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Research Article

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In the intact preparation free of exogenous  $CO_2$ , the dose response curve for acetazolamide showed two plateaus of inhibition, one at 50% and one at more than 80% inhibition. At 50% inhibition (from  $5 \times 10^{-6}$  to  $5 \times 10^{-5}$  M acetazolamide),  $H^+$  secretion was restored or enhanced by  $CO_2$  addition to the same extent as bladders not exposed to acetazolamide. At concentrations of more than  $1 \times 10^{-4}$  M,  $H^+$  secretion was no longer restorable by  $CO_2$ . Unlike acetazolamide, ethoxzolamide caused progressive inhibition of  $H^+$  secretion in the  $CO_2$ -free system. The maximal extent of inhibition with ethoxzolamide and the behavior of inhibition in the presence of 2.5%  $CO_2$  were the same as for acetazolamide. Evidence is presented that all surface epithelial cells secrete  $H^+$  and generate  $OH^-$  within the cell interior. The capacity of cells to dispose of  $OH^-$  by  $CO_2$  [...]

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# Carbonic Anhydrase Function and the Epithelial Organization of $H^+$ Secretion in Turtle Urinary Bladder

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**ABSTRACT** The function of carbonic anhydrase in  $H^+$  secretion by the turtle bladder was studied in vitro. Dose response curves were obtained for the carbonic anhydrase inhibitors, acetazolamide and ethoxzolamide, with and without addition of  $CO_2$  to the system. In addition, carbonic anhydrase was assayed in homogenates of mucosal cells. The activity in the homogenates was  $155 \pm 16$  U/g dry wt, of which only 11 U represented contamination from erythrocytes; after addition of  $5 \times 10^{-6}$  M acetazolamide, no enzyme activity was detectable.

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On the basis of these results and histochemical data on the distribution of carbonic anhydrase within the mucosa, an analysis is presented of the epithelial organization of acidification by the turtle bladder.

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## INTRODUCTION

Previous studies have demonstrated that the isolated turtle bladder acidifies the solution bathing its mucosal surface by a mechanism of  $H^+$  secretion (2-4), and that in the absence of external  $CO_2$  the rate of  $H^+$  secretion is markedly reduced by the carbonic anhydrase inhibitor, acetazolamide (2, 5). Further studies on the role of carbonic anhydrase in acidification by this urinary membrane, however, have yielded results that were inconclusive in several respects. Maren (6) in his review listed turtle bladder as containing no carbonic anhydrase, and Schilb and Brodsky (7) reported in their initial studies of a  $CO_2$ -containing preparation that acidification is unaffected by acetazolamide. Gonzalez and Schilb (8) subsequently showed that acetazolamide does inhibit acidification, but attributed the inhibition to a non-carbonic anhydrase effect on  $HCO_3^-$  transport per se. Other lines of evidence, however, appear to indicate that carbonic anhydrase must play a role in acidification by the turtle bladder. Schwartz and Steinmetz (9) measured the rate of metabolic  $CO_2$  production by the intact bladder in vitro and found that metabolic  $CO_2$  alone is insufficient to support the observed rates of acidification without catalysis. Steinmetz (5) observed inhibition of acidification with the known carbonic anhydrase inhibitors, C1 11,366 and acetazolamide, but not with the inactive, *t*-butyl-substituted analogue of acetazolamide. Evidence for the existence of carbonic anhydrase in turtle bladder mucosa has recently been strengthened greatly by studies by Rosen (10) and Scott, Shamoo, and Brodsky (11) indicating that the enzyme is present in the mucosa in a nonhomogenous distribution and in relatively low over-all concentration.

The studies to be described confirm the presence of carbonic anhydrase in homogenates of bladder mucosa and explore the role of carbonic anhydrase in  $H^+$  secretion by the intact epithelium in vitro. The mode of inhibition of acidification was examined over a wide con-

centration range of inhibitors and at different CO<sub>2</sub> tensions. On the basis of these physiologic studies and histochemical information on the distribution of cytoplasmic carbonic anhydrase, a tentative description is given of the functional organization of acidification within the epithelium of the turtle bladder. According to this analysis, all surface epithelial cells are capable of H<sup>+</sup> secretion, but the secretory rates may vary greatly with the availability of cytoplasmic carbonic anhydrase. This dependence of the rate on carbonic anhydrase was greatest at low CO<sub>2</sub> tensions. The studies also raise the possibility that higher concentrations of inhibitor affect a non-cytoplasmic moiety of carbonic anhydrase or interfere directly with a transport step for H<sup>+</sup> or OH<sup>-</sup> across the luminal membrane.

## METHODS

*Studies of acidification in vitro.* Urinary bladders of adult fresh water turtles, *Pseudemys scripta* were removed with minimal handling, washed with Ringer's solution and mounted in lucite chambers as described previously (2). In all experiments the two sides of the bladder were bathed with identical bicarbonate-free Ringer's solution containing, in millimoles per liter: Na<sup>+</sup>, 115.0; K<sup>+</sup>, 3.5; Ca<sup>++</sup>, 0.9; Cl<sup>-</sup>, 119.7; HPO<sub>4</sub><sup>=</sup>, 0.3; and dextrose, 2.0. The osmolality ranged from 222 to 230 mOsm/kg H<sub>2</sub>O. The mucosal (M) solution was stirred and oxygenated with air which had been passed through three KOH traps to remove all detectable CO<sub>2</sub>. The gas used to bubble the serosal (S) solutions was either this CO<sub>2</sub>-free air or gas obtained from tanks containing 1.4% or 2.5% CO<sub>2</sub> in air (Medical Technical Gases, Inc., Medford, Mass.; the CO<sub>2</sub> content was analyzed by the Scholander method).

All experiments were performed with the bladders in the short-circuited state except for brief intervals when the spontaneous electrical potential difference across the bladder was measured. H<sup>+</sup> secretion was measured by the pH stat technique. CO<sub>2</sub> production was measured by a conductometric method (9, 12).

For the experiments with carbonic anhydrase inhibitors the following sulfonamides were studied: (a) acetazolamide in final concentrations ranging from  $1 \times 10^{-6}$  to  $5 \times 10^{-4}$  M; (b) 2-acetylamine-1,3,4-thiadiazole-5-sulfon-*t*-butylamide (Cl 13,850), an analogue of acetazolamide without carbonic anhydrase inhibitory activity, in a final concentration of  $5 \times 10^{-4}$  M; and (c) ethoxzolamide (Lot no. 673L) in final concentrations from  $1 \times 10^{-8}$  to  $3 \times 10^{-5}$  M. After a control period in the absence of exogenous CO<sub>2</sub> during which the rate of H<sup>+</sup> secretion was measured for 50–60 min with the mucosal pH set at 7.0 and the serosal pH set at 5.4, one of the three compounds was added to S. Once H<sup>+</sup> secretion reached a steady rate for 30 min the gas bubbled through S, was changed to one containing CO<sub>2</sub> as described previously (9). For the dose-response curves for ethoxzolamide in the CO<sub>2</sub>-free system the media were kept at pH 7.4.

The chambers employed in these studies provided an exposed membrane area of 8 cm<sup>2</sup>. As in previous studies all rates are expressed as  $\mu$ moles/hr per 8 cm<sup>2</sup> of membrane area exposed  $\pm$ SEM. The mean dry weight of the bladders exposed in the lucite chamber was  $13.4 \pm 0.6$  mg.

The compound, Cl 13,850, was made available through

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*Assay for carbonic anhydrase and histochemistry.* Homogenates of turtle bladder mucosa were analyzed for carbonic anhydrase activity by a pH indicator method. For each analysis from 3 to 12 bladders were used. After an initial wash in Ringer's solution, the bladders were placed in a large petri dish containing Ringer's solution, and were stretched and scraped with the edge of a glass microslide. To minimize contamination from submucosal tissues each area was scraped only once. Microscopic evaluation of the scrapings stained by Papanicolaou's technique revealed sheets of epithelium. The bladder scrapings were washed three times with Ringer's solution, weighed, resuspended in 3–7 ml of distilled H<sub>2</sub>O, and homogenized. Carbonic anhydrase activity was measured using a micromethod modification of Maren (13) of the technique of Philpot and Philpot (14) and expressed in units according to Maren et al. (15). For the inhibition studies the acetazolamide was added immediately before the buffer so that equilibrium was not attained. The homogenate was analyzed for hemoglobin (16). The carbonic anhydrase activity of hemolyzed turtle erythrocytes was also determined.

The distribution of carbonic anhydrase in turtle bladder mucosa was examined histochemically by the technique of Hansson (17). A report of the application of this technique to turtle bladder has been published (10). The distribution of carbonic anhydrase within individual cells was examined further by electron-micrography of the histochemical preparation; a full report of the histochemistry will be published separately (18).

## RESULTS

*Carbonic anhydrase activity in homogenates of mucosal cells.* The results of 15 assays for carbonic anhydrase on the mucosal scrapings of a total of 106 turtle bladders are shown in Table I. The carbonic anhydrase activity of the homogenates of mucosal cells was  $155 \pm 16$  U/g, dry wt, a value about 14% of the activity of turtle erythrocytes. Since 0.008 g hemoglobin was detectable per gram of bladder homogenate, contamination with hemoglobin can account for only about 7% of the observed activity.

As shown in Table II, the carbonic anhydrase activity was inhibited by low concentrations of acetazolamide, about 50% inhibition being observed at concentrations close to  $1 \times 10^{-7}$  M. At  $5 \times 10^{-6}$  M acetazolamide, enzyme activity was no longer detectable by this method.

*Histochemical distribution of carbonic anhydrase in turtle bladder.* Fig. 1 shows the surface topography of the stretched whole turtle bladder stained for carbonic anhydrase. About 10% of the surface area is covered by carbonic anhydrase-containing cells. All epithelial staining was abolished by  $10^{-6}$  M acetazolamide or  $10^{-7}$  M ethoxzolamide, while staining was unaffected by  $10^{-8}$  M Cl 13,850, the inactive analogue of acetazolamide. After the histochemical procedure for light microscopy, the tissue was directly dehydrated, embedded, cut, and examined

TABLE I  
*Carbonic Anhydrase Activity in Homogenates of Bladder Mucosal Cells and Turtle Erythrocytes*

	Carbonic anhydrase (CA)	Hemoglobin	Erythrocyte CA
	U/g dry wt	g/g dry wt	U/g dry wt
Mucosal cells	155±16	0.008±0.001	11.6±1.5
Erythrocytes	1101±91	0.728±0.007	

The values are expressed as the mean ±SEM for 15 experiments.

with the electron microscope after staining with uranyl acetate. The end product of the histochemical reaction is a finely granular electron dense precipitate, localized to cytoplasmic sap, and delimited by the plasma membrane (Fig. 2).

*Inhibition of H<sup>+</sup> secretion by acetazolamide.* The inhibitory effect of acetazolamide on H<sup>+</sup> secretion was examined in six groups of bladders at concentrations ranging from  $1 \times 10^{-8}$  M to  $5 \times 10^{-4}$  M. The average control rates of H<sup>+</sup> secretion in these experiments, which were carried out in the absence of exogenous CO<sub>2</sub>, ranged from 0.99 to 1.24  $\mu$ moles/hr. In Fig. 3, the per cent inhibition of H<sup>+</sup> secretion is shown for acetazolamide concentrations of  $1 \times 10^{-8}$  to  $5 \times 10^{-4}$  M. The inhibition represents the maximal effect attained at each concentration; the development of a constant level of inhibition re-

TABLE II  
*Inhibition of Mucosal Cell Carbonic Anhydrase by Acetazolamide*

Exp.	Carbonic anhydrase units			
	Acetazolamide 0	Acetazolamide $5 \times 10^{-8}$ M	Acetazolamide $1.25 \times 10^{-7}$ M	Acetazolamide $5 \times 10^{-6}$ M
a	1.6	0.7	0.2	
b	3.2	2.0	1.5	
c	4.1	3.0		
d	2.2	1.5		0
e	1.9			0
f	2.2		1.3*	0
g	2.5		1.3*	0
h	1.4			0

The amount of homogenate added in each experiment was constant and was titrated with varying concentrations of acetazolamide. Zero values indicate that no enzyme was detectable.

\* In experiments f and g the acetazolamide concentrations were  $1.5$  and  $2.0 \times 10^{-7}$  M, respectively.

quired from 30 to 80 min after addition of acetazolamide to S, the longer intervals being required at the lower concentrations. The rate of H<sup>+</sup> secretion was inhibited very close to 50% over a concentration range of at least 10-fold from  $5 \times 10^{-8}$  to  $5 \times 10^{-5}$  M. At  $1 \times 10^{-4}$  and  $5 \times 10^{-4}$  M, H<sup>+</sup> secretion was inhibited 88%. The data for  $5 \times 10^{-5}$  and  $5 \times 10^{-4}$  M acetazolamide are given in detail in the first two columns of Tables III and IV. Since

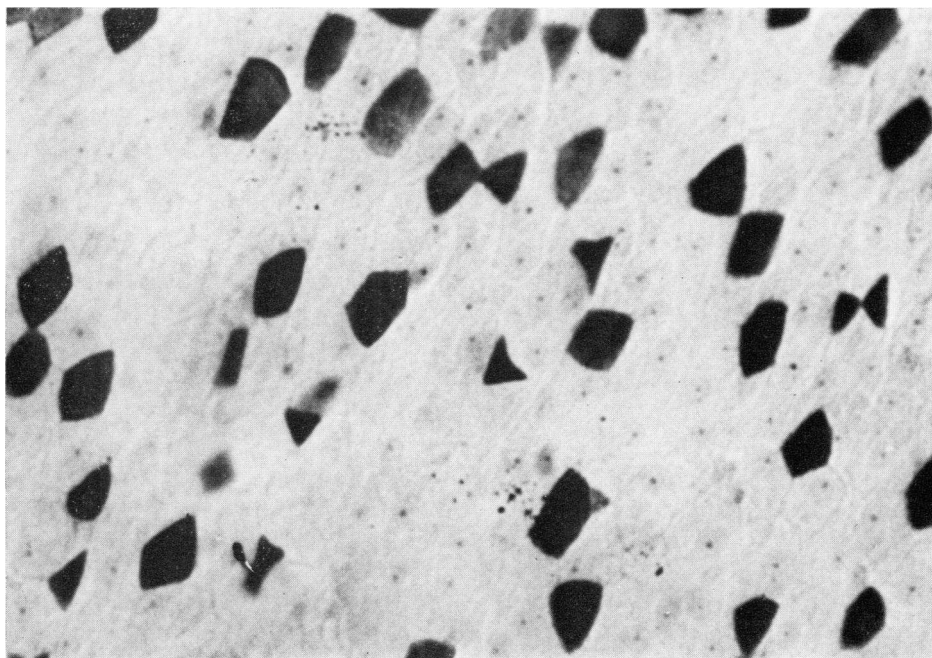


FIGURE 1 Surface view of stretched whole turtle bladder after staining for carbonic anhydrase. The enzyme active cells appear as black polygons. Magnification  $\times 450$ .

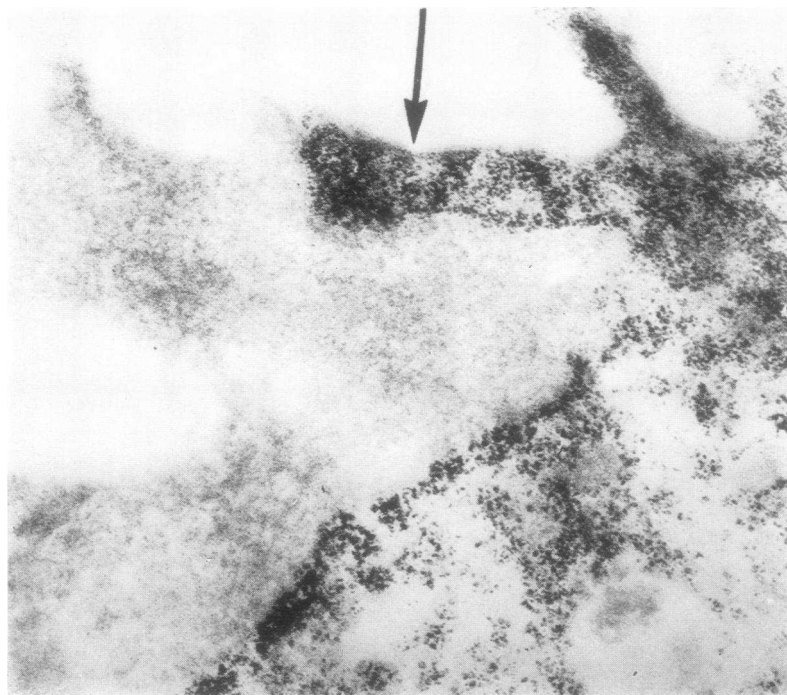


FIGURE 2 High magnification electron micrograph of the luminal region of two epithelial cells stained for carbonic anhydrase. The cell on the right contains a finely granular precipitate indicating carbonic anhydrase activity. The plasma membrane (arrow) is only faintly visible since osmium fixation was not used in this procedure; the precipitate is confined to the cytoplasm. No precipitate is demonstrable in the plasma membrane or in the cell on the left. Magnification  $\times 70,000$ .

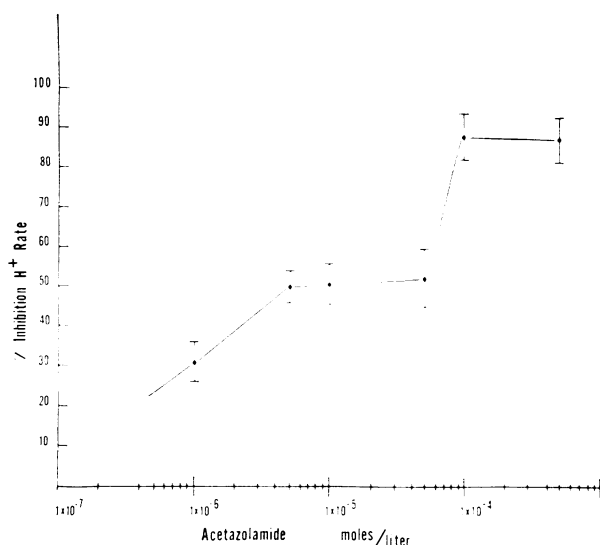


FIGURE 3 Per cent inhibition of  $H^+$  secretion at acetazolamide concentrations ranging from  $1 \times 10^{-6}$  to  $5 \times 10^{-4}$  M. The six groups of experiments were carried out in the absence of exogenous  $CO_2$  and  $HCO_3^-$ . The experiments at  $5 \times 10^{-5}$  and  $5 \times 10^{-4}$  M acetazolamide are given in detail in the first two columns of Tables III and IV.

washout of acetazolamide from the bladder is slow, no recontrol observations were made. Instead the constancy of the rate of  $H^+$  secretion was examined in a series of control studies in which  $H^+$  secretion was measured over prolonged periods of time. In six such studies, in which the initial rate was  $1.12 \pm 0.07$   $\mu$ moles/hr, the rate after 4 hr of secretion was  $1.15 \pm 0.08$   $\mu$ moles/hr. These results are in accord with previous studies in which re-control periods after such experimental procedures as deoxygenation, nullification of potential difference (2), and addition of  $CO_2$  (9) were unaltered from the initial controls after periods of 4-6 hr.

*Effect of exogenous  $CO_2$  on the inhibition by acetazolamide.* Since metabolic  $CO_2$ , the substrate for the reaction catalyzed by carbonic anhydrase, is in short supply in the above experiments, the effects of adding exogenous  $CO_2$  to the serosal solution were explored. At concentrations of  $5 \times 10^{-6}$  and  $5 \times 10^{-5}$  M acetazolamide,  $H^+$  secretion was restored to control values by 1.4%  $CO_2$ . In Table III the results are shown for the experiments with  $5 \times 10^{-5}$  M acetazolamide. The rate was restored by 1.4%  $CO_2$  and increased to 1.89  $\mu$ moles/hr by 2.5%  $CO_2$ . This enhanced rate was 70% greater than the control rate and comparable to the enhancement observed

with 2.5% CO<sub>2</sub> in bladders not treated with acetazolamide (1.84  $\mu$ moles/hr in Table 8 of reference 9). In the presence of 2.5% CO<sub>2</sub>, therefore, concentrations of acetazolamide up to  $5 \times 10^{-5}$  M had no effect on H<sup>+</sup> secretion. No further increase in H<sup>+</sup> secretion was observed with the addition of higher concentrations of CO<sub>2</sub> either in the presence or absence of acetazolamide; in fact H<sup>+</sup> secretion decreased with 5% CO<sub>2</sub>. It is not clear whether this decrease is caused by S to M HCO<sub>3</sub><sup>-</sup> flow resulting from addition of CO<sub>2</sub> with some HCO<sub>3</sub><sup>-</sup> to S, or by a primary decrease in H<sup>+</sup> secretion.

Table IV shows the effects on H<sup>+</sup> secretion of  $5 \times 10^{-4}$  M acetazolamide and of subsequent addition of CO<sub>2</sub> to S. H<sup>+</sup> secretion was inhibited by more than 80%, a degree comparable to that observed previously at similar dose levels (2, 5). When CO<sub>2</sub> was added to the gas bubbled through S, the rate of H<sup>+</sup> secretion was only partially restored to 0.42  $\mu$ moles/hr at 1.4% CO<sub>2</sub> and 0.60  $\mu$ moles/hr at 2.5% CO<sub>2</sub>. This results is in contrast with the data obtained at the lower acetazolamide concentrations at which H<sup>+</sup> secretion was not only restored but increased by CO<sub>2</sub>.

To exclude the possibility that acetazolamide causes nonspecific cytotoxic changes, we examined the effect of an even higher dose,  $5 \times 10^{-3}$  M acetazolamide, on the metabolic rate as measured by CO<sub>2</sub> production. In nine experiments the control rate of CO<sub>2</sub> production was  $2.43 \pm 0.25$   $\mu$ moles/hr and  $2.38 \pm 0.15$   $\mu$ moles/hr after addition of acetazolamide. In addition electron micrographs

TABLE III

*Effect on H<sup>+</sup> Secretion of  $5 \times 10^{-5}$  M Acetazolamide (ACZ) and Addition of Exogenous CO<sub>2</sub>*

Exp.	H <sup>+</sup> secretion			
	No exogenous CO <sub>2</sub>		Exogenous CO <sub>2</sub>	
	Control	ACZ	ACZ + 1.4% CO <sub>2</sub>	ACZ + 2.5% CO <sub>2</sub>
	$\mu$ moles/hr		$\mu$ moles/hr	
a	0.70	0.30	0.75	0.90
b	1.00	0.50	0.80	1.20
c	0.95	0.35	1.10	1.60
d	0.65	0.40	0.60	1.15
e	1.20	0.30	0.85	1.70
f	1.30	0.05	1.10	1.05
g	1.37	0.56	1.27	1.56
h	0.85	0.41	1.25	1.63
i	1.66	1.10	2.09	3.62
j	1.44	1.41	2.99	4.55
Mean	1.11	0.53	1.18	1.89
$\pm$ SEM	0.11	0.13	0.16	0.38
		$P < 0.002$	$P < 0.11$	$P < 0.025$

ACZ and exogenous CO<sub>2</sub> were added to the serosal side only. The rate of H<sup>+</sup> secretion represents the mean of a 30 min period 45 min after the addition of ACZ and CO<sub>2</sub>. The *P* values were obtained from the individual differences between the control and experimental groups.

TABLE IV

*Effect on H<sup>+</sup> Secretion of  $5 \times 10^{-4}$  M Acetazolamide (ACZ) and Addition of Exogenous CO<sub>2</sub>*

Exp.	H <sup>+</sup> secretion			
	No exogenous CO <sub>2</sub>		Exogenous CO <sub>2</sub>	
	Control	ACZ	ACZ + 1.4% CO <sub>2</sub>	ACZ + 2.5% CO <sub>2</sub>
	$\mu$ moles/hr		$\mu$ moles/hr	
a	1.75	0.25	0.60	0.90
b	0.75	0.00	0.00	0.40
c	1.15	0.20	0.40	0.55
d	1.18	0.25	0.35	0.00
e	1.25	0.24	1.05	1.57
f	1.23	0.59	0.85	1.07
g	0.76	0.08	0.24	0.47
h	0.64	0.05	0.10	0.29
i	0.88	0.00	0.17	0.30
j	0.95	0.00	0.41	0.45
Mean	1.05	0.17	0.42	0.60
$\pm$ SEM	0.10	0.06	0.11	0.15
		$P < 0.001$	$P < 0.001$	$P < 0.002$

ACZ and exogenous CO<sub>2</sub> were added to the serosal side only. The rate of H<sup>+</sup> secretion represents the mean of a 30 min period 45 min after the addition of ACZ and CO<sub>2</sub>. The *P* values were obtained from the individual differences between the control and experimental groups.

of tissue exposed to  $5 \times 10^{-4}$  M acetazolamide revealed no morphological change.

To determine whether the specific end group required for carbonic anhydrase inhibition is also required for the second inhibitory effect of acetazolamide at  $5 \times 10^{-4}$  M concentration, the effect of an inactive analogue of acetazolamide, Cl 13,850, in which a tertiary butyl group is substituted, was examined in the same concentration. In accord with previous studies (5), this analogue failed to inhibit H<sup>+</sup> secretion in the absence of exogenous CO<sub>2</sub>. In Table V, one of five experiments is shown indicating that the enhancement of H<sup>+</sup> secretion by CO<sub>2</sub> is unaffected by this compound.

TABLE V

*Effect on H<sup>+</sup> Secretion of an Inactive Analogue of Acetazolamide (Cl 13,850) at Different Serosal CO<sub>2</sub> Concentrations*

	H <sup>+</sup> secretion	
	Control	$5 \times 10^{-4}$ M Cl 13,850
	$\mu$ moles/hr	
No exogenous CO <sub>2</sub>	0.70	0.65
0.6% CO <sub>2</sub>	1.20	1.15
1.2% CO <sub>2</sub>	1.40	1.45
2.5% CO <sub>2</sub>	2.70	2.65

Exogenous CO<sub>2</sub> and Cl 13,850 were added to the serosal side only. The rate of H<sup>+</sup> secretion represents the mean of a 30 min period 45 min after the addition of Cl 13,850 and CO<sub>2</sub>.

*Inhibition of H<sup>+</sup> secretion by ethoxzolamide.* Acetazolamide is a relatively slowly diffusible inhibitor which is known to have greater affinity for the C enzyme of human carbonic anhydrase than for the B isoenzyme (6). Inhibition of the latter requires acetazolamide concentrations some 12 times higher than the former. Since ethoxzolamide has the same affinity for the two isoenzymes and is rapidly diffusible, this inhibitor was used to obtain a series of dose response curves in the same bladders (6). The results of 6 experiments with an average control rate of 0.99  $\mu$ moles/hr are shown in Fig. 4. The solid line depicts the per cent inhibition for the group of experiments carried out in the absence of exogenous CO<sub>2</sub>. H<sup>+</sup> secretion was inhibited progressively, 50% inhibition occurring at about  $3 \times 10^{-7}$  M ethoxzolamide. In contrast to the acetazolamide studies the dose response curve of ethoxzolamide exhibits no plateau at 50% inhibition of H<sup>+</sup> secretion. Inhibition increased to 67% at  $1 \times 10^{-6}$  M, 73% at  $3 \times 10^{-6}$  M, 77% at  $1 \times 10^{-5}$  M, and 82% at  $3 \times 10^{-5}$  M. The last three values were not statistically different. The lower curve (interrupted line) shows the per cent inhibition observed in six different bladders when ethoxzolamide was added in the presence of 2.5% CO<sub>2</sub> in S. In the presence of CO<sub>2</sub>, inhibition was not observed until ethoxzolamide concentrations of more than  $3 \times 10^{-7}$  M were reached. From concentrations from  $1 \times 10^{-6}$  to  $3 \times 10^{-5}$  M, inhibition increases rapidly to 70%, indicating that at these levels the ethoxzolamide inhibition is not completely reversed by CO<sub>2</sub>.

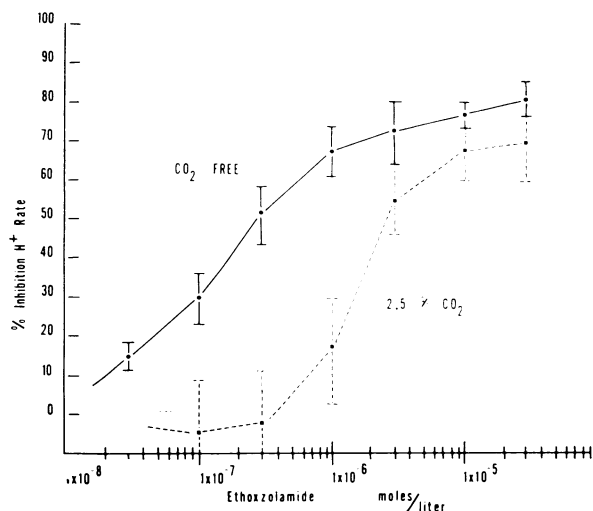


FIGURE 4 Per cent inhibition of H<sup>+</sup> secretion in response to increasing concentrations of ethoxzolamide in six bladders in the absence of exogenous CO<sub>2</sub> (solid line). The interrupted line represents the per cent inhibition in the presence of 2.5% CO<sub>2</sub> in another group of six bladders.

## DISCUSSION

These studies confirm recent reports by Rosen (10) and Scott et al. (11) that the mucosal cells of the turtle bladder contain carbonic anhydrase, and raise several interesting questions about the distribution of carbonic anhydrase and its function in urinary acidification.

The histochemical evidence that the enzyme is demonstrable in only a group of epithelial cells<sup>1</sup> covering some 10% of the mucosal surface is consistent with the relatively low over-all activity observed in homogenates of mucosal cells and may provide an explanation for the difficulties encountered during the initial assays (6) for carbonic anhydrase in whole bladder homogenates. Although the histochemical observations strongly suggest that carbonic anhydrase is abundant in a population of surface cells, they do not exclude that low concentrations of enzyme may be present in the other epithelial cells. Similarly the apparent cytoplasmic localization of the histochemical precipitates does not exclude that some moiety of carbonic anhydrase may be associated with the plasma membrane.

To explore the function of carbonic anhydrase and the significance of its distribution for the organization of acidification within the intact epithelium, the effects of increasing concentrations of carbonic anhydrase inhibitors were studied with and without addition of CO<sub>2</sub>. In the absence of exogenous CO<sub>2</sub>, the dose response curve for acetazolamide has two plateaus. The first occurs at 50% inhibition of H<sup>+</sup> secretion and extends from  $5 \times 10^{-8}$  to  $5 \times 10^{-6}$  M acetazolamide. The second plateau is observed near 80% inhibition at concentrations of  $1 \times 10^{-4}$  M and higher. At the first level of inhibition, addition of CO<sub>2</sub> not only restores H<sup>+</sup> secretion, but increases secretion to the same rates that are reached in the absence of acetazolamide. Ethoxzolamide, a carbonic anhydrase inhibitor with a much higher affinity for the enzyme than acetazolamide, causes a similar degree of maximal inhibition and shows similar features when CO<sub>2</sub> is added at equivalent degrees of inhibition. In contrast to acetazolamide, however, ethoxzolamide gives progressive inhibition in the CO<sub>2</sub>-free system without evidence of plateau in the dose response curve at 50% inhibition.

These results can be interpreted in several ways. The simplest interpretation would be that carbonic anhydrase is present in two concentrations, a high concentration in the cytoplasm of the cells staining histochemically and a low concentration in the rest of the epithelial cells (first hypothesis). Other explanations would be based

<sup>1</sup> Rosen (19) has described a bladder epithelial cell which differs in some respects from the majority of surface cells and corresponds to the cell staining for carbonic anhydrase (20). It is not clear whether this cell represents a transition form between the basal and the surface cell, or a separate cell line.

on the existence of two isoenzymes of carbonic anhydrase with different affinities for acetazolamide. For example, the two isoenzymes may be present in the cytoplasm of the stained cells only (second hypothesis). Or, one isoenzyme may be cytoplasmic and most abundant in the stained cells, and the other isoenzyme may be membrane-associated and involved in transport in all surface cells (third hypothesis).

According to the first interpretation, carbonic anhydrase would be present in high concentrations in the cells staining for the enzyme and in low concentrations in the majority of surface cells that fail to stain. The low-activity cells would be completely inhibited at  $5 \times 10^{-6}$  M acetazolamide, while inhibition in the high-activity cells would become effective only at concentrations near  $1 \times 10^{-4}$  M. Wistrand and Bååthe (21) and Maren and Wiley (22) have examined the inhibition of carbonic anhydrase activity in whole red cells and have observed that in this intact cell system total inhibition of enzyme requires rather high concentrations of inhibitor, about  $10^{-4}$  M ethoxzolamide, reflecting the high carbonic anhydrase concentration in red cell cytoplasm. In turtle bladder, Scott et al. (11) estimated the molar tissue concentration of carbonic anhydrase as  $2.3 \times 10^{-6}$  M in mucosal cells. If the bulk of this was contained in 10% of cells the concentration in these cells would increase to about  $2 \times 10^{-5}$  M. The inhibitor concentrations that were required for full inhibition of acidification in vitro were not inconsistent with such enzyme concentrations. For ethoxzolamide with its high affinity for the enzyme full inhibition was achieved between 1 and  $3 \times 10^{-5}$  M, at equimolar concentrations with the enzyme in high-activity cells. For acetazolamide with its lower affinity for the enzyme this was achieved between 1 and  $5 \times 10^{-4}$  M.

Implicit in this interpretation are two assumptions. One is that all surface epithelial cells are capable of secreting  $H^+$ . Several quantitative considerations to be discussed later suggest that this assumption is correct. The other is that inhibition of  $H^+$  secretion by acetazolamide and ethoxzolamide results only from inhibition of catalyzed  $CO_2$  hydroxylation. The latter assumption, however, has several shortcomings. The rate of  $H^+$  secretion after maximal inhibition is only  $0.17 \mu\text{moles/hr}$  and much of this quantity may be related to organic acid production rather than to  $CO_2$  hydration. Yet the estimated velocity of uncatalyzed  $CO_2$  hydration is 0.5 or  $0.6 \mu\text{moles/hr.}^2$  This suggests that factors other than the uncatalyzed rate have become rate limiting.

<sup>2</sup> The rate of  $0.6 \mu\text{moles/hr}$  is based on a cellular  $P_{CO_2}$  of 2.5 mm Hg (9) and a cell water volume of  $40 \mu\text{l}$ . This volume is an average value (5) instead of the upper estimate given previously (9). For calculating the uncatalyzed rate of hydration a velocity constant of  $0.0375 \text{ sec}^{-1}$  and a  $CO_2$  solubility constant of 0.03501 are assumed (9). To obtain the above estimate of cellular  $P_{CO_2}$  (9) it was as-

The alternative explanation, that the mucosa contains two isoenzymes of carbonic anhydrase, deserves consideration, since it might account for the difference in the dose response curves between acetazolamide and ethoxzolamide. Thus, in primate red cells carbonic anhydrase B and C have different affinities for acetazolamide but not for ethoxzolamide (6). Inhibition of B requires acetazolamide concentrations at least 10 times greater than inhibition of C. The existence of two isoenzymes in the same tissue has recently been reported not only for primate red cells, but also for human lens (6), guinea pig colon, and kidney (23). The existence of a second enzyme has also been suggested for dog kidney (24). If there were two isoenzymes in turtle bladder, they could still be distributed in a variety of ways in the mucosa. For the purposes of our analysis of the physiologic data in the intact epithelium only a few possibilities need be considered. One is that, both isoenzymes might be contained in the cytoplasm of the stained cells only (second hypothesis). This would place the burden of acidification on a small group of epithelial cells. Several lines of evidence, however, indicate that all cells are capable of secreting  $H^+$ . First, in the absence of external  $CO_2$  the acidification process is capable of utilizing the available supply of metabolic  $CO_2$  with extraordinary efficiency. Some 50% of the metabolic  $CO_2$  is utilized in acidification despite the fact that the bladder is maintained as a flat sheet in an open system in which  $CO_2$  is removed rapidly from the bulk solutions (9). If epithelial  $CO_2$  alone is considered this percentage is even higher. It is unlikely, if not impossible, that such a large fraction of all  $CO_2$  could be hydroxylated in a small group of cells making up no more than  $\frac{1}{10}$  of the surface cells. Although the  $P_{CO_2}$  of the carbonic anhydrase-active cell might be lower than that of its neighbors because of rapid  $CO_2$  hydroxylation, the  $P_{CO_2}$  in the two bulk solutions must be even lower and the geometry of  $CO_2$  diffusion in such a flat sheet could not provide enough  $CO_2$  to "1 in 10" cells to account for the observed rates of hydroxylation. Hence, hydroxylation must occur in the bulk of epithelial cells. This argument is further supported by the observation that decreases in  $CO_2$  production brought about by inhibition of sodium transport cause almost stoichiometric decreases in  $H^+$  secretion (9). This relationship suggests that the function of  $H^+$  secretion is distributed in cells that are also involved in sodium transport i.e., in the majority of cells (25).

Other distributions of two isoenzymes in the cytoplasm of all surface cells might account for the data, but the

assumed that all metabolic  $CO_2$  left the cells in the form of free  $CO_2$ . If allowance is made for the portion of metabolic  $CO_2$  that diffuses out of the epithelium in the form of  $HCO_3^-$ , the estimate would be lowered to a  $P_{CO_2}$  of about 2 mm Hg or an uncatalyzed rate of  $0.5 \mu\text{moles/hr}$ .



analysis would resemble our first interpretation and will not be developed further.

The remaining possibility (third hypothesis) is that one isoenzyme is cytoplasmic and the other is associated with the luminal membrane. Carbonic anhydrase has been found in membrane fractions of lens (6), colon (23), and kidney (24). A function for carbonic anhydrase in the transport of ions has been considered in several epithelia, stomach (26, 27), colon (23), gallbladder (28), and also turtle bladder (11). It is conceivable, therefore, that carbonic anhydrase plays a role in the transport process of acidification. For this interpretation, the initial inhibition represents binding to a cytoplasmic enzyme with high affinity for acetazolamide and with greatest abundance in the cells staining for carbonic anhydrase; the final component of inhibition would be binding to a membrane-associated enzyme with low affinity for this inhibitor. Addition of  $\text{CO}_2$  overcomes the inhibition of the cytoplasmic enzyme, but not that of the membrane associated enzyme. If the second component of inhibition of  $\text{H}^+$  secretion represented inhibition of a transport step for  $\text{H}^+$  or  $\text{OH}^-$  and if inhibition of cytoplasmic carbonic anhydrase was complete at the first plateau of the acetazolamide curve (Fig. 3), then the residual rate of acidification should be supported by the uncatalyzed rate of hydroxylation of  $\text{CO}_2$  in the cell water of the epi-

thelium. This rate has been estimated above as about  $0.5 \mu\text{moles/hr.}^2$  This estimate would be very near the residual rate of acidification observed in the presence of  $5 \times 10^{-5} \text{ M}$  acetazolamide. Since the rate of appearance of endogenous  $\text{HCO}_3^-$  in S may be 0.1 or 0.2  $\mu\text{moles/hr}$  less than the rate of  $\text{H}^+$  secretion into M (presumably since some  $\text{OH}^-$  combines with organic acids such as lactic acid), it is probable that the residual rate of acidification can be sustained by the uncatalyzed rate of hydroxylation of metabolic  $\text{CO}_2$ . Similarly, the increments in  $\text{H}^+$  secretion that occur when  $\text{CO}_2$  is added to bladders exposed to  $5 \times 10^{-5} \text{ M}$  acetazolamide can be accounted for by increased uncatalyzed hydroxylation of  $\text{CO}_2$ . The fact that  $\text{H}^+$  secretion is markedly reduced at higher concentrations of either acetazolamide or ethoxzolamide despite the presence of exogenous  $\text{CO}_2$  is consistent with inhibition of some step in the transport process itself.

Whether there is a transport carbonic anhydrase or whether the transport system contains receptors that resemble carbonic anhydrase can not be resolved in the intact preparation. An effect of higher concentrations of acetazolamide on the  $\text{H}^+$  transporting system has also been suggested for the proximal tubule of the rat in a recent report by Radtke, Rumrich, Kinne-Saffran, and Ullrich (29). Such an effect on the transport system might explain the failure of acetazolamide in a previous study (5) to increase the intracellular pH of the epithelium. If acetazolamide had only interfered with the enzymatic hydroxylation of  $\text{CO}_2$  in the cytoplasm, the cells would have become more alkaline, as in the studies of Struyvenberg, Morrison, and Relman (30); in contrast, a direct inhibition of the pump would have reduced the generation of  $\text{OH}^-$  within the cells, and the two effects occurring simultaneously might have caused little net change in cell pH.

Although this hypothesis that the inhibitors act on both the cytoplasmic enzyme and the  $\text{H}^+$  transport system is attractive, the evidence remains circumstantial. The first interpretation that all carbonic anhydrase is cytoplasmic and distributed in two concentrations can not be excluded. It is possible, nevertheless, to arrive at certain general conclusions regarding the epithelial organization of acidification which are independent of the precise distribution of carbonic anhydrase. First, all surface epithelial cells must have the capacity to secrete  $\text{H}^+$ ; this is based on the utilization of metabolic  $\text{CO}_2$  in the acidification process and the close dependence of  $\text{H}^+$  secretion on metabolic  $\text{CO}_2$  production reported previously (9). Second, the capacity of surface epithelial cells to hydrate or hydroxylate  $\text{CO}_2$  is not homogenous, but depends on the quantity of carbonic anhydrase present in the cytoplasm. About 10% of cells have high cytoplasmic enzyme concentrations and as a result can rapidly dispose of  $\text{OH}^-$  generated behind the  $\text{H}^+$  pump. The ma-

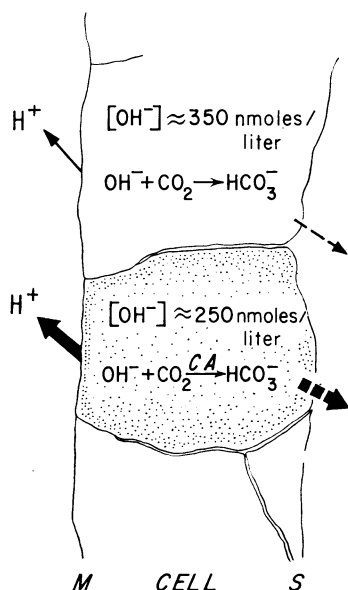


FIGURE 5 Schematic representation of the cellular organization of acidification in the epithelium of the turtle bladder. In the shaded cell carbonic anhydrase (CA) catalyzes the hydroxylation of  $\text{CO}_2$  and thereby limits the accumulation of  $\text{OH}^-$ . When  $\text{CO}_2$  is in short supply, cells without CA (or with CA inhibited at low inhibitor concentrations) become alkaline so that the secretion of  $\text{H}^+$  is reduced by the concentration gradient (see text).

jority of surface cells has much lower cytoplasmic enzyme concentrations and possibly only membrane-associated carbonic anhydrase. These differences in cytoplasmic enzyme affect the capacity to secrete  $H^+$  whenever  $CO_2$  is in short supply. Fig. 5 is a schematic representation of the acid-base relations in the in vitro preparation in which exogenous  $CO_2$  is absent. In the shaded cell the enzymatic hydroxylation of  $CO_2$  limits the accumulation of  $OH^-$  and the development of a steep concentration gradient; hence, a high transport rate can be maintained. In the other cells  $OH^-$  concentrations rise until the pump rate becomes either limited by a concentration gradient or by an effect of intracellular pH on the energetics of transport. The effect of concentration gradients on the pump rate has been defined previously for the whole epithelium (31).

Is such an organization of carbonic anhydrase function unique for the urinary bladder of the water turtle? Histochemical studies of the vertebrate nephron have shown that similar distributions of carbonic anhydrase activity occur in the collecting ducts of several species (32). Thus, the collecting duct of the turtle kidney resembles turtle urinary bladder in staining characteristics for carbonic anhydrase. Similar patterns of stained and unstained cells occur in the rabbit and monkey collecting duct (32).

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