The Chloride Effect of Carbonic Anhydrase Inhibitors

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SUMMARY

Sulfonamide carbonic anhydrase inhibitors not only inhibit carbonic anhydrase(s) but also the active transport of chloride by the gastric mucosa. The concentrations required for the latter effect are 100 or more times that required to block functions ascribed to the enzyme carbonic anhydrase. The slow rate of H^+ ion secretion by the isolated mucosa is not inhibited by sulfonamide inhibitors. Agents that are the most potent inhibitors of carbonic anhydrase exert the greatest effect on chloride transport. Inactive analogs had no discernible effect on either system. For 15 agents, there is a striking parallel between their inhibition of chloride transport and the inhibition of carbonic anhydrase.

INTRODUCTION

Carbonic anhydrase inhibitors have two actions upon the gastric mucosa, one on the stomach of the intact animal and the other on the isolated structure. Moderate amounts, 5–100 mg/kg, given to the intact animal partially suppress formation of HCl (1). The intravenous administration of 40 mg/kg over 20 min results in a plasma concentration of unbound drug of 0.1 mM (2). Greater concentrations of inhibitor, 1–10 mM, bathing the isolated bullfrog gastric mucosa reversibly depress the spontaneous transmucosal electrical potential and short-circuit current due to a selective inhibition of the active transport of chloride (3–5). I shall speak of the latter as the "chloride effect."

The effect of 15 related compounds upon chloride transport is being reported. Evidence is presented that the high concentrations required are not a result of a limitation imposed by diffusion. When due allowance is made for a pharmacologic short-circuiting of the mucosa, it may be concluded that these compounds do not inhibit H^+ ion secretion by the isolated frog gastric mucosa.

METHODS

Bullfrogs, *R. catesbeiana*, were kept in distilled water at 9°. After a frog was pithed, the gastric mucosa was removed and mounted as a flat sheet between plastic chambers. Each surface of two 2.95 cm^2 portions of mucosa was exposed to 20 ml of bathing solution. Serosal surfaces were bathed by a solution having Na^+ 110, K^+ 2.5, Ca^{2+} 2.5, Mg^{2+} 1.0, Cl^- 79.5, HCO_3^- 30, HPO_4^{2-} 3.0, SO_4^{2-} 3.5 mEq and dextrose 28 mM. In most experiments, mucosal surfaces were bathed by a similar saline solution in which HCO_3^- and HPO_4^{2-} were replaced by an equivalent amount of Cl^-. The solutions were gassed with 5% CO_2 and 95% O_2. When H^+ secretion was measured, the mucosal solution was gassed with 100% O_2. The serosal solution was modified if the final concentration of drug exceeded 2 mM. For each millimole of drug, $NaHCO_3$ was increased by 0.5 mM and NaCl reduced by 0.75 mM. Drugs were dissolved in relatively alkaline saline which was then equilibrated with 5% CO_2, 95% O_2, so that the pH was approximately 7.4 prior to introduction into the chamber. In those experiments designed to evaluate the restriction imposed by diffusion (Fig. 2 and Table 5), the solutions bathing the two surfaces were identical; they were buffered, gassed by 5% CO_2, 95% O_2 and had the same concentration of methazolamide.

Transmucosal potential differences were measured with matched salt-bridge calomel

cell Radiometer (K401) pairs and a sensitive self-balancing potentiometer (6). Initial values are the stable spontaneous potential differences attained 1–2 hr after isolation. Although the transmucosal potential difference is such that the mucosal surface is negative with respect to the serosal surface, for simplicity the negative sign has been omitted from the tables since in no instance was there a reversal of the normal polarity.

H^+ secretion was measured as previously (6). Approximate standard errors, ~SE, were computed as range/n, and when $n >$ 16, the appropriate correction was made (7). Conventional estimates of variance were used for the t test. The electrophysi-

TABLE 1

Course of the potential difference with prolonged exposure to 1 mM methazolamide (4 pairs)

Time (min)	Half-mucosa A (mV)	Half-mucosa B (mV)
−1	30 ± 5	31 ± 5
0	Added 1 mM methazolamide	Change of control saline
60	18 ± 5	32 ± 3
120	25 ± 5	34 ± 4
180	27 ± 4	36 ± 4
240	28 ± 3	38 ± 3
241	Changed to control saline	Change of control saline
330	37 ± 2	39 ± 3

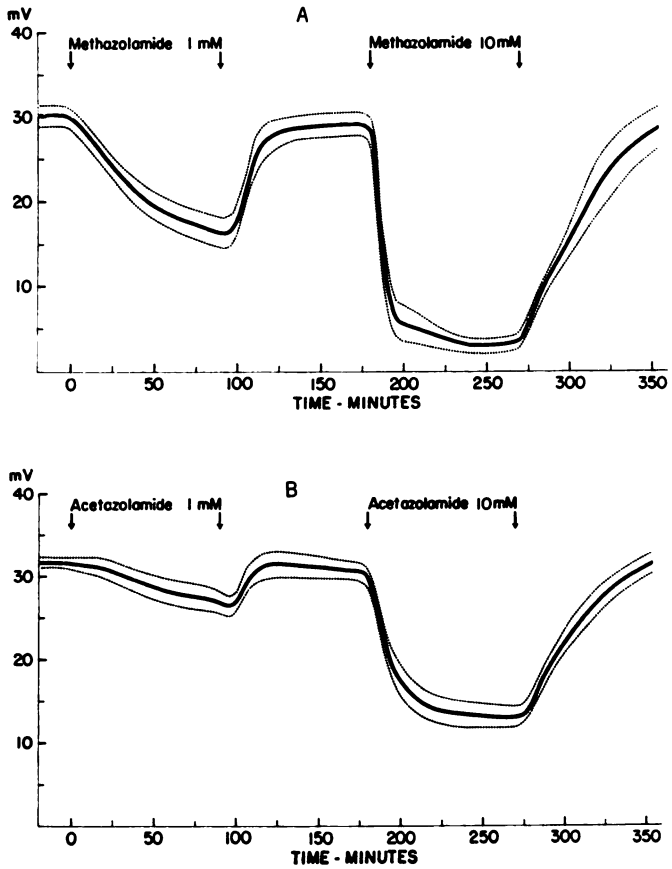

FIG. 1. *Change in the spontaneous transmucosal potential difference (mV) following exposure of the serosal surface to carbonic anhydrase inhibitors*

The results are from 8 paired portions of gastric mucosa. One portion was exposed to methazolamide (A), the other to acetazolamide (B). Dotted lines designate ± ~SE.

ology of the isolated bullfrog gastric mucosa has been described (8).

The structural formulas of the drugs have been given elsewhere (2, 9). The lot numbers and the manufacturers who graciously supplied the drugs were: acetazolamide 0363-K9919, benzothiazole-2-sulfonamide 3766B-16B, Cl 11366 5417B-168A, Cl 13475 S-1540-93, Cl 13580 3939B-9-2, N^5-methyl acetazolamide R3957-62, methazolamide 1328-K4176, sulfadiazine 3917-323, N^5-*t*-butyl acetazolamide 3822B-19A, Lederle; chlorothiazide 30380, dichlorphenamide 32603, sulfanilamide 60162, Merck; *p*-toluenesulfonamide 423, Eastman; sulfacetamide 8715-SX1175, Matheson; ethoxzolamide 788F, Upjohn.

RESULTS

Depression of the spontaneous transmucosal potential difference by methazolamide and acetazolamide is displayed in Fig. 1. A maximal inhibition is reached after about 90 min of exposure to 1 mM methazolamide (Table 1). After 90 min the potential usually increases somewhat in

TABLE 2

Paired comparisons between methazolamide and carbonic anhydrase inhibitors or inactive analogs on the spontaneous potential difference across the isolated bullfrog gastric mucosa

For relative potencies against carbonic anhydrase see Table 4.

Potential differences were taken after 90 minutes of exposure to the agent and after 90 minutes of recovery. Exposure to and recovery from benzothiazole-2-sulfonamide were continued for 180 minutes each, but the recorded values were those obtained at 90 minutes. Exposure and recovery times for Cl 13475 were 110 minutes each. Values are $\bar{x} \pm \sim$SE. When less than 10 mM was employed, the concentrations used approximate solubility at pH 7.4 and 25°C.

	Concentration	mv Control	mv Inhibition	mv Recovery	% Change	mv Control	mv Inhibition	mv Recovery	% Change	n
	Effect of Agent listed on Half-mucosa A					Effect of 1mM Methazolamide on Half-mucosa B				
Ethoxzolamide	0.3mM	30±3	15±3	16±2	49	29±2	18±3	31±2	36	6
Cl 11366	3.0mM	38±2	30±4	36±2	20	39±3	32±5	39±3	18	4
Cl 13580	1.0mM	27±4	10±3	29±6	64	28±3	12±2	28±1	58	4
p-toluenesulfonamide	5.0mM	38±3	22±2	37±2	44	37±3	32±2	39±3	13	8
Dichlorphenamide	5.0mM	31±1	18±1	12±2	43	33±2	28±2	32±2	16	6
Acetazolamide	1.0mM	32±1	27±1	30±1	14	30±1	16±2	29±1	45	8
Chlorothiazide	5.0mM	45±2	43±2	43±2	3	44±2	36±3	41±2	17	6
N^5-methyl acetazolamide	2.0mM	33±2	31±1	35±1	5	34±2	26±2	32±2	22	10
Sulfacetamide	10.0mM	35±3	33±3	36±2	7	36±2	30±4	35±3	17	6
Sulfadiazine	2.0mM	27±2	28±3	31±3	-3	26±3	21±3	26±3	20	6
						Effect of 2mM Methazolamide on Half-mucosa B				
Benzothiazole-2-sulfonamide	2.0mM	36±3	11±1	15±2	70	39±3	19±4	32±2	51	6
Cl 13475	2.0mM	37±2	35±3	37±2	8	34±3	18±3	34±2	46	6
Sulfanilamide	10.0mM	32±2	22±2	35±1	31	31±2	13±2	30±2	60	8
N^5-t-butyl acetazolamide	2.0mM	35±2	33±2	38±2	4	37±1	22±3	34±1	40	8
						Effect of 10mM Methazolamide on Half-mucosa B				
Acetazolamide	10.0mM	30±1	13±1	32±1	57	29±1	4±1	29±2	88	8
						Effect of change of Control Saline on Half-mucosa B				
Sulfanilamide	10.0mM	38±4	27±2	37±3	29	37±3	37±3	38±2	1	8
N^5-t-butyl acetazolamide	2.0mM	34±2	32±2	33±2	3	34±2	32±2	32±2	5	6
Sulfacetamide	10.0mM	32±2	30±2	31±3	4	33±2	31±2	31±2	5	6

spite of the continued presence of the drug. Almost identical results were obtained with acetazolamide.

The results of experiments in which other agents were compared to methazolamide or to a control change of saline are given in Table 2 (included in this table are results given in Fig. 1). Recovery from exposure to ethoxzolamide was delayed, Table 3.

TABLE 3
Recovery of the potential difference after exposure to ethoxzolamide (4 pairs)

Time (min)	Half-mucosa A (mV)	Half-mucosa B (mV)
−1	36 ± 4	35 ± 4
0	Added 0.3 mM ethoxzolamide	Added 0.3 mM ethoxzolamide
90	17 ± 5	13 ± 4
91	No change	Change to control saline
150	14 ± 6	19 ± 3
210	13 ± 6	25 ± 3
270	11 ± 5	28 ± 2
330	11 ± 5	30 ± 2
331	Changed to control saline	Changed to control saline
370	14 ± 5	32 ± 1

Inhibition by benzothiazole-2-sulfonamide and dichlorphenamide was not reversible. All the drugs except chlorothiazide, N^5-methyl acetazolamide, N^5-*t*-butyl acetazolamide, sulfacetamide, and sulfadiazine reduced the spontaneous potential.

Depression of the transmucosal potential difference and the inhibition of erythrocyte carbonic anhydrase by the agents cited in Table 2 are compared in Table 4. In general, the similarities are more striking than the differences. The assumption that drug concentration and depression of potential difference are proportional is admittedly crude, but sufficient for the purpose. Different assays of the inhibition of carbonic anhydrase by acetazolamide and sulfanilamide have yielded ratios of activities from 50 to 350 (2). Nevertheless *p*-toluenesulfonamide and sulfanilamide have a greater effect on chloride transport than would have been expected from their inhibition of carbonic anhydrase. Methazolamide is somewhat more effective than acetazolamide. Cl 13475 is clearly far less active than would be predicted from its effect on carbonic anhydrase. Unfortunately only one lot of this drug has been manufactured so we were unable to confirm that the material used by us still retained its inhibitory activity toward carbonic anhydrase. One cannot ascribe its lack of effect to its pK_a, 4.2, since another compound Cl 11366 which has a pK_a of 3.2 is potent. The values given in Table 4 for chlorothiazide correspond fairly well. However another approach to assaying the inhibition of carbonic anhydrase, preceded by equilibration of drug and enzyme, assigns to chlorothiazide an activity that is as

TABLE 4
Relative Potency of Carbonic Anhydrase Inhibitors
Values for "Cl^- effect" computed from Table 2 as:

$$\frac{\%\text{ PD depression by agent}}{\%\text{ PD depression by methazolamide}} \times \frac{\text{conc. of methazolamide}}{\text{conc. of agent}}$$

Inhibition of carbonic anhydrase relative to methazolamide obtained from (2) and for benzothiazole-2-sulfonamide and *p*-toluenesulfonamide relative to acetazolamide from (10).

Inhibitor	Relative "Cl^- effect"	Inhibition of carbonic anhydrase
1. Ethoxzolamide	4.2	3.3
2. Cl 11366	2.8	3.3
3. Benzothiazole-2-sulfonamide	1.4	2.4
4. Cl 13580	1.1	2.5
5. Methazolamide	*1.0*	*1.0*
6. *p*-Toluenesulfonamide	0.7	0.04
7. Dichlorphenamide	0.5	0.43
8. Acetazolamide	0.3	1.1
9. Cl 13475	<0.2	2.8
10. Sulfanilamide	0.1	0.004
11. Chlorothiazide	<0.02	0.005
12. N^5-Methyl acetazolamide	<0.05	Negligible
13. N^5-*t*-Butyl acetazolamide	<0.05	Negligible
14. Sulfacetamide	<0.01	Negligible
15. Sulfadiazine	<0.05	Negligible

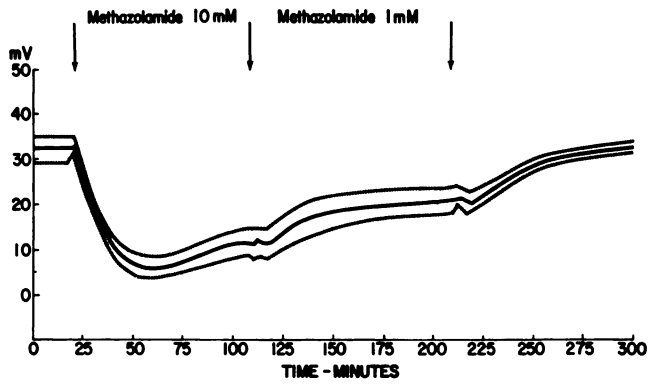

FIG. 2. *The partial restoration of the spontaneous transmucosal potential that follows from first exposing the mucosa to 10 mM methazolamide and then to 1 mM methazolamide*

Both surfaces were exposed to identical bicarbonate saline solutions including specified concentration of drug. $N = 4$; dotted lines designate ± ~SE.

much as one-tenth to one-fifth that of acetazolamide (11).

The extent to which diffusion limits access of the drug to the site of its action was examined by first exposing the mucosa to 10 mM methazolamide and then to 1 mM methazolamide (Fig. 2). After a maximal depression of the potential difference had been attained with 10 mM inhibitor, reduction of the concentration to 1 mM was followed by a partial restitution of the potential difference. This was more amply documented in a series of experiments where one portion of mucosa remained exposed to 10 mM drug while the concentration for the other portion of mucosa was reduced to 1 mM (Table 5). The potential difference increased by 49% after reduction of the concentration to 1 mM. The potential difference of the other portion of mucosa increased by only 15%. Two other sets of experiments differing somewhat in design led to the same conclusion. Since detoxication of methazolamide is negligible (11), we can confidently assert that the extracellular concentration necessary for a maximal chloride effect must be greater than 1 mM.

TABLE 5

Partial recovery of the spontaneous potential difference when exposure to 1 mM methazolamide followed 10 mM methazolamide (10 pairs)

Both surfaces were bathed by identical bicarbonate saline with or without drug.

Time (min)	Half-mucosa A (mV)	Half-mucosa B (mV)
−1	35 ± 2	35 ± 1
0	Added 10 mM methazolamide	Added 10 mM methazolamide
90	15 ± 2	13 ± 1
91	Replaced with 1 mM methazolamide	No change
180	22 ± 2	15 ± 1
181	Changed to control saline	Changed to control saline
270	36 ± 2	33 ± 2

Methazolamide, 0.1 millimole/kg, was injected into the dorsal lymph sac of 4 frogs, 15–34 minutes before their gastric mucosae were removed and mounted in saline with 0.1 mM methazolamide. The mucosae from all 4 frogs developed a normal potential difference which was later depressed by 10 mM methazolamide.

Previously we had only been able to infer that these inhibitors, even when employed in high concentration, did not reduce the H^+ ion secretion of the short-circuited frog gastric mucosa (3). Electrically short-circuiting the mucosa, that is, reducing the transmucosal potential difference to zero, reduces the rate of H^+ ion secretion by as much as 50%. Because these agents also reduce the transmucosal potential difference, the secretory rates have been compared when the transmucosal potential differences were held at a predetermined

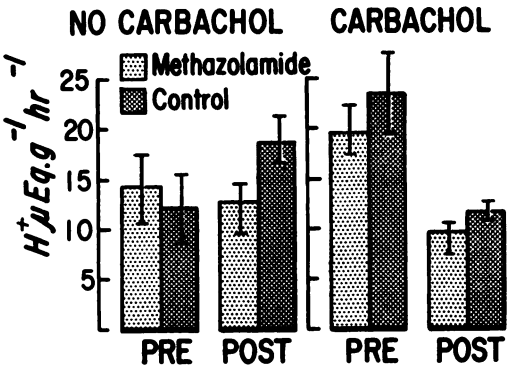

FIG. 3. *Paired rates of H^+ ion secretion by the short-circuited gastric mucosa*

Rates of paired portions of mucosa from one frog during a 1.5-hr pretreatment period (*PRE*). Subsequently one portion of mucosa of each pair was exposed to 10 mM methazolamide. Values are given for the spontaneous secretion of mucosae from 6 frogs (*NO CARBACHOL*); posttreatment period 2.5 hr (*POST*). Secretion of mucosae from another 11 frogs was stimulated by 10 μM carbamylcholine (*CARBACHOL*); 3-hr posttreatment period. Bars indicate ± ~SE.

value. With our apparatus it is simpler to compare rates at 0 mV than at the spontaneous potential difference. H^+ ion secretion rates of paired portions of mucosa were measured over a pretreatment period of 1.5 hr. One portion of mucosa served as a control and the other portion was exposed to a drug. Posttreatment secretion was followed for an additional 2.5 or 3.0 hr. The consequences of adding 10 mM methazolamide with or without prior stimulation with carbachol are set forth in Fig. 3. These results together with those for acetazolamide, ethoxzolamide, *p*-toluenesulfonamide, and Cl 13580 are summarized in Table 6, as well as those obtained with methazolamide when the transmucosal potential difference was fixed at 30 mV. It is clear that the carbonic anhydrase inhibitors did not inhibit these low rates of H^+ ion secretion. It is debatable whether carbonic anhydrase inhibitors increased H^+ ion secretion, perhaps by reducing energy required for active transport of chloride. In most experiments, a confident analysis is thwarted by the circumstance that the mean initial secretory rate of the portions that were to be treated was by chance less than that of the control portion.

TABLE 6

Effect of carbonic anhydrase inhibitors on H^+ ion secretion of paired portions of frog gastric mucosa

Agent	mmole/l	Pretreatment Control A_1	Pretreatment Exptl. B_1	Posttreatment Control A_2	Posttreatment Exptl. B_2	n	Paired differences $\Delta\bar{x}$	SE	P
		H^+ μEq cm^{-2} hr^{-1}							
		0 – 1.5 hr		1.5 – 4.0 hr					
Spontaneous secretion; 0 mV									
Methazolamide	10	1.8 ± 0.4	1.4 ± 0.4	1.5 ± 0.3	2.0 ± 0.1	6	+0.88	±0.18	<0.005
Acetazolamide	10	1.3 ± 0.3	1.2 ± 0.4	0.6 ± 0.2	1.1 ± 0.2	6	+0.58	±0.96	>0.50
Ethoxzolamide	0.3	1.5 ± 0.4	1.1 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	4	+0.62	±0.17	<0.05
Cl 13580	1.0	2.1 ± 0.6	1.6 ± 0.3	1.1 ± 0.2	1.3 ± 0.2	4	+0.75	±0.31	<0.10
		0 – 1.5 hr		1.5 – 4.5 hr					
p-Toluenesulfonamide	5	1.7 ± 0.2	1.7 ± 0.2	1.2 ± 0.2	1.7 ± 0.2	4	+0.55	±0.04	<0.001
Carbamylcholine 10 μM; 0 mV									
Methazolamide	10	2.3 ± 0.2	2.9 ± 0.3	1.2 ± 0.3	1.5 ± 0.1	11	−0.26	±0.45	>0.50
Spontaneous secretion; 30 mV									
Methazolamide	10	1.8 ± 0.2	1.6 ± 0.2	1.2 ± 0.2	1.0 ± 0.1	6	+0.01	±0.67	>0.50

* $\bar{x}$ ± SE; paired differences analysis derived from $(B_2 - B_1) - (A_2 - A_1)$ for each pair of half-mucosae.

The following incidental observations are cited. The combination of 0.3 mM ethoxzolamide with 10 mM methazolamide was associated with less depression of the spontaneous potential than with methazolamide alone (Table 7). When the solutions bathing the mucosae had half the usual concentration of Cl^- (the anion deficit made up with either glucuronate or isethionate), the

TABLE 7
Response of the potential difference to a combination of methazolamide and ethoxzolamide (4 pairs)

Time (min)	Half-mucosa A (mV)	Half-mucosa B (mV)
−1	26 ± 4	29 ± 4
0	Added 10 mM methazolamide	Added 10 mM methazolamide
90	3 ± 2	3 ± 2
91	Changed to 0.3 mM ethoxzolamide + 10 mM methazolamide	No change
180	8 ± 1	3 ± 1
181	Changed to control saline	Changed to control saline
270	14 ± 3	25 ± 2

absolute reduction of the potential difference induced by 1 mM methazolamide did not differ from that of controls. However, the spontaneous potential developed in the presence of a reduced chloride concentration was less so that the percentage depression was 50, in contrast to 26 for the controls.

DISCUSSION

The objectives of this study were to clarify why extraordinarily high concentrations of carbonic anhydrase inhibitors are necessary to achieve the "Cl^- effect" and to test whether the Cl^- effect might be due to a chemical property of these agents other than the structure responsible for inhibition of carbonic anhydrase. We have since found that carbonic anhydrase inhibitors also reduce the active transport of Cl^- by the bullfrog cornea even though carbonic anhydrase cannot be detected in this structure (12).

Evidence has been developed in this study that the extraordinary concentrations of sulfonamide inhibitors required to achieve the Cl^- effect cannot be attributed to a failure of the drug to reach its site of action. When a maximal effect is attained, there must be a steady state of drug distribution. If it is assumed that the cell membrane is impermeable to the ionized moiety, the steady state distribution of a weak electrolyte is given by:

$$\frac{\text{intracellular concentration}}{\text{extracellular concentration}} = \frac{1 + 10^{(pH_i - pK_a)}}{1 + 10^{(7.4 - pK_a)}}$$

where the pH of the extracellular fluid is 7.4 and that of the intracellular fluid designated by pH_i (13). For methazolamide with a pK_a of 7.2 (2), the ratio would not be less than 0.5 even if the intracellular pH were improbably low. In a more rigorous treatment, it would be necessary to allow for the cell membrane being somewhat permeable to the ionized moiety, raising the ratio toward unity, and to allow for a membrane potential difference which would oppose that tendency.

Though we can establish limits for the intracellular concentration of drug, we can only estimate the extent of carbonic anhydrase inhibition by extrapolation. The kinetics of interaction between inhibitor and enzyme are known for a "test tube" reaction. Maren and Wiley (14) find that test tube kinetics can be extrapolated to the intact erythrocyte. If extrapolation is also applicable to the gastric oxyntic cell, from the dissociation constant, K_I, of the complex, EI, of inhibitor, I, and enzyme, E:

$$K_I = [E]\cdot[I]/[EI]$$

so that:

$$K_I/[I] = [E]/[EI]$$

Since the K_I of methazolamide is 3.5×10^{-8} M (15), an intracellular concentration of 0.1 mM could inhibit more than 99.9% of enzyme. The carbonic anhydrase concentration of the bullfrog gastric oxyntic cell is 30 μM (16).

Thus part of the findings reported here are in accord with our observations of the isolated cornea. In this study we find that the concentration of sulfonamide inhibitor

necessary for the Cl^- effect is much more than should be needed for effective inhibition of carbonic anhydrase. Active transport of chloride by the cornea is inhibited by the same agents even though carbonic anhydrase is not detectable.

Nevertheless the susceptibilities of the Cl^- transport mechanism and carbonic anhydrase to agents of different structure are remarkably similar. Granted the basis of comparison that I have used, I have noted that sulfanilamide and *p*-toluenesulfonamide have relatively more effect upon chloride transport and the compound Cl 13475 more upon carbonic anhydrase. I choose to stress that for the variety of chemical structures assayed, it is surprising that greater discrepancies were not encountered.

It is doubtful that the Cl^- effect is involved in any other recognized action of the carbonic anhydrase inhibitors because the concentrations needed for the effect are excessive. Carbonic anhydrase is implicated in many epithelial secretory and absorptive processes. I have not presented any reason for reconsidering the action of the sulfonamide inhibitors on such processes as the formation of gastric juice, urine, pancreatic juice, cerebrospinal fluid, or aqueous fluid. There is no basis to link the present studies to sulfonamide inhibition of chloride transport by the fish gill (17) or to inhibition of gas secretion by the swimbladder (18). Rehm and colleagues have made relevant observations on the canine stomach wall segment whose blood supply is intact (19). Intravenous administration of acetazolamide, 40 mg/kg or more, reduced the transmural potential difference by 17 mV when the stomach was secreting. When the stomach was not secreting, the segment was not influenced by acetazolamide.

Until further knowledge is developed, the significance of the Cl^- effect may be that we will have a tool that can be used to identify a component of the chloride transport system. If the sulfonamide inhibitors bind to that component, it may become possible to isolate it.

The results given here should serve to repudiate any assertion that H^+ secretion is absolutely dependent upon carbonic anhydrase (20). Concentrations of inhibitor more than sufficient to complex carbonic anhydrase do not block H^+ secretion by the isolated gastric mucosa. However, the rate of H^+ secretion by the isolated gastric mucosae of many species, ca. 2 μEq cm^{-2} hr^{-1}, is much less than that which can be attained in the intact mammal, up to 200 μEq cm^{-2} hr^{-1}. It has been claimed that carbonic anhydrase inhibitors render the gastric mucosa more susceptible to damage (21). Such damage was not encountered in these experiments, where the effects were readily reversed.

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REFERENCES

1. F. M. Byers, Jr., P. H. Jordan, Jr. and T. H. Maren, *Am. J. Physiol.* **202**, 429 (1962).
2. P. Wistrand, J. A. Rawls, Jr. and T. H. Maren, *Acta Pharmacol. Toxicol. Kbh.* **17**, 337 (1961).
3. C. A. M. Hogben, *in* "Electrolytes in Biological Systems" (A. M. Shanes, ed.). Am. Physiol. Soc., Washington, D.C., 1955.
4. R. P. Durbin and E. Heinz, *J. Gen. Physiol.* **41**, 1035 (1958).
5. S. Kitahara and A. Imamura, *Life Sci.* **5**, 215 (1966).
6. S. Kaneko-Mohammed and C. A. M. Hogben, *Am. J. Physiol.* **207**, 1173 (1964).
7. Documenta Geigy: Scientific Tables, 6th ed., p. 47. Ardsley, New York, 1962.
8. C. A. M. Hogben, *Federation Proc.* **24**, 1353 (1965).
9. T. H. Maren, *J. Pharmacol. Exptl. Therap.* **117**, 385 (1956).
10. W. H. Miller, A. M. Dessert and R. O. Roblin, Jr., *J. Am. Chem. Soc.* **72**, 4893 (1950).
11. T. H. Maren, *Physiol. Rev.* (1967) in press.
12. S. Kitahara, K. R. Fox and C. A. M. Hogben, *Nature* (1967) in press.
13. P. A. Shore, B. B. Brodie and C. A. M. Hogben, *J. Pharmacol. Exptl. Therap.* **119**, 361 (1957).
14. T. H. Maren and C. E. Wiley, *Federation Proc.* **25**, 320 (1966).
15. T. H. Maren, *J. Pharmacol. Exptl. Therap.* **139**, 129 (1963).

16. T. H. Maren and C. E. Wiley, *Bull. Mt. Desert Island Biol. Lab.* **5**, 28 (1965).
17. J. Maetz and F. G. Romeu, *J. Gen. Physiol.* **47**, 1209 (1964).
18. R. Fange, *Acta Physiol. Scand.* **30**, Suppl. 110 (1953).
19. W. S. Rehm, C. A. Canosa, H. S. Schlesinger, W. K. Chandler and W. H. Dennis, *Am. J. Physiol.* **200**, 1074 (1961).
20. R. E. Davies and J. Edelman, *Biochem. J.* **50**, 190 (1952).
21. J. L. Werther, F. Hollander and M. Altamirano, *Am. J. Physiol.* **209**, 127 (1965).