethoxolamide. The very low-activity carbonic anhydrase III is an exception, but we do not yet know the function of this protein, and it is not certain that its physiological role is indeed the catalysis of $CO_2 + OH^- \leftrightharpoons HCO_3^-$. Included in the sweep of sulfonamide sensitivity are carbonic anhydrases from bacteria and algae. So far as is now known, these soluble carbonic anhydrases (except in mollusc serum and algae, which are polymers) have these structural features in common: molecular weight of about 30,000, one zinc atom, single polypeptide chain, and some homology in amino acid sequence. In the cases of carbonic anhydrase I and II from human red cells and the bovine rumen and red cells, the complete amino acid sequence is known. Partial sequence is known for some other primates and the turtle.

The plant kingdom enzyme is quite different, sulfonamide sensitivity being 10^3–10^4-fold less than for animals. This is clearly a reflection of a different protein, the plant enzyme having a molecular weight of 180,000 and being a hexamer of the animal type. Full details of this structure are not yet known. The specific activity for CO_2 hydration of this enzyme is high.

The question of anion sensitivity is more subtle and perhaps more interesting and revealing. The type of carbonic anhydrase II, which includes the major respiratory and secretory enzyme in animals, is relatively insensitive to anions, meaning that Cl^- has K_I of 0.1 M or more. Where high concentrations of NaCl are involved in the secretory process (rectal gland, renal cell membranes), this value may exceed 1 M. The order of activity of the various ions are the same against the different tissues, Cl^- being one of the weakest and HS^-, CN^-, and CNO^- the strongest; the range is about 10^4 and follows an activity sequence similar to that found for inhibition of several other enzyme systems [(17), Scheme A1]. The other carbonic anhydrases (I and III), whose functions are obscure, have high anion sensitivity, nearly 100 times that of II. This is also the case for the plant enzyme. The primary question remaining is whether this high anion affinity indicates a function for these carbonic anhydrases, as yet unknown and lying outside the realm of CO_2 or HCO_3^- reactions.

The discerning reader may ask: If sulfonamide affinity is indeed a special case of anion affinity, why do their activities not run parallel for the several isozymes? The answer probably lies in the fact that there are two modes of binding, and that the lipophilic and ionic modes may vary independently

toward these isozymes. Thus with type II the lipophilic mode predominates; sulfonamide sensitivity is high whereas ionic sensitivity is low. The other extreme is type III, with a weak lipophilic component, leading to poor sulfonamide binding, but a strong ionic component.

In closing, it is appropriate to emphasize the crucial role of specific inhibitors and an intimate knowledge of their properties in elucidating the role of an enzyme in physiological and biochemical processes (2). Speaking as a pharmacologist and as a chemist, it is gratifying to write this review, and to see how greatly these professions contribute to their sister sciences.

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