Adaptation to Respiratory Acidosis in the Turtle Bladder (43123)

DANIEL KNIAZ AND JOSE A. L. ARRUDA
Section of Nephrology, University of Illinois College of Medicine at Chicago, Chicago, Illinois and West Side Veterans Administration Medical Center, Chicago, Illinois 60612

Abstract. **The effect of *in vivo* respiratory acidosis for 4 and 48 hr was examined in the turtle bladder by placing turtles in hypercapnic chambers. Blood pH was significantly lowered and pCO_2 was significantly elevated over control values both at 4 and 48 hr, while blood bicarbonate was only increased after 48 hr. *In vitro* rates for H^+ secretion determined by the reverse short-circuit current were significantly greater in bladders from 48 hr of respiratory acidosis than those of controls (27.3 ± 2.7 vs 20.6 ± 1.7 μA, $P < 0.05$). *In vitro* rates for HCO_3^- secretion determined by pH stat were not altered. Fluorescence microscopy was used to study cell morphology. The number of carbonic anhydrase cells (corrected for the total number of cells) as determined by four different fluorescence stains (6-carboxyfluorescein, rhodamine 123, acridine orange, and 3,3′-diethyloxacarbocyaninine iodide) was increased both after 4 and 48 hr of respiratory acidosis. However, the number of HCO_3^--secreting (β subtype) carbonic anhydrase cells, determined by a probe for the anion exchanger, NBD-taurine, was not increased. *In vitro* 1% CO_2 for 4 hr also resulted in an increase in H^+ secretion and in the number of 6-carboxyfluorescein-positive cells, both of which could be blocked with SITS pretreatment. We conclude that CO_2 changes the mucosal cells more toward the carbonic anhydrase phenotype, and that if NBD-taurine accurately identifies the β cells, that the adaptation produces or recruits more α-carbonic anhydrase cells.**
[P.S.E.B.M. 1990, Vol 195]

Respiratory acidosis results in increased renal acid secretion and generation of bicarbonate. The adaptation is thought to occur, at least in part, in the distal nephron but the mechanism responsible is still largely unknown (1, 2). The turtle bladder has been used as an analog of mammalian distal nephron and acidification by this membrane is enhanced by CO_2 (3–5).

The turtle bladder epithelium is composed of two major cell types (6, 7). The great majority are granular cells, postulated to be responsible for sodium transport (7). Most of the remaining cells, corresponding to the intercalated cells of the distal tubule and the mitochondria-rich cells of the toad bladder, are rich in carbonic anhydrase (8–10) and are postulated to be the source of acid and bicarbonate secretion (11, 12). Ultrastructural studies have defined two subpopulations of carbonic anhydrase cells based on apical membrane characteristics (12) and suggest that the one subtype (α) secretes hydrogen and the other subtype (β), with reversed polarity of its membrane elements, secretes bicarbonate.

Studies of the mechanisms on adaptation of this epithelium to chronic hypercapnia have been limited. Insertion of H^+ pumps into the apical membrane by exocytosis and a role for intracellular pH have been demonstrated in response to acute CO_2 stimulation and correlates very closely to the increased rate of acidification by this epithelium (13–16). Moreover, by freeze-fracture electron microscopy, the α-carbonic anhydrase-rich cells have been demonstrated to respond to a high CO_2 tension by change in apical membrane characteristics and increased surface area, and this has been proposed to be associated with the insertion of proton pumps and increased acid secretion (12). An increased number of proton pumps per cell could be responsible for the adaptation to respiratory acidosis in the chronic state as well. Alternatively, there could be an increase in the number of α-carbonic anhydrase cells. In support of this hypothesis is the demonstration that in chronic metabolic acidosis, there is an increased number of mitochondrial rich cells both in the toad

Received March 7, 1990. [P.S.E.B.M. 1990, Vol 195]
Accepted May 10, 1990.

0037-9727/90/1951-0084$2.00/0
Copyright © 1990 by the Society for Experimental Biology and Medicine

bladder and in the turtle bladder (17, 18). In this study we report on the adaptive changes in transport activity of the turtle bladder in response to acute (4 hr) and chronic (48 hr) respiratory acidosis and on the changes in turtle bladder cell morphology as determined by fluorescence microscopic probes which allow us to examine numerous experimental conditions.

Materials and Methods

Turtles used were *Pseudemys scripta elegans* obtained from Mogul, Oshkosh, WI. When a state of acute or chronic respiratory acidosis was desired, it was induced by placing the turtles in hypercapnic chambers (10% CO_2 in compressed air) for 4 or 48 hr, respectively. To confirm a state of respiratory acidosis, 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, pCO_2, and ammonia. The blood bicarbonate concentration was calculated from the pH and pCO_2. Ammonia was measured according to the Berthelot method.

In vitro H^+ or HCO_3^- secretion was determined in bladders from control turtles and turtles with 4 hr or 48 hr of hypercapnia. Hemibladders were mounted on Ussing chambers 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 (19). In experiments where acidification was measured, the two sides of the bladder were bathed in bicarbonate-free Ringer's solution containing the following composition (in millimoles/liter): sodium chloride, 114.4; potassium chloride, 3.5; dibasic sodium phosphate, 2.0; dextrose, 5; magnesium chloride, 0.5; and calcium chloride, 1.8 (pH 7.4; osmolality 249 mosmol/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 and then the effect of 1% serosal CO_2 was determined over 30 min.

In the experiments where the rate of alkali entry into the mucosa 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 (in mmol/liter): sodium sulfate, 13.3; sodium chloride, 54.4; choline chloride, 40; potassium chloride, 3.5; magnesium chloride, 0.5; dibasic sodium phosphate, 2.0; calcium chloride, 1.8; and dextrose, 5 (pH 7.4; osmolality, 236 mosmol/kg H_2O). The second bicarbonate Ringer solution contained sodium bicarbonate, 20; sodium chloride, 54.4; choline chloride, 40; potassium chloride, 3.5; magnesium chloride, 0.5; dibasic sodium phosphate, 2.0; calcium chloride, 1.8; and dextrose, 5 (pH 8; osmolality, 236 mosmol/kg H_2O). In these experiments, the bladders were mounted and bathed with the control Ringer solution. The pH of the serosal solution was maintained at 8, whereas the pH of the mucosal solution was lowered below 5 with hydrochloric acid. The pH of the mucosal solution at which net hydrogen ion secretion became zero was determined (end point). This value was determined when the pH of the mucosal solution remained stable, at which point the net movement of either acid or alkali was not detectable (<0.075 μmol/hr) (19). 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's 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.

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-secreting cells with respiratory acidosis. The hemibladders were divided into four to six 1-cm^2 sections, mounted (mucosa up) unstripped across pipette tips (diameter, 0.6 cm), and then placed in PO_4 turtle Ringer's 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 (pH 7.40 unless stated otherwise) and added to the mucosal surface. Acetazolamide (10^{-3} *M*) dissolved in turtle Ringer's was added to the mucosal surface 60 min before staining with 6-carboxyfluorescein diacetate.

Table I summarizes the fluorescence probes used to quantify turtle bladder cells. Acridine orange staining was done as described by Graber *et al.* (8) by exposing the bladders to 100 μ*M* for 1 min. Acridine orange is a fluorescent probe that partitions across membranes according to pH gradient. As the concentration of the dye changes, its emission wavelength shifts. It accumulates in acid areas and appears red, whereas it appears green in more alkaline areas. Rhodamine 123 (20 μg/ml for 90 min) is a fluorescent mitochondrial dye which results in bright red staining of the "mitochondria-rich" cells (8, 20). 6-Carboxyfluorescein diacetate (35 μ*M* for 30 sec, pH 6.20) is a fluorescent stain in which trapping of the dye is dependent on the presence of active carbonic anhydrase enzyme activity (8, 18). Mucosal staining with 6-carboxyfluorescein for 30 sec results in the appearance of bright green cells with very little back-

Table I. Fluorescence Probes Used to Identify Acid- and Alkali-Secreting Turtle Bladder Cells

Stain	Probable mechanism	Appearance	Cells identified
Acridine orange	pH Sensitive, accumulates in acid areas	Reddish-orange vesicles	Carbonic anhydrase cells
3,3′-$DiOC_2$	Potential-sensitive, binds endoplasmic reticulum and mitochondria	Bright red cytoplasm	Carbonic anhydrase cells
Rhodamine 123	Mitochondrial dye	Red cytoplasm	"Mitochondrial rich" cells
6-CF	pH-sensitive, accumulates by hydrolysis with carbonic anhydrase	Bright green cytoplasm	Carbonic anhydrase cells
NBD-taurine	Substrate for anion exchange	Bright green cytoplasm	Mucosal β-subtype carbonic anhydrase cells
Ethidium bromide	Binds DNA	Bright red nuclei	Total cell counts

ground uptake. The potential sensitive cationic dye, 3,3′-diethyloxacarbocyaninine iodide (3,3′-$DiOC_2$), has also been used to identify the mitochondrial rich cells of the turtle bladder (8). This dye, like other permeant cationic fluorescent probes, selectively accumulates in mitochondria of living cells and is dependent on high transmembrane potential maintained by functional mitochondria (20, 21). Mucosal staining with 3,3′-$DiOC_2$ (25 μg/ml for 4 min) also resulted in the appearance of brightly stained cytoplasm in a minority of the total cell population. NBD-taurine (100 μM for 2 hr) is a fluorescent probe shown previously to bind to the anion exchanger of red cells (22). For this stain hemibladders were mounted in Ussing chambers and the stain was added only to the mucosal side to prevent staining of the anion exchanger on the serosal surface of the α cells. After staining, the tissue was washed three times in fresh turtle Ringer's and the tissue subsequently mounted on pipette tips for fluorescence microscopy.

Microscopy was carried out with a Nikon Diaphot microscope equipped with epifluorescence capability. Acridine orange, 6-carboxyfluorescein, and NBD-taurine were observed under excitation set at 460–485 nm and emission at 515 nm. Rhodamine 123 and ethidium bromide were observed by excitation set at 535–550 nm and emission at 580 nm. For each stain, 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 (23, 24) (100 μM for 60 min) and again taking four random photographs. Acid-secreting cells and total cell counts were later scored on randomized slides by four persons having no knowledge of the experimental conditions to which the turtles had been exposed.

***In Vitro* Stimulation with CO_2.** The effect of *in vitro* hypercapnia on acid secretion and cell morphology was investigated. Bladders were hemisected and 1-cm peripheral portions taken from each hemibladder for determining baseline-positive fluorescence staining with 6-carboxyfluorescein or NBD-taurine. The bladders were then mounted on lucite chambers and baseline rates for acid secretion determined as described previously. Bladders were then bathed in Ringer's gassed with 100% compressed air which was maintained for 4 hr (controls). The other hemibladder was gassed with compressed air followed by 1% serosal CO_2 for 4 hr. After this period of time, the bladders were removed from the chamber and peripheral sections were again obtained for staining with 6-carboxyfluorescein or NBD-taurine as described previously.

Results

Validation of Fluorescence Probe Specificity. Figure 1 shows the typical appearance of the bladder epithelium from control (A) and hypercapnic (B) turtles after staining with the nuclear fluorescence probe ethidium bromide. All cells were easily identified and counted by the four observers with less than 10% variability. The average number of cells per photograph determined by this technique was not different between control and hypercapnic turtle bladders. The vast number of turtle bladder cells (80–90%) were granular in appearance and were not believed to play a significant role in acid or alkali secretion. Carbonic anhydrase-rich cells made up 10–20% of the total cell population and were made up of two subpopulations or cell types. The α type is believed to be responsible for acid secretion and would be the expected population of cells to be increased under hypercapnic conditions. The β type of carbonic anhydrase cells is believed to play a role in bicarbonate secretion and likewise would not be expected to be increased under these conditions. To test this hypothesis, we stained the mucosa of turtle bladders from control and hypercapnic turtles with several distinct probes for the carbonic anhydrase cells and with the fluorescence probe specific for the anion exchanger, NBD-taurine (Table 1). Figure 2A demonstrates the typical appearance of a control bladder stained with 6-

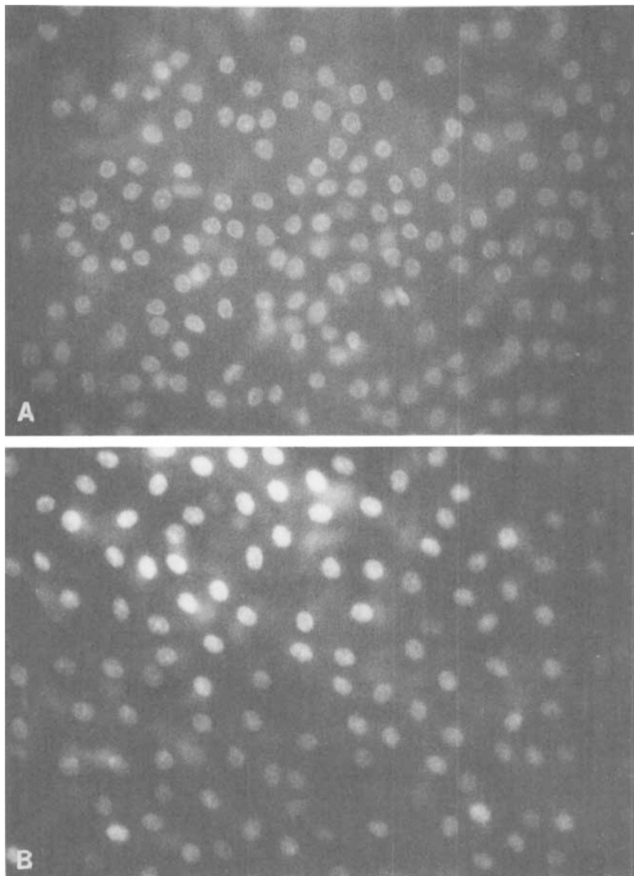


Figure 1. Appearance of turtle bladders from control (A) and hypercapnic (B) turtles stained with ethidium bromide (100 μM for 60 min) under fluorescence microscopy (excitation 535–550 nm, emission 580 nm; original magnification ×400). The cells out of focus cells in A are not mucosal cells.

carboxyfluorescein. The brightly green-stained cells were consistently seen in only a minority of the total cells per field as would be expected for carbonic anhydrase cells. Moreover, in paired bladders which were preincubated for 1 hr with the carbonic anhydrase enzyme inhibitor, acetazolamide (10^{-3} M), before staining with 6-carboxyfluorescein, this characteristic staining was completely abolished (Fig. 2B).

Rhodamine 123 is a cationic fluorescent probe at physiologic pH which has been used for localization of mitochondria in living cells (20, 21). The staining by rhodamine 123 is thought to be related to the high negative electric potential across mitochondrial membranes and therefore would be expected to stain the mitochondrial rich cells (carbonic anhydrase cells) with special intensity (20). Bladders that were double stained with 6-carboxyfluorescein and rhodamine and then observed under different excitation and emission wavelengths revealed a close correlation in the identification of positively stained cells by these two probes (Fig. 2C and D). The identification of positive cells with rhodamine 123 was the least distinctive, because with very short incubation cells stained only faintly whereas with longer incubation time all cells demonstrated some positive fluorescence. Thus, only the brighter cells were counted as "positively stained," and this correlated closely with positive cells seen with 6-carboxyfluorescein. Acridine orange was used to quantify carbonic anhydrase cells because of its property of concentrating in acidic vesicles and appearing bright reddish orange. These areas of red fluorescence can be rapidly dissipated by NH_4Cl solution at pH 8.0 (18), indicating they represent accumulation of acridine orange in intracellular vesicles of low pH. Figure 4E is a representative photograph of the acridine orange-stained bladder cells from a control bladder. The pattern of heavy concentration of red-orange pockets was seen only in a small percentage of cells, as would be expected if only carbonic anhydrase cells were identified. Figure 4G illustrates the typical appearance of a control turtle bladder stained with the potential sensitive catonic dye, 3,3′-$DiOC_2$. The positively stained bright red cells again were only a minority of the total cells per field and resembled cells stained by 6-carboxyfluorescein or rhodamine, suggesting that a group of cells, probably the carbonic anhydrase cells, were preferentially stained.

To quantify the subset of carbonic anhydrase cells that secrete bicarbonate (β type), we stained the mucosal surface with the fluorescence probe for the anion exchanger, NBD-taurine. As shown in Table II, addition of mucosal NBD-taurine (10^{-4} M) for 2 hr resulted in a significant inhibition of the rate of bicarbonate secretion. The effect of NBD-taurine was specific, as acid secretion was not affected. Apical staining with NBD-taurine for 2 hr resulted in the appearance of faintly green cells by fluorescence microscopy. When paired tissues from the same bladder were stained with NBD-taurine, NBD-taurine-positive fluorescence was present in roughly 50% of cells of the total number of cells stained with 6-carboxyfluorescein. This supports the claim that only a subset of carbonic anhydrase cells is being identified. The difference between the total number of carbonic anhydrase cells, (6-carboxyfluorescein-positive cells, 5.7 ± 0.3%) and the number of β cells (NBD-taurine-positive cells, 3.4 ± 0.1%) allows an indirect means of estimating the number of H^+ secreting cells (α-carbonic anhydrase cells, 2.2 ± 0.3%).

Effect of *In Vivo* Respiratory Acidosis. Turtles placed in hypercapnic chambers for 4 hr or 48 hr had a significant elevation in their blood pCO_2 and a decrease in blood pH as compared with control turtles, although the decreased pH no longer reached significance by 48 hr because of a larger standard deviation (Table III). As can be seen in Table III, the blood bicarbonate concentration was increased above that of control turtles in the chronic state (35.3 ± 2.0 vs 25.7 ± 1.4 mEq/liter, $P < 0.0005$). Furthermore, only the chronically hypercapnic turtles had a significantly lower urine pH ($P < 0.05$) and higher urine ammonia con-

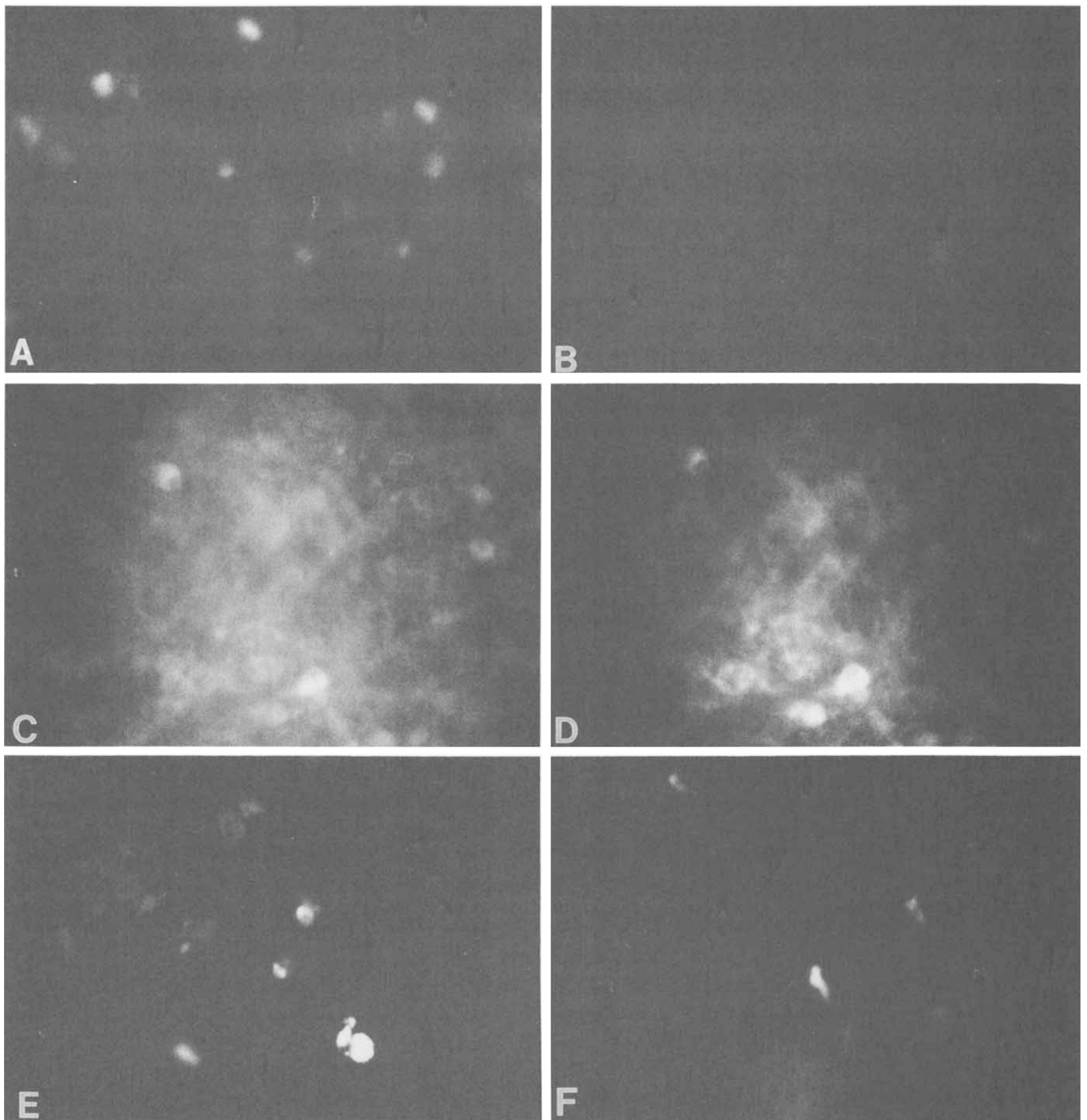


Figure 2. Appearance of turtle bladders from control turtles under fluorescence microscopy (original magnification ×400). 6-Carboxyfluorescein alone (A) or 6-carboxyfluorescein after preincubation with acetazolamide (10^{-3} *M*) for 1 hr (B). Same field photographed after coincubation with 6-carboxyfluorescein and rhodamine 123, excitation 460–485 nm (C) and excitation 535–550 nm (D). Paired tissue from same bladder stained with 6-carboxyfluorescein (E) or NBD-taurine (F).

centration ($P < 0.05$) as compared with controls (Table III). Thus, a significant degree of metabolic compensation to hypercapnia is evident only in the chronic state.

Bladders from turtles exposed to 4 hr of hypercapnia tended to have an increase in the rate of hydrogen secretion *in vitro* over that of controls, but this did not achieve statistical significance. Figure 3 shows that bladders from chronically hypercapnic turtles had significantly higher rates of hydrogen secretion (both at baseline and after stimulation with 1% serosal CO_2 for 30 min) than did bladders from control turtles, despite similar *in vitro* conditions. HCO_3^- secretion, however, was not different between bladders from control and hypercapnic turtles (1.5 ± 0.5 vs 1.1 ± 0.2 μmole/hr), indicating a selective effect of respiratory acidosis on H^+ secretion.

Table II. Effect of Mucosal Addition of NBD-Taurine on Bicarbonate and Proton Secretion in Two Different Sets of Hemibladders[a]

Secretion	Basal	P	NBD-Taurine (10^{-4} M)
HCO_3^- [b] (μmol/hr)	0.79 ± 0.08	<0.001	0.41 ± 0.09
Acid[c] (μA)	27.9 ± 3.9	NS	27.7 ± 4.0

[a] Values are mean ± SE. Acid secretion and HCO_3^- secretion was determined in two different sets of hemibladders from normal turtles.
[b] $n = 7$.
[c] $n = 12$.

Quantification of the Acid-Secreting Cells with Acridine Orange, Rhodamine 123, 3,3′-DiOC₂, and 6-Carboxyfluorescein Diacetate. Figure 4 is a representative photograph of control (left) and hypercapnic (right) turtle bladders stained with 6-carboxyfluorescein (4A and B), rhodamine 123 (4C and D), acridine orange (4E and F), and 3,3′-$DiOC_2$ (4G and H). Hypercapnia resulted in an increase in the number of positively stained cells with each of these independent probes. The mean number of 6-carboxyfluorescein-positive cells increased from 5 ± 1% in control bladders to 13 ± 3% after 4 hr of hypercapnia and to 15 ± 2% after 48 hr of hypercapnia (Table IV). The results with 6-carboxyfluorescein paralleled the results with the other three probes; however, the percentage of positive cells identified by this probe was lower both in hypercapnic and controls. Rhodamine 123-positive staining increased from 10 ± 1% in control turtle bladders to 17 ± 4% in bladders from 4 hr of hypercapnia ($P < 0.05$) and to 20 ± 4% in bladders from 48 hr of hypercapnia ($P < 0.025$) (Table IV). Figure 4E and F represent photographs after staining with acridine orange. In hypercapnic turtles (right), there is a dramatic increase in the apparent number of cells containing red granules. The presence of acridine orange-positive cells appeared in clusters in different areas of the bladder. Table IV shows that there was a significant increase in the percentage of acridine orange-positive cells from 11 ± 3% in control bladders to 20 ± 3% in bladders after 48 hr of *in vivo* hypercapnia ($P < 0.05$). The mean percentage after 4 hr of hypercapnia also increased to 15 ± 2%; however, this increase did not reach statistical significance.

Representative photographs of 3,3′-$DiOC_2$-stained bladders from a control and hypercapnic turtle are seen in Figure 4G and H, respectively. The appearance of bright red cytoplasm in a minority of cells was easily detectable under fluorescence microscopy utilizing the "green" filter block. As with the acridine orange-stained cells, 48 hr of hypercapnia resulted in a noticeable increase in the number of positively stained cells per field. From Table IV, the percentage of 3,3′-$DiOC_2$-positive cells increased from 11 ± 1% in control bladders to 18 ± 3% after 48 hr of hypercapnia ($P < 0.01$).

Finally, the percentage of NBD-taurine-positive cells was less than 5% under control conditions and was not different in bladders from chronic hypercapnic turtles (Table IV).

Effect of *In Vitro* CO_2 on Turtle Bladder Cell Morphology Determined by 6-Carboxyfluoescein and NBD-Taurine. There was a 2-fold increase in the rate of H^+ secretion *in vitro* over 4 hr of 1% serosal CO_2 stimulation from a baseline current of 17.0 ± 1.5 μA to 39.6 ± 5.0 μA ($P < 0.005$) (Fig. 5, left). There was no significant increase in H^+ secretion in control bladders bubbled with compressed air only (Fig. 5, left). The middle of Figure 5 shows the change in the number of 6-carboxyfluorescein-positive cells before and after gassing with 1% CO_2 or with 100% compressed air. Bladders stimulated with 1% CO_2 had a 2.5-fold increase from a baseline value of 5.0 ± 0.8% to 14.7 ± 2.6% ($P < 0.02$). Bladders exposed to 100% compressed air had a small but significant increase in 6-carboxyfluorescein-positive cells, from 6.9 ± 0.1 to 9.0 ± 1.2% ($P < 0.05$). However, this increment was significantly less than the increment in 6-carboxyfluorescein-positive cells with 1% CO_2 ($P < 0.01$). In contrast to the dramatic increase in 6-carboxyfluorescein-positive cells after 4 hr of *in vitro* 1% CO_2, neither 1% CO_2 nor air had an effect on the percentage of NBD-taurine-positive cells (Fig. 5, right).

Table III. Blood and Urine Data from Control and Hypercapnic Turtles[a]

	Control	4-Hr respiratory acidosis	48-Hr respiratory acidosis
Blood pH	7.38 ± 0.02	7.24 ± 0.01[b]	7.25 ± 0.18
Blood pCO_2 (mm Hg)	44.4 ± 1.0	70.9 ± 5.1[b]	82.5 ± 3.0[b]
Blood HCO_3^- (mEq/liter)	25.7 ± 1.4	29.3 ± 1.7	35.3 ± 2.0[b]
Urine pH	6.97 ± 0.08	6.57 ± 0.42	5.23 ± 1.25[c]
Urine NH_4^+ (mM)	1.6 ± 0.2	1.5 ± 0.3	2.9 ± 0.7[c]

[a] Values are mean ± SE, n = 6–15 observations.
[b] Significantly different from controls at $P < 0.001$.
[c] Significantly different from controls at $P < 0.05$.

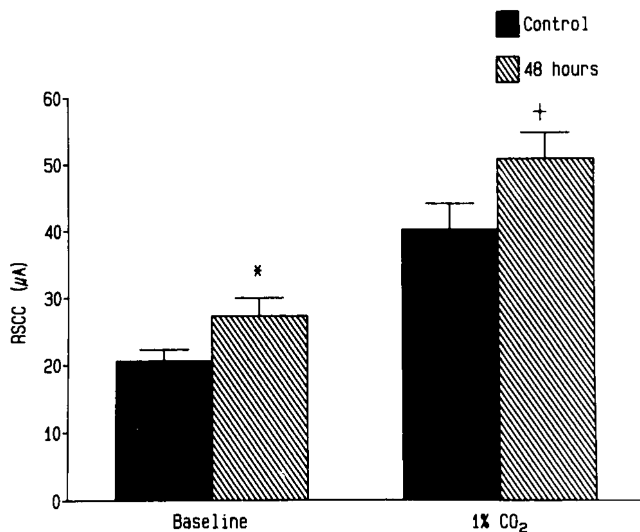


Figure 3. *In vitro* H^+ secretion determined by the reverse short-circuit current (RSCC) in control bladders and bladders from turtles after 48 hr of respiratory acidosis at baseline and after stimulation with 1% CO_2 for 30 min, $n = 10$–13 for each observation. Significantly different from controls at $P < 0.05$ (*) or $P < 0.01$ (+).

The effect of 1% CO_2 *in vitro* on H^+ secretion and the number of carbonic anhydrase cells was also studied in the presence of SITS (5×10^{-4} *M*). SITS reduced baseline H^+ secretion from 18.3 ± 3.1 to 7.3 ± 0.8 μA ($P < 0.01$). Furthermore, SITS completely blocked the increase in the number of 6-carboxyfluorescein-positive cells induced by 4 hr of 1% CO_2 (4.2 ± 0.5 vs 5.5 ± 1.2%).

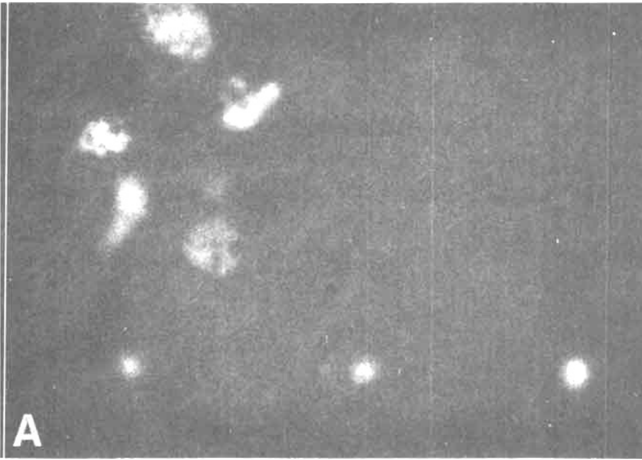
Discussion

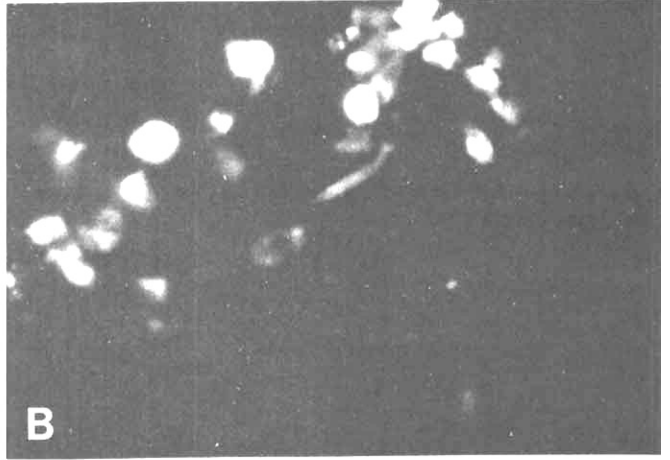
Our data using fluorescence probes support a role for an increase in α-carbonic anhydrase (CA) cells in the turtle bladder in the adaptation to acute and chronic hypercapnia. The use of fluorescence staining for identifying carbonic anhydrase cells in the turtle bladder allows for a rapid and easy means of assessing changes in the relative number of these cells under various experimental conditions. Numerous investigators have previously reported on the staining characteristics of CA cells under control conditions with 6-carboxyfluorescein, acridine orange, rhodamine 123, and potential sensitive dyes (8, 15, 18, 23). As a group these probes are believed to identify CA cells and there was a close correlation of the number of cells identified by these various probes with double staining (8, 15, 18). 6-Carboxyfluorescein (6-CF) diacetate is preferentially sequestered into CA cells because they are enriched with the carbonic anhydrase enzyme which has potent esterase activity (8). The absence of 6-CF staining after pretreatment with acetazolamide in our studies attests to the specificity of this probe (8, 18). That the number of 6-CF-positive cells increases with metabolic acidosis (18) or in response to 1% CO_2 in this study provides further support for specific staining of CA cells. Rhodamine 123 identifies CA cells because its staining is dependent on increased mitochondrial activity. In the present study, we have demonstrated a close correlation between 6-CF-positive and rhodamine-positive cells in double-labeling experiments and previously we have demonstrated parallel increases in the number of rhodamine-positive cells and 6-CF-positive cells under conditions of metabolic acidosis (18).

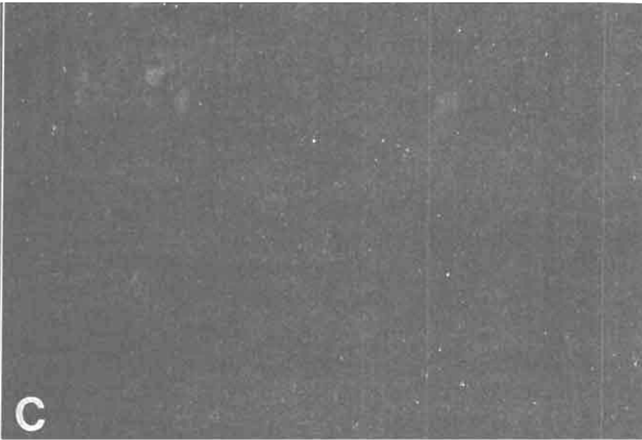
Perhaps the strongest evidence that the four probes used in this study identify CA cells is the striking consistency in the percentage of positive cells under control and hypercapnic conditions. Rhodamine, acridine orange, and 3,3′-$DiOC_2$ all stained close to 10% of the cells in control bladders and 20% in bladders from hypercapnic turtles. The number of CA-positive cells quantitated in control bladders using fluorescence microscopy in our laboratory was similar to that identified by scanning electron microscopy (9, 25). Under control conditions the number of 6-CF-positive cells identified was lower than that identified with the other three fluorescence probes, but the increase with hypercapnia was of the same order of magnitude. It is unclear why the numbers of cells identified by 6-CF staining were lower than with the other three probes but it may be related to the very short incubation used with 6-CF in order to prevent background fluorescence, to variability in carbonic anhydrase activity from one cell to another, or to the dependence of 6-CF staining on other factors in addition to cell number, such as membrane properties or cell surface area (26).

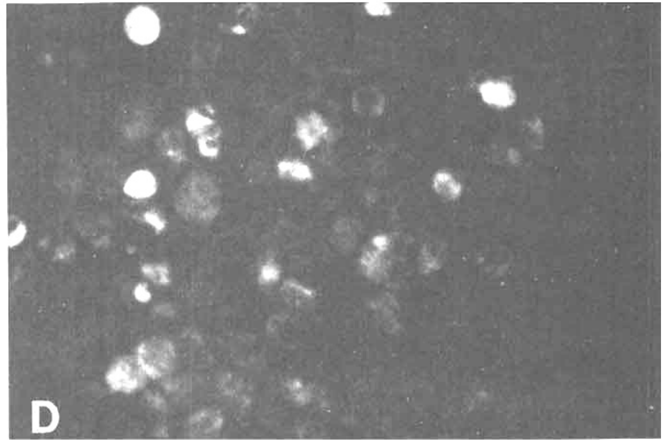
To quantify changes in the subpopulations of CA cells with hypercapnia, we stained the mucosal side of turtle bladders with a fluoroprobe for the chloride-bicarbonate exchanger, NBD-taurine. By allowing contact only with the mucosal side, we believe we can identify the bicarbonate secreting or β-CA cells. With this stain we consistently identified only a portion of the total number of cells stained with 6-CF or the other three CA-specific probes. Palmisano *et al.* (23) initially suggested that NBD staining could be used to identify the β-carbonic anhydrase cells in the turtle bladder. In a subsequent report (24), these investigators indicated that addition of NBD-taurine to the apical surface resulted in staining only when added at high concentration (500 μ*M*). These investigators concluded that at this high concentration NBD-taurine stained 22% of the cells and, based on studies utilizing carboxyfluorescein staining, they concluded that addition of NBD-

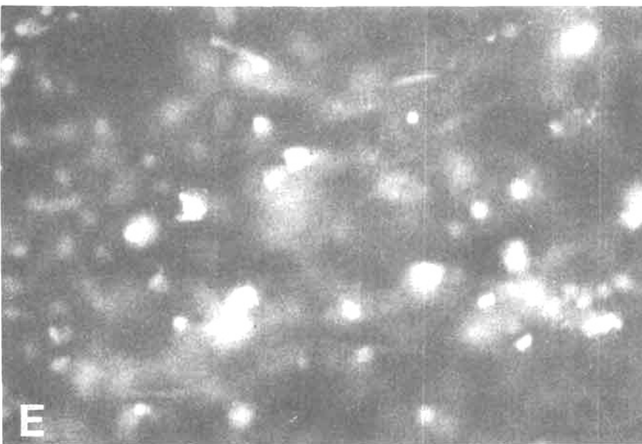
Figure 4. Appearance of turtle bladders from control (left) and hypercapnic (right) turtles after staining with: (A and B) 6-carboxyfluorescein, (C and D) rhodamine 123, (E and F) acridine orange, and (G and H) 3,3′-$DiOC_2$ (original magnification ×400).

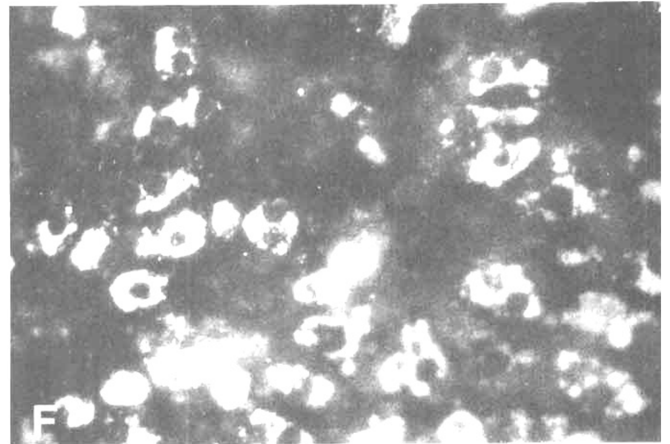

A

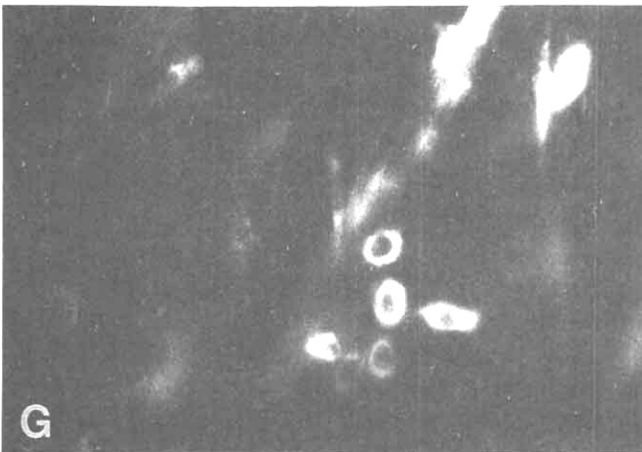

B


C


D


E


F


G

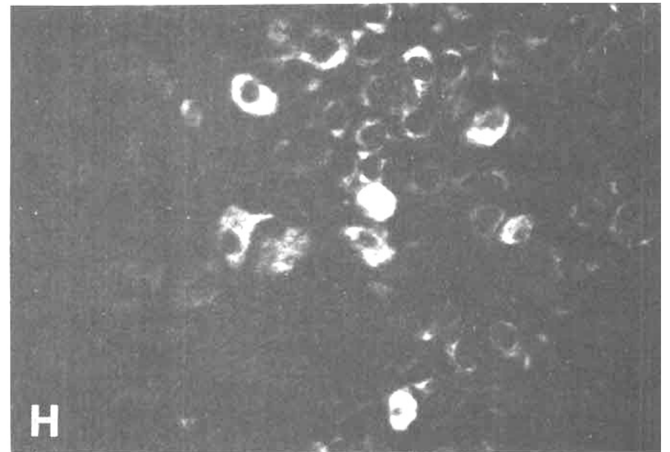

H

Table IV. Percentage of Positively Stained Cells Determined by Five Different Fluorescence Probes[a]

	Control	4-Hr respiratory acidosis	48-Hr respiratory acidosis
6-CF	5 ± 1	13 ± 3[b]	15 ± 2[c]
Rhodamine	10 ± 1	17 ± 4[d]	20 ± 4[d]
Acridine orange	11 ± 3	15 ± 2	20 ± 3[d]
$DiOC_2$	11 ± 1	12 ± 5	18 ± 1[c]
NBD-Taurine	3.5 ± 0.1	—	3.2 ± 0.2

[a] Values are mean ± SE, $n = 6$–11.
[b] Significantly different from controls at $P < 0.01$.
[c] Significantly different from controls at $P < 0.001$.
[d] Significantly different from controls at $P < 0.05$.

taurine stained mainly the α-carbonic anhydrase 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 carboxyfluorescein staining. In addition, this dose of NBD-taurine inhibited HCO_3^- secretion without affecting H^+ secretion, suggesting a selective interaction of NBD-taurine with a specific type of carbonic anhydrase cells. In this regard, it is also of interest that CO_2 stimulation of H^+ secretion increased the total number of carbonic anhydrase-rich cells without changing the number of NBD-taurine-positive cells, again suggesting that the NBD-taurine staining as performed in our studies labels the β-carbonic anhydrase cells. Thus, we believe that 6-CF, rhodamine, 3,3′-$DiOC_2$, and acridine orange stain the entire CA population, while NBD-taurine stains only the β-CA cells.

The difference between the number of total CA cells and β-CA cells can be used indirectly to estimate the number of α-CA cells. This conclusion is supported by the doubling of the number of positively stained cells by the CA fluoroprobes with *in vivo* hypercapnia without any increase in the number of cells stained with NBD-taurine. Moreover, as mentioned above, *in vitro* 1% CO_2 resulted in an increase in 6-CF-positive cells without a change in the number of NBD-taurine-positive cells. In these *in vitro* studies, a small but significant increase in 6-CF-positive cells was noted in control (air-treated) bladders as well. However, the increase with 1% CO_2 was significantly greater than that in controls. The reason for the increase in 6-CF-positive cells in air-treated bladders, despite no change in the rate of acid secretion, is unknown. Acetazolamide was unable to inhibit a small percentage of cell staining after 4 hr *in vitro* under these conditions. One explanation is that cells *in vitro* develop alterations in membrane permeability over time which lead to nonspecific cellular uptake of 6-CF. Alternatively, it is possible that during the 4-hr incubation *in vitro* with optimal oxygenation and substrate, the hemibladders recovered from initial trauma of mounting and therefore the 6-CF staining was more reliable.

The difference between the number of total CA cells and β-CA cells can be used indirectly to estimate the number of α-CA cells. This conclusion is supported by the doubling of the number of positively stained cells by the CA fluoroprobes with *in vivo* hypercapnia without any increase in the number of cells stained with NBD-taurine. Moreover, as mentioned above, *in vitro* 1% CO_2 resulted in an increase in 6-CF-positive cells without a change in the number of NBD-taurine-positive cells. In these *in vitro* studies, a small but significant increase in 6-CF-positive cells was noted in control (air-treated) bladders as well. However, the increase with 1% CO_2 was significantly greater than that in controls. The reason for the increase in 6-CF-positive cells in air-treated bladders, despite no change in the rate of acid secretion, is unknown. Acetazolamide was unable to inhibit a small percentage of cell staining after 4 hr *in vitro* under these conditions. One explanation is that cells *in vivo* develop alterations in membrane permeability over time which lead to nonspecific cellular uptake of 6-CF. Alternatively, it is possible that during the 4-hr incubation *in vitro* with optimal, oxygenation and substrate, the hemibladders recovered from initial trauma of mounting and therefore the 6-CF staining was more reliable.

The observations from these studies clearly indicate an increase in the number of carbonic anhydrase-rich cells with chronic *in vivo* hypercapnia. After 48 hr, bladders demonstrated a consistent doubling in the percentage of cells to approximately 20% per field. In addition, the intensity and the number of acid vesicles staining with acridine orange and the intensity of mitochondrial staining with rhodamine 123 were appreciably greater in bladders from chronically hypercapnic turtles, suggesting increased activity per cell as well. Thus, our findings are consistent with an adaptive process that includes both an increase in proton secretion per cell as well as an increase in total number of acid-secreting cells. These two processes are not necessarily mutually exclusive. Wheeler and Arruda (18) have previously reported data supporting parallel mechanisms of adaptation to chronic metabolic acidosis in the turtle bladder. In that study an increase in the number of CA cells as well as an increase in the number of acidic vesicles was detected by fluorescence staining techniques, suggesting a dual mode of adaptation. The toad urinary bladder also increased its rate of acid secretion under conditions of metabolic acidosis. Frazier (17) has demonstrated an increase in the number of mitochondria-rich cells after 48 hr of *in vivo* acid loading in association with an increased capacity for acid excretion. However, acid excretion was also in-

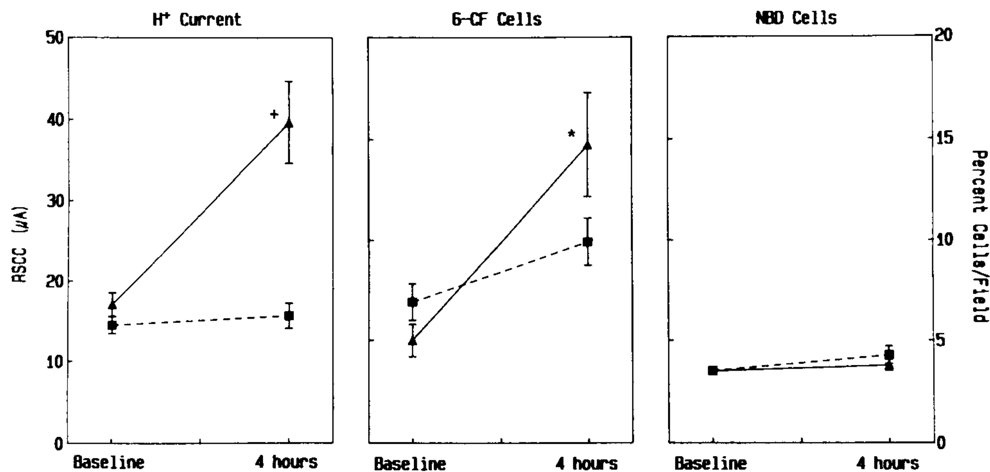


Figure 5. Effect of 4-hr *in vitro* 1% CO_2 (▲) versus air (■) on H^+ secretion (RSCC) and cell morphology (% 6-CF or NBD cells/field), $n = 6–7$ for each observation. Significantly different from controls at $P < 0.05$ (*) or $P < 0.025$ (+).

creased after only 24 hr of acid loading, without a demonstrable increase in mitochondria-rich cells.

We observed that after 4 hr *in vivo* hypercapnia, there was a tendency toward an increase in hydrogen ion secretion *in vitro* as well as an increase in the number of CA-positive cells, but because of increased variability in these data, the results did not achieve statistical significance for all of the fluorescence probes. However, the results of the *in vitro* 4 hr CO_2 stimulation clearly show an increase in hydrogen ion secretion and the number of 6-CF cells, thus supporting the contention that the *in vivo* results at 4 hr are similar to those observed after 48 hr. Frazier (17) hypothesized that this early adaptive process represented an increase in the hydrogen secretion rate of existing mitochondria-rich cells. The cellular process for the early adaptive increase in hydrogen secretion per cell hypothesized by Frazier is almost certainly mediated by the fusion of vesicles containing proton pumps to the luminal membrane (15). Ultrastructural studies of the turtle urinary bladder have equated this process with characteristic membrane changes identified by scanning electron microscopy as increased apical surface area and microplicae. Two hours of serosal SITS which inhibits acid secretion results in a decline in luminal microplicae without affecting cell number (25), while high CO_2 tension which increases acid secretion results in an increase in these membrane particles (12). Both of these studies examined the acute changes in cell morphology at a point when an increase or decrease in α-CA number would not be expected based on Frazier's findings in the toad bladder. Madsen and Tisher (27) have also studied the morphologic response of intercalated cells in rat medullary collecting duct to respiratory acidosis. The intercalated cells of the distal nephron are structurally analogous to the mitochondria-rich cells of the toad bladder and the carbonic anhydrase-rich cells of the turtle urinary bladder. Their studies, again using electron microscopy, revealed an increase in the surface density of the luminal membrane in these cells but did not detect a change in the number of intercalated cells. With acute *in vivo* hypercapnia the surface changes were proposed to represent insertion of hydrogen pumps into the luminal membrane.

Our fluorescence data for chronic hypercapnia are in full agreement with the conclusions of Frazier (17) and Wheeler and Arruda (18) in chronic metabolic acidosis, that an increase in the number of α-CA cells plays a prominent role in the adaptive process. In addition, we demonstrated an increase in the number of 6-CF-positive and rhodamine-positive cells with only 4 hr of *in vivo* hypercapnia as well as with 1% CO_2 *in vitro.* These conditions were not associated with any change in the number of NBD-taurine-positive cells. Moreover, the increase in 6-CF-positive cells *in vitro* is completely inhibited by serosal SITS which also prevents the increase in acid secretion induced by increased CO_2 tension. The change in the number of positively stained cells with acute hypercapnia may indicate a very rapid increase in the number of acid-secreting (α-CA) cells, which, so far, has gone undetected by ultrastructural techniques. In addition, changes in CA cell surface area have been induced *in vitro* under conditions associated with an increase (12, 27, 28) or decrease (25) in acid secretion, and therefore it is possible that an increase in membrane surface area may contribute to the increased number of cells staining with 6-CF seen at 4 hr of *in vivo* hypercapnia. This second conclusion would be in agreement with the acute studies of Madsen and Tisher (27) and Stetson and Steinmetz (12) with acute *in vivo* respiratory or metabolic acidosis and with *in vitro* CO_2 stimulation, respectively. If the number of α-CA cells is increased by hypercapnia after only 4 hr, the mechanism for this increase would likely be a transformation of existing cells preordained for acid secretion rather than a proliferation of α-cells through

mitosis. This recruitment of cells through transformation is not a reflection of the exocytosis but is an additional independent adaptive process. In support of this hypothesis is the recent evidence for the existence of a subluminal (basal layer) cell population in the turtle bladder with carbonic anhydrase activity (29). One can speculate that these cells may be dormant under basal conditions but be recruited to lumen under conditions associated with increased acid secretion. Our data are in partial agreement with a recent study in the rabbit cortical correcting tubule which has shown that an acidic environment induces an increase in the number of cells responsible for acid secretion with a corresponding reduction in the number of cells responsible for HCO_3^- secretion (30). This study did not, however, find an increase in the total number of intercalated cells as we did. The reason for this difference is not clear but it may be related to species difference.

The most reasonable conclusion of our studies is that CO_2 changes the mucosal cells more toward the CA phenotype and that if NBD-taurine accurately marks the β cells, that the adaptation produces or recruits more α-CA cells. These results do not exclude the possibility that increased surface area and exocytosis also play a role in the adaptation to chronic hypercapnia.

This research was supported by a Merit Review program from the Veterans Administration Central Office (J. A. L. A.), by Grant DK36-253 from the National Institute of Health (J. A. L. A.), and by a grant from the National Kidney Foundation of Illinois (D. K.).

The authors acknowledge the technical assistance of Roger B. Mola.

1. Bengele HH, Schwartz JH, McNamara ER, et al. Effect of buffer infusion during acute respiratory acidosis. Am J Physiol **250**:F115–F119, 1986.
2. Pollak A, Haynie GD, Hays RM, et al. Effects of chronic hypercapnia on electrolyte and acid-base equilibrium. 1. Adaptation. J Clin Invest **40**:1223–1237, 1961.
3. Brodsky WA, Schilb TP. Mechanisms of acidification in turtle bladder. Fed Proc **26**:1314–1321, 1967.
4. Schwartz JH. H^+ current response to CO_2 and carbonic anhydrase inhibition in turtle bladder. Am J Physiol **231**:565–572, 1976.
5. Steinmetz PR. Characteristics of hydrogen ion transport in the urinary bladder of water turtle. J Clin Invest **46**:1531–1540, 1967.
6. Rosen S. Localization of carbonic anhydrase activity in the turtle and toad urinary bladder mucosa. J Histochem Cytochem **20**:548–551, 1972.
7. Schwartz JH, Bethencourt D, Rosen S. Specialized function of carbonic anhydrase-rich and granular cells of turtle bladder. Am J Physiol **242**:F627–F633, 1982.
8. Graber M, Dixon TE, Coachman D, Herring K, Ruenes A, Gardener T, Rastoriza-Munoz E. Fluorescence identifies an alkaline cell in turtle bladder. Am J Physiol **250**:F159–F168, 1986.
9. Rosen S. The turtle bladder in morphological studies under varying conditions of fixation. Exp Mol Pathol **12**:286–296, 1970.
10. Schwartz JH, Rosen S, Steinmetz PR. Carbonic anhydrase function and the epithelial organization of H^+ secretion in turtle urinary bladder. J Clin Invest **51**:2653–2662, 1972.
11. Stetson DL, Beauwens R, Palmisano J, Mitchell PP, Steinmetz PR. A double-membrane model for urinary bicarbonate secretion. Am J Physiol **249**:F546–F552, 1985.
12. Stetson DL, Steinmetz PR. α and β types of carbonic anhydrase-rich cells in the turtle bladder. Am J Physiol **249**:F553–F565, 1985.
13. Cannon C, Van Adelsgberg J, Kelly S, Al-Awqati Q. Carbon dioxide induced exocytic insertion of H^+ pumps in turtle bladder luminal membrane: role of cell pH and calcium. Nature Lond **313**:443–446, 1985.
14. Cohen LH, Steinmetz PR. Control of active proton transport in turtle urinary bladder by cell pH. J Gen Physiol **76**:381–393, 1980.
15. Gluck S, Cannon C, Al-Awqati Q. Exocytosis regulates urinary acidification in turtle bladder by rapid insertion of H^+ pumps into the luminal membrane. Proc Natl Acad Sci USA **79**:4327–4331, 1982.
16. Wheeler RP, Arruda JAL. Intracellular pH of the turtle bladder [Abstract]. Clin Res **30**:865, 1983.
17. Frazier L. Cellular changes in the toad urinary bladder in response to metabolic acidosis. J Membr Biol **40**:165–177, 1978.
18. Wheeler RR, Arruda JAL. Adaptation to metabolic acidosis by turtle urinary bladder. Am J Physiol **252**:F256–F266, 1987.
19. Arruda JAL, Dytko G, Mola R, Kurtzman NA. The mechanism of lithium induced distal and renal tubular acidosis: studies in the turtle bladder. Kidney Int **17**:196–204, 1980.
20. Johnson LV, Walsh ML, Chen LB. Localization of mitochondria in living cells with rhodamine 123. Proc Natl Acad Sci USA **771**:990–994, 1980.
21. Johnson LV, Walsh ML, Bockus BJ, Chen LB. Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. J Cell Biol **88**:526–535, 1981.
22. Macara IG, Cantley LC. Mechanism of anion exchange across the red cell membrane by band 3: Interactions between stilbene disulfonate and NAP-taurine binding sites. Biochemistry **20**:5659–5701, 1981.
23. Palmisano J, Mitchell P, Steinmetz PR. Fluorescence microscopy of carbonic anhydrase cells of the β type in turtle bladder [Abstract]. Kidney Int **31**:413, 1987.
24. Palmisano J, Mitchell PP, Steinmetz PR. NBD-taurine uptake by α-type carbonic anhydrase cells of turtle bladder. Am J Physiol **257**:F1015–1020, 1980.
25. Husted RF, Mueller AL, Kessel RG, Steinmetz PR. Surface characteristics of carbonic anhydrase-rich cells in turtle urinary bladder. Kidney Int **19**:491–502, 1981.
26. Weiner DI, Ham LL. Use of the fluorescent dye BCECF to measure intracellular pH in cortical collecting tubule. Am J Physiol **256**:F957–F964, 1989.
27. Madsen KM, Tisher CC. Cellular response to acute respiratory acidosis in rat medullary collecting duct. Am J Physiol **245**:F670–F679, 1983.
28. Madsen KM, Tisher CC. Response of intercalated cells of rat outer medullary collecting duct to chronic metabolic acidosis. Lab Invest **51**:286–276, 1984.
29. Fritsche C, Kleinman JG, Bain JLW, Heinen RR, Riley DA. Improved carbonic anhydrase staining reveals a reactive subluminal cell and unique distribution of enzyme activity in the turtle bladder mitochondrial-rich cells [Abstract]. Kidney Int **33**:399, 1988.
30. Satlin LM, Schwartz GJ. Cellular remodeling of HCO_3^--secreting cells in rabbit renal collecting duct in response to an acidic environment. J Cell Biol **109**:1279–1288, 1989.