Acid-Base Relations in Epithelium of Turtle Bladder: Site of Active Step in Acidification and Role of Metabolic CO₂

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ABSTRACT The acid-base relations across the two surfaces of the epithelium of the turtle bladder were examined. By means of the 5,5-dimethyl-2,4-oxazolidinedione (DMO) technique the intracellular OH⁻ concentration was measured in the presence and absence of a transepithelial pH gradient.

When both sides of the bladder were bathed with solutions free of exogenous CO₂ and bicarbonate at pH 7.41 ($[OH^-] = 239$ nmoles/liter), the epithelial cells were alkaline, the mean intracellular [OH-] being 347 nmoles/liter. This alkalinity of the cells was preserved in bladders that secreted H⁺ against a gradient of over 2 pH units. In bathing solutions stirred with 4.85% CO₂ and buffered with 25 mM HCOs⁻ at pH 7.41 the intracellular [OH-] was lower than in CO₂-free solutions and close to the extracellular [OH-]. In the CO2-free system anaerobiosis caused increased alkalinity of the cells and inhibition of H⁺ secretion presumably by decreased metabolic CO2 production. Carbonic acid inhibitors reduced H⁺ secretion, but had no significant effect on the alkalinity of the cells. An inactive analogue of acetazolamide had no effect on H⁺ secretion. The results indicate that the active step in acidification is located near the mucosal surface of the epithelium and that the alkali formed within the epithelial cells moves passively into the serosal solution along an electrochemical gradient. The inhibitory effect of certain sulfonamides on H⁺ secretion by the bladder is directly correlated with their known carbonic anhydrase inhibitory activity, but not associated with a measurable change in the mean intracellular $[OH^-]$.

INTRODUCTION

Previous studies (2-4) have shown that the isolated urinary bladder of the water turtle secretes hydrogen ion into the solution bathing its mucosal surface and that this process is not directly dependent on the transport of sodium or other electrolytes. Observations made during these studies suggest that hydrogen ion transport depends on the metabolic production of CO₂ and that the mucosal membrane of the epithelium is the probable site of the active step in acidification.

In the present investigation these observations were further explored. The intracellular pH of the epithelial cells was estimated from the distribution of the weak acid, 5,5-dimethyl-2,4-oxazolidinedione (DMO), and the acid-base relations across the two borders of the epithelium were examined under a variety of conditions.

The results indicate that the epithelial cells are relatively alkaline and that hydrogen ion transport across the mucosal surface of the epithelium takes place against a steep concentration gradient. In the absence of exogenous CO_2 in the system metabolic CO_2 plays an important role in the disposition of hydroxyl ions generated behind the hydrogen ion pump.¹ Reduction of CO_2 production results in greater alkalinity of the cells, but such increased alkalinity could not be demonstrated after addition of carbonic anhydrase inhibitors. The mechanism by which acetazolamide and other sulfonamides inhibit hydrogen ion secretion by the turtle bladder

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¹The term hydrogen ion pump will be employed to describe an active process in which H⁺ and OH⁻ are dissociated and one of the two ions is transported across the membrane.

remains to be clarified. The inhibitory effect on hydrogen ion secretion, however, was observed only with compounds known to inhibit carbonic anhydrase.

METHODS

Urinary bladders of adult water turtles, Pseudemys scripta elegans, were removed with a minimum of handling, washed with Ringer's solution, and mounted between two halves of a lucite chamber which provided an exposed membrane area of 8 cm². The spontaneous electrical potential difference (PD) across the bladder, the short circuit current (SCC), and the rate of hydrogen ion secretion into the compartment bathing the mucosal surface of the bladder were measured as described previously (2). In some experiments bladders were mounted on plastic frames and bathed with Ringer's solution in a beaker. Two Ringer's solutions were employed: (a) bicarbonate-free Ringer's of the following composition: Na⁺, 115.0 mEq/liter; K⁺, 3.5 mEq/ liter; Ca⁺⁺, 0.9 mmole/liter; Cl⁻, 119.7 mEq/liter; HPO₄⁻ 0.3 mmole/liter; dextrose, 2.0 mmole/liter; osmolality, 227 mOsm/kg H₂O; gas phase, air passed through 3 м KOH to trap CO₂; (b) bicarbonate Ringer's of the following composition: Na⁺, 117.0 mEq/liter; K⁺, 3.5 mEq/liter; Ca⁺⁺, 0.9 mmole/liter; Cl⁻, 97.3 mEq/liter; HCO3⁻, 25 mEq/liter; dextrose 2.0 mmole/liter; osmolality, 228 mOsm/kg H2O; gas phase, 4.85% CO2 in air. After mounting of the bladders the Ringer's solutions were exchanged from three to four times over a 2 hr period. The final solution was equilibrated with the gas phase for 10 min, and its pH adjusted to 7.40 with either HCl or NaOH.

The isotopic compounds, inulin-14C and DMO-14C (5,5dimethyl-2,4-oxazolidinedione-14C), were obtained from the New England Nuclear Corp., Boston, Mass. and were counted in a Nuclear-Chicago model 6801-S liquid scintillation counter. Before the counting, the samples of bladder tissue were solubilized and left overnight in liquid scintillation medium. Quenching corrections were made by counting each sample before and after addition of a known number of counts. The water content of the tissues was obtained as the difference between the wet weight of the blotted bladder and the weight after 24 hr of drying in an oven at 100°C. The ratio of dry weight to wet weight was 0.171 ± 0.008 (SEM) for the inulin experiments and 0.179 ± 0.006 (SEM) for the DMO experiments. The mean dry weight of the exposed bladder in the lucite chamber was $13.9 \pm 0.8 \text{ mg}$ (SEM).

To measure the fraction of tissue water that was extracellular two series of experiments were carried out with inulin-¹⁴C. In six experiments inulin-¹⁴C was added to the serosal compartment of the lucite chamber and in six other experiments it was added to the beaker fluid bathing both sides of the membrane. The inulin space of the tissues was measured after 2 hr of exposure to inulin-¹⁴C in the bathing fluid. Since the inulin space differed by less than 2% in the two sets of experiments, the results were pooled. The mean inulin space was $40 \pm 3\%$ (SEM).

The distribution of DMO-¹⁴C between cell water and extracellular fluid was studied under the same experimental conditions. In all but one set of experiments both sides of the bladder were exposed to DMO-²⁴C. In the experiments in which the mucosal (M) solution was acidified to a pH c¹ose to 5 the label was added only to the serosal (S) solution, the equilibrium concentrations in M being negligible compared to S. For the calculation of the extracellular fraction of tissue water the inulin space was used, and the DMO-14C concentration was considered to be identical in the extracellular fluid and in the bathing fluid. The extracellular fluid pH was taken from the mean bathing fluid pH which was kept close to 7.40 during 2 hr of equilibration with DMO-14C in the bathing fluid. This time interval was considered to be sufficient to reach an equilibrium distribution of DMO in the epithelium since, in a separate series of experiments, the DMO flux from S to M reached steadystate values 43 ± 5 min after addition of DMO to S; the mean permeability coefficient was $88 \pm 19 \times 10^{-7}$ cm sec⁻¹/8 cm². An apparent mean intracellular pH was calculated according to Waddell and Butler (5) from the extracellular pH, the DMO-14C concentrations in bathing fluid and tissue water, and the information obtained above that 60% of tissue water represented cellular water. The room temperature was maintained close to 23°C and the pK of DMO at 23°C was extrapolated from data by Waddell and Butler (5) at 37°C and by Waddell and Hardmann (6) at 25°C. The extrapolated value at 23°C of 6.27 was in good agreement with a value of 6.28 obtained experimentally by Gulyassy (7) at 22°C. The pKw at 23°C was taken as 14.04 (8). The limitations of the DMO method in estimating the intracellular pH in the turtle bladder epithelium are discussed below.

For the experiments with carbonic anhydrase inhibitors the following sulfonamides were studied: (a) acetazolamide in a final concentration of 4×10^{-4} moles/liter in the serosal solution, (b) 2-acetylamino-1,3,4-thiadiazole-5-sulfont-butylamide, CL 13,850 (batch 8044B-145A), an analogue of acetazolamide which has no carbonic anhydrase inhibitory activity in the same final concentration as acetazolamide, (c)2-benzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide, CL 11,-366, a potent carbonic anhydrase inhibitor used in a concentration of 8×10^{-5} moles/liter, or one-fifth that of acetazolamide.² The effects on H⁺ secretion of these compounds was studied by exchanging the serosal solution after a control period of 60-90 min during continuous pH stat recording of acid secretion on the mucosal side of the bladder. In several experiments the effects of the inactive analogue and acetazolamide were studied in sequence in the same preparation.

The effects of the two carbonic anhydrase inhibitors, acetazolamide and CL 11,366, on the intracellular pH were examined by carrying out the above described DMO experiments in the presence of acetazolamide or CL 11,366.

Limitations of the DMO method in relation to the structure of the bladder epithelium. The theory of the determination of intracellular pH from the distribution of DMO and other weak electrolytes has been discussed recently by several authors (5, 9-12). In a multicompartment system a weak acid indicator, such as HDMO, permits determination of the true mean OH⁻ concentration in the system, but the mean H⁺ concentration^{*} calculated does not represent a mean value in the mathematical sense, but rather an intermediate value between regions of different pH (11, 12). For this study the results will be expressed in terms of OH⁻ concentrations, as well as in terms of mean apparent pH values.

The cellular water in the turtle bladder was measured as the fraction of tissue water from which inulin was excluded. This fraction comprised the epithelial cell layer as well as

² Compounds CL 13,850 and CL 11,366 were made available through the generosity of Dr. Selby B. Davis of Lederle Laboratories, Pearl River, N. Y.

^aFor the purposes of our discussions no distinctions will be made between concentrations and activities. some cellular elements contained in the loose tissue layer on the serosal side of the epithelium, i.e., muscle and connective tissue cells. The serosal lining cells did not survive in the mounted preparation and hence did not contribute to cell water (13). It was not feasible to remove the loose tissue layer from a large enough (about 8 cm^2) sheet of epithelium to permit studies of acid secretion on the epithelial cell layer alone. The epithelial cell layer, however, comprised the bulk of cell mass on cross-sectional microscopy.

Studies by light and electron microscopy⁴ indicate that the mucosal border of the epithelium is formed by a row of homogeneous epithelial cells that are connected by tight junctions. In the stretched and mounted bladder the mucosal surface of the epithelium is flat except for microvilli detectable only by electron microscopy. The cytoplasmic region below this mucosal surface contains numerous large granules with the staining characteristics of mucopolysaccharides. The basal regions of the epithelial cells are relatively agranular, but contain mitochondria and a fairly prominent endoplasmic reticulum. Interspersed between the basal aspects of the surface cells are basal cells which have a cytoplasm which resembles the cytoplasm of the deeper regions of the surface cells.

To examine the extent to which the mean $[OH^-]$ that can be determined for the over-all cellular compartment by the DMO technique is representative of the $[OH^-]$ in the bulk phase of cytoplasm a number of calculations were made. For a given mean $[OH^-]$ of 288 nmoles/liter, a value in the range of our experimental results, the effect was explored of assuming a region of different acidity on the remaining compartment. In Table I is shown that if the pH of a 10% compartment is varied from 5 to 8 the alkalinity of the main compartment is not much affected. Only a marked alkalinity in a compartment as large as 25% would alter the pH of the main compartment appreciably.

Aside from the limitation imposed on the method by the heterogeneity of the cells there are two potential theoretical limitations, namely that the distribution of DMO is affected by (a) binding of the DMO anion to charged polyelectrolytes in the tissue, or by (b) active transport of DMO across the cell membrane. For a variety of tissues it has been shown that these limitations do not apply (9). Studies with carrier DMO will be presented which uncover no evidence that DMO is either bound or actively transported by the turtle bladder epithelium.

In the text and in the tables, values for the mean and SEM have been calculated directly from the individual values, whether they were concentrations in nanomoles or pH values. All mean values are given with the standard error of the mean (SEM).

RESULTS

Concentration profile of H^+ and OH^- across the cell borders of the epithelium during acidification against a transepithelial gradient. The active step in acidification may be considered a reaction in which H^+ and OH^- are dissociated and one of the two ions is transported across the cell membrane. If there is a single active transport system for acidification at one of the surfaces of the epithelium then the transport across the opposite surface should be accounted for by passive electrochemi-

⁴ Rosen, S. Unpublished observations.

1260 P. R. Steinmetz

TABLE I Possible Ranges of Acid-Base Heterogeneity in Two-Compartment System for a Given Mean [OH-]

Management	a	Assumed acidity of smaller compart-	Effect on re- maining com- partment of	
acid indicator	25	% or 10%	75%	90%
	pН	5.00	7.62	7.55
	OH ⁻ , nmoles/liter	0.91	384	320
[OH ⁻] = 288 nmoles/liter	pН	6.00	7.62	7.55
pH apparent = 7.50	OH ⁻ , nmoles/liter	9.12	381	319
	pН	7.00	7.59	7.53
	OH ⁻ , nmoles/liter	91.2	354	310
	pН	8.00	6.94	7.34
	OH ⁻ , nmoles/liter	912	80	219

cal forces. In the short-circuited bladder in which acidification takes place against a concentration gradient an analysis of the concentration profile across the two borders of the epithelium might indicate at which cell border the active step is located and in addition provide information on the concentration driving force available for passive transport across the other surface.

In 14 experiments such a profile was obtained from the distribution of DMO-14C between cell water and the serosal bathing solution. Bicarbonate-free Ringer's solutions were employed (see Methods). The pH of the serosal solution was controlled close to 7.40, the mean serosal $[OH^-]$ being 237 ±16 nmoles/liter. The mucosal fluid pH ranged from 5.0 to 5.6 with a mean value of 5.27 \pm 0.04. The mean intracellular [OH⁻] in this group of experiments was 311 ± 34 nmoles/liter representing an apparent intracellular pH of 7.49 ± 0.04 . Despite the acidity of the mucosal solution the epithelial cells were more alkaline than the serosal solution. The mean of the individual differences of cell [OH-] minus serosal [OH-] was 74.5 ± 26.9 nmoles/liter, a value significantly different from zero (P < 0.02). In Fig. 1 the concentration profile for OH- is indicated across the serosal surface of the epithelium. A concentration driving force is present for the movement of alkali into the serosal compartment. At the other surface of the epithelium the movement of H⁺ into the mucosal solution takes place against a steep concentration gradient, the mean apparent intracellular [H⁺] being 30 ± 3 nmoles/liter (SEM) and the mean mucosal fluid [H⁺] being $5370 \pm$ 390 nmoles/liter (SEM).

Role of CO₂ in acid-base behavior of epithelium. Our pH stat measurements of acid secretion by the turtle bladder have been carried out in CO₂-free⁵ and HCO₃-

⁵ The term "CO₂-free" indicates that the Ringer's solutions were prepared without CO_2 and were bubbled with gas that had passed in fine bubbles through 3 M KOH. In



FIGURE 1 Concentration profile of H^+ and OH^- across the mucosal and serosal borders of the epithelium. H^+ transport into the mucosal fluid takes place against a steep concentration gradient. OH^- ions move into the serosal fluid down a concentration gradient.

free Ringer's solutions buffered with 0.3 mM Na₃HPO₄ at pH 7.40. Under these experimental conditions only metabolic CO₂ is available within the epithelial cells. In Table II the mean cellular [OH⁻] in the CO₂-free system is compared with that in a system buffered with 4.85% CO₂ and 25 mM HCO₃. In both instances the pH of the bathing solutions was kept close to 7.4. For the

the presence of the bladder the media contained trace amounts of metabolic CO₂.

TABLE II Mean Cellular [OH⁻] with and without Exogenous CO₂ in the Bathing Solutions

Bathing solution			Cell	Cell-ECF	Cell	
CO2	нсо-	a pH	OH-	ОН	ОН	pН
%	mEq/ liter		nmoles/ liter	nmoles/ liter	nmoles/ liter	
0	0	7.41 ± 0.02	239 ± 12	347 ± 29	108 ±23	7.56 ± 0.04
4.85	25	$7.41\ \pm 0.01$	232 ± 3	267 ±15	15 ± 14	7.42 ± 0.03

Mean values \pm SEM are given for 14 experiments in each group. Both sides of the bladder were bathed with the same solutions.

same extracellular pH the cell water was more alkaline in the CO₂-free system than in the media with 4.85% CO2. In the latter experiments the mean cellular [OH-] was not significantly different from the [OH-] of the bathing solutions. The increased alkalinity of the cells observed in the absence of exogenous CO₂ suggests that endogenous CO2 may be in short supply in our open system. The dependence of cellular acid-base regulation on metabolic CO2 production had been demonstrated previously by the observation that acute reduction of CO₂ production by deoxygenation with Ns caused transient alkalinization of the serosal compartment (2). In Table III further evidence is presented that deoxygenation causes increased alkalinity of the epithelial cells. In seven experiments bladders were equilibrated with DMO-14C and the solutions stirred with CO2-free air. As in previous experiments deoxygenation resulted in alkalinization of the serosal solution whereas the mucosal compartment remained at 7.40 (in some experiments acid secretion into this compartment continued at very low rates). At 25 min the intracellular DMO concentration was compared with that in the serosal fluid and

			25 min after N2					
	Control, Serosal fluid (S)		Serosal fluid (S)		0.114 0			
Turtle	pH	[OH-]	pH	[OH-]	[OH-]	Cell* [OH-]	Cell* pH	
		nmoles/liter		nmoles/liter	nmoles/liter	nmoles/liter	/	
а	7.49	282	7.65	407	254	661	7.86	
b	7.39	224	7.50	288	84	372	7.61	
с	7.42	240	7.67	427	265	692	7.88	
d	7.46	263	7.65	407	196	603	7.82	
е	7.44	251	7.48	275	105	380	7.62	
f	7.46	263	7.78	550	244	794	7.94	
g	7.45	257	7.48	275	193	468	7.71	
Mean ±sем	7.44 ± 0.01	254 ± 7	7.60 ± 0.05	376 ± 39	192 ± 27	567 ± 62	7.78 ± 0.05	

 TABLE III

 Increased Alkalinity of Epithelial Cells after Deoxygenation in CO₂-Free Ringer's Solution

* Non-steady-state values.

		Bathing solution			Cell-ECF [OH-]	Cell pH
		pH [OH-]		Cell [OH-]		
			nmoles/liter	nmoles/liter	nmoles/liter	
CO ₂ -free media	Control	7.41 ± 0.02	239 ± 12	347 ± 29	108 ± 23	7.56 ± 0.04
	Acetazolamide	7.39 ± 0.01	226 ± 1	316 ± 24	90 ± 32	7.52 ± 0.03
	CL 11,366	7.40 ± 0.01	227 ± 2	294 ± 15	67 ± 14	7.50 ± 0.02
CO ₂ , 4.8	Control	7.41 ± 0.01	232 ± 3	267 ± 15	15 ± 14	7.42 ± 0.03
HCO-3 25 mmoles/liter	Acetazolamide	7.41 ± 0.01	235 ± 4	247 ± 15	12 ± 15	7.42 ± 0.03

 TABLE IV

 Mean Cellular [OH⁻] with and without Carbonic Anhydrase Inhibitors

Mean values \pm SEM are given for from 14 to 16 experiments in each group. Both sides of the bladder were bathed with the same solutions.

the mean cellular [OH⁻] was estimated on the basis of these nonsteady-state values. Because the interval is too short to yield a steady-state distribution of DMO, the calculated values do not give quantitative information on the cellular [OH⁻] but they provide evidence for a change in the direction of increased alkalinity.

Effect of acetazolamide and CL 11,366 on intracellular pH. To elucidate the inhibitory effect of acetazolamide on acid secretion by the bladder the effect of acetazolamide on the intracellular pH was examined in both the CO₂-free and the CO₂-containing media. Inhibition of carbonic anhydrase would be expected to interfere with

the disposition of OH^- by the catalyzed hydroxylation of CO_a and hence result in increased alkalinity of the cell. On the other hand, an effect of acetazolamide directly on the transport system might decrease the alkalinity of the cell. The results in Table IV demonstrate that the intracellular pH as estimated by the DMO method was little affected by acetazolamide in either the presence of absence of exogenous CO_a. In the presence of CL 11,366 the intracellular pH was slightly lower but the difference was not statistically significant.

Effect of carrier DMO on distribution of DMO-¹⁴C. To examine the possibility of binding of the DMO



FIGURE 2 Effects of CL 13,850, an analogue of acetazolamide without carbonic anhydrase inhibitory activity, and acetazolamide on cumulative H⁺ secretion. The rate of the secretion is given by the slope of the tracing. Both compounds were added to the serosal fluid in a final concentration of 4×10^{-4} moles/liter.

 TABLE V

 Effect of Carbonic Anhydrase Inhibitors and an Inactive Analogue on H⁺ Secretion

Turtle No.	Control					
H^+ secretion μ moles/hr						
Acetazolamide 4.10 ⁻⁴ moles/liter						
		Acetazolamide				
1	1.20	0.20				
2	1.23	0.30				
3	1.27	0.35				
4	1.72	0.86				
5	0.70	0.27				
6	0.89	0.00				
7	0.59	0.16				
8	1.18	0.29				
9	0.99	0.28				
10	1.34	0.55				
Mean ±SEM	1.20 ± 0.11	0.33 ± 0.07				
Inactive analogue: CL 13,850 4.10 ⁻⁴ moles/liter						
		CL 13,850				
11	1.37	1.50				
12	0.98	0.75				
13	0.83	1.08				
14	0.75	0.75				
15	0.86	0.85				
16	0.53	0.46				
17	1.08	0.96				
18	1.34	1.40				
Mean ±sем	0.97 ± 0.10	0.97 ± 0.12				
Potent inhibitor: CL 11,366 8.10 ⁻⁵ moles/liter						
		CL 11,366				
19	1.40	0.45				
20	0.95	0.50				
21	1.02	0.43				
22	2.12	1.00				
23	1.13	0.32				
24	1.30	0.76				
25	2.12	0.29				
26	0.65	0.22				
27	0.95	0.26				
Mean ±SEM	1.29 ± 0.17	0.47 ± 0.09				

anion to charged polyelectrolytes in the epithelium, the distribution of DMO-¹⁴C was determined in nine bladders that had been exposed to cold DMO in concentrations 400 times that of the labeled compound. In CO-free solutions the mean serosal fluid pH in this group of experiments was 7.43 ± 0.02 and the mean serosal [OH⁻] 246 ±13 nmoles/liter. The mean apparent intracellular pH was 7.55 ± 0.06 and the mean cellular [OH⁻] was 350 ± 46 , values comparable to those obtained in the absence of carrier DMO (Table II).

Effect of carbonic anhydrase inhibitors and an inactive analogue of acetazolamide on H^+ secretion. Since the DMO studies failed to clarify the precise mechanism by which acetazolamide inhibits H⁺ secretion in our system and since the existence of carbonic anhydrase in the epithelium of the turtle bladder is not certain (13), the inhibitory effect of acetazolamide was reexamined and compared with the effect of an acetazolamide analogue, CL 13,850, without carbonic anhydrase inhibitory activity. As indicated in Table V acetazolamide and CL 13,850 were added in equimolar concentrations. In addition, data are presented on another carbonic anhydrase inhibitor, CL 11,366, which is more potent than acetazolamide and can be used in lower concentrations at which nonspecific sulfonamide effects should be reduced. It is clear from Table V that acetazolamide causes a marked and consistent inhibition of H⁺ secretion, whereas the t-butyl analogue, CL 13,-850, has no inhibitory effect. In Fig. 2 an experiment is shown in which CL 13,850 and acetazolamide were added to the serosal solution in sequence. The rate of secretion (given by the slope of the curve) was unaffected by CL 13,850 and markedly reduced by acetazolamide.

The average reduction of H⁺ secretion by acetazolamide was 72% (Table V) and that by CL 11,366, the known inhibitor of carbonic anhydrase, 64%. In these experiments the inhibitors were added to the serosal compartment. CL 11,366 had little or no effect on H⁺ secretion (four experiments) when added to the mucosal solution alone.

DISCUSSION

These studies indicate that the epithelial cells of the urinary bladder of the water turtle are relatively alkaline. This alkalinity was demonstrated in epithelia that were secreting acid while the pH of the mucosal solution was kept near 7.4 by the pH stat technique, as well as in preparations that were secreting against concentration gradients of 2 or 3 pH units in which the pH of the mucosal solution was close to 5. The uphill transport of hydrogen ion from cell interior to mucosal solution is best attributed to an active transport system located at or near the mucosal membrane of the epithelial cell layer. The alternative possibility of passive H⁺ transport across the mucosal surface would require that the cell interior was electrically positive by more than 150 mv with respect to the mucosal solution. Although the electrical driving forces across the mucosal membrane per se could not be controlled in our experiments, the transcellular potential difference was nullified, and under these circumstances, it is most unlikely that the cytoplasm would be so positive. In the shortcircuited toad bladder, for example, there is only a small potential step from mucosal solution to cell interior, which is negative rather than positive (14).

To account for acidification of the mucosal solution with associated alkalinization of the serosal solution in the presence of the concentration gradients observed across the two surfaces of the epithelium, a single active transport system located at the mucosal surface would be sufficient. So long as the energy-producing reaction remains unknown, such a system may be described in its general form as a pump dissociating H⁺ and OH⁻. The movement of OH⁻ or other equivalent anion across the serosal surface would take place passively along an electrochemical gradient. This analysis of the active and passive steps in acidification on the basis of the concentration gradients is in accord with previous observations that appeared to indicate a mucosal location of the H⁺ pump (2).

That cells secreting acid have an alkaline cytoplasm has been suggested in the past for the parietal cells of the gastric mucosa (15) and for yeast cells (16) on basis of observations made with indicator dyes. Recently Struyvenberg, Morrison, and Relman (17) reported that the cells of separated renal tubules in the dog are relatively alkaline as studied by the DMO technique, a result comparable to that obtained in the turtle bladder. In contrast, cell types that are not specialized in acid secretion have a lower pH by the DMO method (5, 12), as well as by measurements with glass microelectrodes (11, 18).

In the experiments discussed so far the turtle bladders were bathed in media prepared without the volatile CO₂-HCO₃ buffer system. These experimental conditions permitted reproducible measurements of the rates of acid secretion but differed from in vivo conditions in which extracellular pH is controlled via the CO₂-HCO₃ buffer system. For a given extracellular pH of 7.4 the intracellular pH was between 0.1 and 0.2 units higher in our CO₂-free system than in media that contained physiologic quantities of CO2 and HCO3-. Despite the somewhat higher intracellular pH in our CO2-free system the epithelium carries out its functions of sodium transport and acid secretion and maintains its electrical resistance for periods well over 8 hr. How the control of cellular pH depends on metabolic production of CO2 is further demonstrated by the increased alkalinity when CO₂ production is reduced acutely by deoxygenation. Although it was impossible to make steady-state observations during deoxygenation, the simultaneous occurrences of an outflow of alkali from the bladder into the serosal solution and increased cellular DMO concentration indicate clearly a directional change toward alkalinity when CO₂ production is reduced.

Less clear is the effect of carbonic anhydrase inhibitors on the intracellular pH. If carbonic anhydrase plays

a role in the rapid hydroxylation of CO₂ formed from metabolism,⁶ one would expect that inhibition of the enzyme would reduce the fraction of CO₂ that can be trapped within the cell to buffer OH⁻ and therefore that the cell would become more alkaline. On the other hand, if the inhibitory effect of acetazolamide on acid secretion were not mediated through carbonic anhydrase but represented a direct inhibitory effect of the sulfonamide on the H⁺ pump, a lower cellular pH would be expected since less OH- would be generated in the cytoplasm. Unfortunately, no significant change in cellular pH was observed in the presence of acetazolamide in either the CO₂-free system, in which the inhibitory effect on acid secretion was clearly present, or in media containing exogenous CO₂ and HCO₃-. It is possible that the method was not sufficiently sensitive to reveal a small regional change in the vicinity of the pump or that the acetazolamide in the tissue had a buffer effect minimizing any change in mean [OH-]. Struyvenberg, Morrison, and Relman (17) did observe a slightly higher intracellular pH in separated canine renal tubules in the presence of 10⁻⁵ M acetazolamide, a concentration lower than that required to inhibit acid secretion in the turtle bladder. In their studies the acetazolamide effect on cellular pH was greatest at high pCO2. Their preparation, however, did not permit measurement of acid secretion so that correlation between the observed pH differences and actual inhibition of acid secretion by acetazolamide was not possible.

Since the presence of carbonic anhydrase in turtle bladder has not been established (13) and since DMO studies of the acetazolamide effect were inconclusive, the inhibitory effect of acetazolamide on H^+ secretion was further explored by comparison with an analogue, CL 13,850, which has no carbonic anhydrase inhibitory activity, and with a very potent inhibitor that can be administered at a lower concentration of sulfonamide.

In accord with previous results acid secretion was reduced by about 72% by 4.10^{-4} M acetazolamide; the inactive analogue in the same concentration had no effect on acid secretion by the bladder. On the other hand, the potent inhibitor, CL 11,366, reduced acid secretion in a fifth of the concentration of acetazolamide. These results indicate that the inhibition of acid secretion does not represent a nonspecific sulfonamide effect. Whatever the precise mechanism of action of these carbonic anhydrase inhibitors in the turtle bladder, the effect on acid excretion appears to depend on the presence of the same specific end groups that are needed for carbonic anhydrase inhibition in other systems. Hogben (19) and Kitahara, Fox, and Hogben (20) observed that Cl⁻ transport in the frog stomach and in the

^eFor this analysis we have assumed that metabolic CO₂ is generated as the anhydrous form.

cornea is inhibited by the "true" carbonic anhydrase inhibitors and not by the analogues. The concentrations used by these investigators, however, were some 20– 30 times higher than the ones used in the turtle bladder.

In our preparation the inhibition of H⁺ secretion is most easily ascribed to inhibition of carbonic anhydrase. Although little or no enzyme was found in unpublished studies cited by Maren (13), small quantities of enzyme might escape detection by the indicator techniques (21) or might be obscured by inhibitor substances contaminating the epithelium from urine or cloacal contents. Since the hydration reaction requires only very small quantities of enzyme, a rather exhaustive search with sensitive techniques is needed to exclude its presence. If the enzyme proves to be absent from the turtle bladder, alternative mechanisms must be invoked. The inhibitors, for example, could act in one of the energyproducing reactions involved in the extrusion of H⁺ through the mucosal membrane or in the step by which OH⁻ is generated in the cell.

Brodsky and Schilb (22) have suggested that carbonic anhydrase might be present at the mucosal surface of the turtle bladder and that in a CO₂- and HCO₃-free system, such as ours, metabolic CO2 might be hydroxylated to HCO₃⁻ in the unstirred layer of fluid along the mucosal surface of the membrane. The HCO3⁻ so formed would then be transported from the mucosal to the serosal side of the membrane as proposed in previous studies by these authors (23). Our failure to find inhibition of acid secretion with CL 11,366 added to the mucosal instead of the serosal medium makes it unlikely that acidification would be dependent on the catalyzed hydroxylation of CO₂ in a fluid layer adjacent to the mucosal membrane. It appears best to describe the active transport step at the mucosal membrane as a reaction in which H⁺ and OH⁺ are dissociated. H⁺ is extruded directly through the membrane into the mucosal solution and OH⁻ is generated at some site behind the pump and buffered by metabolic CO2 or by organic acids formed from metabolism. Whether the inhibitory effect of carbonic anhydrase inhibitors is mediated through carbonic anhydrase or another reaction in the transport of H⁺ or the generation of OH⁻ remains to be determined.

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