The *in Vitro* Activity of Sulfonamides against Red Cell Carbonic Anhydrases. Effect of Ionic and Substrate Variation on the Hydration Reaction[1]

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The activity of 28 sulfonamides against dog red cell carbonic anhydrase was studied. I_{50} ranged from 10^{-4} to 10^{-9} *M*. Aliphatic compounds were the least, and heterocyclic compounds the most, active. Attention was given to order of addition, equilibration, buffer system, and temperature. Selected compounds were assayed against red cells of other species and pure enzyme from human red cells. The inhibitory effect of high ionic concentration upon enzyme activity, and upon the drug–enzyme interaction, was probably not enough to alter *in vivo* relations in the varying milieu of body cells and fluid compartments. Kinetics of inhibition of the hydration reaction by acetazolamide, sulfanilamide, and ethoxzolamide was noncompetitive.

In the course of studying red cell carbonic anhydrase inhibition by sulfonamides *in vitro* over some 15 years, the usual array of variables was encountered. We wish to report upon these in systematic fashion, with particular emphasis on those factors which are applicable to *in vivo* inhibition.

The following will be considered.

(1) The effect of variations in organic structure upon enzyme activity in standard test systems in which the enzyme source was dog red cells and the temperature 0°. In these test systems, two buffers, carbonate and barbital, were used. Each possessed advantages in differing situations: carbonate because of greater solubility allowed higher concentrations and longer reaction times, making estimations of I_{50} easier; barbital was more suitable for kinetic work because the pH change was minimal.[2a] Analysis of data showed that the activity of drugs was not significantly different between the two systems.

(2) A significant circumstance was whether drug was added to enzyme in the presence of substrate (SEI), or incubated with enzyme before substrate (EI). The former method simulates *in vivo* events and was suitable for rapid screening; the latter ensures equilibrium of enzyme and inhibitor. As will be shown, differences in activity of certain drugs between the two allows speculation as to interaction of substrate and inhibitor at the active site.

(3) The effect of raising the temperature to 37°.

(4) The effect of sulfonamides upon red cells of the various species.

(5) The effects of ions on drug activity.

(6) The effect of substrate variation upon reaction rates.

Extensive references to the literature will not be made, since a review including this subject has just been published.[3]

Methods

The analytical system for carbonic anhydrase is that reported previously,[2] with certain additions. The earlier method, here designated SEI (order of addition is substrate–enzyme–inhibitor) is in principle as follows. CO_2 gas at various concentrations was bubbled through a solution of phenol red. For the uncatalyzed rate, buffer was then added and the time for the formation of acid was measured. For the catalyzed or catalyzed-inhibited rate, enzyme or enzyme and inhibitor were added in that order to the phenol red solution; in all cases the timed reaction was started by the addition of buffer. Equilibration of this system was done by allowing all reactants to mix for the appropriate time (determined for each drug) before the addition of buffer. The new method designated EI (enzyme–inhibitor) was identical chemically to SEI, but here enzyme and inhibitor were preequilibrated outside the reaction vessel, usually in 1-ml volume, and then added to the CO_2–phenol red solution. Again the reaction was started by addition of inhibitor.

Red cell enzyme from the various species was prepared by lysing whole blood in distilled water usually at a concentration of 1:100. The concentration of enzyme in the test was $2–5 \times 10^{-9}$ *M*.[2a] The pure human enzymes HCA-B and HCA-C were obtained through the courtesy of Dr. Sven Lindskog. Their properties have been reviewed.[3] Dog and bovine purified fractions were prepared by Byvoet and Gotti.[4]

The drugs were made up in aqueous solution; in some cases it was necessary to add NaOH to effect solution. Drugs of classes A–D in Table I were obtained from the American Cyanamid Co. through the courtesy of Dr. Selby Davis. Class E drugs were obtained from the appropriate pharmaceutical company. Class F was synthesized for us by Peninsular Chemresearch, Inc., Gainesville, Fla.

Results and Discussion

The activity of drugs is expressed as the I_{50}, the molar concentration which inhibits the catalytic rate 50%. The relation of this value to the K_I, the dissociation constant of the enzyme–inhibitor complex, is given by the relation $K_I = I_{50} - {}^1/_2E_0$, where E_0 is the molar concentration of active enzyme originally present. In the case of most of the drugs of Table I, $I_{50} \gg E_0$ so that the latter term drops out of the equation, and $I_{50} \simeq K_I$. For drugs with $K_I < 10^{-8}$ *M*, the I_{50} is influenced by the magnitude of E_0. As noted in the footnote to Table I, however, E_0 is constant in these tests, so the I_{50} remains a reasonable estimate of activity. The K_I may be calculated from the given values of I_{50} and E_0. These relations are developed in detail in ref 2a.

Effect of Variation in Structure upon Activity.—Table I shows the activity against crude dog red cell carbonic anhydrase at 0° of 28 sulfonamides. They are divided into six groups.

A.—The simple benzene sulfonamides, by the SEI system, have activity in the 10^{-5}–10^{-6} *M* range. With incubation (EI), activity increases. Starting from the

(1) Supported by Grant NB01297 from National Institutes of Health.

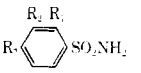
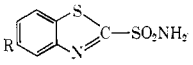
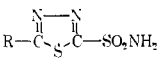
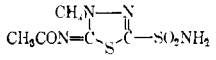
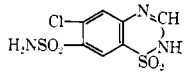
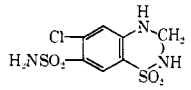
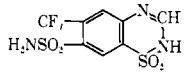
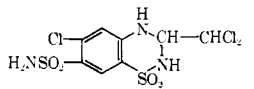
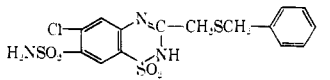
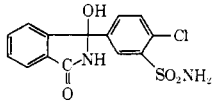
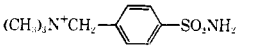
(2) (a) T. H. Maren, A. L. Parcell, and M. W. Malik, *J. Pharmacol. Exp. Ther.*, **130**, 389 (1960); (b) T. H. Maren, V. I. Ash, and E. M. Bailey, Jr., *Bull. Johns Hopkins Hosp.*, **95**, 244 (1954); (c) T. H. Maren, *J. Pharmacol. Exp. Ther.*, **139**, 129 (1963).

(3) T. H. Maren, *Physiol. Rev.*, **47**, 595 (1967).

(4) P. Byvoet and A. Gotti, *Mol. Pharmacol.*, **3**, 142 (1967).

TABLE I

I_{50} ($\times 10^7$) OF SULFONAMIDES AGAINST CRUDE DOG RBC CARBONIC ANHYDRASE AT 0.3°[a]

Class and compd	SEI Carbonate	SEI Barbital	SEI Time, min	EI Carbonate	EI Barbital	EI Time, min
A. Benzenesulfonamides: 						
R_1 R_2 R_3						
H H H	19	...	Ne	2	...	4
H H CH_3CONH	5	13	2	0.2	0.2	2
H H CH_3	113	...	2	2.0	...	2
H NH_2 H	34	...	Ne	2.7	...	2
NH_2 H H	40	...	Ne	1.3	...	Ne
H H NH_2	28	57	Ne	6.0	4.0	2
B. Aliphatic Sulfonamides: $RCH_2SO_2NH_2$						
R						
H	3000	...	Ne	3000	...	2
CH_3	7200	...	Ne	7200	...	2
C_6H_5	600	...	Ne	300	...	2
C. Benzothiazole Sulfonamides: 						
R						
H	0.04	...	15	...	...	...
OC_2H_5 (ethoxzolamide)	0.04	0.03	10	0.01	0.02	2
D. Thiadiazole Sulfonamides: 						
R						
NH_2	0.6	...	2	...	...	2
CH_3CONH (acetazolamide)	0.2	0.2	2	0.03	0.02	2
$C_6H_5SO_2NH$ (I)	0.03	0.03	2	0.02	0.025	2
p-$NH_2C_6H_4SO_2NH$	0.04	...	2	...	...	2
o-ClC_6H_4 (II)	0.03	0.03	1	0.03	0.02	2
 (methazolamide)	0.17	0.12	2	0.07	0.07	5
E. Disulfonamides (Saluretics) and Congeners						
1,3-Disulfamylbenzene	21	...	Ne	0.08	...	2
1,4-Disulfamylbenzene	14	...	Ne	0.06	...	2
1,3-Disulfamyl-4,5-dichlorobenzene (dichlorphenamide)	0.7	0.7	10	0.03	0.03	2
5-Chloro-2,4-disulfamylaniline (salamid)	22	...	5	...	...	...
 (chlorothiazide)	18	14	5	0.07	0.06	5
 (hydrochlorothiazide)	225	240	10	1.8	1.2	5
 (flumethiazide)	123	...	Ne	4	...	2
 (trichlormethiazide)	350	...	2	0.2	...	2
 (benzthiazide)	14	16	2	0.03	0.03	20
 (chlorthalidone)	2	...	Ne	0.1	...	...
F. Quaternary Ammonium Sulfonamide						
	300	...	2	9	...	2

[a] Procedure is given in ref 2 and Methods section. The time given is the minimum needed for equilibration. Ne means <15 sec. The enzyme used was 0.25 ml of a 1:100 suspension of whole dog blood in water. This is approximately 5×10^{-9} *M*, and equivalent to 2 enzyme units in the carbonate system and 5 in barbital.[2a]

simplest and prototype drug, $C_6H_5SO_2NH_2$, it appears that *para* amination does not alter activity, but *para* methylation reduces it. The isomers of sulfanilamide all have the same activity. It is of interest that acetylation of the amino group of sulfanilamide increases activity; it appears (class D below) that this may be a general phenomenon. Whether this alteration is related to the marked lowering of pK is not known; if this is the case it would only apply within a given series, since drugs of widely varying pK_a have maximal activity in this test (classes C and D below). Within class A, moving from the SEI to EI system increases activity 5–50-fold. Further experiments were done to see whether equilibria in the EI system could be displaced by prolonged contact with CO_2. Normally, in this test, CO_2 is bubbled into the enzyme inhibitor mixture for about 5–10 sec before addition of buffer. This exposure was increased to 10 min, and the I_{50} of seven of the drugs tested then fell into two groups. For five of the drugs, the I_{50} reverted to the SEI value; these are acetazolamide, methazolamide, chlorthalidone, sulfanilamide, and hydrochlorothiazide. Two drugs, benzthiazide and dichlorphenamide, retained their very high activity (EI test, Table I) even after exposure to saturated CO_2 for 30 min. Clearly there appear to be quite different kinetics between these two classes of drugs. It seems likely that the present finding is related to the observation of Leibman, *et al.*,[5a] that benzthiazide, unlike acetazolamide, displays notable departure from reversible inhibition kinetics.

B.—The aliphatic sulfonamides have very weak activity in both EI and SEI tests with I_{50} of the order of 10^{-3} M. The question may arise as to whether activity in this class may be due to contamination with trace amounts of aromatic sulfonamides—as little as 0.1% could produce an inhibition which would appear active in the range observed. But the fact that the aliphatic compounds give the same values for the SEI and EI test argues strongly that they are active as such, since the aromatic sulfonamides are all more active in the latter test.

C.—A few simple benzothiazolesulfonamides are listed. These are maximally active, when it is considered that enzyme concentration is $2–5 \times 10^{-9}$ M in the test. Thus there can be little difference between SEI and EI tests.

D.—Several thiadiazoles and related compounds are shown. All are active in the 10^{-8}–10^{-9} M range; those with I_{50} of 10^{-3} in SEI attain potency of 10^{-9} with EI (*cf.* acetazolamide). The data reported in parts A–D of this table agree with earlier studies in which benzenesulfonamides, aliphatic sulfonamides, and heterocyclic sulfonamides have been compared.[6] The data from the literature on these and other structural types have been documented.[3] It is not possible yet to relate structure or chemical or physical properties to activity in any meaningful way. Two of the most active compounds, ethoxzolamide and 2-benzenesulfonamidothiadiazole-5-sulfonamide (I), are antipodal in character; the former is highly lipid soluble and un-ionized, the latter ionic in character at pH 7.4.[3] Attention has not, however, been given to the charge surrounding the SO_2NH_2 group itself.

E.—Disulfonamides and salureties of the thiazide and related types are listed together. All salureties of this type contain a 1,3-disulfamyl or 1-sulfamyl-3-carbamoyl function. It is characteristic of these drugs that they are quite weak carbonic anhydrase inhibitors in the SEI system, but potent in EI. This is not to imply that the latter explains their renal activity; it has been shown elsewhere that this is unrelated to carbonic anhydrase inhibition.[7a] Flumethiazide, for example, is a potent diuretic (at 0.1 mg/kg)[7b] and, at this dose, even the EI potency of $I_{50} = 4 \times 10^{-7}$ M would not be expected to yield any reasonable fraction of renal enzyme inhibition *in vivo*.[8] In fact, the urine is not alkalinized at this dose.[7b] By contrast, acetazolamide is at least ten times more potent *in vitro*, yet requires 25 times this dose for the diuretic (and alkalinizing) effect.[7,8] However, the very large (up to 2000-fold) increase in activity in the EI test shown by some of these drugs is of kinetic interest. As noted above for benzthiazide, the effect seems to bear on the question of reversibility.[5a] Comparison of compounds in which SEI $\simeq$ EI with those in which SEI $>$ EI may yield significantly different values for the individual constants in $K_1 = k_{-1}/k_1$.

F.—This class is represented by only one compound, but is of importance since it is the first cationic sulfonamide to be reported in this context. Activity is comparable to the parent (and at pH 7.4, uncharged), *p*-toluenesulfonamide.

Effect of Raising the Temperature to 37° (Table II).

TABLE II

I_{50} ($\times 10^8$ M) OF SULFONAMIDES AT 37°[a]

Compound	SEI	EI
II	0.03	0.03
Ethoxzolamide	0.04 (0.06)	0.04
I	0.05 (0.03)	0.05
Dichlorphenamide	0.09	0.03
Benzthiazide	0.27	0.03
Methazolamide	0.37 (0.45)	0.37
Acetazolamide	0.51 (0.7)	0.43 (0.21)
Chlorothiazide	11	4
Hydrochlorothiazide	56	59
Sulfanilamide	66 (100)	91

[a] Dog red cell (0.25 ml of 1:100 hemolysate) was used as enzyme source. All drugs were equilibrated either with enzyme (EI) or enzyme and substrate (SEI). Equilibration time varied with the drug, from 15 sec to 5 min. CO_2 was delivered from a 26% mixture in air, yielding 6 mM in solution; data in parentheses are from CO_2 concentration of 3 mM. Barbital buffer.

In general, this does not alter activity greatly from the standard 0° (SEI) system. Equilibration time is generally reduced. The principal change is that the difference between SEI and EI systems observed at 0° is nearly obliterated for all the drugs except benzthiazide. Even this is reduced from about 500-fold (Table I) to ninefold. It must also be pointed out that in experiments of Table II the CO_2 concentration was reduced (from 100% gas in the 0° test to 26% and in a few cases to 12%) in order to bring the reaction into a

(5) (a) K. C. Leibman, D. Alford, and R. A. Boudet, *J. Pharmacol. Exp. Ther.*, **131**, 271 (1961); (b) R. P. Davis, *J. Am. Chem. Soc.*, **81**, 5674 (1959).

(6) (a) T. Mann and D. Keilin, *Nature*, **146**, 164 (1940); (b) H. A. Krebs, *Biochem. J.*, **43**, 525 (1948); (c) W. H. Miller, A. M. Dossert, and R. O. Roblin, *J. Am. Chem. Soc.*, **72**, 4893 (1950).

(7) (a) T. H. Maren and C. E. Wiley, *J. Pharmacol. Exp. Ther.*, **143**, 203 (1964); (b) K. H. Beyer and J. E. Baer, *Pharmacol. Rev.*, **13**, 517 (1961).

(8) T. H. Maren, *J. Pharmacol. Exp. Ther.*, **139**, 140 (1963).

TABLE III

I_{50} ($\times 10^7$ M) OF SIX SULFONAMIDES AGAINST RED CELL CARBONIC ANHYDRASE FROM VARIOUS SOURCES[a]

Compound	Human RBC SEI	Human RBC EI	HCA-B SEI	HCA-B EI	HCA-C SEI	HCA-C EI	Cord RBC SEI	Purified dog RBC SEI	Bovine RBC SEI	Semipurified bovine RBC SEI	Semipurified bovine RBC EI	Rat RBC SEI
Acetazolamide	0.34	0.07	3.2	3.9	0.46	0.05	0.32	0.21	0.25	1.2	0.06	0.26
Sulfanilamide	35	18	234	280	31	32	35	53	83	128	7.5	35
II	0.07	...	0.44	...	0.06	...	0.1	0.06	0.06	0.10	...	0.03
I	0.15	...	0.32	...	0.10	...	0.14	0.14	0.03	...	...	0.07
Methazolamide	0.48	...	0.77	...	0.25	...	0.38	...	0.35	...	...	0.4
Ethoxzolamide	0.05	0.05	0.60	...	0.04	...	0.05	...	0.04	...	...	...

[a] At 0° with carbonate buffer. One to two enzyme units ($2–4 \times 10^{-9}$ M) were used in all cases except HCA-B, where these units are equivalent to $20–40 \times 10^{-9}$ M.[3] Enzyme and drug were equilibrated throughout.

TABLE IV

EFFECT OF IONS ON ENZYME AND DRUG ACTIVITY

T, °C	Ion concn, mM	(S), mM	Method	Drug[a]	$I_{50} \times 10^7$ M Carbonate	$I_{50} \times 10^7$ M Barbital
0	143 KCl	60	SEI	...	V_{cat}[b] unchanged	V_{cat}, 40% inhib
				A	0.6	
				S	31	
37	286 KCl	3	SEI	...		V_{cat}, 46% inhib
				A		0.5
				S		75
				B		0.1
	143 KCl			...		V_{cat}, unchanged
0	143 KCl	60	EI	...		V_{cat}, 44% inhib
				A		0.2
				S		200
	143 NaCl			...		V_{cat}, 70% inhib
0	143 $NaHCO_3$	60	EI	...		V_{cat}, 73% inhib
				A		0.06
				S		16
				II		0.03
				C		0.1

[a] A = acetazolamide, S = sulfanilamide, C = chlorothiazide, B = benzolamide (I). [b] Catalyzed velocity.

feasible time range. In solution, the CO_2 was 3–6 mM. The SEI test at this temperature and substrate concentration then represents the closest approach to the *in vivo* situation, since substrate and enzyme are present in the blood, and inhibitor added or injected last. The values of Table II for 37° and SEI have been used for calculation of *in vivo* inhibition kinetics.[8]

Effect of Sulfonamides upon Red Cell Carbonic Anhydrase of Various Species.—Table III shows the inhibitory data (I_{50}) of six representative sulfonamides on red cell enzyme from human, newborn, dog, rat, and ox. In addition, data are given for the two pure enzymes from human blood. With the exception of certain of the values for HCA-B, it is evident that each drug has about the same activity for the different red cell species.

The discrimination between HCA-B and HCA-C is particularly instructive in the case of sulfanilamide, since it is a comparatively weak inhibitor. In this case, $I_{50} \gg E_0$, and I_{50} is not influenced by the concentration of enzyme present. This is important, because much more enzyme is used in HCA-B assay than for HCA-C and other enzymes, because it has lower activity (*cf.* legend to Table III; see also ref 3). Acetazolamide, although stronger than sulfanilamide, still has $I_{50} > E_0$, so the same principle applies. Use of these two drugs then permits a decision as to whether an enzyme source is akin to HCA-B or to HCA-C. In cord blood for example, Table III shows a pattern similar to HCA-C; this agrees with the comparison based on reaction rates.[9]

On the other hand, the group of strongest inhibitors, ethoxzolamide and 2-benzenesulfonamido- (I) and 2-*o*-chlorophenylthiadiazole-5-sulfonamide (II), does not discriminate between HCA-B and HCA-C. The differences observed in I_{50} against the two enzymes for each of these drugs is due to the larger amount of HCA-B present, since the I_{50} is essentially an equimolar titration of enzyme.[2a]

The Effect of Ions on Enzyme and on Drug Action.—Table IV sets out various experiments showing the effect of ions on enzyme and drug activity. In the high concentrations of ions used, there was up to 73% inhibition of the catalyzed rate. The following gives additional data on the fractional inhibition (i) at various concentrations of KCl in the EI system using barbital buffer: 35 mM, 0.25; 71 mM, 0.36; 143 mM, 0.44; 214 mM, 0.62. For NaCl there is more inhibition as follows: 35 mM, 0.42; 71 mM, 0.63; 143 mM, 0.70; 214 mM, 0.78. $NaHCO_3$ yields at 28 mM, 0.28; 70 mM, 0.4; 105 mM, 0.58; 142 mM, 0.64. Clearly, the ions are all inhibitory. Previous work on this subject has also been reviewed.[3] It is important that the chief intracellular cation, K^+, does not inhibit more than about 40% at the approximate concentration that is found *in vivo*, and in view of the very large excess of enzyme normally found,[8] it is likely that this has no physiological implications. In the presence of KCl, inhibitory action of sulfonamides at 0° in the EI system is reduced. The data of Table IV may be compared with that of Table I and Table II. Perhaps the

(9) B. H. Gibbons and J. T. Edsall, *J. Biol. Chem.*, **239**, 2539 (1964).

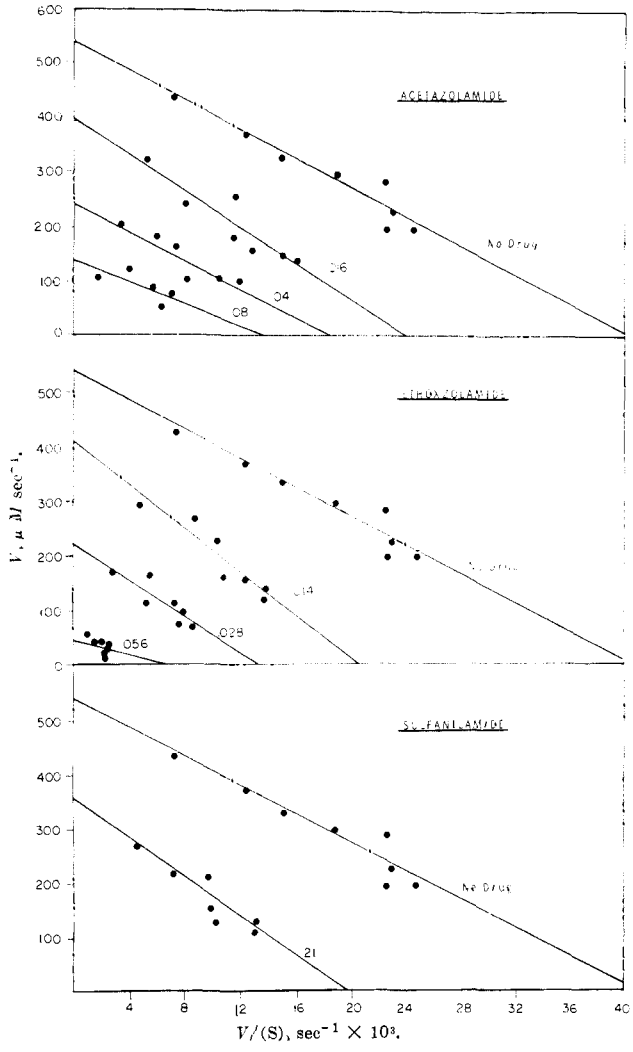


Figure 1.—Inhibition of purified canine red cell carbonic anhydrase[4] by sulfonamides. Velocities (V) are those of the catalyzed reaction: total rate minus uncatalyzed rate. Numbers on lines are drug concentrations in units of 10^{-7} M. All experiments were at 0° in barbital buffer, using EI system.

most important comparison in the *in vivo* context is that for the 37° low substrate SEI system. The data of Table IV, for 286 mM KCl, agree within twofold with that of Table II, for no KCl, for the three drugs tested.

Table IV also shows (compare Table I) that very high concentration of HCO_3^- does not appreciably alter the I_{50} of the sulfonamides in the enzymic hydration reaction. This supports the idea that the enzymic site for CO_2 and for HCO_3^- are not identical (discussed in ref 3).

The Effect of Substrate on Inhibition.—Figure 1 shows plots for several of the drugs, to test the nature of the inhibition. Following the careful critique of Dowd and Riggs,[10] we have used the plot of V (velocity) against $V/[CO_2]$, rather than the usual Lineweaver–Burk plot. In the method used, the slope is $-K_m$ and the ordinal intercept is V_{max}. If experimental lines representing uninhibited and inhibited reactions intersect at the ordinate, the kinetics are competitive; if the lines are parallel, kinetics are noncompetitive.

The EI system was used, in which drug and enzyme were equilibrated for 2 min in 1-ml volume, then added to the phenol red–CO_2 mixture (5 ml) for 5–10 sec before addition of buffer. Exploratory tests showed that the SEI system failed to yield any consistent pattern for acetazolamide. Presumably, this would also be the case if CO_2 exposure time to the EI mixture was prolonged, since, as noted above, this caused, for acetazolamide and sulfanilamide, reversion of the EI-determined I_{50} to that of SEI. However, for ethoxzolamide, the EI and SEI inhibition were the same (Table I).

Figure 1 shows the kinetics of all three drugs to be noncompetitive. The K_m was 16–20 mM. Under these conditions the K_I's of the three drugs ($\times 10^{-7}$) were acetazolamide, 0.03; ethoxzolamide, 0.02; sulfanilamide, 30.

The data on acetazolamide and sulfanilamide agree with those of Leibman, *et al.*, and of Davis.[5] Those for ethoxzolamide are new and are of particular interest since, as indicated, they apply to any order of addition or time of exposure to CO_2.

In spite of the rather clear kinetic plots, the displacement of enzyme–inhibitor equilibria by CO_2 in the cases of sulfanilamide and acetazolamide (and certain of the other drugs of Table I) leaves some doubt as to whether the pattern is one of classical noncompetition. It is likely that the true picture is more complex than this, and that substrate and inhibitor sites on enzyme are sufficiently close so that mutual perturbations are possible. They are not observed with ethoxzolamide or others of the ultrapotent group (see identity of SEI and EI data in several drugs of Table I) because of very high affinity of drug and enzyme in these cases. Such affinity is about 10^7 greater than substrate–enzyme affinity, K_m being about 20 mM.[3] The perturbations observed for the weaker inhibitors may reflect the close spacial relation between the Zn site and the inhibitor site, as revealed by X-ray diffraction.[11]

(10) J. E. Dowd and D. S. Riggs, *J. Biol. Chem.*, **240**, 863 (1965).

(11) K. Fridborg, K. K. Kannan, A. Liljas, J. Lundin, B. Strandberg, R. Strandberg, B. Tilander, and G. Wirén, *J. Mol. Biol.*, **25**, 505 (1967).