THE BINDING OF INHIBITORS TO CARBONIC ANHYDRASE *IN VIVO*: DRUGS AS MARKERS FOR ENZYME

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UNITS of activity of carbonic anhydrase in tissues or solutions may be converted to molar terms, as a result of an analysis of inhibition kinetics[1]. *In vitro*, lens suspensions and, under certain conditions red cells, bind potent carbonic anhydrase inhibitors in concentrations equivalent to original enzyme[2]. The present study shows that acetazolamide, an inhibitor of $K_I = 6{\cdot}4 \times 10^{-8}$, binds stoichiometrically to carbonic anhydrase — containing tissues *in vivo*. If conditions are properly selected, (EI), the bound form of enzyme, might be used as a marker for (E_o), enzyme orginally present.

METHODS AND MATERIALS

Enzyme activity was determined in dog tissue homogenates[3] and units converted to molar terms[1,2] by the relation: Enzyme units/g $\times$ 0·017 = μmol/kg. The kidneys were thoroughly perfused with saline (200–100 ml) through the renal arteries; the choroid plexus was not perfused. When the head was tilted up following death, the vessels of the choroid emptied. Perfusions and dissections were carried out following deep pentobarbital or thiopental anesthesia. Tissues were immediately removed and suspended in distilled water, and enzyme assays carried out. Tests for blood in tissues by the benzidine reaction[4] always showed less than 1%; usually less than 0·2%. It was determined that enzyme was not washed out by continued perfusion.

Acetazolamide was determined by the same technique[3] but it is emphasized that the assay for the enzyme and for inhibitor are based on entirely different standards, and should be regarded as independent estimates.

Dogs were injected with 5 mg/kg acetazolamide by vein. Blood was drawn into heparinized syringes and immediately centrifuged. Tissues were taken as described above, except that in this case only 50 ml saline was used to perfuse the kidneys, since it was recognized that washing might dissociate the enzyme-inhibitor complex. Urine

was collected by catheter. The fluids and tissues were diluted with water, homogenized where necessary, and analyzed for total content of acetazolamide. The analysis depends upon the carbonic anhydrase inhibiting potency of the tissue; however only a small fraction of acetazolamide is metabolized in the dog, and the gravimetric recovery and isolation of drug from urine agrees with recovery as measured by this enzymatic method[3].

The K_I of acetazolamide was determined as described previously[1] except that measurements were made at 37°. The barbital buffer system was used. The term K_I is retained from enzymological use; strictly this is the dissociation constant of the enzyme-inhibitor complex K_{EI}.

Other terms used are: (E_o), molar concentration of carbonic anhydrase in untreated dogs; (I), total concentration of acetazolamide found by analysis; (I_f), concentration of unbound or free acetazolamide; (EI) concentration of enzyme-inhibitor complex, or bound or inhibited enzyme; b = fractional binding, EI/E_o; i = fractional inhibition, formally the same as b, but computed as previously[5], $= I_f/(I_f + K_I)$. Where $I \gg E_o$, $i = I/(I + K_I)$.

RESULTS AND DISCUSSION

1. E_o

Table I shows the concentration of enzyme in the tissues. Because of the relatively low concentration in renal medulla, with the attendant dangers of red cell contamination particularly in drug experiments where washing was limited, no further data are given. The relations in enzyme concentration between renal cortex and medulla are nevertheless

TABLE I

Carbonic anhydrase concentration in untreated dogs: (E_o)

	Renal cortex	Renal medulla	Choroid plexus	Red cells
Units/g ± S. E.	546 ± 11	64 ± 18	386 ± 61	1400 ± 120
μmol/kg ± S. E.	9·2 ± 0·2	1·1 ± 0·3	6·6 ± 1·0	24 ± 2
(n)	(14)	(11)	(5)	(5)

Tissues were perfused with saline until free of blood.
Analysis by changing pH method[3]. Units/g × 0·017 = μmol/kg(1).

of interest. The tissues studied are among the highest in carbonic anhydrase concentration; others in this category are lens[4] and epithelium of inner ear[6] which for anatomical reasons are not so suitable for drug distribution work.

2. *Plasma*

Fig. 1 shows the decay of acetazolamide from plasma, red cells, renal cortex, and choroid plexus. These will be considered in the present and the following three sections. Section 6 concerns calculated free drug (I_f) in the various compartments.

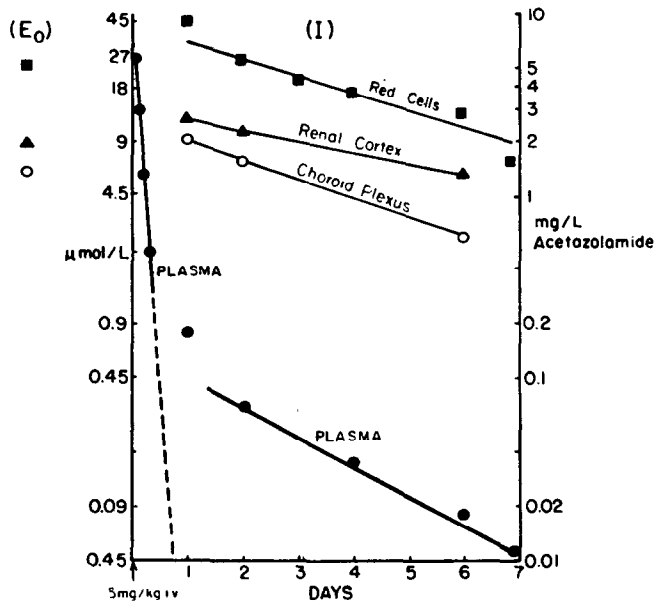

FIG. 1. On the left of the ordinate are shown enzyme concentrations (E_0) in untreated dogs: in red cells ■, renal cortex ▲, and choroid plexus ○. On the right of the ordinate are (I) total concentrations of acetazolamide found in these organs.
The dotted line shows the theoretical plasma decay for the initial curve, if it were a simple first order process.

For the first 6 hr there is a first order decay curve with half-life of 105 min. The first order rate constant is then, $k = 0{\cdot}693\, t^{1/2} = 0{\cdot}4\text{hr}^{-1}$. These data agree with earlier work which include a range of doses and times and in which it appeared that this decay is largely due to renal excretion[7]. Following this phase the shape of the decay curve changes radically, as was noted in a qualitative way in our previous studies[7]. At plasma concentrations about 0·3 µg/ml. the analytical method is less accurate, and 0·1 µg/ml. is the lower limit. The plasma concentrations of Fig. 1 past 6 hr are calculated from urinary excretion, taking the clearance (UV/F), of free drug to be 30 ml./min[7] and the plasma binding to be 80%. Plasma concentration (bound and unbound) was thus equal to [U(µg/ml.) × V(ml./min)]/6(ml./min). UV (av. of 4 dogs at each of the times indicated) are: 24 hr. 1·1 µg/min; 48 hr, 0·4 µg/min; 96 hr,

0·21 μg/min; 144 hr, 0·11 μg/min; 168 hr, 0·065 μg/min. Urine flow was about 0·2 ml./min.

Using these data, a second plasma decay curve is obtained between 2 and 7 days for which $k = 0{\cdot}3$ days^{-1}. The profound difference between the early and late curve is illustrated by the fact that the early curve (following dashed line) according to the formula $C_t = C_o e^{-kt}$ yields at time $t = 48$ hr, the concentration (C_t) of approximately 10^{-3} to 10^{-4} μg/l. The actual concentration (Fig. 1) at this time is some 50,000 times greater. It appears that factors other than renal excretion, which govern the first curve, have come into play (see section 6).

3. *Red Cells*

The uptake of acetazolamide into red cells *in vivo* and *in vitro* has been the subject of an earlier study[2]. Fifteen minutes after injection (Table II), red cells contain a substantial amount of diffusable drug. After a dose of 50 mg/kg, large amounts rapidly enter and leave cells.

TABLE II

I_f in tissues, and plasma concentrations following acetazolamide (μmol/kg)

Time	50 mg/kg			5 mg/kg			
	Pl total	RBC I_f	RBC Bound	Pl total	RBC I_f	Kid. I_f	RBC Bound
15 min	820	320	144	40	9	58	45
1 hr	540	225	135	32	7	45	96
4 hr	180	55	120	13	7	—	90
24 hr	5	<2	45	—	—	—	40
48 hr	(·3)	1·2*	23	(·3)	1·2*	0·8*	25
144 hr	(·06)	0·07*	7	(·06)	0·07*	0·1*	7

* Calculated, see Table III

( ) From urinary concentrations: see text.

The exit of this fraction proceeds at a rate roughly that of initial plasma decay (1–4 hr data). Fig. 1 confirms the finding that the half-life in red cells of bound drug is approximately 3 days; $k = 0{\cdot}23$ days^{-1}. It will be noted that the 1 day value falls above this curve, in agreement with the finding that the decay of acetazolamide bound to red cells is biphasic, there being probably two receptors. The first is largely responsible for the decay of bound drug before 2 days; 2–7 day curve appears to reflect dissociation and elimination of drug bound to carbonic anhydrase[2]. Fig. 1 shows that (I) in red cells at 48 hr has approximately the same value as (E_o). Presumably (I) is in the form (EI), whence (EI) $\cong$ (E_o). Based on these relationships the fractional binding, designated b, is readily

calculated; b = (EI)/(E) Table III. This is formally the same as fractional inhibition; however, calculation of b or i in precise terms from (EI) and (E_o) is not feasible since (E_o) is somewhat variable. Precise estimations of i, particularly for values over 0·90, are readily calculable from measured (I_f), and are considered elsewhere[1,5].

The decay of (I) — presumably from (EI) in red cells — in such that about 50% of enzyme is still bound to inhibitor after 7 days. Although the reaction $EI \rightleftharpoons I_f + E_f$ is readily reversible *in vitro*[7,8] and (I_f) initially in plasma declines rapidly (Fig. 1) (I_f) within red cells declines but slowly *in vivo* (Table II). The slow decline of drug in red cells is ultimately accountable to renal excretion, operating on the small amounts of (I_f) liberated into the plasma. Thus on day 2, the dog (av. wt. 10 kg) has 400 ml. red cells × 5·6 μg/ml. drug = 2240 μg/red cell mass. This probably represents well over 90% of drug remaining in

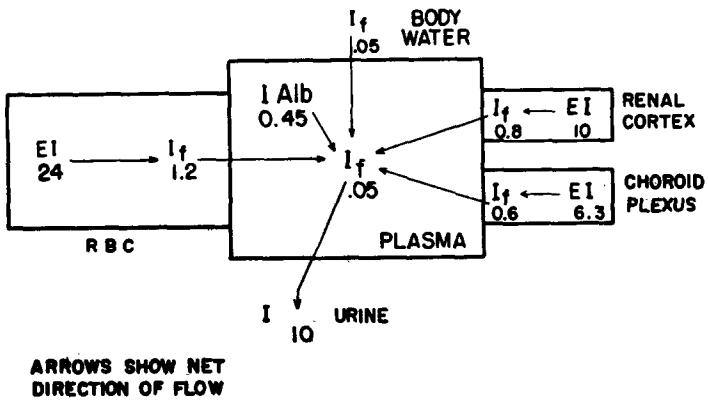

FIG. 2. Derivation of the values for I_f in tissues is given in Table III. It is recognized that I_f is also considered to be in equilibrium with EI; in this model however, the arrows are arranged to show the flow of drug away from equilibrium.
The following are assumed: 90% binding to plasma protein; UV/F = 30; urine flow 0·15 ml./min; unbound drug freely diffusible through body water.

the body. The rate of urinary excretion is about 0·4 μg/min, or 600 μg/day; it is from this figure that the plasma concentration of Fig. 1 is calculated. This urinary excretion rate satisfactorily accounts for the red cell decay curve. It should be emphasized that if the initial decay of plasma continued to 48 hr, (see above), the theoretical concentration would be 10^{-3} μg/l.) and no drug would be detectable in the urine.

These data indicate that (I_f) dissociated from (EI) within red cells is only slowly liberated into the circulation. The overall relations between (I_f) in the various compartments are illustrated in Fig. 2 and amplified in section 6 below.

4. *Renal Cortex*

The initial uptake of drug from plasma (1 hr after injection of 5 mg/kg) results in a concentration in renal cortex of about 12 μg/g or 54 μmol/kg tissue. Since (E_o) is 9 μmol/kg (Table I), (I_f) is 45 μmol/kg, i. e. somewhat higher than the simultaneously determined plasma concentration (Table II). Fig. 1 shows that at 48 hr, (I) in renal cortex is approximately equal to (E_o). The decay rate of (I) from 24 hr on in this tissue is somewhat slower than those of the others studied. Closer analysis of relations between (I) and (E_o) are subject to error, since the kidney continues to excrete drug. At 48 hr for example urinary concentration is 2 μg/ml.; if this component were not thoroughly washed out in the perfusion it would add substantially to the drug found. It is probably for this reason that (I) appears slightly greater than (E_o). There is no evidence to rule out another receptor in kidney, with relatively few binding sites, but it is not demanded by present data. At any rate, it is

TABLE III

Fractional binding (b) of acetazolamide to carbonic anhydrase in tissues

$b = EI/E_o$. See Table I for E_o values

	48 hr				144 hr			
	I_{total}	I_f	EI	b	I_{total}	I_f	EI	b
	(μmol/kg)				(μmol/kg)			
Renal cortex	10·8	0·8	10	1·09	6·3	0·12	6·2	·68
Choroid plexus	6·9	0·6	6·3	·96	2·7	0·06	2·6	·40
Red cells	25	1·2	23·8	·99	13	0·07	13	·54

$EI = I_{total} - I_f \cdot I_{total}$ is measured directly.

From $K_I = I_f \cdot E_f/EI$, I_f is calculated as follows: — At 48 hr, formation of EI from E_o is essentially complete and there is no excess I_f or E_f. Thus $I_f = E_f = \sqrt{K_I \cdot \int EI \cdot EI}$ is approximated by E_o or I_{total}. — At 144 hr, E_f is obtained from $E_o - EI$, using for EI its close approximation I_{total}. Then $I_f = (K_I \cdot EI)/E_f$.

provisionally assumed that (I) at 48 and 144 hr is equivalent to (EI) + (I_f). The latter term is small enough so that (I) $\cong$ (EI) $\cong$ (E_o) (Table III). The fractional binding is given in Table III; these are subject to the errors discussed above, and as noted for red cells cannot be regarded as an accurate measure of i.

(I_f) in the kidney at 48 hr compared with the 1 hr value (Table II) is instructive. The decline is 70 fold. The theoretical decline for I_f in plasma if there were no binding would be 10^7 fold. Clearly the release

of free drug from cells is slow enough to account for the radical difference in shapes of the 2 plasma decay curves; the controlling element of the first being renal clearance (when bulk of the drug is largely in plasma and extracellular fluid) and of the second, release from cells (remaining drug bound to carbonic anhydrase within tissues, as red cells and kidney .

5. *Choroid Plexus*

As for the other tissues, (I) at 48 hr in choroid plexus approximates (E_o). In this case, considering the standard error of (E_o) (Table I), (I) is as close to (E_o) as methodology involved permits; the presumptive conclusion again is that (I) $\cong$ (EI) $\cong$ (E_o) and that b is close to 1 (Table III). Since the choroid is not an excretory organ (cf. kidney) and since no other receptor appears dominantly involved (as red cells prior to 2 days), analysis of this tissue appears most satisfactory in terms of finding (EI), the union of carbonic anhydrase and acetazolamide.

The decay of drug in choroid is parallel to that of the second curve for plasma; half-life is 2·5 days and $k = 0{\cdot}28$ days^{-1}.

6. I_f *in Tissues*

Table III shows the calculated (I_f) in red cells and tissues at 48 and 144 hr. These values are all extremely small, due to the facts that the large excess (I_f) originally present (Table II) has disappeared in the first phase of plasma decay, and that the (I_f) theoretically demanded for the equilibrium with (EI) is very small, since K_I is small.

(EI) in tissues at 48 hr and after is thus the source of (I_f) in the body; the latter may be thought of as continuously discharging from the store of (EI). The *concentration* of (I_f) in red cell cytoplasm is now fixed by the magnitudes of (E_o), (EI) and K_I. As noted above, the *rate* of discharge of (I_f) from cells is slow in these later periods, since (I_f) — the driving force for outward movement — is maintained low due to the buffering action of the enzyme-binding reaction. It is recognized that since equilibrium between (E) and (I) is rapid and reversible, the time consuming element in discharge of (I_f) is not this reaction, but the gradient of (I_f) between red cell water and plasma. This depends not only on concentrations involved, but on resistance to diffusion and permeability. A quantitative treatment of the outward movement of drug assessing the relative importance of the rate constant of the binding reaction, the K_I, the magnitude of E, and the rate constants for drug diffusion and permeability is beyond the scope of this paper.

As a first approximation, however, we may consider the magnitude of K_I in relation to the release of (I_f). Methazolamide, a drug of the same K_I as acetazolamide but with superior red cell penetrability [2]

showed the same red cell decay curve as that for acetazolamide, i. e. $k = 0{\cdot}35$ days^{-1}. This suggests that the binding constant is in fact the controlling factor. Comparison between acetazolamide and sulfanilamide, whose K_I is 100 times greater, is of interest. Acetazolamide bound to red cells did not measurably diffuse from a dialysis bag into a very large reservoir within 24 hr; under the same conditions sulfanilamide was completely released [2]. Acetazolamide in free aqueous solution under similar conditions has a half-life of 1-2 hr. Comparison of red cell decay of these two drugs *in vivo* is illuminating. For acetazolamide, as noted in Fig. 1, the half-life is about 2 days. Table III shows that (I_f) in equilibrium with essentially fully reacted (E) in red cells (the situation at 48 hr is equal to 1·2 mol/kg = $\sqrt{K_I} \times (EI)$. If the rate of discharge of (I_f) from combined E, or (EI), is essentially a function of K_I, it will vary as $\sqrt{K_I}$, since $I_f \propto \sqrt{K_I}$. The saturation value, (EI), is the same in any experiment, being the same as E_o. From these considerations, sulfanilamide should decay 10 times as rapidly as acetazolamide, i. e. the red cell half-life should be about 5 hr. This is roughly the case.[2]

It may also be shown that for an inhibitor 100 times weaker than acetazolamide, saturation of (EI) requires 10 times the value of (I_f) noted in Table III. This means that a considerable fraction of (I_{total}) would be (I_f). In such case the use of (I_{total}) as a marker for (EI) would be improper. For a drug to be a marker for enzyme in this system, K_I cannot exceed 10^{-7}M. Table IV makes the additional point that for acetazolamide, (I_f) is too high in the first four hours to permit (I_{total}) to be used as a measure of (EI).

TABLE IV

Inhibition and binding of acetazolamide and carbonic anhydrase in vivo

Time after drug	I_f Relative to EI	E_f	i or b	Physiol. effects	$I \cong EI$
2 hr	High	Very low	$>.99$	+	No
48 hr	Low	Low	·90—·95	0	Yes
144 hr	Very low	$\cong$ EI	·4—·7	0	Yes

Acetazolamide initially gains entrance well into red cells and kidney (Table II), accounting for the physiological inhibition of enzyme observed after injection. For several hours after injection of 5 mg/kg, percent inhibition, calculated as a function of (I_f) in tissues, exceeds 99·9%. Dose-response curves show that only at this order of inhibition are observable effects (typically alkalinization of urine) elicited [5]. By

6 hr the renal effect is finished; Fig. 1 shows the inhibitor concentration in plasma to be $4{\cdot}5 \times 10^{-6}$ M. Taking this as I_f percent inhibition is about 99%: (I_f) within tissues may be higher than this. Up to this time, free drug enters and leaves the cell by diffusion, and decay from plasma is a function of renal excretion. Thereafter, decay of (I_f) from tissues is (see above and section 4) largely a function of the very small K_I, with probable minor roles for diffusion and cell permeability. Table III shows that at 48 hr binding is still over 90%, and at 144 hr about 50%. It is emphasized that these degrees of binding, although chemically and perhaps histologically interesting, are far less than required for detectable physiological effects. Table IV summarizes these relationships.

SUMMARY AND CONCLUSION

The fate of acetazolamide in the dog (5 mg/kg *i. v.*) has been studied as consisting of two distinct phases.

In the *first* phase (about 6 hr) drug reaches a concentration in plasma which suggests some general distribution into tissues, and declines with a half-life of 105 min, a function of its renal clearance. During this period, drug in at least two carbonic anhydrase containing tissues, red cells and kidney exists free (I_f) at a concentration of the order of that in plasma. Based on the conventional expression for drug enzyme equilibria, about 99·9% of carbonic anhydrase is inhibited; this is the period of pharmacological response. Since (I_f) exceeds and masks (EI) in the period, measured drug concentration in tissues is not correlated with or localized to carbonic anhydrase.

In the second phase (2–7 days), plasma concentration has declined to about 1% that of the first day and the half-life is very much less. The concentration of drug in tissues is now virtually equivalent (at 2 days) to that of carbonic anhydrase. (I_f) in these tissues is now too small to be directly measurable; calculation from the equilibrium expression shows that (I_f) is less than 10% total (I) in tissues. Thus $(I_{total}) \cong (EI) \cong (E_o)$. In this phase ($I_f$) in tissues declines very slowly, and appears to be the source of the small, slowly decaying plasma concentration. The chief ultimate source of (I_f) in the body is red cell (EI), the largest site of carbonic anhydrase. (I_f) approaches reversible equilibrium with (EI); but because of very small dissociation of (EI) and slow rate of loss of (I_{free}) from cells, (EI) is maintained within tissues for at least 7 days. During the last five of these days, the localization of inhibitor (total I) is felt to be equivalent to the localization of enzyme originally present (E_o).

These data would appear conducive to further studies, in which acetazolamide or other suitable inhibitors are labelled for initimate cytological localization of carbonic anhydrase.

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REFERENCES

1. T. H. MAREN, A. L. PARCELL and M. N. MALIK; *J. Pharm. Exp. Therap.* **130** 389 (1960).
2. T. H. MAREN, B. ROBINSON, R. F. PALMER and M. E. GRIFFITH; *Biochem. Pharm.*, **6**, 21 (1961).
3. T. H. MAREN, V. I. ASH and E. M. BAILEY; *Bull. Johns Hopkins Hosp.* **95** 244 (1954).
4. E. J. BALLINTINE and T. H. MAREN; *Amer. J. Ophthal.* **40** 148 (1955).
5. T. H. MAREN; *Fed. Proc.* **20** 227 (1961).
6. S. ERULKAR and T. H. MAREN: *Nature, Lond.* **189** 459 (1961).
7. T. H. MAREN, E. MAYER and B. C. WADSWORTH; *Bull. Johns Hopkins Hosp.* 95 199 (1954).
8. K. C. LEIBMAN, D. A. ALFORD and R. A. BOUDET; *J. Pharm. Expt. Therap.* **131** 271 (1961).