Adaptation to Metabolic Alkalosis by the Turtle Urinary Bladder (43214)

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Abstract. **We studied the mechanism of adaptation to metabolic alkalosis by the turtle urinary bladder *in vitro*. Turtles were made alkalotic by administration of oral $NaHCO_3$. Bladders removed from alkalotic turtles had an increased rate of HCO_3^- secretion *in vitro* as compared with that of control. H^+ secretion, however, was not different, indicating that metabolic alkalosis selectively increases HCO_3^- secretion. Fluorescence microscopy was used to quantify the carbonic anhydrase cells. The total number of carbonic anhydrase cells was determined by mucosal staining of the bladder with 6-carboxyfluorescein diacetate. The number of HCO_3^--secreting cells (β cells) was quantified by mucosal staining with NBD-taurine and the number of H^+-secreting cells (α cells) was calculated from the difference between the two. Metabolic alkalosis significantly increased the total number of 6-carboxyfluorescein positive cells and NBD-taurine-positive cells. The increase in the number of 6-carboxyfluorescein positive cells was totally accounted for by the increase in the NBD-taurine-positive cells without change in the number of α cells. If NBD-taurine accurately reflects the number of β cells, these studies show that the adaptation to metabolic alkalosis is mediated, at least in part, by an increase in the number of HCO_3^--secreting (β) cells.** [P.S.E.B.M. 1991, Vol 196]

The turtle urinary bladder is capable of H^+ secretion and HCO_3^- secretion *in vitro* (1, 2). Bladders removed from turtles with metabolic or respiratory acidosis *in vivo* have increased rates of H^+ secretion *in vitro* without changes in HCO_3^- secretion (3, 4). Conversely, bladders removed from alkalotic turtles have increased rate of HCO_3^- secretion *in vitro* (5). Similar adaptation to acid base disorders has been described in toad bladder and in the frog skin (6, 7). These changes in transport were observed in the presence of identical *in vitro* pH, indicating that extracellular pH cannot account for the increased rate of transport observed *in vitro* in bladders from turtle with acid base disorders.

H^+ and HCO_3^- secretion by the turtle urinary bladder is mediated by the carbonic anhydrase-rich cells (8, 9). These cells are divided in two subtypes: the α cells are thought to be responsible for H^+ secretion, whereas the β cells are responsible for HCO_3^- secretion (10). We have previously shown that fluorescence microscopy with different probes is capable of identifying these cell subtypes (3, 4). Metabolic and respiratory acidoses were associated with increased numbers of total carbonic anhydrase cells and this increase in the number of carbonic anhydrase cells was accounted for by an increase in the number of α subtype, without change in the number of β subtype (3, 4). In the present investigation, we studied the mechanism of adaptation to metabolic alkalosis by the turtle urinary bladder by quantifying the number of carbonic anhydrase cells and their subtypes by fluorescence microscopy.

Materials and Methods

Turtles used were *Pseudemys scripta elegans* obtained from Mogul, Oshkosh, WI. Metabolic alkalosis was induced by feeding the turtles 20 ml of 1 *M* $NaHCO_3$ daily by gavage for 3 days. To confirm a state of metabolic alkalosis, arterial blood was collected by cardiac puncture for measurement of pH and pCO_2 immediately after the turtle was killed. Urine was also collected from the bladder for measurement of pH and pCO_2. The blood bicarbonate concentration was calculated from the pH and pCO_2.

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In vitro H^+ or HCO_3^- secretion was determined in bladders from control turtles and turtles with metabolic alkalosis. Hemibladders were mounted on Ussing chambers with an exposed area of 8 cm^2, and hydrogen ion secretion was determined by the reverse short circuit current technique and HCO_3^- secretion by the pH stat technique as described previously (1). In experiments where acidification was measured, the two sides of the bladder were bathed in bicarbonate-free Ringer's solution containing the following composition (mmol/liter): 114.4 sodium chloride, 3.5 potassium chloride, 2.0 dibasic sodium phosphate, 5 dextrose, 0.5 magnesium chloride, and 1.8 calcium chloride (pH 7.4, osmolality 249 mOsm/kg H_2O). Bladders that failed to maintain an initial resistance of greater than 400Ω × cm^2 were discarded. Thereafter, ouabain (1×10^{-4} *M*) was added to the serosal solution and the bladders were short circuited throughout the experiment except for brief periods during which the potential difference was measured. One hour was allowed for stabilization of the reverse short circuit current.

In the experiments where the rate of HCO_3^- secretion into the musocal fluid was measured, the pH stat technique was used, with 0.01 *N* hydrochloric acid as the titrant. In these experiments, two Ringer solutions with identical chloride concentrations and osmolalities were used. The first Ringer solution was the control solution and contained the following (mmol/liter): 13.3 sodium sulfate, 54.4 sodium chloride, 40 choline chloride, 3.5 potassium chloride, 0.5 magnesium chloride, 2.0 dibasic sodium phosphate, 1.8 calcium chloride, and 5 dextrose (pH 7.4; osmolality, 236 mOsmol/kg H_2O). The second bicarbonate Ringer solution contained 20 sodium bicarbonate, 54.4 sodium chloride, 40 choline chloride, 3.5 potassium chloride, 0.5 magnesium chloride, 20 dibasic sodium phosphate, 1.8 calcium chloride, and 5 dextrose (pH 8; osmolality, 236 mOsm/kg H_2O). In these experiments, the bladders were mounted and bathed with the control Ringer solution. The pH of the serosal fluid was maintained at 8.0, whereas the pH of the mucosal fluid was lowered below 5.0 with hydrochloric acid. The pH of the musoca at which net hydrogen ion secretion became zero was determined (end point). This value was determined when the pH of the mucosal remained stable, and no net movement of either acid or alkali was detectable (<0.075 μmol/hr). Usually, 3–4 hr were required for the end point value to be achieved. After the end point was achieved, the serosal solution was replaced with bicarbonate Ringer solution, and the rate of 0.01 *N* hydrochloric acid required to maintain the pH at the end point level was measured. Only rates of bicarbonate secretion that remained stable for at least 30 min were analyzed.

Electrogenic HCO_3^- secretion was measured as described by Durham and his colleages (11, 12). The bladder was bathed at both surfaces by a choline sulfate Ringer's solution containing the following (mmol/liter): 20 choline bicarbonate, 40 choline sulfate, 0.8 $MgSO_4$, 2 K_2SO_4, 0.53 K_2HPO_4, 0.22 KH_2PO_4, 2 $CaSO_4$, 11 glucose; osmolality after addition of 220 mOsm sucrose, pH 7.2. The mucosal and serosal solutions were gassed with 95% O_2 and 5% CO_2. Quabain (5×10^{-4} *M*) was added to the serosal solution. Under these conditions control bladders and bladders from alkalotic turtles have a negative current (in reference to the serosal solution) which is thought to be due to H^+ secretion. Addition of 5×10^{-4} *M* serosal 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid results in reversal of the current (serosal positive) and this current is a measurement of electrogenic HCO_3^- secretion (13).

Determination of the Percentage of Carbonic Anhydrase Cells by Fluorescence Microscopy. Parallel experiments using fluorescence microscopy were performed in the corresponding hemibladders to determine any changes in the number of acid and HCO_3^--secreting cells with metabolic alkalosis. Table I shows a summary of the stains used in this study to quantify the total number of carbonic anhydrase cells, the β subtype of carbonic anhydrase cells, and the total number of cells. The whole hemibladders were divided into four to 6, six 1-cm^2 sections, mounted with mucosal side up, across pipette tips (diameter 0.6 cm), and then placed in PO_4 turtle Ringer's solution on ice until used. Care was taken so that the mucosal surface was handled as little as possible and that staining was done within 5 min of excision of the tissue. In unstained bladders no autofluorescence was observed. Fluorescence stains were dissolved in turtle Ringer's solution (pH 7.40 unless stated otherwise). 6-Carboxyfluorescein diacetate (35 μ*M* for 30 sec, pH 6.20) is a permeant fluorescent precursor which is trapped after hydrolysis by carbonic anhydrase enzyme activity to form the more polar 6-carboxyfluorescein (4, 14). Mucosal staining with 6-carboxyfluorescein for 30 sec results in the appearance of bright green cells with very little background uptake. The NBD-taurine (100 μ*M* for 2 hr) is a fluorescent probe shown previously to bind to the anion exchanger of red blood cells (15). For this stain hemibladders were mounted on Ussing chambers and the stain was added only to the mucosal side to prevent staining of the anion exchanger on the serosal surface of α-subtype cells. After staining, the tissue was washed three times in fresh turtle Ringer's solution and the tissue was subsequently mounted on pipette tips for fluorescence microscopy.

Microscopy was carried out with a Nikon Diaphot microscope equipped with epifluorescence capability. 6-Carboxyfluorescein and NBD-taurine were observed with excitation at 460–485 nm and emission at 515 nm. Ethidium bromide was observed by excitation at 535–550 nm and emission at 580 nm. For each stain,

Table I. Fluorescence Microscopy

Cell identified/quantified[a]	Stain[b]	Mechanism	Appearance	Excitation/ emission (nm)
All carbonic anhydrase cells	6-carboxyfluorescein	pH-sensitive, accumulates in presence of carbonic anhydrase enzyme	Bright green cytoplasm	460–485/515
HCO_3^--secreting cells[b] (β-carbonic anhydrase)	NBD-taurine	Substrate for mucosal anion exchange	Bright green cytoplasm	460–485/515
All cells (granular + carbonic anhydrase) (used to standardize number of carbonic anhydrase cells as % of total cells/field)	Ethidium bromide	Binds DNA	Bright red nuclei	535–550/480

[a] H^+-secreting cells (α-carbonic anhydrase): determined from the difference between number of all carbonic anhydrase cells and the number of β-carbonic anhydrase cells.
[b] Random photographs were taken after staining either with 6-carboxyfluorescein or NBD-taurine, then restained with ethidium bromide and photographed again.

four to eight random fields were photographed by means of a 35-mm camera with high speed (ASA = 400) Ektachrome film. Each tissue was subsequently standardized to the total numbers of cells per field by restaining with the nuclear stain ethidium bromide (100 μM for 60 min) and again taking four random photographs. 6-Carboxyfluorescein- and NBD-taurine-positive cell counts were later scored on randomized slides by four persons having no knowledge of the experimental condition to which the turtles had been exposed. The four individuals scoring the slides were instructed as how to score the slides, and the cells were considered positive if they appeared bright and distinctly different than the background cells. For each slide scored there was good agreement among the four persons.

Results are presented as mean ± SE. The *t* test for unpaired data was used to analyze the data with significance level of 0.05 or less. Blood pH, pCO_2, and HCO_3^- levels are expressed at 36°C.

Results

Table II shows that administration of $NaHCO_3$ *in vivo* was associated with a significant increase in blood and urine pH, indicating that the turtles developed metabolic alkalosis. Plasma HCO_3^- concentration was significantly higher in alkalotic turtles than in controls (65.0 ± 10.0 mEq/liter vs 30.0 ± 0.5 mEq/liter, $P < 0.001$). Plasma pCO_2 was also higher, reflecting the respiratory compensation to metabolic alkalosis. Table II also shows the rate of HCO_3^- secretion and H^+ secretion *in vitro* in bladders removed from alkalotic and control turtles. HCO_3^- secretion was determined in one set of hemibladders while the remaining hemibladders were used to measure the rate of H^+ secretion. The *in vitro* conditions used to determine the rates of HCO_3^- or H^+ secretion were identical for both control and alkalotic bladders. It can be seen that bladders removed from alkalotic turtles had increased rates of HCO_3^- secretion *in vitro* as compared with those of control bladders. Electrogenic HCO_3^- secretion was also increased in the alkalotic bladders as compared with that of controls (5.2 ± 1.3 μA vs 0.7 ± 0.8 μA, $P < 0.05$). This increase in HCO_3^- secretion *in vitro* was observed despite solutions of the same pH on both sides. In contrast, H^+ secretion was not different between bladders from control and alkalotic turtles, indicating that metabolic alkalosis induces a selective increase in HCO_3^- secretion.

Table II. *In Vivo* and *In Vitro* Acid Base Parameter in Control and Alkalotic Turtles

Group[a]	*In vivo*		*In vitro*	
	Blood pH	Urine pH	HCO_3^- secretion (μmol/hr)	H^+ secretion (μA)
Control	7.44 ± 0.02	6.36 ± 0.17	0.73 ± 0.05	35.0 ± 5.0
P	<0.005	<0.005	<0.005	NS
Alkalotic	7.57 ± 0.03	7.17 ± 0.15	1.23 ± 0.15	38.0 ± 5.0

[a] n = 6–13.

Figure 1 shows the staining with 6-carboxyfluorescein of urinary bladder from a control turtle (Fig. 1A) and from a turtle with metabolic alkalosis (Fig. 1B). The number of cells positive for 6-carboxyfluorescein (shown as bright cells) was increased in the bladder from the alkalotic turtle. Figure 2 shows the staining of the same bladder with the fluorescent probe NBD-taurine. Again, the bladder from the alkalotic turtle (Fig. 2B) had an increased number of NBD-taurine-positive cells (bright cells) as compared with those of control bladder (Fig. 2A). Figure 3 summarizes the results of the fluorescence microscopic experiments performed in bladders from six control and six alkalotic turtles. The total number of carbonic anhydrase cells

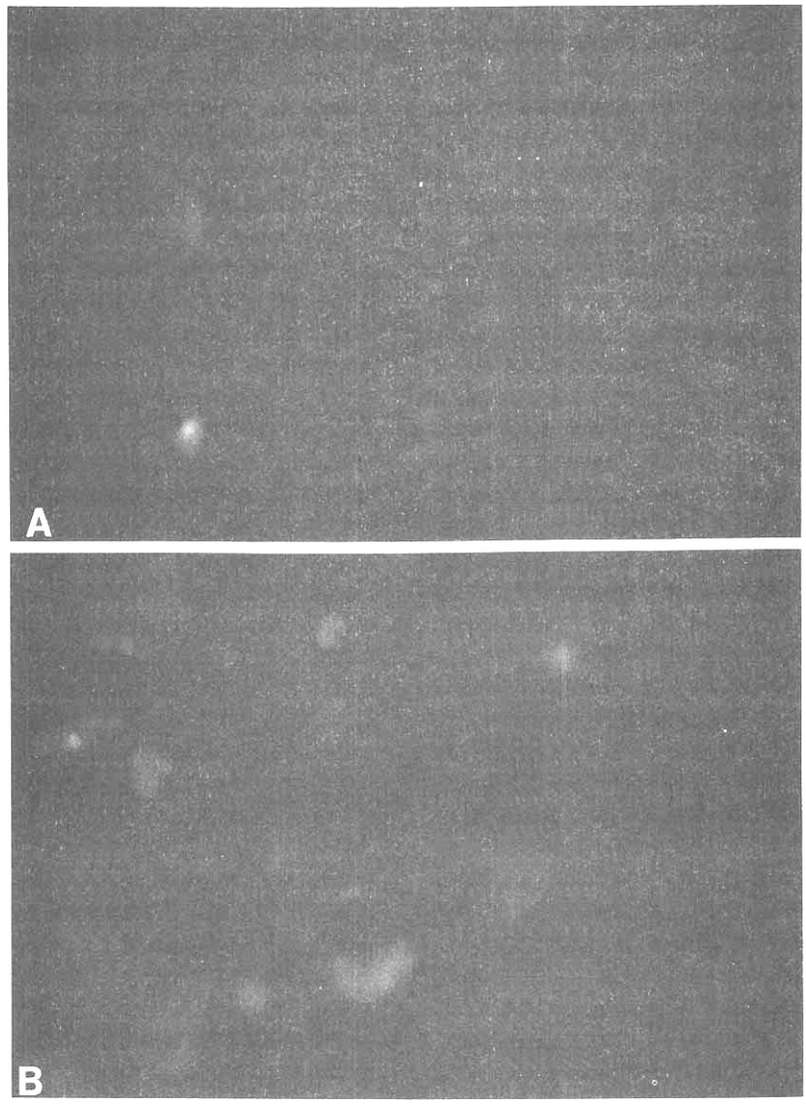

Figure 1. Appearance of turtle bladders from control (A) and alkalotic (B) turtles stained with 6-carboxyfluorescein (35 μM for 30 sec) under fluorescence microscopy (excitation 460–485 nm, emission at 515 nm; original magnification ×400).

was determined from the number of cells positive for 6-carboxyfluorescein. The NBD-taurine-positive cells were assumed to be the HCO_3^--secreting cells (β cells) and the difference between the two was assumed to be the α cells. Figure 3 shows that metabolic alkalosis is associated with an increased number of carbonic anhydrase-positive cells as compared with that of controls ($6.5 \pm 0.7\%$ vs $4.6 \pm 0.3\%$, $P < 0.01$) and this increase is accounted for by an increase in number of β cells ($4.9 \pm 0.5\%$ vs $3.0 \pm 0.3\%$, $P < 0.01$) without a change in the number of α cells ($1.8 \pm 0.7\%$ vs $1.6 \pm 0.3\%$, not significant).

Discussion

This study was aimed at identifying the mechanism responsible for adaptation to metabolic alkalosis by the turtle urinary bladder. Our results show that urinary bladders from turtles with metabolic alkalosis have increased rates of HCO_3^- secretion *in vitro* without a change in H^+ secretion. The increased rate of HCO_3^- secretion was observed *in vitro* in the presence of identical extracellular pH, indicating that pH is not responsible for the increased secretion of HCO_3^-. These findings are similar to those observed in bladders from turtles and toads with metabolic acidosis and in bladders from turtles with respiratory acidosis in that these bladders show increased rates of H^+ secretion *in vitro* without change in HCO_3^- secretion (3, 4, 6). These results indicate that bladders "remember" the metabolic environment from which they originated.

It has been well established that carbonic anhydrase-rich cells are responsible for H^+ and HCO_3^- secretion in turtle bladder epithelium, with the α subtype being involved in H^+ secretion while the β subtype is thought to be responsible for HCO_3^- secretion (9, 10). Adaptation to metabolic and respiratory acidosis is

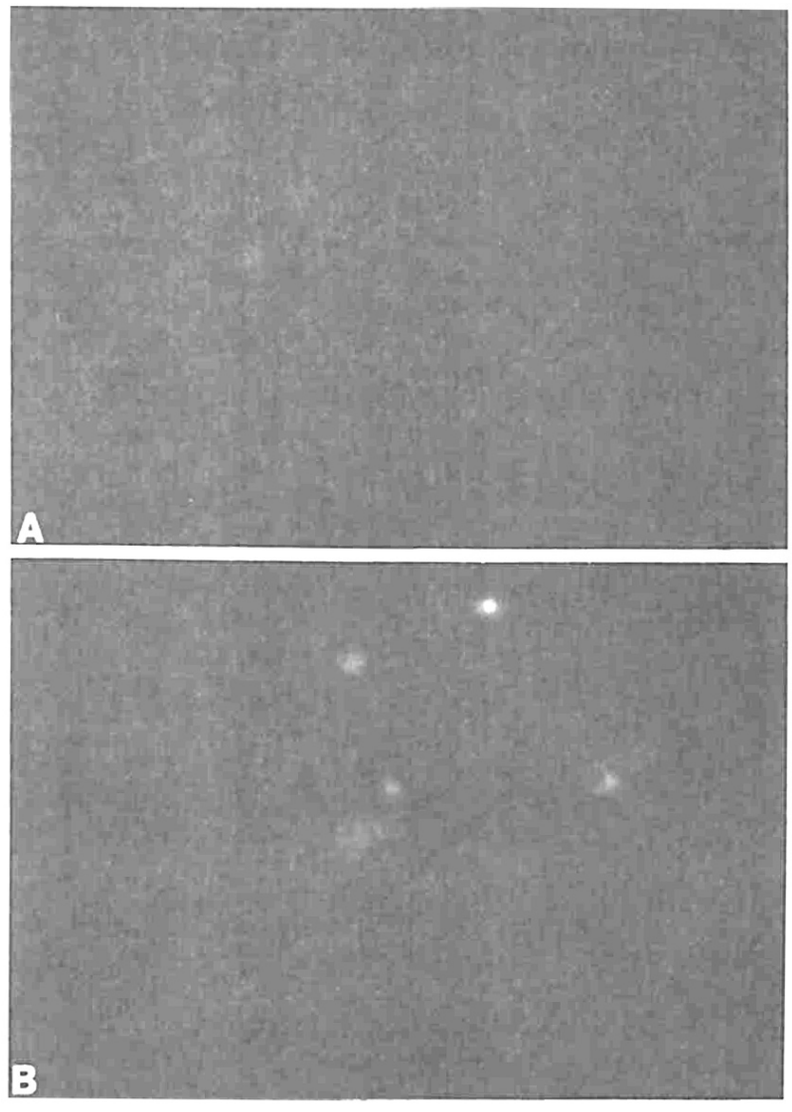

Figure 2. Appearance of turtle bladders from control (A) and alkalotic (B) turtles stained with NBD-taurine (100 μM for 2 hr) under fluorescence microscopy (excitation 460–485 nm, emission at 515 nm; original magnification ×400).

associated with increased numbers of carbonic anhydrase-rich cells and this increase is thought to mediate, at least in part, the adaptation to these disorders (3, 4). We reasoned that metabolic alkalosis should be associated with increased number of the β subtype of carbonic anhydrase-rich cells. We quantified the total number of carbonic anhydrase-rich cells with the fluorescent probe 6-carboxyfluorescein (3, 4, 14). This probe stains the carbonic anhydrase-rich cells as evidenced by the fact that the carbonic anhydrase inhibitor acetazolamide totally blocks the staining with 6-carboxyfluorescein (3, 14). In previous studied, we have utilized different fluorescent probes, 6-carboxyfluorescein, rhodamine-123, acridine orange, and 3-3′-diethyloxacarbocyanimine iodine to quantify the carbonic anhydrase cells. The rationale for the use of these probes is discussed in detail in our previous studies (3, 4) and it suffices to say that double labeling experiments indicated that these probes identified the same cell type and disclosed a comparable increase in the number of cells in response to acidosis (3, 4).

In this study, we chose 6-carboxyfluorescein to quantify the carbonic anhydrase cells because this probe is simple to use and gave very consistent and reproducible results. Since, in previous studies, the results obtained with 6-carboxyfluorscein were similar to those obtained with other probes, we did not feel it necessary to use other probes to quantify the carbonic anhydrase cells. An additional point that needs to be discussed is the number of cells identified by 6-carboxyfluorescein. The number of carbonic anhydrase cells determined by electron microscopy ranges from 5 to 20% (16). Staining with 6-carboxyfluorescein revealed a lower number of cells while other fluorescent probes disclosed a higher number of positive cells. Graber *et al.* (14), using 4-methylumbelifelrylacetate and 6-carboxyfluorescein,

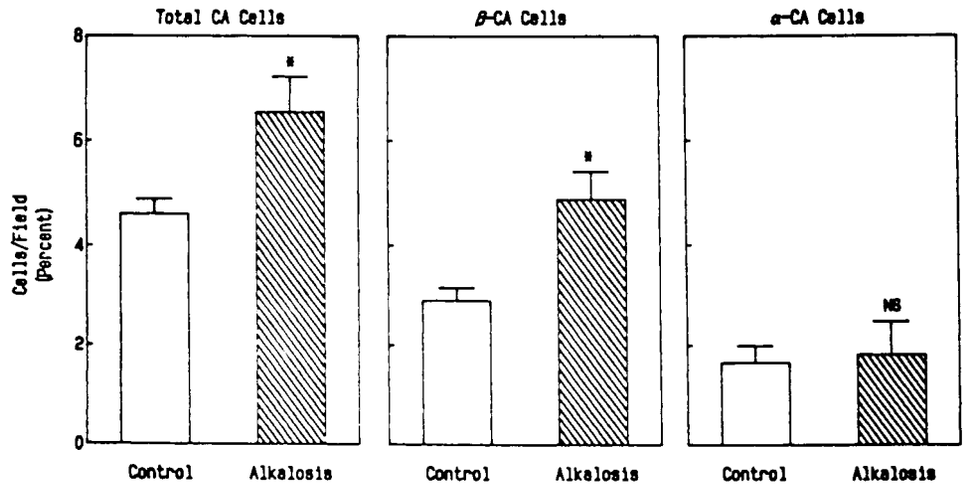

Figure 3. Percentage of 6-carboxyfluorescein-positive cells (left panel, CA cells), NBD-positive cells (middle panel, β cells), and the difference between 6-carboxyfluorescein-positive cells and NBD-positive cells (right panel, α cells).* $P < 0.01$. The mean was calculated from individual differences in each experiment. Cells are expressed as percentage of the total number of cells determined in the same tissue with the nuclear stain ethidium bromide. CA, carbonic anhydrase.

found 5–25% of these cells to be carbonic anhydrase cells. The lower number found in our study may have been due to the fact that the photographs were taken in a random fashion without looking through the microscope to find the positive cells. The objective of our study was not to identify the true frequency of carbonic anhydrase which is best done by electron microscopy, but rather to disclose whether alkalosis was associated with an increased number of carbonic anhydrase cells.

NBD-taurine binds to the anion exchanger and was proposed to be a probe for the β cells when added to the mucosal solution where it would bind to the apical HCO_3^--Cl exchanger from the β cell (3, 15). We have previously shown that NBD-taurine added in the mucosal solution inhibits HCO_3^- secretion without affecting H^+ secretion (3). The selective inhibition of HCO_3^- secretion suggests a specific interaction with β cells. Palmisano *et al.* (17) indicated that addition of NBD-taurine to the apical surface resulted in staining only when added at high concentration (500 μM). These investigators concluded that at this high concentration NBD-taurine stained 22% of the cells and, based on studies using carboxyfluorescein staining, they concluded that NBD-taurine was endocytosed by the α-carbonic anhydrase cells and therefore stained these cells. In contrast, in studies performed in the same tissue, we observed consistent staining with 100 μM NBD-taurine in the apical solution and the number of cells identified with this stain was approximately 50% of the total number of cells identified with 6-carboxyfluorescein staining. Despite prolonged incubation with NBD-taurine, we observed staining of a much lower proportion of cells than did Palmisano *et al.* (17), suggesting that under our experimental condition NBD may not be endocytosed by the α cells but rather labels the β cells. It should be pointed out, however, that our studies suggest but do not unequivocally demonstrate that NBD-taurine labels the β cells.

Notwithstanding this difficulty it is clear, however, that metabolic alkalosis was associated with an increased number of 6-carboxyfluorescein-positive cells. If staining with NBD-taurine indeed labels the β cells, then our studies suggest that this increase was entirely accounted for by an increase in the number of β cells without change in the number of α cells. It should be pointed out that the number of β cells may have been undercounted in the control state, and higher count obtained after alkalosis because the β cells could have a more alkaline cell pH and thus appear brighter. If this hypothesis is correct, then there may be little or no true recruitment of β cells. This hypothesis cannot be excluded since intracellular pH was not measured in the present experiments. Our results are different than those reported in a recent study (18) which showed that in the rabbit cortical collecting tubule an acidic stimulus increases the number of cells responsible for acid secretion with a corresponding reduction in the number of cells responsible for HCO_3^- secretion, leaving the total number of cells unchanged. This suggests that in rabbit cortical collecting tubule adaptation to acid base disorder would involve interconversion of cell types whereas in the turtle bladder the increased number of carbonic anhydrase cells would be mediated by cell recruitment. Thus, our results showing an increased number of carbonic anhydrase cells in bladders from turtles with metabolic alkalosis suggest that the adaptation to this disorder is mediated, at least in part, by recruitment of additional β cells, perhaps, from the basal layer to the surface of the epithelium (19) where these cells would effect increased HCO_3^- secretion. It should be emphasized, however, that the present studies, although showing a role for an increased number

of carbonic anhydrase cells, do not exclude a role of exocytosis, intracellular pH, alterations in H^+-ATPase activity, and hormonal changes in the adaptation to this disorder (4, 20–22).

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