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Direct Measurements of the Rate Constants of Sulfonamides with Carbonic Anhydrase

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SUMMARY

Kinetic values for 14 sulfonamides and carbonic anhydrase (equivalent of isozyme II) were determined directly by measurement of association rate constants (k_{on}) and equilibrium constants (K_I), yielding dissociation rate constants, k_{off}. Values for k_{on} (in liter/mol sec^{-1}) ranged from 0.003 to 31 $\times$ 10^6, whereas K_I ranged from 0.7 to 17,000 $\times$ 10^{-9} M. The k_{off} range was very small, 0.01–0.05 sec^{-1}. Thus, the activity, which is usually thought of as reflecting K_I, is entirely a function of the association rate. This is not the common situation in enzyme-inhibitor reactions. The k_{off} range is faster by several orders of magnitude than drug decay from plasma, so equilibrium is always achieved at the enzyme site *in vivo*, after parenteral administration of acetazolamide, methazolamide, or ethoxzolamide. For topical administration, as for MK-927 to reduce intraocular flow and pressure, the quantitative relation between free drug in tissue and that bound to enzyme in ciliary process is not so clear. Thus, k_{off} might be an independent factor in pharmacological activity. The reaction of anions with carbonic anhydrase is entirely different, in that variations in K_I are chiefly determined by k_{off}.

The remarkable power of unsubstituted heteroaromatic sulfonamides to inhibit the isozymes of carbonic anhydrase (albeit with markedly different quantitative effect, depending on both drug and particular enzyme) has been the subject of intensive study for over 50 years. Most of the acquired data have been in terms of the equilibrium dissociation constant (K_I) or the I_{50} determined at equilibrium, whence the simple relation $K_I = I_{50} - \frac{1}{2}E_0$, with the latter term being the initial enzyme concentration.

I turn now to measurement of the rate constants (k) that underlie K_I ($K_I = k_{off}/k_{on}$). These should yield fundamental information on the nature of the drug-enzyme reaction, particularly when sulfonamides of differing physicochemical properties are studied and comparison is made also to rates of anion binding. A pharmacological goal of this work was to search for compounds with very slow off-rates, with k_{off} being perhaps 10^{-4} sec^{-1}, whence the half-life of the drug-enzyme complex would be about 2 hr. Such rates are known for other enzyme systems (1). We could then inquire whether these compounds have any pharmacological value, compared with compounds (see Table 1A) whose dissociation half-time from enzyme is approximately 10 sec.

The only paper in the literature directly comparable to the present work is that of Kernohan (2), in which rates were measured for acetazolamide and benzene sulfonamide, using a stop-flow technique in which the degree of inhibition of the catalytic reaction was timed. Lindskog (3) made measurements of several compounds using inhibition of the esterase reaction. The major work was by Taylor *et al.* (4) and King and Burgen (5), who used displacement of fluorescent sulfonamide markers to measure rates. This work yields a wealth of information on the effects of 24 compounds against carbonic anhydrase I and II, but only one compound (acetazolamide) was of therapeutic interest. Selected data from these three laboratories are shown in Table 1A, to invite comparison with the present study. The significant findings to emerge were the general ranges of k_{on} and k_{off} for the sulfonamides and the conclusion (3, 4) that, for this type of compound, potency at equilibrium over a 5000-fold range (K_I) was largely determined by k_{on}. k_{off} values generally varied only about 8-fold (Table 1A)[1] and are on the order of 0.05 sec^{-1} which is intermediate in the range of slow to fast reactions (1). In sharp contrast, nonsulfonamide anions, as shown in Table 1B, have faster off-rates by many orders of magnitude and can vary by 10^4 (6–9). I shall return to this important point in the Discussion.

This study invokes the classical equations and manipulations for second- and first-order reversible reactions. The usual textbook treatment for A + B $\rightleftharpoons$ C, however, makes the caveat that

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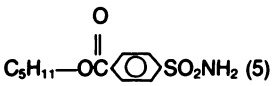
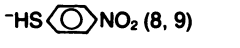
[1] In this study of 24 compounds (4), k_{off} appeared to vary 80-fold, but, if two closely related compounds (**21** and **22**) are excluded, the range is only 8-fold. New examination of compound **21**, 2-NO_2-4Cl benzene sulfonamide, failed to confirm the high k_{off} (1.5 sec^{-1}) of Ref. 4. Our value is 0.035 sec^{-1}, similar to others of Ref. 4 and present Table 5. The true range of k_{off} values in Ref. 4 may thus be about the same as ours.

ABBREVIATION: EU, enzyme unit.

TABLE 1

Representative literature data on rate constants for sulfonamides and anions against carbonic anhydrase

Compound and reference (see Table 3 for structures)	$K_I \cdot 10^9$	$k_{on} \cdot 10^{-6}$	k_{off}
	M	M^{-1} sec^{-1}	sec^{-1}
A. Sulfonamides			
Sulfanilamide (3)	2000	0.07	0.15
Benzene sulfonamide (2)	1000	0.16	0.16
(4)	1600	0.10	0.16
Chlorothiazide (3)	540	0.04	0.02
Acetazolamide (2)	20	5	0.1
(4)	14	5	0.07
(3)	25	4	0.01
Benzolamide (3)	9	6	0.05
Ethoxzolamide (3)	1.6	30	0.05
 (5)	0.4	47	0.02
B. Anions			
Cl^- (6)	7×10^6	140	10^6
CNO^- (7, 20)	4×10^4, 10^5	10, 100	400, 10^4
 (8, 9)	2.5×10^7	700	170
CN^- (20)	1600	3000	5×10^3
SCN^- (20)	3500	2000	7×10^3
$HCOO^-$[a] (21)	6.5×10^6	390	6×10^4

[a] Data also given for fluoracetates, in the same range as formate.

concentrations A = B and implies that the solution is difficult where A ≠ B. In the present case, concentrations of a relatively weak inhibitor (A) will be much greater than of enzyme (B), so it was necessary to modify the existing equations, as given in Ref. 10 and elsewhere. The new derivation is given in the Appendix.

The method depends on incubating enzyme with inhibitor for incremental periods, from 15 sec to equilibrium. At various time intervals, an aliquot of the mixture is withdrawn and analyzed for its enzyme activity. The measure of increasing inhibition (i and iE_0) as drug reacts is the increase in hydration time of CO_2 in our standard system (Fig. 1). Details are given below and a model experiment in Table 2.

Compounds were selected on the basis of varied structures and activity (Table 3). Three (compounds **8**, **9**, and **14**) have been in parenteral use, originally as diuretics but largely for the treatment or prevention of glaucoma, mountain sickness, and certain other conditions. One (compound **11**) is a specific renal drug. Compounds **3** and **5** go back to the original discovery of aromatic sulfonamides as carbonic anhydrase inhibitors. Compounds **12** and **13** are more recent experimental compounds. Five (compounds **1**, **2**, **4**, **6**, **7**) are "chloruretics," which are used in the treatment of cardiac edema and in the doses used have renal mechanisms quite apart from carbonic anhydrase inhibition. However, they also have the free

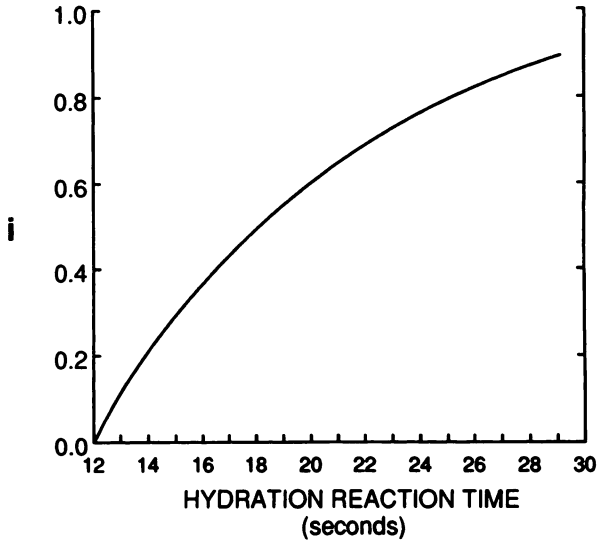


Fig. 1. Fractional inhibition (i) as measured by reaction time (t) of CO_2 hydration in the presence of increasing concentrations of inhibitor. T = 25°, E_0 = 2 nM, uncatalyzed time = 36 sec, and catalyzed time = 12 sec.

TABLE 2

Sample experiment for acetazolamide, showing method for determination of rate constants for inhibitors of carbonic anhydrase

Conditions were: 24°, 6 mM CO_2, E_0 = 2 nM, I_0 (acetazolamide) = 7 nM, uncatalyzed time (T_{unc}) = 34 sec, and catalyzed time (T_{cat}) = 11.6 sec. See text for other details. Values given for E_0, I_0, x, x_e, and t are put into eq. 2 and yield two sets of numbers, corresponding to the 15- and 30-sec data: k_{on} = 3.3 and 2.3 × 10^8 M^{-1} sec^{-1}. K_I calculated from the equilibrium position (x_e) = 6.8 × 10^{-9} M. k_{off} = $k_{on} \cdot K_I$ = 0.022 and 0.016 sec^{-1}.

Incubation time, E + I	Hydration reaction times (T_{pi})[a]	i (eq. 1)	$x = iE_0$
sec, t	*sec*		
15	13.7	0.24	0.48
30	14.4	0.30	0.60
60	16.8	0.47	0.94[b]
120	16.8	0.47	0.94[b]

[a] Mean of three experiments.
[b] x_e.

$-SO_2NH_2$ group. Compound **10** is the type now being developed for topical treatment of glaucoma. Most of these relationships are discussed in Refs. 11 and 12. Ref. 11 summarizes data showing that inhibition is noncompetitive and reversible.

Experimental Procedures

Methods. The principle of this method is the measurement of enzyme activity as it declines, due to progressive inhibition by the sulfonamide with time. This is best shown in model form (Table 2). The terms are as follows: I_0 = initial drug concentration; E_0 = initial enzyme concentration (this is calculated for dog hemolysate according to Ref. 13; see below); and i = fractional inhibition of enzyme [this is calculated from the relations among the observed catalyzed time (T_{cat}), the uncatalyzed time (T_{unc}), and the partially inhibited time (T_{pi})]. In the control reaction, 1 EU halves the reaction time or doubles the rate of the uncatalyzed reaction: EU(control) = $(T_{unc} - T_{cat})/T_{cat}$. The units for the partially inhibited reaction are correspondingly EU(pi) = $(T_{unc} - T_{pi}/T_{cat}$. The fractional activity (a) of the enzyme-inhibitor complex is the ratio of EU(pi)/EU(control), and the fractional inhibition (i) is $1 - a$. Numbers are shown in Table 2; appropriate algebra yields the relation:

$$i = \frac{T_{unc}(T_{pi} - T_{cat})}{(T_{unc} - T_{cat})T_{pi}} \quad (1)$$

$iE_0 = EI = x$ [concentration of enzyme inhibited = concentration of drug (x) transferred from I_0 to enzyme] at time t. $x_e = iE_0 = EI = x$ at equilibrium.

The equations are (see Appendix):

$$k_{on} = \frac{1}{t} \cdot \frac{x_e}{E_0 I_0 - x_e^2} \ln \frac{x_e(E_0 I_0 - x\, x_e)}{E_0 I_0 (x_e - x)} \quad (2)$$

$$K_I = \frac{(I_0 - x_e)(E_0 - x_e)}{x_e} \quad (3)$$

$$k_{off} = k_{on} \cdot K_I \quad (4)$$

The procedure follows. Enzyme (E_0) (1–2 nM, 0.1–0.2 ml of 1/100 dog blood), inhibitor (I_0), and indicator (phenol red or bromthymol blue) (25 mg/liter) are mixed in 5 ml for differing periods (0.25–6 min). The solution is transferred to the reaction vessel (14, 15), into which CO_2 is flowing; no dilution is involved. The reaction is started immediately by the addition of 2 ml of 50 mM barbital buffer at its pK_a of 7.9. The transfer of solution and buffer addition take 3–5 sec. The usual experiment is done at room temperature (23–25°) with 16% CO_2 (6 mM). Each point is done in triplicate; data are only acceptable if agreement is <0.5 sec. Under these conditions, the uncatalyzed time is 34–36 sec and the catalyzed is 11–13 sec. When drug is added, the time increases to 14–30 sec, depending on concentration and time of the incubation reaction (Fig. 1). The end point is determined visually, red to yellow (phenol red) or yellow to blue (bromthymol blue), using a stop watch. The pH range is 7.9 to 7.2. The catalysis described corresponds to about three enzyme units, $T_{unc}/(T_{unc} - T_{cat})$, in our standard 0° system using barbital buffer and 100% CO_2. This is equivalent to 2 nM carbonic anhydrase II, as determined by titration with the very powerful inhibitor ethoxzolamide, according to methods in Ref. 13. Dog red cells contain both carbonic anhydrase I and II (16), but 80% of the activity is due to isozyme II (17). The hemolysate of dog red cells is used because it is stable and inexpensive, in view of the thousands of determinations done. Results have been checked against pure human carbonic anhydrase II or pure bovine red cell enzyme and found to be identical.[2] The overall reliability of the rate and equilibrium constants is judged at about ±25%.

Chloroform/water partition coefficients were determined by shaking dilute solutions of drug (10–100 μM), in pH 7 buffer, with equal volumes of solvent. Each phase was analyzed by our enzymic method (14, 15). Solubility at pH 7 was determined by analyses of saturated aqueous solution. pK_a was determined by titration against NaOH or HCl. Where there are two acidic pK_a values, the higher one is assigned to the —SO_2NH— group, by analogy to compounds (e.g., benzolamide and furosemide) in which it is clear that the "other" pK_a is much lower.[3]

Materials. Sources of the sulfonamides are given in Table 3. Enzyme from dog blood (1% in water) was prepared fresh each week. Such a solution at 2° is stable for months. The CO_2 source was tanks of 16% of the gas in air or nitrogen. Indicators and buffers were purchased from Aldrich (Milwaukee, WI).

Results

Procedure and theory (eqs. 1 and 2). Fig. 1 plots fractional inhibition (i) versus time (t), given the particular case where T_{unc} = 36 sec and T_{cat} = 12 sec. Eq. 1 is the general case, yielding i for any values of catalyzed, uncatalyzed, and inhibited times. Because E_0 is known, iE_0 then gives the concentration of enzyme reacted, which is equivalent to x or x_e, the concentration of drug transferred to enzyme at time t or at equilibrium. The values E_0, E_0, x, x_e, and t (for values of x) are entered in eq. 2. Table 2 gives the details of an experiment for acetazolamide.

Physicochemical properties of sulfonamides. Table 3 gives the structures of the 14 sulfonamides used in this study. Table 4 gives ionization and solubility characteristics. Further chemical and pharmacological data on some of these compounds are given in Ref. 11. These compounds display a very wide range of physicochemical and structural properties. As shown below (Table 5), the most active compounds can be either lipid or water soluble. Thus, in benzolamide (compound **11**) the pK_a of the phenylsulfamyl proton is low (about 3), so that this compound is ionized and soluble at pH 7; lipid solubility is vanishingly small. Such an ionizing group is absent in ethoxzolamide (compound **14**); the pK_a of 8 and the ether linkage dictate very high lipid and low water solubility. But as

[2] These experiments would have been impossible using pure carbonic anhydrase II, because of its instability, particularly when 4-min incubation was demanded. Dog hemolysate is very stable, and we have never observed a difference in K_I for any drug between these two preparations. The carbonic anhydrase I in dog hemolysate contributes only 20% to the reaction rate (17) and, for nearly all sulfonamides, shows less inhibition (12, 19) (unpublished data from this laboratory).

[3] A caveat must be made with respect to the compounds showing two acidic pK_a values within 2 units of each other. Lindskog (3) (personal communication) finds that in compounds such as **2**, **4**, **7**, and **9** the assignation of pK_a to sulfonamide or "other" proton is not clear, as revealed by measurement of the microscopic pK_a values.

TABLE 3

Structures

Sources of the sulfonamides are *a*, Hoechst-Roussel (Summerville, NJ); *b*, Merck Sharp & Dohme Research Laboratories (West Point, PA); *c*, American Cyanamid Co. (Pearl River, NY); *d*, Pfizer Laboratories (Groton, CT) and A. H. Robins (Richmond, VA); *e*, Dr. Rodney King, National Institute for Medical Research (Mill Hill, England); *f*, Wallace Laboratories (Cranbury, NJ); or *g*, Upjohn Laboratories (Kalamazoo, MI).

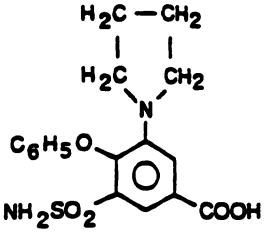
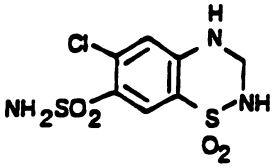
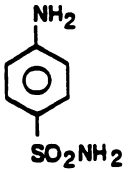
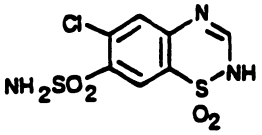
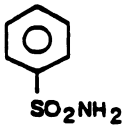
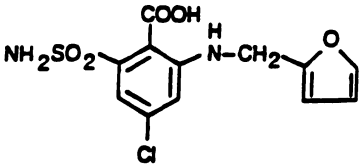
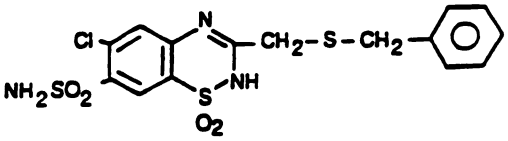
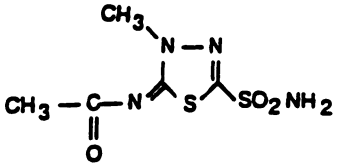
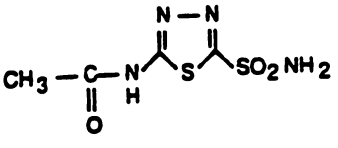
		Compound	Molecular weight	Source
1		Piretanide	362	*a*
2		Hydrochlorthiazide	298	*b*
3		Sulfanilamide	172	*c*
4		Chlorthiazide	296	*b*
5		Benzenesulfonamide	157	*c*
6		Furosemide	331	*a*
7		Benzthiazide	432	*d*
8		Methazolamide	234	*c*
9		Acetazolamide	222	*c*

TABLE 3—Continued

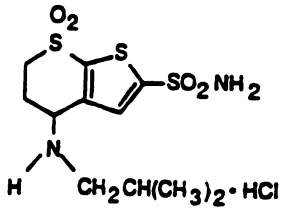
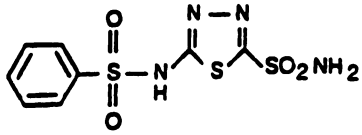
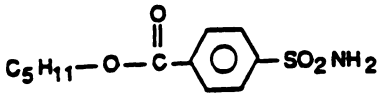
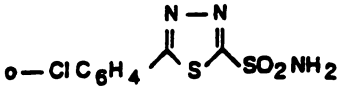
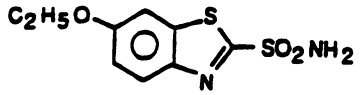
		Compound	Molecular weight	Source
10		MK-927	376	*b*
11		Benzolamide	330	*c, f*
12		C_5 ester of *p*-carboxybenzenesulfonamide	271	*e*
13		Chlorzolamide	276	*c*
14		Ethoxzolamide	258	*c, g*

TABLE 4
Ionization and solubility characteristics of 14 sulfonamides

Compound	pK_a[a]		SO_2NH^- at pH 7.4	Solubility at pH 7[b]	$CHCl_3$/buffer partition coefficient at pH 7
	$-SO_2NH_2$	Other (acidic)			
			% of total	mM	
1. Piretinide	9.9	5.2	0.25	s	0.05
2. Hydrochlorthiazide	9.2	7.0	1.6	i	0.01
3. Sulfanilamide	10.1		0.2	50	0.02
4. Chlorthiazide	9.1	7.5	2	2	0.04
5. Benzenesulfonamide	10.0		0.25	16	1.1
6. Furosemide	10.1	5.6	0.2	s	0.03
7. Benzthiazide	9.6	8.1	0.6	i	0.16
8. Methazolamide	7.4		50	5	0.06
8. Acetazolamide	9.1	7.4	2	3	0.001
10. MK-927	8.3	5.8[c]	12	15	2
11. Benzolamide	9.1	3.2	2	>100	$<10^{-5}$
12. C_5 ester of *p*-carboxybenzenesulfonamide	10.1		0.2	i	0.3
13. Chlorzolamide	7.3		56	0.4	10
14. Ethoxzolamide	8.0		25	0.04	25

[a] See Experimental Procedures for comment on identification of the two pK_a values.
[b] s, >50 mM; i, <1 mM.
[c] Basic group (12).

appears below (Table 5), equilibrium and rate constants for these two disparate compounds are within 2-fold of each other.

Rate constants for sulfonamides and carbonic anhydrase. Kinetic data for the activity of 14 sulfonamides are shown in Table 5. The range in equilibrium constants is 24,000, and it is seen at once that this is virtually all a function of the k_{on} values, which vary 10,400-fold, and in about the same order. Over this enormous range of equilibrium constants, which are the usual measure of potency, k_{off} varies only 5-fold.

It will be observed that the k_{on} constants approach 10^8 liter/mol sec^{-1}, which is near the usual limit of 10^9 liter/mol sec^{-1} placed on diffusion-controled reactions. It may be predicted that, if k_{off} is indeed constant, we cannot expect there to be inhibitors that are more than about 10 times as potent as shown here.

TABLE 5

Equilibrium and rate constants of 14 carbonic anhydrase inhibitors at 25°, 6 mM CO_2, and 2 nM E_0

Compound	$K_I \times 10^9$	$k_{on} \cdot 10^{-6}$	k_{off}
	M	liter/mol sec^{-1}	sec^{-1}
1. Piretinide	17,000	0.003	0.050
2. Hydrochlorthiazide	2,350	0.013	0.030
3. Sulfanilamide	750	0.033	0.024
4. Chlorthiazide	460	0.066	0.030
5. Benzenesulfonamide	440	0.10	0.044
6. Furosemide	80	0.3	0.024
7. Benzthiazide	16	0.6	0.010
8. Methazolamide	13	3.5	0.042
9. Acetazolamide	7	3.0	0.021
10. MK-927	4	4.4	0.020
11. Benzolamide	0.9	31	0.028
12. C_5 ester of *p*-carboxy-benzenesulfonamide	0.8	15	0.012
13. Chlorzolamide	0.8	30	0.024
14. Ethoxzolamide	0.7	14	0.010

Relation to pharmacological activity. Do rate constants influence pharmacological activity, independent of equilibrium constant? This question has rarely been asked; potencies of drugs are usually given in terms of the K_I. This is reasonable if drug and enzyme are in equilibrium at the active site. Using the data at hand, I attempt to answer this question.

Fig. 2 gives the construct for sulfonamides for which we have pharmacological information (11). The model uses K_I of 10^{-8} M, k_{on} of 10^6 M^{-1} sec^{-1}, k_{off} of 0.01 sec^{-1}, and plasma half-life *in vivo* of 1 hr. Enzyme concentration (E_0) is 1×10^{-6} M (in Fig. 3, we use 1.001×10^{-6} M for convenience) and drug concentration (I_0) is 10^{-5} M. This corresponds roughly to measured values for acetazolamide or methazolamide and for carbonic anhydrase in rabbit ciliary process.

The model of Fig. 2 shows that there is equilibrium between drug and enzyme at the active site. The basis for this is the comparison between k_{off} (0.01–0.05 sec^{-1}; Table 5) and the drug-elimination constant. Given a plasma half-life of 60 min (11), decay (k_{pl}) is on the order of 10^{-4} sec^{-1}, some hundred times slower than k_{off} (Fig. 2). Thus, the equilibrium condition holds for parenteral administration, and the K_I appears to be a proper measure of drug potency, assuming that drug is well distributed in the tissue.

Fig. 2 shows that, 15 min after drug administration and at the peak of activity in reducing aqueous flow (11), the fractional inhibition of enzyme at the active site is $i = 0.999$. Free drug in plasma and tissue is 10 μM, and at the tissue site 10 nM drug/sec is moving between bound and free form. At 6 hr, free drug has declined to 0.1 μM; the affinity between drug and enzyme dictates that now $i = 0.90$, whereas drug movement is only reduced to 9 nM/sec. The system is still at equilibrium, but now the pharmacological effect has disappeared, as is always the case in carbonic anhydrase-dependent systems when i is <0.99 (11).

Discussion

I shall follow the same four divisions as given in Results. The main finding concerns the measurement of rate constants for 14 sulfonamides and the constancy of k_{off} values, k_{on} varying greatly. The other sections relate to procedure, with characterization of the drugs, and application to a pharmacological model.

Procedure and theory. The method is a direct one, in which drug and enzyme are incubated for various periods of time and then the fractional inhibition is measured. A model experiment and data applied to the appropriate eqs. 1 and 2 are shown in Table 2. It was necessary to depart from the usual textbook treatment for the opposing reversible reactions A + B ⇌ C in which the chemist sets A = B. The enzymologist cannot do this, because, in E + I ⇌ EI, E is usually <I. The modification of the classical equation to fit this situation is given in the Appendix.

Physicochemical properties of sulfonamides. A study of Tables 3–5 does not reveal any central characteristics for high activity or, more specifically, the great variation in k_{on}. It may be noted, however, that the pK_a range of the sulfonamide group is not great. Surprisingly, small molecules, even uncharged (benzene-sulfonamide and sulfanilamide), do not have high k_{on} rates. Clearly, factors other than charge and diffusion into the active site are at work; X-ray crystallography has revealed binding not only to the Zn atom, yielding the Zn-$NHSO_2R$ bond, but also at the lipophilic cavity (18). Within a homologous series of benzene sulfonamides and their esters and amides, King and Burgen (5) did find a relation between octanol/water partition coefficient and k_{on}. In the present study, however, with a wide diversity of aromatic and heteroaromatic structures, no relation was observed between k_{on} and the chloroform/water partition coefficient. It might be noted in passing that octanol/water partitions are enormously greater than those for chloroform and water; for compound **12**, the octanol value is 510 (5) and the chloroform value is 0.3 (Table 4).

Rate constants and contrast with anions. The great range of k_{on} values does not yield a simple explanation based on the physical chemistry of these compounds.

Turning to k_{off} values, it is perhaps not surprising to find them nearly equal, despite the large differences in structure among these 14 compounds. These rates must ultimately be related to binding at the EZnOH site, which may be about equal in the compounds studied, i.e., once the bond is formed, its strength is similar among the compounds. Fig. 3 shows an energy diagram for three compounds of low, middle, and high activity. The activation energy $\Delta G‡$ is remarkably low for compound **14**, the most active inhibitor, reflecting low K_I and high k_{on}. Values of ($\Delta G + \Delta G‡$) are similar for the three compounds, reflecting similar k_{off} values.

Following the argument of King and Burgen (5), release of EI is limited by transition between two internal complexes; the first step (slower) is the breaking of the Zn-N bond in the final product EI, and the second (fast) is diffusion of the products from the active site. Thus, the k_{off} represents the lifetime of the final product EI. Their paper (5) should be consulted.

The data on anion binding (Table 1B) are instructive. The k_{on} rates are far too fast to measure with our techniques, but the literature provides data on anions of different type. All are striking examples of potency being dictated by off-rates, with that for chloride being nearly 10^8 times greater than those of the sulfonamides and that for cyanate being about 10^4 times greater. These large differences are reflected in the K_I values. Because anionic ligands, unlike those of the sulfonamides, are all different, it may be expected that there are large differences in their dissociation constants. The final enzyme-anion com-

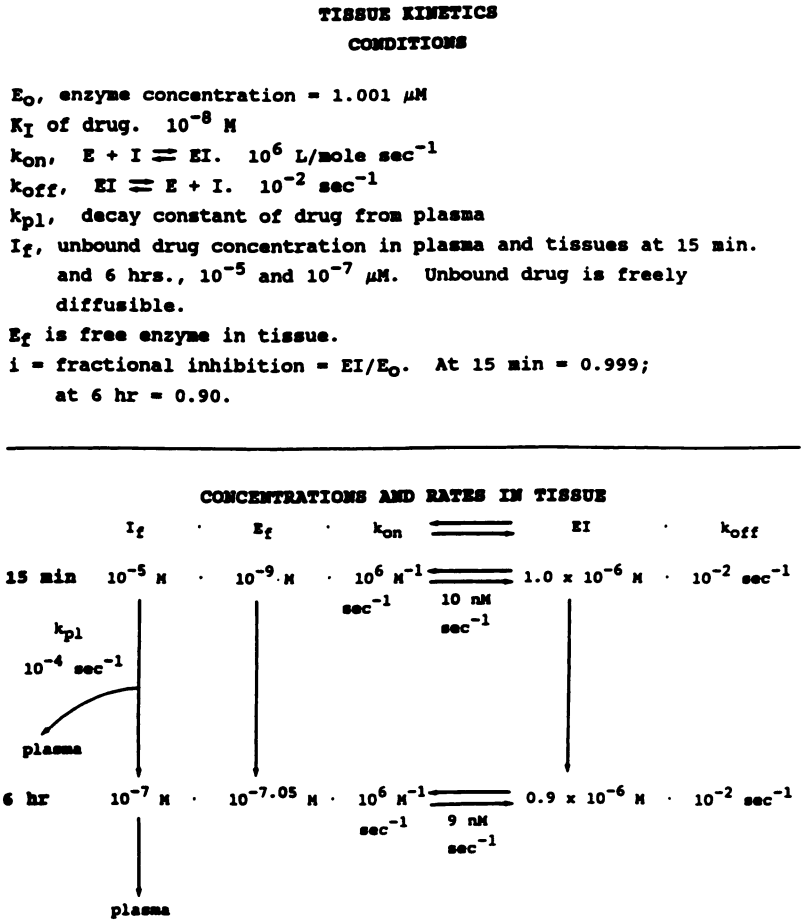


Fig. 2. Model for kinetics of carbonic anhydrase inhibition in tissue after systemic administration of sulfonamide.

plex is far less stable than the sulfonamide complex and suggests that anion binding is of a different type than the Zn-N association of the enzyme-sulfonamide complex. Indeed, recent X-ray crystallography suggests that some anions may not bind to the zinc,[4] and most anions are not capable of displacing Zn-bound H_2O/OH from the active site, as are sulfonamides (18). Their association constants reflect approach to the diffusion-limited rate between such small ions and the 30-kDa protein; thus, for anions there appear to be slight or no energies of activation, relative to diffusion control.

Relation to pharmacological activity. From the analysis of Fig. 2, it appears that equilibrium constants are a proper measure of drug potency, as long as k_{off} is much faster than plasma decay; so far, it seems that this would always apply for systemic administration in the carbonic anhydrase system. However, for other enzyme-inhibited systems, where k_{off} is 10^{-4} sec^{-1} or less (1), a different analysis and conclusion may be demanded. Also, a different analysis may apply to other routes of administration in the carbonic anhydrase system, as follows.

Topical administration of a carbonic anhydrase inhibitor has now become feasible, with the advent of sulfonamides applied to the cornea, for inhibition of the enzyme at the ciliary process. This reduces aqueous flow and pressure and is designed for the treatment of glaucoma (literature and data cited in Ref. 12). The prototype drug is MK-927 (compound **10**). As in Fig. 2, EI is formed at the ciliary process, but now there is no free drug in plasma to drive the reaction to the right. This would not appear to be an equilibrium situation. Given k_{off} in the range of 0.01–0.05 sec^{-1} (Table 5) and the very high blood flow to the tissue, it would seem that here the pharmacological effect might dissipate in several minutes and that a truly effective compound would require that k_{off} be 10^{-4} sec^{-1} or less.

However, MK-927, with k_{off} = 0.02 sec^{-1}, is active for 5 hr (12). The explanation seems to be that after topical administration, there are depots within the eye that may serve (roughly) the same function as drug in plasma. The chief depot is the corneal stroma, where the 1-hr concentration of MK-927 is 7 times that in ciliary process and the half-life is 3 hr (12). Presently, there is not enough information to quantify relations between drug in cornea (or other sites) and ciliary process. It is still conceivable that, for locally acting compounds, drastic reduction in k_{off} would be highly effective, but this has not yet been accomplished.

In conclusion, it is shown that the equilibrium potency (K_I) of sulfonamides against carbonic anhydrase in a range of 24,000-fold and depends essentially on the rate of binding (k_{on}) of drug to enzyme. This contrasts sharply with the reaction of enzyme with anions, where the dissociation rate (k_{off}) appears critical. The data for sulfonamides are used to suggest models in which either K_I or k_{off} is the controlling element for activity *in vivo*.

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[4] M. Lindahl, A. Svensson, and A. Lilyas. Metal poisons and the inhibition of carbonic anhydrase. Submitted for publication.

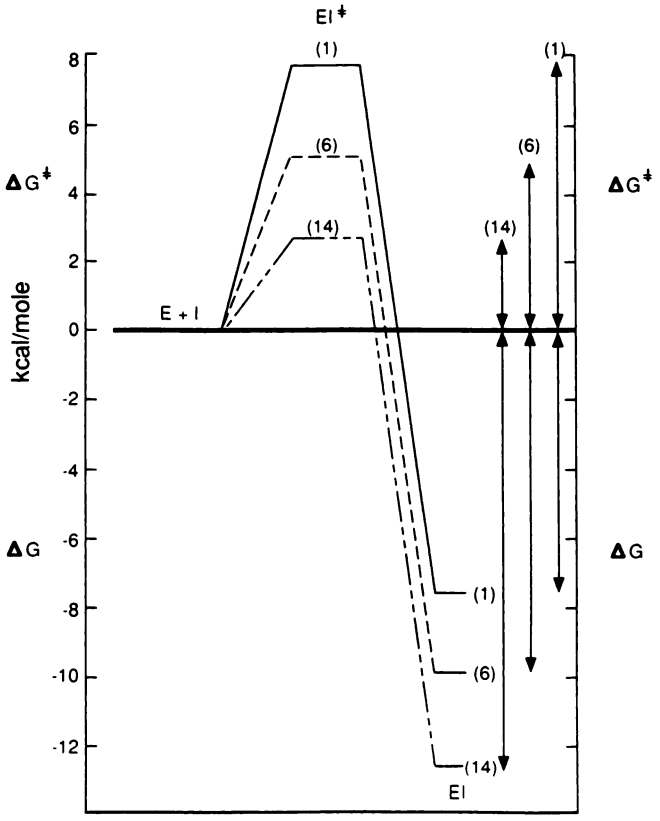

Fig. 3. Energy diagram for reaction of three sulfonamides (compounds **1**, **6**, and **14**) (Table 3) with carbonic anhydrase (*E*). $\Delta G = -RT \ln K_{IA}$, where *R* and *T* have their usual meanings and K_{IA} is the equilibrium association constant (from K_i of Table 5). The activation energy is that relevant to a diffusion-limited reaction: $\Delta G‡ = -RT \ln k_{on}/k_{diff}$. Values of k_{on} are from Table 5, and k_{diff} = diffusion-limited rate for molecules of size of E and I, 10^9 liter/mol sec^{-1}.

equations. I am particularly grateful to Dr. David Silverman for valuable criticism and contribution of Fig. 3.

Appendix

Eq. 2 is derived as follows, following the treatment in Ref. 10 but using the general case where the reactants E_0 and I_0 have different concentrations.

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI$$

at time 0, $E_0 \quad I_0 \quad 0$ (a)

at time t, $(E_0 - x)(I_0 - x)x$

$$\frac{dx}{dt} = k_{on}(E_0 - x)(I_0 - x) - k_{off}(x) \quad \text{(b)}$$

at equilibrium where $x = x_e$, $dx/dt = 0$, then

$$\frac{k_{on}(E_0 - x_e)(I_0 - x_e)}{x_e} = k_{off} \quad \text{(c)}$$

Substituting the expression for k_{off} in eq. c into eq. b yields

$$k_{on}dt = \frac{x_e dx}{(x_e - x)(E_0 I_0 - x_e x)} \quad \text{(d)}$$

Eq. d is integrated after resolution into partial fractions, ultimately yielding

$$k_{on} = \frac{1}{t} \cdot \frac{x_e}{E_0 I_0 - (x_e)^2} \ln \frac{x_e(E_0 I_0 - x\, x_e)}{E_0 I_0 (x_e - x)} \quad \text{(eq. 2 of text)}$$

I am indebted to Dr. Keith Laidler of the University of Ottawa for pointing out that the departure from existing equations (see Ref. 10) in which $A_0 = B_0$ (or $E_0 = I_0$) need only involve substituting the concentration of each reactant (as above) for the term A_0^2 generally used. Dr. Kenneth Steiglitz of Princeton also was most helpful in discussion of the mathematics.

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