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Cellular Changes in the Toad Urinary Bladder in Response to Metabolic Acidosis

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Summary. The urinary bladder of *Bufo marinus* excretes H^+ and NH_4^+, and the H^+ excretion is increased when the animal is placed in metabolic acidosis. The mitochondria-rich (MR) cells mediate the H^+ excretion by the bladder. The purpose of this study was to determine if there is a change in MR cells of the bladder during metabolic acidosis. Bladders from normal toads and from toads that had been placed in metabolic acidosis were used. The bladders were mounted between plastic chambers and H^+ excretion measured. The bladder was then fixed and prepared for scanning (SEM) and transmission (TEM) electron micrograph studies. SEM's at low magnification were used to count the various cell types and the TEM's were used to confirm the different cell types. Fields were randomly selected and a total of 2500 cells counted in each group. The bladders from toads in metabolic acidosis had a consistently higher ratio of MR cells to granular cells than did the normal bladders. These results indicate that during metabolic acidosis there is an increased number of MR cells in the bladder, and this increases the bladder's capacity to excrete H^+.

It has been known for some time that the urinary bladder of the Colombian toad can acidify the urine (Frazier & Vanatta, 1971; Ludens & Fanestil, 1972). Frazier and Vanatta (1971) also demonstrated that bladders from toads in chronic metabolic acidosis had a significantly increased capacity to acidify the mucosal bathing fluid. In the mucosal layer of the toad urinary bladder four cell types have been described (Pak Poy & Bentley, 1960; Peachy & Rasmussen, 1961): granular cells (GR), mitochondia-rich cells (MR), goblet cells (GO), and basal cells. Scott and Sapirstein (1974) and Rosen, Oliver and Steinmetz (1974) have been able to partition epithelial function in the toad bladder into the different cell types. Their study indicates that most of the carbonic anhydrase activity of the bladder is localized to the MR cells and that it is the MR cell that is the mediator of H^+ transport.

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The purpose of this study was to determine if there is a change in the number of MR cells of the bladder during chronic metabolic acidosis. Bladders from normal toads and toads which had been in metabolic acidosis for either 24, 48, or 96 hr were used in the experiments. H^+ excretion was measured, and then the bladders were fixed and prepared for scanning (SEM) and transmission (TEM) electron micrograph studies. We found a consistent increase in the MR cells in the bladder from toads which had been in acidosis for 48 or 96 hr.

Materials and Methods

The toads used in these studies were *Bufo marinus* of Colombian origin and were supplied by Charles P. Chase of Miami, Florida. The routine care of toads, solutions, the procedure of inducing acidosis, and the method of measuring H^+ and NH_4^+ excretion were as previously described (Frazier & Vanatta, 1973). In all experiments, the H^+ excretion was calculated from change in pH and the concentration of buffer in the mucosal solution. The H^+ excretion was calculated using a pK_a for the phosphate buffer pair of 6.50. The NH_4^+ was determined colorimetrically. A Radiometer model PHM 64 digital pH meter was used for all pH determinations. One hundred percent humidified O_2 was bubbled into the mucosal medium throughout each experiment. Ringer's solution bathed both surfaces of the bladder throughout the experiment. The Ringer's solution contained (in mM): NaCl, 114.5; KCl, 3.0; $CaCl_2$, 0.9; and sodium phosphate (dibasic), 1.5; the final pH was 6.80. All statistics on H^+ and NH_4^+ excretion were performed as the difference between sample groups using Student's *t* test.

Hemibladders from both normal toads and toads in NH_4Cl-induced acidosis were mounted as sheets between plastic chambers. The chambers were 2 ml in volume, and the exposed area of each hemibladder was 1.98 cm^2. Both the mucosal and serosal chamber contained the 1.5 mM phosphate buffered Ringer's solution. The flux period was for 120 min. NH_4^+ and pH were determined on each mucosal and serosal sample both before and after the flux period. The flux period was started after a 15-min equilibration period.

After the flux period, the bladders were fixed by addition of 50% glutaraldehyde to both the mucosal and serosal chambers to give a final glutaraldehyde concentration of 2%. The tissue was fixed for 1 hr, the exposed area then cut from the chamber and placed in 2% glutaraldehyde in 0.1 M cacodylate buffer and postosmicated (90 min) in 1% osmium tetroxide, 0.1 M cacodylate buffer containing 5 mM $CaCl_2$. After washing, tissues were dehydrated in graded acetones and prepared for scanning electron microscopy by the critical-point CO_2 method (Anderson, 1951). Specimens were glued on SEM studs and shadow cast with gold-palladium on a rotating table. Each specimen was examined in an AMR scanning electron microscope operating at 20 kV. SEM's at low magnification (1–2,000 ×) were used to count the various cell types on the mucosal surface of the bladder. Fields were randomly selected and SEM's taken. All of the SEM's were coded, mixed, the cells counted and then decoded. Counting was done until at least 2,500 cells had been counted in each experimental group of animals. All of the cell counts were analyzed statistically by the chi-square test.

The other half of each specimen was washed in 0.1 M cacodylate buffer. One to 2-mm fragments were postosmicated the same as above for the SEM. They were then washed and dehydrated in a graded series of ethanol and embedded in Epon 812. Sections for

electron microscopy were cut on a Porter-Blum ultramicrotome. The sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Venable & Coggeshall, 1965) and examined in a Philips EM 300 electron microscope.

An additional series of bladders from both normal and acidotic toads were done. H^+ excretion was determined as above and then each bladder was stained for succinic acid dehydrogenase while still stretched on the chamber. The staining was done by the method of Nachlas *et al.* (1975). The tissue was then dehydrated rapidly in a series of graded ethanol and mounted in Cuparal on glass slides for light microscopic examination.

Results

The experiments were done on normal toads and toads which had been in metabolic acidosis for 24, 48 and 96 hr. Table 1 shows the H^+ excretion of the urinary bladder from these four different groups. H^+ excretion was significantly increased in all of the bladders from toads which had been in metabolic acidosis for 24, 48 or 96 hr ($p<0.005$, $p<0.01$, and $p<0.001$, respectively). There was no difference in the rate of H^+ excretion between the bladders of 24 and 48 hr acidosis ($p>0.50$). However, the H^+ excretion rate was significantly greater in the 96 hr group compared to the 24 and 48 hr groups ($p<0.05$).

In a similar response NH_4^+ excretion was increased in all of the bladders from toads which had been in metabolic acidosis for 24, 48 and 96 hr ($p<0.05$, $p<0.005$, and $p<0.001$, respectively).

SEM's were used to count the different cell types on the mucosal surface of the bladder according to procedures given above in *Methods.* Figures 1 and 2 are typical SEM's of the toad bladder mucosal surface from which the counts were made. It is apparent that the mucosal surface of both the normal and acidotic bladders contain three distinct and

Table 1. H^+ and NH_4^+ excretion by the urinary bladder in normal toads and toads in metabolic acidosis

Acid-base state	H^+ excretion (nmoles/ hr × cm^2)	*P* value[a]	NH_4^+ excretion (nmoles/ hr × cm^2)	*P* value[a]
Normal[b]	25.2 ± 3.0[c]		3.18 ± 0.57	
24-hr metabolic acidosis[b]	49.5 ± 5.6	<0.005	4.75 ± 0.37	<0.05
48-hr metabolic acidosis[b]	46.5 ± 5.8	<0.01	8.16 ± 1.12	<0.005
96-hr metabolic acidosis[b]	77.5 ± 5.0	<0.001	10.27 ± 1.30	<0.001

[a] Calculated as the difference from the normal group.
[b] $n=10$.
[c] ±1 SEM.

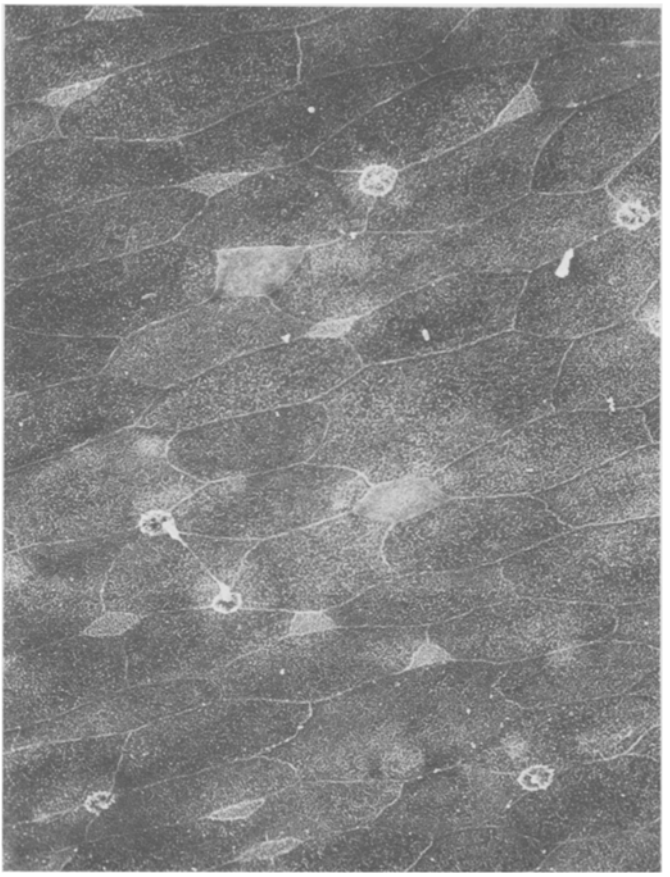

Fig. 1. Scanning electron-micrograph of the surface of a moderately stretched bladder from a toad in metabolic acidosis. The large polygonal cells corresponds to the GR cells of the transmission electron micrographs. The MR cells are scattered among the GR cells. They have a smaller surface area, and their microvilli are erect and densely packed. The GO cells are recognized by their long filamentous microvilli. Two GO cells in the secreting stage are seen near the left center of figure. (1,600×)

easily identifiable cell types: (i) the large polygonal GR cell that is present in greater number, but with fewer and shorter microvilli than the other two cell types; (ii) the MR cell with smaller apparent surface area but with more densely packed and longer microvilli protruding from the mucosal surface; (iii) the GO cell with smaller surface area and long filamentous microvilli. Figure 3 is a higher magnification SEM of the bladder surface from an acidotic toad showing these characteristics in greater detail.

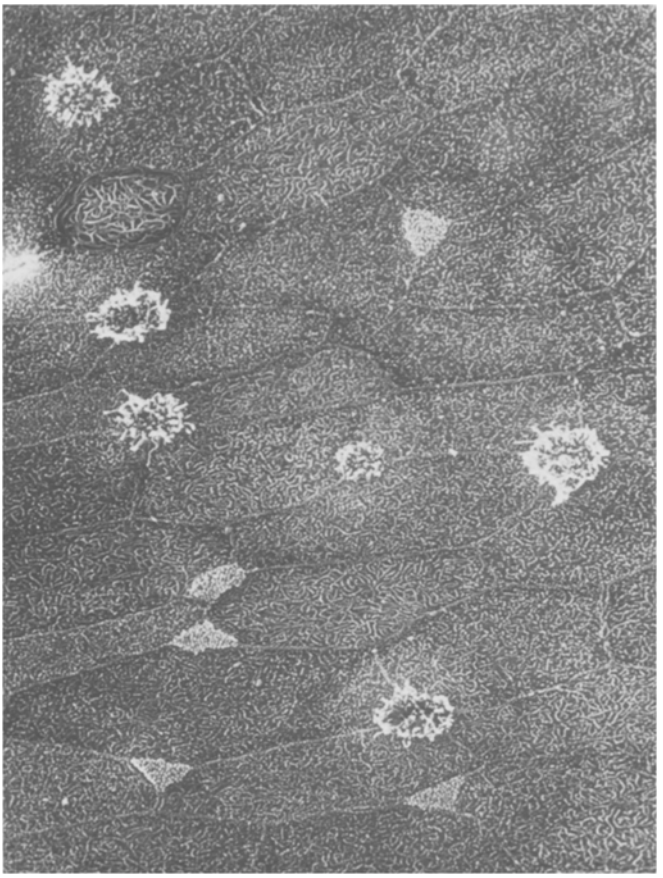

Fig. 2. Scanning electron-micrograph of the surface of a moderately stretched bladder from a normal toad. The same cell types are present as in Fig. 1, and the surface anatomy is the same as in Figs. 1 and 3. (2,000×)

Figure 4 is a TEM of an MR cell confirming that the MR cell has longer and more abundant microvilli and, in addition, less surface area than the GR cell. Likewise, Figure 5 is a TEM of a GO cell, confirming that it has a smaller mucosal area than the GR cell and has much longer filamentous microvilli than either the GR or MR cell.

Table 2 shows the ratio as well as the % of cell types found on the mucosal surface in the SEM's of bladders from both normal and acidotic toads. There is clearly an increase in the number of MR cells in the bladder from toads when in metabolic acidosis for 48 or 96 hr ($p < 0.001$ and $p < 0.001$). However, there was no increase above the normals in the bladders from toads which had been in metabolic acidosis for 24 hr.

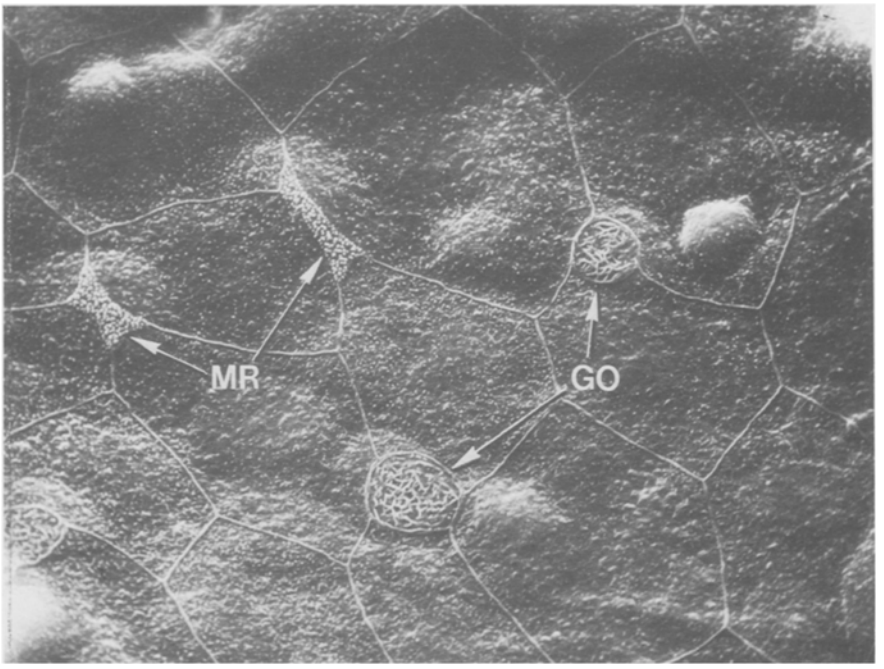

Fig. 3

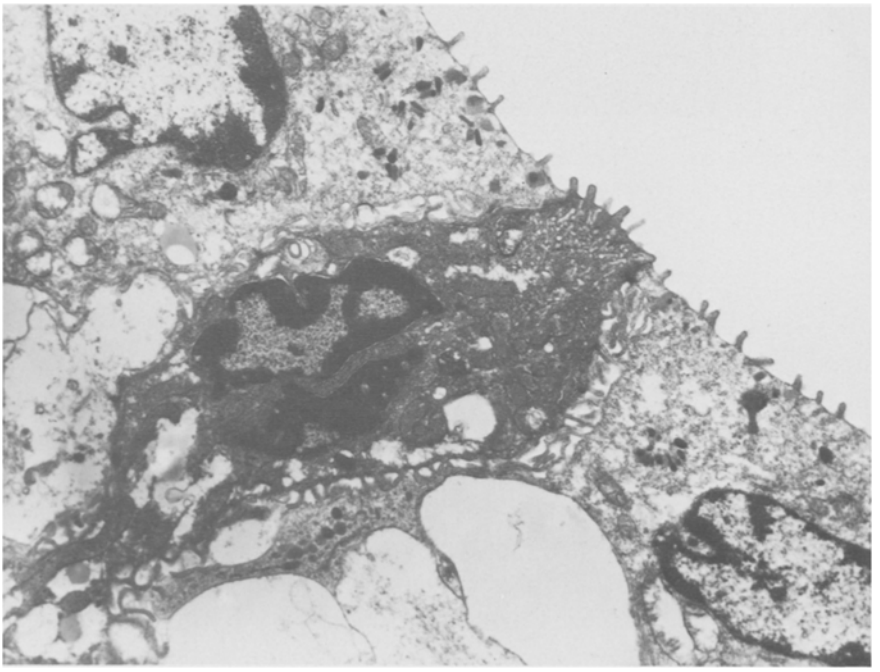

Fig. 4

An additional experiment was done in which normal bladders and bladders from toads in metabolic acidosis for 48 hr were examined histochemically for succinic acid dehydrogenase. Succinic acid dehydrogenase is a mitochondrial enzyme, and therefore should be localized to the MR cells, while only small amounts would be present in the GR and GO cells. If the total number of MR cells increase during acidosis

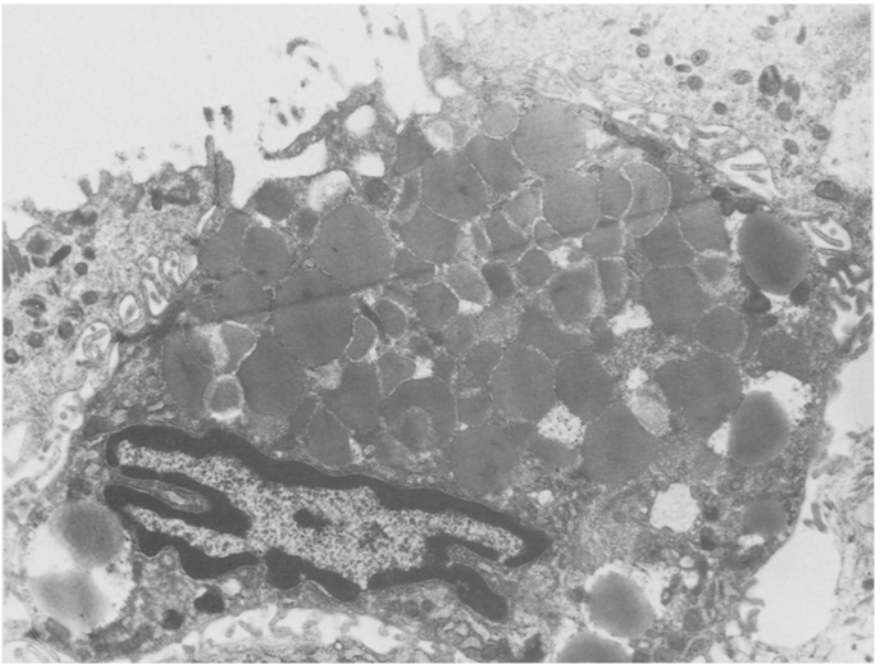

Fig. 5. Transmission electron micrograph of a GO cell between two GR cells. The microvilli of the GO cell are much longer than the microvilli of the GR cell, confirming the SEM observations. Note the many mucous-containing granules of the GO cell. (8,000 ×)

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Fig. 3. High magnification scanning electron micrograph of a moderately stretched bladder from a toad in metabolic acidosis. Note the smaller surface area of the MR cell compared to the GR cell and also the microvilli of the MR cell are more erect and closely packed. The GO cells on the other hand have long filamentous microvilli. (2,600 ×)

Fig. 4. Transmission electronmicrograph showing an MR cell between two GR cells. Note the rounded, thick and closely packed microvilli of the MR cell as compared with the few irregular microvilli of the GR cells. Although fairly large, only a small area of the MR cell is exposed on the mucosal surface of the bladder compared to the GR cells. The bulk of the cell seems to lie beneath the GR cells. This is consistent with the SEM observations. (5,500 ×)

Table 2. Changes in cell population in the toad urinary bladder in response to a metabolic acidosis (from scanning electron micrographs)

Bladder	Ratio of cell types		
	Granular /	Mitochondrial-rich /	Goblet
Normal[a] ($n=10$)	1.0 (80%)[b]	0.12 (9%)	0.14 (11%)
24-hr metabolic acidosis[a] ($n=10$)	1.0 (80%)	0.10 (8%) NS[c]	0.15 (12%)
48-hr metabolic acidosis[a] ($n=10$)	1.0 (73%)	0.22 (16%) $P<0.001$[c]	0.15 (11%)
96-hr metabolic acidosis[a] ($n=10$)	1.0 (77%)	0.16 (13%) $P<0.001$[c]	0.13 (10%)

[a] Represents a total of 2,500 cells counted.
[b] Number in parenthesis is the % that cell represents of the total number counted.
[c] P value determined by the chi-square test on the actual cells counted in each group compared to the counts in the normal group. NS = nonsignificance.

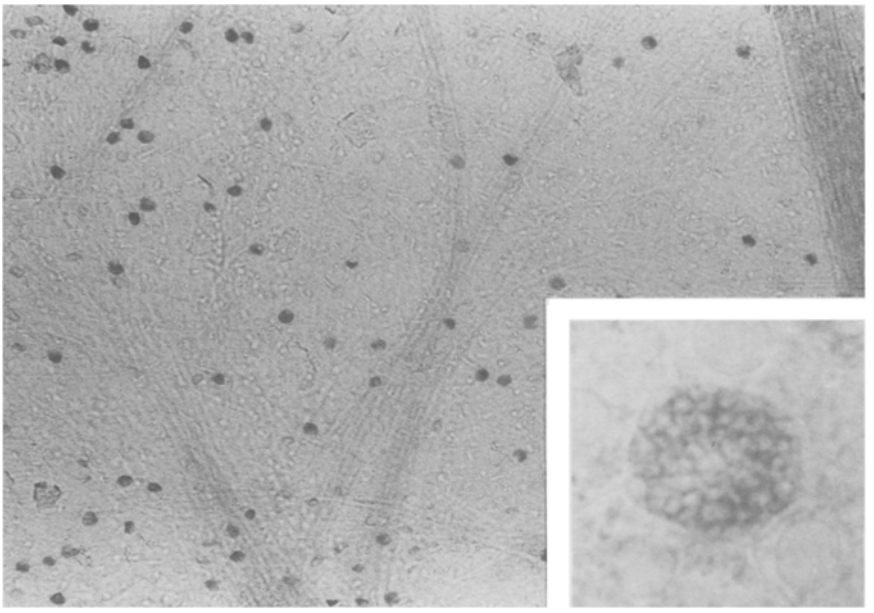

Fig. 6. Histochemical demonstration of succinic acid dehydrogenase activity in a moderately stretched bladder of a normal toad. A single cell is shown at high magnification in the inset. The clear, lighter polygonal central area is the luminal surface of the cell and the darker peripheral area is staining deeper in the cell. (125×, 1,250×)

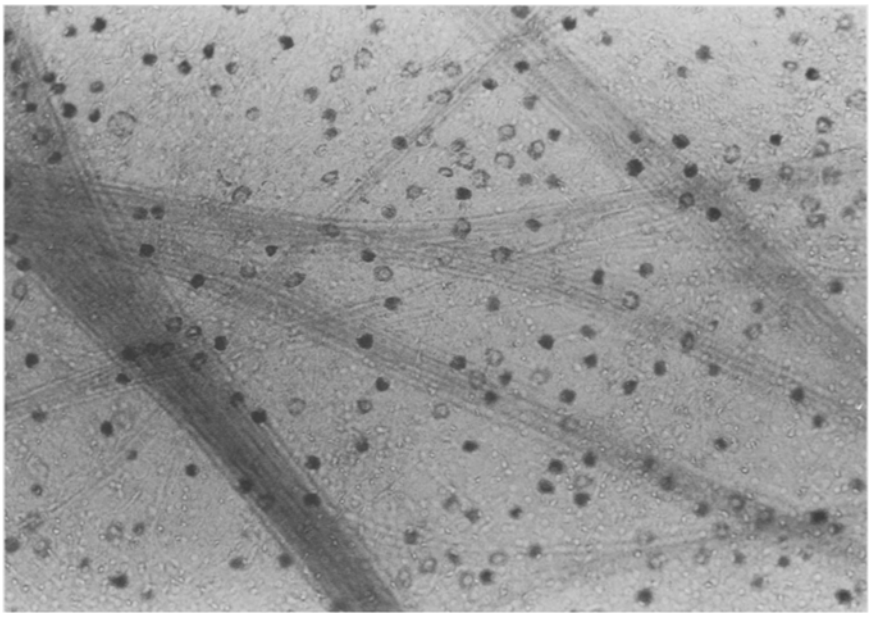

Fig. 7. Histochemical demonstration of succinic acid dehydrogenase activity in a moderately stretched bladder from a toad in metabolic acidosis. Note that more cells are stained in this preparation than in Fig. 6, indicating a greater number of MR cells. (125 ×)

then this should be reflected by an increase in succinic acid dehydrogenase activity in the acidotic bladder. Figure 6 shows a typical field in a normal bladder stained for succinic acid dehydrogenase. Figure 7 is the same stain in a bladder from a toad after 48 hr in metabolic acidosis. It is clearly shown that the succinic acid dehydrogenase activity is increased in the bladder from the acidotic toad. This supports the SEM data that the number of MR cells is increased in response to a metabolic acidosis.

Discussion

Frazier and Vanatta (1971) have previously shown that the urinary bladder is capable of excreting H^+ and NH_4^+ and that the H^+ excretion is increased when the animal was placed in a chronic metabolic acidosis. More recent studies have indicated that the MR cell of the Colombian toad bladder contains carbonic anhydrase and that it is the MR cell which mediates the H^+ excretion (Rosen *et al.*, 1974; Scott & Sapirstein,

1974). In light of these reports, our present studies were done to determine if the observed increase in H^+ excretion during acidosis might be a result of an increased number of MR cells.

Our studies reveal that at 24, 48, and 96 hr after the animal had been placed in acidosis the H^+ excretion by the bladder is increased significantly over the control group. This increased H^+ excretion seen at 48 and 96 hr is accompanied by an increase in the number of MR cells. Our data support the concept that the ability of the urinary bladder to increase H^+ excretion during chronic metabolic acidosis is dependent on the adaptive cellular changes that occur in the bladder. This adaptation occurs as an increase in the number of MR cells in the bladder.

These findings raise an interesting question. Could the mammalian nephron increase its ability to acidify the urine in response to a chronic acid load by a similar adaptive mechanism? It is generally held that the function of the toad urinary bladder is analogous to the mammalian distal nephron. Andrews and Porter (1974) have shown that the collecting tubules of the rat contain at least two cell types: One similar to the GR cell of the toad bladder, containing very few mitochondria, and the second, fewer in number, containing many mitochondria in the cytoplasm (dark cells). Other studies by Zufarov and Gontmakher (1974) have implicated the dark cells (mitochondria-rich cells) of the collecting tubules of rats as the cell possessing acidification function. It would be tempting to answer the above question in the affirmative. However, until more experiments are done with the mammalian nephron we can only speculate about the adaptation of the mammalian nephron during acidosis.

We have found three cell types present on the mucosal surface of the toad urinary bladder using the SEM examination. This is in agreement with several other laboratories (Ferguson & Heap, 1970: Danon, Strum & Edelman, 1974; Spinelli, Grosso & de Sousa, 1975) which have reported a similar description of the mucosal surface of the urinary bladder. In addition, we have found that there is an increase in the number of MR cells on the mucosal surface of the bladder when the animal is faced with a chronic acid load.

There are two apparent discrepancies between our present work and that reported by Danon *et al.* (1974) and Wade (1976) that should be noted. Firstly, Danon *et al.* (1974) report the following ratios for GR/MR/GO cells as 1.0:0.25:0.19 in normal toads, compared to our findings of 1.0:0.12:0.14. In the study by Danon *et al.* (1974) the geographical origin of the toads used was not reported. If they were from other than

Colombian origin this might account for the difference observed in cell populations of the bladder as suggested by Rosen *et al.* (1974). In addition, it should be pointed out that an earlier light microscopy study (Choi, 1963) on toad urinary bladder reported the GR/MR/GO cell ratios as 1.0:0.12:0.12, which is in agreement with our findings. Secondly, Danon *et al.* (1974) and Wade (1976) report the presence of two types of MR cells on the mucosal surface of toad urinary bladder, one with fingerlike microvilli and a second type with ridgelike microvilli. After a very careful examination and comparison between our SEM's and TEM's we were unable to find any evidence for two populations of MR cells. All MR cells in TEM's had the characteristic short, finger-like microvilli as shown in Fig. 4. Further, all cells observed in the TEM's with longer or ridge-like microvilli were characteristic of the cell seen in Fig. 5. The two cells labeled GO cells in our Fig. 3 are very similar to the cell shown in Fig. 16 of the study by Danon *et al.* (1974). They report this cell to be a degenerating form of an MR cell or a GO cell prior to initiation of secretory activity. The results of our study would support the latter of the two statements. Our present study does not reveal any evidence for the presence of two types of MR cell in the toad urinary bladder. We feel, however, that additional studies with more stringent criteria must be done before an unequivocal statement can be made.

It should be noted that in the H^+ excretion studies the rate of acidification was significantly increased after 24 hr of acid load. This occurred without an apparent increase in the number of MR cells in the bladder. This would indicate that the existing MR cells of the bladder were stimulated early in the acid loading to increase their intrinsic ability to excrete H^+. It could be that the bladder responds in two different ways to acidosis; (i) initially in acidosis the bladder could be stimulated by a hormone to increase H^+ excretion, and (ii) when faced with a chronic acidosis it is induced to increase the number of MR cells present on the surface. At 48 hr the H^+ excretion per cell was decreased. This could be due to the fact that the "new" MR cells are now present on the bladder surface but perhaps are not secreting at their maximal rate. Then by 96 hr both the old and "new" MR cells are stimulated and secreting at a near maximal rate under influence of hormonal stimulation. Parathyroid hormone has been shown to increase H^+ excretion by the toad urinary bladder after a 30-min exposure (Frazier, 1976). Similarly, Ludens and Fanestil (1974) have shown a stimulation of acidification of urine by the urinary bladder following a 2-hr exposure to aldosterone. However, until more experiments are performed to measure the levels of

parathyroid hormone and/or aldosterone during acidosis in the toad, we can only surmise such a control mechanism in the toad urinary bladder.

It is also interesting that NH_4^+ excretion increased in a similar manner to H^+ excretion during acidosis. If NH_4^+ excretion in the bladder occurs by the ionic trapping mechanisms described by Pitts (1974) for the mammalian nephron, then this NH_4^+ would represent a net acid excretion. At the present time, the mechanism of NH_4^+ excretion in the toad bladder has not been elucidated. A preliminary report by Melton and Vanatta (1977) indicates that excretion may be as an independent NH_4^+ carrier-mediated process. Our results would tend to indicate a similar relationship between NH_4^+ excretion and the MR cell population as exists for H^+ excretion. There is no evidence available at the present time that would indicate what cell in the toad urinary bladder is responsible for NH_4^+ excretion. This should not affect the interpretation of any results in our study since the NH_4^+ excretion is a relatively small portion of the total acid excretion.

These present studies do not clearly reveal the origin of the new MR cells that appear during acidosis. They could arise from: (i) division of an existing MR cell; (ii) differentiation of an existing GO cell; (iii) differentiation of a sub-mucosal stem cell, the basal cell (Choi, 1963); and finally (iv) we must consider the possibility that some MR cells on the mucosal surface are very small and hidden in the unstimulated state and hence missed in the counting procedure. When the bladder is acid-stimulated, these cells could increase their exposed surface area and appear as "new" cells on the mucosal surface. Studies are currently under way which we hope will help elucidate the origin of the new MR cells seen during adaptation to acidosis.

In summary, our studies have shown: (i) the rate of acidification and NH_4^+ excretion is increased in urinary bladders from toads in chronic metabolic acidosis; (ii) there are three distinct cell types on the bladder mucosal surface in both normal bladders and bladders from toads in metabolic acidosis; and (iii) the toad urinary bladder undergoes an adaptive change which leads to an increase in the number of MR cells in the bladder during acidosis. This increase in MR cells appears to be associated with an increase in the bladder's capacity to acidify the urine.

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