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Urinary Acidification and Carbonic Anhydrase Distribution in Bladders of Dominican and Colombian Toads*

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Summary. A comparative analysis of the acidifying capacity and distribution of carbonic anhydrase was made in isolated bladders of toads, *Bufo marinus*, obtained from the Dominican Republic (D) and Colombia (C). In the absence of exogenous CO_2 and HCO_3^-, mucosal acidification was observed in 10 of the 18 C toad bladders examined and in only one of 18 D toad bladders. In approximately half of the C toad bladders the rate of acidification was measurable by means of the pH stat method; the average rate in this group was 0.22 ± 0.03 μmoles/hour in the absence of exogenous CO_2 and 0.35 ± 0.04 μmoles/hour in the presence of 1% CO_2. Rates were not measurable in D toad bladders with or without CO_2. Staining of whole stretched bladders for carbonic anhydrase revealed a population of enzyme-containing mucosal cells accounting for 4.6% of the surface area in C toad bladders and 0.8% in D toad bladders. Electron histochemistry of both bladders identified the carbonic anhydrase-containing cells as mitochondria-rich cells.

Conflicting observations on the acidifying ability of the urinary bladder of the toad, *Bufo marinus*, have been attributed to the different geographical origin of the toads studied [16]. Whereas Leaf and coworkers [14, 15] using toads obtained in the Dominican Republic [4] found no evidence of acidification, Frazier and Vanatta [9] have recently reported urinary acidification in bladders of toads originating from Colombia.

The present investigation was stimulated by two observations. The first is by Ludens and Fanestil [16] who reported that acetazolamide inhibited

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acidification by the toad bladder. The second is an observation from this laboratory [19, 20] which demonstrated carbonic anhydrase activity by a histochemical method in a small population of mucosal cells in the bladder of the Dominican toad.

The studies to be described explore the relationship between acidifying ability and the distribution of carbonic anhydrase in Colombian and Dominican toad bladders.

Materials and Methods

Adult toads, *Bufo marinus*, from the Dominican Republic and Colombia were obtained from National Reagents, Bridgeport, Connecticut and Tarpon Zoo, Tarpon Springs, Florida, respectively. All toads were kept at room temperature on sand moistened with tap water, and fed weekly with ground chuck.

Studies of Acidification in vitro

Urinary bladders from pithed toads were removed with a minimum of handling, washed with Ringer's solution and mounted in Lucite chambers which provided an exposed area of 8 cm^2. The spontaneous electrical potential difference (PD) across the bladder and the short-circuit current (SCC) were monitored as previously described [27]. The two sides of the bladder were bathed with identical Ringer's solutions containing in millimoles per liter: NaCl 114.4; KCl 3.5; Na_2HPO_4 0.1; CaCl 0.9; dextrose 2.0. Osmolality ranged from 222 to 230 mOsm/Kg H_2O. The solutions in the mucosal (M) and serosal (S) compartments were stirred and oxygenated with air which had been passed through three 3-M KOH traps to remove all detectable CO_2. The rate of mucosal acidification was measured by the pH stat method [27]. All rates were expressed as μmoles/hr/8 cm^2 of membrane area ± standard error of the mean. Since rates of less than 0.10 μmoles/hr cannot accurately be measured, only acidification rates of more than 0.10 μmoles/hr were accepted as evidence for acidification.

The rate of acidification was also measured in experiments in which the gas phase contained 1% CO_2 in air (Medical Technical Gases, Inc., Medford, Mass). To avoid interference with the pH stat measurements in the mucosal compartment from the introduction of a volatile buffer system, exogenous CO_2 was added only to the serosal solution [26], while its pH was kept at 7.0 by titration with NaOH. After addition of CO_2 to S, a transitory phase of nonsteady titration which lasted less than 30 min was observed in the bladders of both groups of toads. Since this phase represents CO_2 diffusion to M with secondary formation of HCO_3^- to match the new CO_2 concentration of the mucosal bulk solution, all reported measurements were obtained after a steady-state had been reached.

Histochemistry for Carbonic Anhydrase

Bladders were maximally stretched and mounted between two halves of a Lucite chamber so that the apical portion of the horn was the area exposed. They were washed with Ringer's solution, and fixed in situ with 3% glutaraldehyde buffered [23] with 0.17 M cacodylate for 30 to 60 min. The whole bladder was then divided into portions,

picked up on Millipore filters, and briefly stored in petri dishes until the histochemical reaction was carried out. Sections of 10-μ thickness of fixed and unfixed contracted bladders were also examined. A modified Hansson technique [12, 19, 20] was used to demonstrate carbonic anhydrase activity. For each group of bladders studied, a simultaneous incubation was carried out with bladder segments in the presence of 1×10^{-5} M acetazolamide. After the procedure the tissue was dehydrated and mounted for light microscopy.

Although the specificity of this technique for the demonstration of carbonic anhydrase has been questioned [18], considerable evidence has accumulated in its support [12, 22]. The staining is inhibited by low concentrations of acetazolamide, but not by an analogue without carbonic anhydrase inhibitory activity [12, 19, 22]. The presence of carbonic anhydrase by the histochemical method correlates well with its demonstration by biochemical methods [12, 22]. In tissues with low enzyme content as determined biochemically the histochemical technique has revealed activity in a minority of cells, which may account for the low overall enzyme content [21, 25].

As reviewed in a recent analysis of the distribution of carbonic anhydrase in turtle urinary bladder the absence of staining does not necessarily exclude the presence of low concentrations of enzyme [25]. As in all histochemical techniques, factors of enzyme diffusion and inactivation may play a part in the distribution of the precipitate. The similarity of staining patterns in fixed and unfixed tissues, however, suggests that the staining patterns presented in this study reflect closely the distribution of the enzyme in the epithelium [12, 19].

For electron-microscopic observations, whole bladders were washed after the completion of the histochemical staining reaction. The tissues were then directly dehydrated, and embedded in Epon 812 [17]. Since osmium tetroxide solubilizes the precipitate, postfixation could not be used. Survey sections were cut at 1 μ to locate the stained cells by light microscopy. Blocks containing these cells were trimmed further and ultrathin sections were examined after staining with uranyl acetate [29] in a Philips EM 200 electron-microscope.

Results

Acidification of Mucosal Medium

The acidifying ability was examined in 18 bladders of Colombian toads (C) and in 18 bladders of Dominican toads (D) in the presence of the spontaneous potential difference (PD). Identical Ringer's solutions with an initial pH of 7.0 ± 0.1 were employed on both sides of the bladder. Mucosal acidification was observed in 10 C and in only one D bladder. The time required to reach the maximal pH gradient varied from 2 to 8 hr. Table 1 shows the maximal pH gradients generated in these 11 experiments. Since acidification was taking place in the direction of the electrical field, the pH gradients that could be expected from the effect of the PD alone are indicated in the last column. The discrepancy between the observed and expected gradients is consistent with an active mechanism of acidification.

Measurement of Acidification in the Absence of Electrochemical Gradients

In the absence of concentration gradients and with continuous nullification of the PD, the rate of acidification was determined with the pH

Table 1. Maximal pH gradient across the toad bladder in the presence of spontaneous potential difference (PD)

Toad bladder	pH		pH gradient	
	Mucosal	Serosal	Observed	Expected from PD
Colombian				
a	5.7	8.8	3.1	0.5
b	6.4	8.6	2.2	0.2
c	5.4	8.6	3.2	0.7
d	5.8	7.8	2.0	0.1
e	5.3	7.6	2.3	0.4
f	6.4	7.3	0.9	0.1
g	6.6	7.3	0.7	0.1
h	5.1	8.6	3.5	0.7
i	4.4	7.9	3.5	0.1
j	6.4	7.8	1.4	0.1
Dominican				
a	6.2	7.7	1.5	1.0

18 Colombian and 18 Dominican toad bladders were examined. Values are listed for those bladders in which acidification occurred.

stat method applied to the solution bathing the mucosal surface of the bladder. Rates were measurable in approximately half of the bladders from C toads. The average rate in 12 acidifying bladders was 0.22 ± 0.03 μmoles/hr. Acidification, on the other hand, was not measurable in any of the bladders from the 15 D toads studied.

Effect of Exogenous CO_2 on Acidification

From the foregoing experiments it is clear that acidification in the C toad bladder does not require the presence of exogenous CO_2 and HCO_3^- in the bulk solutions. Urinary acidification, however, has been found to be dependent on the availability of CO_2 in the epithelia that have been studied [25, 26]. Since in our conditions only metabolic CO_2 produced by the bladder was present, the dependence of acidification on the availability of CO_2 was examined by increasing the supply of CO_2 to the epithelium.

In 12 C bladders in which 1% exogenous CO_2 had been added to the gas phase of the serosal solution, the rate of acidification averaged 0.35 ± 0.04 μmoles/hr, a value significantly greater than the one obtained in the absence of CO_2 ($p < 0.05$). The addition of exogenous CO_2 had no effect in D bladders.

Histochemical Distribution of Carbonic Anhydrase

In sections of both fixed and unfixed toad bladders certain mucosal cells were stained intensely and completely, whereas the majority of cells remained unstained (Fig. 1). No staining was observed in the control tissues incubated in the presence of acetazolamide. To more accurately examine the relationship of stained cells to the mucosa, whole stretched bladder segments were analyzed for carbonic anhydrase activity. As previously described [20] D bladders revealed a small population of noncontiguous mucosal cells with carbonic anhydrase activity (Fig. 2). Since the reaction product was densest in the apical portion of these cells, their individual surface areas appeared sharply delineated as small polygonal forms (Fig. 2). Observations of C bladders strongly suggested that the cells demonstrating enzymatic activity were more numerous and had larger individual surface areas (Fig. 3). To quantitate these observations the bladders were photographed and the number of stained cells/mm^2 counted. The surface area of each stained cell was estimated by cutting out and weighing its image from the print. The results are given in Table 2. As shown in the last column of the table, the surface area accounted for by the stained cells is about six times greater in the C bladders than in the D bladders. In D bladders all stained cells had small areas of surface representation, whereas in C bladders the surface areas of individual cells varied widely, as shown in Fig. 4. Although these results do not provide a precise overall estimate of the carbonic anhydrase content of the mucosal layer, they do suggest that the enzyme is more

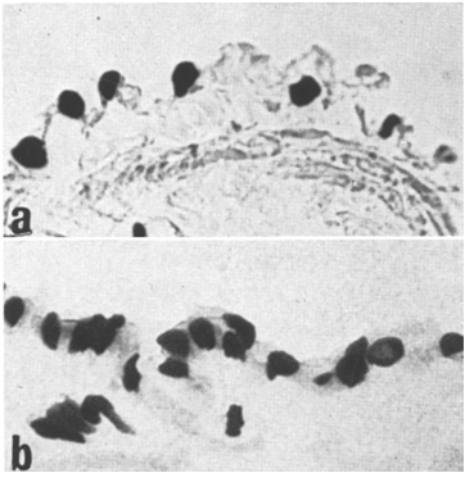

Fig. 1. Histochemical demonstration of carbonic anhydrase activity in sections (10 μ) of contracted Dominican (*a*) and Colombian (*b*) toad bladders. More mucosal cells show enzymatic activity in *b* than *a*. 315 ×, 315 ×

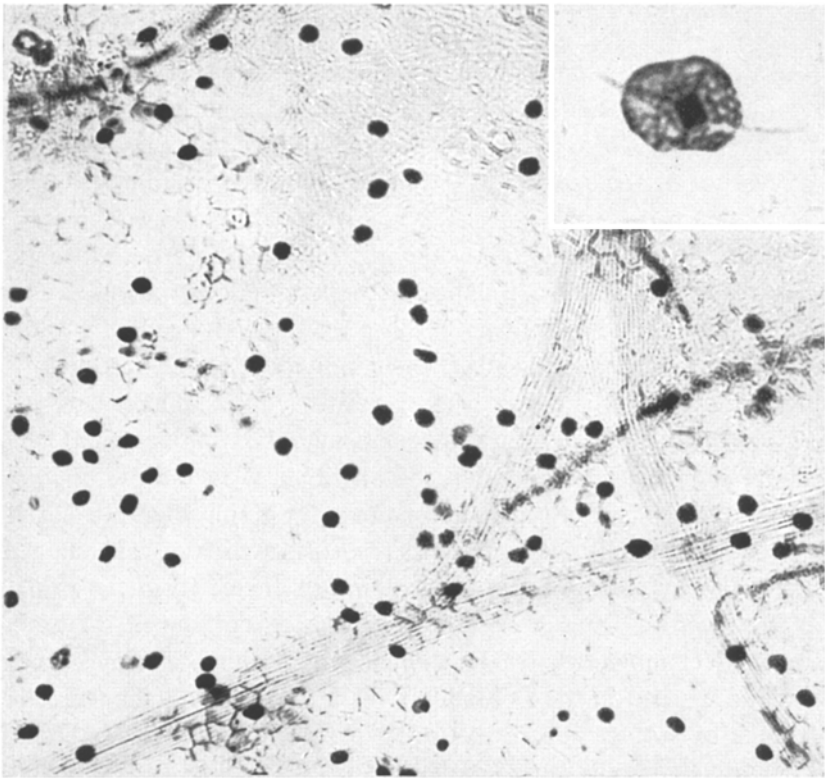

Fig. 2. Histochemical demonstration of carbonic anhydrase activity in whole stretched bladder of Dominican toad. A single cell is shown at high magnification in the inset. The black polygonal central area is the luminal surface of the cell and the lighter peripheral area is staining deeper in the cell. 125×, 800×

Table 2. Characteristics of mucosal cells stained for carbonic anhydrase in the toad bladder

	Number/mm^2 [a]	Individual surface [b] area (1.09×10^{-5} mm^2)	% Surface area covered
Colombian	560 ± 89 [c]	7.8 ± 1.1	4.6 ± 0.8
Dominican	224 ± 51	3.0 ± 0.3	0.8 ± 0.3
p	<0.01	<0.005	<0.005

[a] Number cells counted: 6,184 for C and 2,160 for D.
[b] Number of cells measured: 86 for C and 37 for D.
[c] All values are means ± SEM.

abundant in C bladders. This result is consistent with a preliminary report by Ziegler, Ludens and Fanestil [30] that biochemical assays of C bladders reveal more enzyme activity than D bladders.

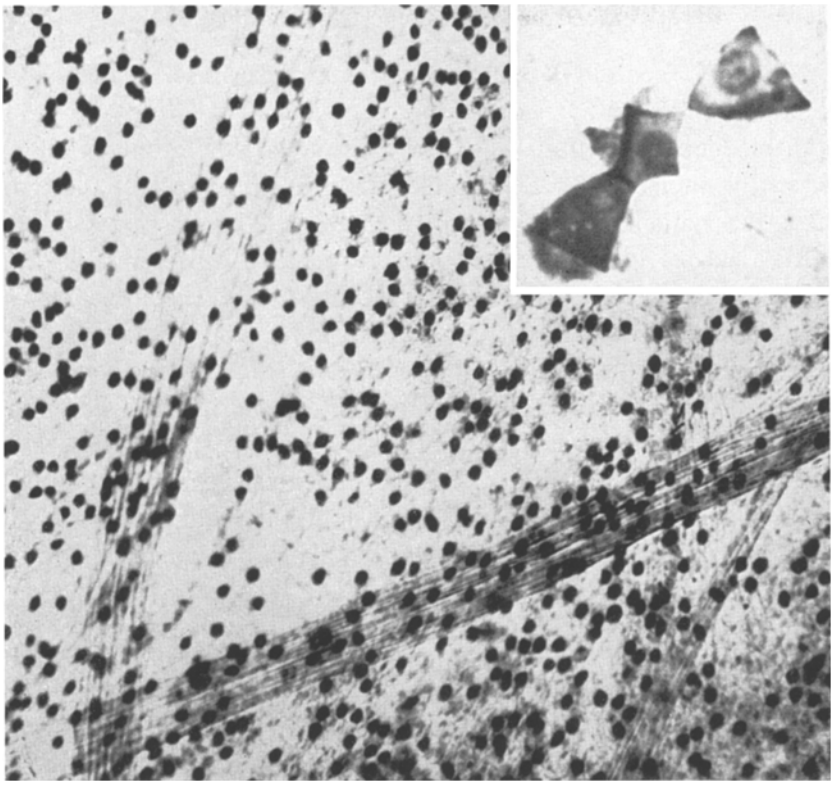

Fig. 3. Histochemical demonstration of carbonic anhydrase activity in whole stretched bladder of Colombian toad. More cells are stained in this preparation than in Fig. 2. Three cells are shown at high magnification in the inset. The luminal surface area of these cells is larger than that of the cell in Fig. 2. 125 ×, 800 ×

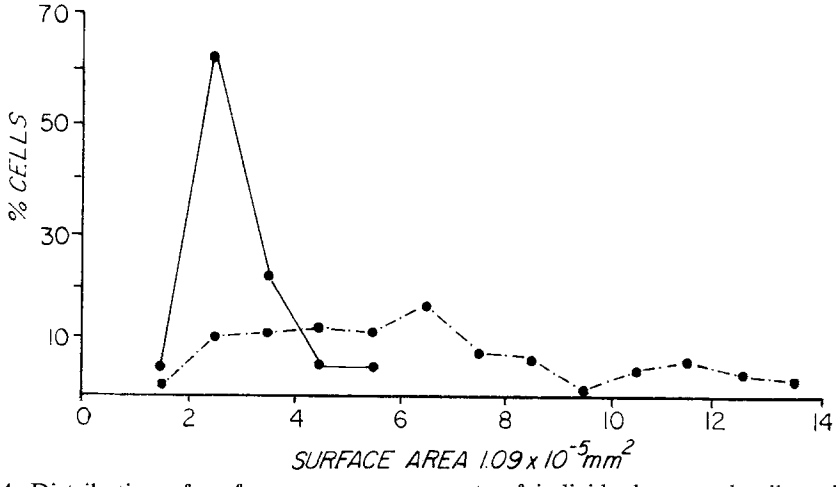

Fig. 4. Distribution of surface area measurements of individual mucosal cells stained for carbonic anhydrase activity in Dominican (solid line) and Colombian (dotted line) toad bladders (*see* Table 2)

Electron-Microscopic Observations

To identify the cells containing carbonic anhydrase, stained bladder segments were studied by electron-microscopy. Studies of the fine structure of both bladders reveal four cell types [3, 5, 7, 24]: the granular cell characterized by numerous apical granules and covering most of the luminal surface; the mitochondria-rich cell distinguished by its abundant mitochondria, lack of granules, small luminal contact, apical vesicles, and a prominent villous pattern; mucous and basal cells. Analysis of whole stained bladders of both toads revealed that the reaction product is present in the cytoplasm of the mitochondria-rich cells (Fig. 5). All the 15 stained cells examined in D bladders were of the mitochondria-rich type. None of the other cells seen, about 150 of the three other types, contained the precipitate. In C bladders, similarly, the 11 stained cells examined were mitochondria-rich cells, whereas about 100 others were free of precipitate. These observations do not reflect the greater abundance of stained cells in the C

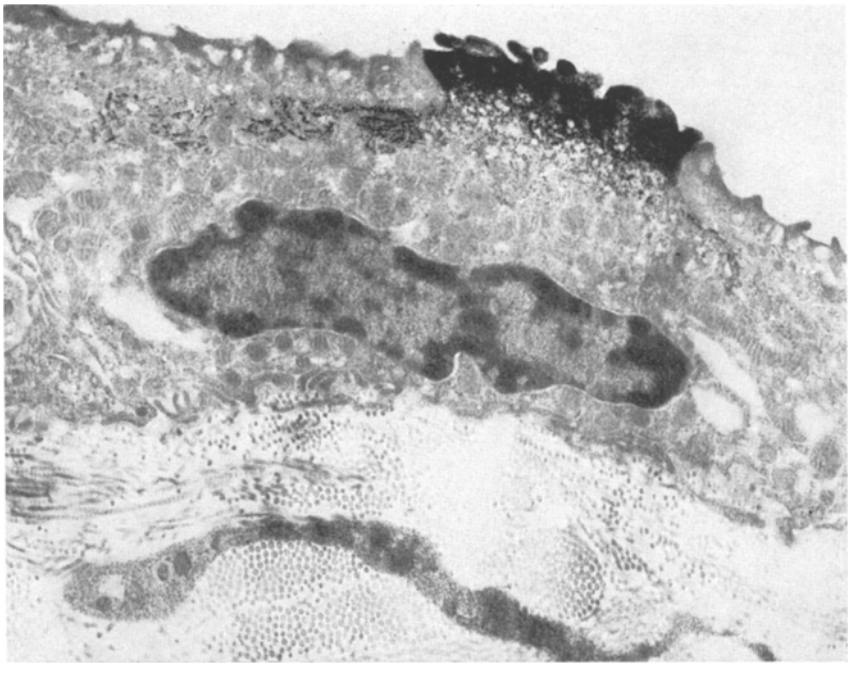

Fig. 5. Electron histochemistry of a carbonic anhydrase-containing cell of a Colombian toad bladder. The reaction product is densest in the apical area of this flask-shaped mitochondria-rich cell. Note that the adjacent cells are unstained. Because of the lack of osmium post fixation, plasma membranes are not visualized and the mitochondrial cristae appear as white lines [23]. × 21,000

toad bladder since the sections were taken to include only one stained cell and its immediate neighbors.

Although these studies are limited by the number of cells examined, they strongly suggest that the cells containing carbonic anhydrase are mitochondria-rich cells. In the D bladder, the validity of this correlation is supported by other lines of evidence. As shown in Fig. 4 the carbonic anhydrase-containing cells uniformly have a small surface area, suggesting that a single population of cells is stained. Moreover, the small and polygonal surface area of these cells as observed in whole bladder preparations (Fig. 2) is quite similar to that of the mitochondria-rich cells as observed by transmission and scanning[1] electron-microscopy [5, 7]. Finally, Keller[2] [13] estimated that in whole bladders stained for the demonstration of mitochondria, the surface area accounted for by the mitochondria-rich cells was 1%, an area almost identical to the one reported here for the carbonic anhydrase-containing cells. In the C bladder, however, although our observations appear to indicate that the mitochondria-rich cells are the ones stained for carbonic anhydrase, the surface area measurements of the individual cells reveal wide variations (Fig. 4). Thus, either the mitochondria-rich cells have variable surface areas, or different cell types stain for carbonic anhydrase. The present studies, although not conclusive, support the former possibility.

Discussion

These results confirm the studies by Frazier and Vanatta [9] and Ludens and Fanestil [16] that the urinary bladder of the toad, *Bufo marinus*, is able to acidify the solution bathing its mucosal surface, and document further by direct measurements of acid secretion that this ability depends upon the geographic origin of the toad. Bladders of toads of Colombian origin have acidifying capacity, whereas those from the Dominican Republic with rare exceptions have not. The present study defines another difference between the Dominican and Colombian toad bladders, the extent to which their mucosa stains for carbonic anhydrase. Since 4.6% of the surface area of the C bladder and 0.8% of the D bladder is accounted for by enzyme-containing

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1 Although Ferguson and Heap [7] examined bladders of toads from Jamaica, scanning electron-microscopy of bladders from Dominican toads demonstrated that their mitochondria-rich cells also have a small and polygonal surface area (Rosen, *unpublished observations*).

2 Keller's studies [13] were carried out in bladders of Dominican toads *(personal communication from the author)*.

cells, it is apparent that this difference parallels the difference in acidifying capacity of the bladders.

These findings are consistent with other observations that carbonic anhydrase plays a role in acidification by the C bladder. Acetazolamide has been shown to inhibit acidification when the bathing media contain low tensions of CO_2 [10, 16]. Moreover, this inhibitory effect may be partially overcome by increasing the supply of CO_2 to the bladder [10, 30]. In the present study, in which pH stat measurements were made in the Ussing chamber, the rate of acidification was greater in the presence of exogenous CO_2 than with endogenous CO_2 alone. In the latter case, therefore, the availability of CO_2 appears to be rate limiting for acidification.

Since a quantitative analysis of the production and fate of metabolic CO_2 by the toad bladder is not available, the precise dependence of acidification on CO_2 and its enzymatic hydration cannot be fully explored. Such an analysis has been made for the isolated turtle bladder [26], an epithelial membrane extensively studied for its acidifying capacity [1, 25, 27]. In this tissue, the rate of metabolic CO_2 is too low to account for the observed rates of acid secretion without catalysis by carbonic anhydrase [26]. Although the available information allows no definitive conclusion, it seems to suggest that this is also the case in the toad bladder.

What then is the significance of the parallelism between the extent of mucosal staining for carbonic anhydrase and acidifying capacity in C and D toad bladders? Are only the enzyme-containing cells capable of acid secretion? Answers to these questions must remain largely inferential. First of all, the cytoplasmic carbonic anhydrase being detected by the histochemical technique is probably not directly responsible for acid or alkali transport across the cell membrane. The enzyme is thought to permit the rapid disposition of OH^- (or formation of H^+) and thereby to prevent the development of steep local H^+ gradients. In turtle bladder [25] quantitative studies suggest that the cells with abundant carbonic anhydrase are capable of higher secretion rates than the majority of cells in which no enzyme is detectable; in this reptilian tissue all cells have secretory capacity as judged from the utilization of metabolic CO_2. Secondly, comparison of toad and turtle bladder is of additional interest in that the latter has not only greater secretory capacity, but also greater representation of enzyme-containing cels on the luminal surface; the stained area is close to 10%. This overall corlrelation between extent of staining and secretory capacity in bladders of toads of different origin and the turtle appears to indicate that the enzyme-containing cells indeed must be better equipped for acid secretion than the majority of surface cells. The toad bladder differs from the turtle bladder in

that the cells containing carbonic anhydrase are distinctly rich in mitochondria [3, 20]. These cells may also be specialized in terms of the transport system and energy supply for acidification.

The different physiologic and histochemical characteristics of bladders of Colombian and Dominican toads may provide an explanation for certain small but important differences observed in earlier studies. Leaf, Anderson and Page [14] observed originally that the short-circuit current (SCC) in the isolated toad bladder was quantitatively accounted for by the active transport of Na^+. Further studies by Essig and Leaf [6] demonstrated that K^+ removal from the bathing media virtually abolished the net transport of Na^+ and thereby the PD and SCC. Finn, Handler and Orloff [8], however, observed in a subsequent study that the same maneuver caused reversal of the PD and SCC. They attributed this reversal to active Cl^- absorption. It was pointed out by Davies, Martin and Sharp [4] that the effect of K^+ removal on the electrical activity of the bladder was different depending on the geographic origin of the toads. They demonstrated that K^+ removal caused current reversal in the C but not in the D bladder. This difference has been confirmed by Ludens and Fanestil [16] who attributed the reversed SCC to acidification rather than to Cl^- transport. They observed that the reversed SCC was not dependent on the presence of Cl^- and that it closely matched the rate of acidification. Their observations as well as unpublished results in our own laboratory suggest that mucosal acidification in the C bladder is associated with a transfer of charge. The electrical characteristics of acidification in the C toad bladder, therefore, are comparable to those described previously for turtle bladder [28]. For the D toad bladder the absence of an acidification current provides certain experimental advantages for the study of sodium transport per se.

Little is known about geographical differences among the large neotropical toads of South America. Cei [2] refers to this toad population as the *Bufo marinus* complex. Within this complex, the *Bufo marinus marinus* inhabits the northern part of South America including Colombia, Mexico and Central America [2]. The toads from the Dominican Republic are part of the *Bufo marinus* complex. They probably originated in the northern part of Brazil and were established on the island of the Dominican Republic by 1938 [4]. The available information suggests that the mainland toads from Colombia and probably Brazil [11] are capable of acidifying bladder urine, whereas the Dominican toads have lost this ability.

Whether this difference is determined by selection of genetic factors or by adaptive changes to the environment remains to be determined.

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