BBA 76092

MECHANISM OF ACIDIFICATION OF THE MUCOSAL FLUID BY THE TOAD URINARY BLADDER

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(Received May 30th, 1972)

SUMMARY

1. Studies were performed to determine the mechanism of acidification of the mucosal fluid by the toad urinary bladder

2. Experiments performed in the absence of exogenous CO_2 and HCO_3^- showed that the acidification process is not due to selective readsorption of HPO_4^{2-} or secretion of $H_2PO_4^-$.

3 The acidification process was found to be dependent on carbonic anhydrase activity. But this dependence was not apparent when exogenous CO_2 5% was present in the serosal medium.

4. Measurements of pCO_2 and pH in hemibladder sacs showed that concurrent with acidification there was an increase in mucosal pCO_2. This finding was taken as evidence that mucosal acidification in the toad urinary bladder occurs by the process of H^+ excretion *per se*.

5. A one to one stoichiometry was not found between mucosal H^+ excretion and serosal base excretion. The serosal base excretion being only about 25% of the mucosal H^+ excretion.

INTRODUCTION

Previous experiments from this laboratory have shown that the urinary bladder of the toad *Bufo marinus* is capable of acidifying the urine[1]. This acidification of the mucosal fluid occurred concurrently with an alkalization of the serosal medium. These experiments were performed *in vitro* in the absence of exogenous CO_2 and bicarbonate in either the mucosal or serosal fluid. Under these conditions acidification could occur by one of the following mechanisms: (1) The selective reabsorption of the HPO_4^{2-} or selective excretion of the $H_2PO_4^-$ moiety of the buffer pair; (2) Diffusion into or formation of H_2CO_3 in the mucosal medium and subsequent reabsorption of HCO_3^-; (3) The excretion of H^+ from the cell into the mucosal fluid.

Schilb and Brodsky[2,3] have reported that the urinary bladder of the water turtle acidifies the mucosal fluid by the process of HCO_3^- reabsorption. While on

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the other hand Steinmetz[4] and Steinmetz *et al.*[5], report that the acidification of the mucosal fluid is by the process of H^+ excretion.

The experiments reported here were performed to determine: (1) If selective phosphate reabsorption or secretion can account for the acidification of the mucosal fluid by the toad bladder; (2) If the acidifying process is dependent on carbonic anhydrase activity; and (3) If H^+ excretion *per se* or HCO_3^- reabsorption is responsible for the acidification of the mucosal fluid.

MATERIALS AND METHODS

The source and the routine care of toads, instrumentation, solutions, the procedure for inducing acidosis and the methods of measuring H^+ excretion were as previously described[1,6]. In all experiments, the H^+ excretion was calculated from change in pH and the concentration of buffer in the mucosal solution, unless noted otherwise. Titratable acidity was determined by titrating an aliquot of the mucosal bath back to the pH at zero time. A Corning Model 10 pH meter was used for this and all pH determinations. Inorganic phosphate was determined by the colorimetric method of Fiske and SubbaRow[7] using a Bausch and Lomb spectrophotometer.

The Ringer solutions used contained in mM: NaCl, 114.0; KCl 3.0; $CaCl_2$, 0.9; sodium phosphate was added to the desired concentration with adjustment in the NaCl concentration to maintain the total osmolarity at 237 mosmoles/l. The final pH was 6.80. The Ringers solution used in the experiments measuring pCO_2 contained in mM: NaCl, 114.5; KCl, 3.0; $CaCl_2$, 0.9, $NaHCO_3$, 0.5; and sodium phosphate, 0.5; the final pH was 7 00. The serosal solution in the acetazolamide experiments using exogenous CO_2 contained in mM: NaCl, 89.5, KCl, 3.0; $CaCl_2$, 0.9; $NaHCO_3$, 25.0, the solution was then equilibrated with 5% CO_2 and the final pH was 7.20. 100% O_2 was bubbled into the mucosal medium throughout each experiment, unless noted otherwise. In separate experiments this was found adequate to maintain a steady potential difference across the bladder. Each flux was for a 2-h period unless otherwise indicated. All statistics were performed as the mean difference on paired hemibladders or between two consecutive time periods on the same hemibladder.

Experiments on phosphate movement

The *in vitro* experiments were performed on toads which had been placed in a metabolic acidosis by loading with NH_4Cl for 48 h. The toads were then sacrificed by pithing. The hemibladders were removed and mounted between lucite chambers each of which held 2 ml of a 1.5 mM phosphate Ringer solution. The bladders equilibrated for at least 15 min before the flux period was begun. At the end of the flux period the pH and inorganic phosphate was determined on each mucosal and serosal sample.

Acetazolamide, effect on H^+ excretion

Acetazolamide was obtained from Lederle Laboratories. Paired hemibladders were used, the control hemibladder was not treated while the experimental hemibladder received 0 2 mM acetazolamide in the serosal medium. The mucosal medium for all experiments was a 1.5 mM phosphate Ringer solution. The serosal bath in one group of bladders was a 1.5 mM phosphate Ringer solution, while another group of

bladders had a 25.0 mM HCO_3^- Ringer solution equilibrated with 5% CO_2 as the serosal medium In the latter group 5% CO_2 was bubbled through the serosal medium during the flux period In both groups the only difference between the control and experimental hemibladders was the presence of acetazolamide in the experimental serosal medium.

Experiments measuring pCO_2 and pH in bladder sacs

Bladders from metabolic acidotic toads were used. The bladders were dissected out and a hemibladder tied around the outlet of the chamber as shown in Fig 1 The pH and pCO_2 electrodes were placed in position. The chamber and bladder were filled with 5 ml of the 0.5 mM phosphate and 0.5 mM HCO_3^- Ringer solution Air bubbles were removed from the chamber and bladder by forcing the air out of the syringe outlet. A 5-ml syringe was then placed in position and the flux period begun. The flux period was for 60 min. Every 3 min during the flux period the fluid (approximately 2.5 ml) was pulled up into the syringe and reinjected into the bladder and chamber. This was done to mix the bladder contents and to obtain readings of the pH and pCO_2.

All pCO_2 measurements were made using a pCO_2 electrode obtained from Instrumentation Laboratory Inc. The pCO_2 was read on a Corning Model 10 pH meter The pH meter was calibrated with commercially analyzed mixtures of gases whose CO_2 composition were known to an accuracy of 0 01% The readings were converted to pCO_2 in mm Hg by the method of Severinghaus and Bradley[8] The theoretical error in determining pCO_2 was calculated from the observed reproducibility of reading the pH meter attached to the pCO_2 electrode, which was $\pm$ 0 01 pH unit. The error was 1.00 $\pm$ 0.05 mm Hg, and 0.24 $\pm$ 0 10 mm Hg, which were the highest and lowest values for the initial pCO_2 In this experiment 100% O_2 was bubbled into the serosal medium and not the mucosal medium. The final pH

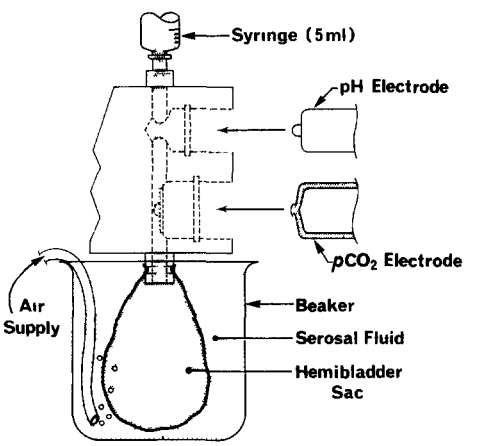

Fig 1 Schematic diagram of the chamber used for measuring simultaneous pH and pCO_2 of the mucosal bath in hemibladder sacs The interior volume of the chamber is approximately 1 ml, excluding the volume of bladder and syringe As is shown, the chamber has an adapter for a leur lock syringe, an outlet on which to tie the hemibladder, and two inlets with rubber "O" rings for the pH and pCO_2 electrodes A layer of mineral oil is placed between the syringe plunger and barrel

of the serosal medium ranged from pH 7.05–7.15. This increase in pH of the serosal medium would be expected and could arise from two causes: (1) The bubbling of 100% O_2 into the medium would bubble off any CO_2 present resulting in the following reactions, $H_2CO_3 \rightarrow CO_2 + H_2O$ and $NaHCO_3 + NaH_2PO_4 \rightarrow Na_2HPO_4 + H_2CO_3 \uparrow$; (2) The excretion of a basic substance by the bladder into the serosal medium during the acidification process.

Two consecutive flux periods were performed on each bladder. During the first period the mucosal solution was 5.0 ml of a 0.5 mM phosphate and 0.5 mM HCO_3^- Ringer solution and the serosal medium was 20.0 ml of the same solution. The second flux period was the same except the serosal solution contained 0.4 mM acetazolamide. The pH and pCO_2 were read every 5 min during the flux period. The changes in pH observed over the 60-min period were used to calculate H^+ excretion. The changes in pCO_2 were determined as described above. H^+ excretion was calculated as described previously using the pK_a for the phosphate system of 6.50 and for the HCO_3^- system of 6.10. A slight error in H^+ excretion would occur if CO_2 was lost from the mucosal medium by diffusion into or through the bladder. This error would be small in magnitude, and should not alter the interpretation of the results

Experiments on change in pH versus titratable acidity and serosal base excretion

In vitro experiments were performed in general as given above under the experiments dealing with phosphate movement. Hemibladders from metabolic acidotic toads were used. Both the serosal and mucosal medium was a 1.5 mM phosphate Ringer solution. Mucosal H^+ excretion was measured both as titratable acidity and as calculated from the observed change in pH. Serosal base excretion was calculated from the change in pH using the Henderson–Hasselbach relationship.

RESULTS

Experiments on phosphate movement

As was mentioned earlier, acidification of the mucosal fluid could occur by selective reabsorption of the basic or excretion of the acidic form of the phosphate buffer pair. In order to determine if phosphate movement was occurring, we did *in vitro* studies in which inorganic phosphate was determined before and after acidification of the mucosal fluid.

In Table I are shown the results of the *in vitro* studies in which the total phosphate of the mucosal and serosal medium was measured both before and after acidification. The average acidification by the bladders is also shown. It can be seen that during the acidification period there was no significant gain or loss of phosphate from the mucosal ($P > 0.10$) or the serosal ($P > 0.10$) medium. These findings also indicate that the acidification process is not due to selective phosphate reabsorption or excretion. Although no significant increase was found, it may be that the increase observed was real and was due to phosphate being leached from the bladders.

The effect of acetazolamide on H^+ excretion

In order to determine if carbonic anhydrase was involved in the excretion of H^+ we used the inhibitor acetazolamide. The effect was tested in both the presence

TABLE I

CHANGES IN AMOUNT OF PHOSPHATE IN THE MUCOSAL AND SEROSAL MEDIUM OF ACIDOTIC BLADDERS *in vitro*

Both the mucosal and serosal chamber contained 2 ml of a 1 5 mM phosphate Ringer solution The flux period was for 120 min Mucosal H^+ excretion averaged 16 5 ± 2 04 nmoles/100 mg bladder wet weight per min

N	*Mucosal phosphate before* (*μmoles*)	*Mucosal phosphate after* (*μmoles*)	*Serosal phosphate before* (*μmoles*)	*Serosal phosphate after* (*μmoles*)
5	3 00*	3 00 ± 0 01**	3 04*	3 14 ± 0 08**
	$P > 0$ 10		$P > 0$ 10	

* Single determination, 1 50 μmoles/ml in 2 ml; 1 52 μmoles/ml in 2 ml
** Average ± S E

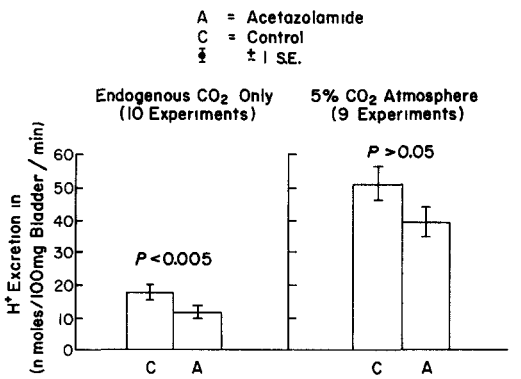

Fig 2 Graph showing the effect of acetazolamide (0 2 mM) on H^+ excretion in the toad urinary bladder The mucosal medium was 2 0 ml of a 1 5 mM phosphate Ringer solution in both groups The serosal medium in the endogenous CO_2 experiments was 2 0 ml of a 1 5 mM phosphate Ringer solution, while the serosal medium of the exogenous CO_2 group contained 2 0 ml of a 25 0 mM HCO_3^- Ringer solution equilibrated with 5 % CO_2 The experimental hemibladder received 0 2 mM acetazolamide in the serosal medium

and absence of exogenous CO_2 In Fig. 2 we report that the endogenous CO_2 experiments had a control excretion rate of 15.70 ± 3.28 nmoles/100 mg bladder wet weight per min. Acetazolamide treatment was found to reduce this rate to 11.60 ± 2.80. Analyzing these experiments as paired data this was a significant decrease ($P < 0.005$). In the system with a 5 % CO_2 atmosphere the H^+ excretion in the control bladders averaged 52.90 ± 12.00 nmoles/100 mg bladder per min, while the acetazolamide treatment reduced this to 38.60 ± 7.50. Treating these experiments as paired data gave a mean difference of 14.3 ± 8.86 nmoles/100 mg bladder per min which was not a significant reduction of H^+ excretion ($P > 0.05$) Time control experiments have been performed with regard to H^+ excretion and it has been found that the H^+ excretion remains constant up to a 180-min period. This latter finding was not surprising since carbonic anhydrase only catalyzes the reaction $CO_2 + H_2O \rightarrow H_2CO_3$. Therefore, by supplying CO_2 to the system and driving the reaction to the right the importance of carbonic anhydrase should be reduced

Experiments measuring pH and pCO_2 in bladder sacs

Acidification of the mucosal fluid could also occur by either H^+ excretion *per se* or HCO_3^- reabsorption. The HCO_3^- for reabsorption would arise from CO_2 diffusing out of the bladder into the mucosal medium and subsequent hydration to carbonic acid. The next experiment was done to determine if H^+ excretion was occurring by the toad bladder during acidification. If H^+ excretion was occurring in the *in vitro* closed bladder sac preparation, as described previously under Methods, then one should observe an increase in pCO_2 concurrent with mucosal acidification. On the other hand, if HCO_3^- reabsorption is the cause of the acidification the pCO_2 should remain constant or decrease slightly during the process of acidification.

The basis for this argument follows from the consideration given below. Our buffer system contained the following buffer pairs: $NaHCO_3$–H_2CO_3 and Na_2HPO_4–NaH_2PO_4. If HCO_3^- was reabsorbed from the bladder then the reaction $H_2CO_3 \rightleftarrows H^+ + HCO_3^-$ would proceed to the right and the pH would decrease. However, the ratio of the phosphate buffer system would also be upset according to the reaction $H_2CO_3 + Na_2HPO_4 \rightarrow NaHCO_3 + NaH_2PO_4$ (isohydric principle). Both reactions would result in a decrease in pCO_2 concurrent with acidification and at the same time the latter reaction would supply more HCO_3^- for reabsorption

If on the other hand H^+ excretion occurred then the reaction $H^+ + NaHCO_3 \rightleftarrows H_2CO_3 + Na^+$ would proceed to the right. The reaction $H^+ + Na_2HPO_4 \rightarrow NaH_2PO_4 + Na^+$ would also occur, resulting in a change in the phosphate buffer ratio. However, in this case there would be an increase in pCO_2 concurrent with acidification.

TABLE II

EXPERIMENT TO DETERMINE IF ACIDIFICATION IS BY H^+ EXCRETION OR BICARBONATE REABSORPTION

Excretion reported in nmoles/100 mg bladder wet weight per min, pCO_2 in mm Hg. The mucosal medium was 5 0 ml of a 0 5 mM HCO_3^- and 0 5 mM phosphate Ringer solution The serosal medium was 20 ml of the same solution The initial pCO_2 in the bladder sacs averaged 0 58 ± 0.07 mm Hg The ΔpCO_2 of the control period represents approximately a 172% increase pCO_2, while that of the experimental a 74% increase pCO_2 + represents increase and − represents decrease in pCO_2

Experiment No	*H^+ excretion*		*ΔpCO_2*	
	*Control**	*Experimental***	*Control**	*Experimental***
1	4.91	1 79	+0 90	+0 05
2	48 10	9 29	+0 02	0 00
3	9 22	2 65	+2 80	+1.20
4	15 60	2 09	+0 75	+0 25
5	7 25	2 30	+0 65	−0 95
6	12 50	4 22	+1 10	+1 00
7	9 03	1 84	+0 80	+0 74
8	8 75	2 63	+0 55	+0 45
9	8 33	4 26	+0 23	+0 63
10	6 29	3 09	+2 10	+0 95
Mean	13 00	3 42	+1 00	+0 43
Mean difference ± S E	9 58 ± 3 39 $P < 0$ 01		0 57 ± 0 22 $P < 0$ 025	

* Without acetazolamide in the serosal medium
** 0 4 mM acetazolamide in the serosal medium

In Table II the results of ten closed bladder sac preparations are shown. As can be seen in the table there was an increase in mucosal pCO_2 concurrent with mucosal acidification. The pCO_2 was also found to increase with time throughout the experimental flux period. Acetazolamide was found to inhibit H^+ excretion in every case ($P < 0.01$). The increase in pCO_2 of the mucosal fluid was also inhibited in nine out of ten cases by the addition of acetazolamide ($P < 0.025$).

Experiments on change in pH versus titratable acidity and serosal base excretion

In previous experiments it was found that there was an alkalization of the serosal medium concurrent with mucosal acidification[1]. It was also possible that another buffer system might be leaking from the bladder during the flux period, thereby inducing an error in the calculation of H^+ excretion from change in pH. The next experiment reported was designed to answer two questions: (1) Was the serosal base excretion quantitatively similar to mucosal H^+ excretion?; and (2) Is the method of measuring H^+ excretion by pH change equivalent to measuring it as titratable acidity?

TABLE III

COMPARISON OF TITRATABLE ACIDITY DETERMINED BOTH BY TITRATION AND BY CALCULATION FROM pH CHANGE AND COMPARISON OF MUCOSAL ACIDIFICATION WITH SEROSAL ALKALIZATION

Excretion reported in nmoles/100 mg bladder wet weight per min Both the mucosal and serosal medium was 2 ml of a 1 5 mM phosphate Ringer solution Averages ± S E are given

N	*Mucosal H^+ excretion*		*Serosal base excretion*
	As titratable acidity	*As calculated from pH change*	
10	11 52 ± 1 63	11 58 ± 1 16	3 21 ± 0 92
	$P > 0$ 90	$P < 0$ 005	

In Table III the results of this experiment are given. H^+ excretion was first calculated from the change in pH and then determined on the same sample as titratable acidity. As can be seen in the table, correlation between the two methods was very good ($P > 0$ 90). A correlation coefficient between the two methods was determined ($r = +0.70$). It can also be seen in Table III that the serosal base excretion is significantly lower than the mucosal H^+ excretion ($P < 0$ 005). The serosal base excretion was only about 25 % of the mucosal H^+ excretion.

DISCUSSION

Our *in vitro* studies in which phosphate concentrations were determined show that the acidification of the bladder urine is not due to a selective reabsorption of HPO_4^{2-} or secretion of $H_2PO_4^-$. The possibility of an ionic exchange involving mucosal HPO_4^{2-} and serosal $H_2PO_4^-$ cannot be ruled out by these data, but such an exchange seems an unlikely way for a biological system to excrete acid.

Carbonic anhydrase is obviously involved in H^+ excretion in the toad bladder. This is similar to other epithelia, such as gastric and renal tissue, which also excrete

H^+ and have a dependence on carbonic anhydrase. The fact that carbonic anhydrase is involved in the H^+ excretion is another argument for intracellular hydration of CO_2 with subsequent H^+ excretion. However, one cannot exclude entirely on the basis of the present evidence, that the carbonic anhydrase is on the mucosal membrane and has access to the mucosal contents as in the case of the proximal tubule[9].

Maren[10] using a biochemical analysis found no carbonic anhydrase activity in the toad bladder when using a bicarbonate buffer but found very low levels of activity when using a barbital buffer system. Rosen[11] using a very sensitive histochemical technique has also demonstrated carbonic anhydrase in the toad bladder epithelium. He further found the enzyme to be localized in the mitochondrial rich cells of the mucosal epithelium. Their experiments showed that carbonic anhydrase was present in the toad bladder; however, the importance was not apparent since these studies preceded our earlier reports on the ability of the toad bladder to acidify the mucosal fluid[1]. Our present experiments with acetazolamide support the findings of Maren[10] and Rosen[11], that carbonic anhydrase is present in the toad urinary bladder.

It could be argued that the experiment shown in Fig. 2 with acetazolamide might be invalid because the serosal medium contained 5 mM HCO_3^- equilibrated with 5 % CO_2. The CO_2 diffusing from the serosal to mucosal fluid would then give increased H^+ excretion either as a decrease in pH or increase in titratable acidity. Two facts argue against this occurring: (1) 100 % O_2 was bubbled concurrently into the mucosal medium and this would be expected to keep the CO_2 of the mucosal fluid at essentially zero; (2) In other experiments performed in our laboratory and to be reported in a subsequent paper, we found a drastic inhibition of H^+ excretion by CN^-. The experiments were run in a manner identical to those given for the acetazolamide experiments except CN^- (1 mM) was in the serosal medium in place of acetazolamide. Under these conditions, H^+ excretion was only 7 % of the H^+ excretion observed in paired control bladders. This was interpreted as representing the maximum rate of acidification due to CO_2 diffusing from serosal to mucosal fluid. Since this represents only a small per cent of the total H^+ excretion, no correction was made for the amount of CO_2 diffusing across the bladder in the acetazolamide experiment.

In the experiments measuring pH and pCO_2 in the bladder sacs, acetazolamide could have inhibited either H^+ excretion or HCO_3^- reabsorption as reported in the turtle bladder[12]. As mentioned above, the hydration of CO_2 is only catalyzed by carbonic anhydrase and even in the absence of this enzyme a finite amount of CO_2 will continue to be hydrated. The observed pCO_2 seen during the acetazolamide inhibition could come from two sources: (1) Metabolic CO_2 from the cell diffusing into the mucosal medium; (2) H^+ excretion, limited because hydration of CO_2 in the cell is proceeding at a slower rate and the intracellular H^+ limits the excretion. The H^+ excreted reacts with HCO_3^- in the mucosal medium to produce CO_2. Therefore, the difference in pCO_2 between the experimental and control period was treated as the pCO_2 arising from excreted H^+ reacting with HCO_3^- in the mucosal fluid. A correlation did not exist between the increase in pCO_2 and rate of acidification. This could conceivably result from the fact that each bladder was of a different weight; hence, a variable area for diffusion of CO_2.

Schilb and Brodsky[3] in a recent study on the acidification process of the urinary bladder of the turtle report that increases in mucosal pCO_2 are inevitable whenever the rate of leak of metabolic CO_2 from the cell to mucosal fluid exceeds

the concomitant rate of hydration of CO_2 in the mucosal fluid. They further provide evidence showing that if the serosal CO_2 is near zero levels then mucosal CO_2 would always exceed that of the serosal CO_2. However, because of the design of our experiments these arguments would not hold. In the case of our experiments each hemibladder served as its own control, the serosal solutions and CO_2 being the same in both the experimental and control periods except for the presence of acetazolamide. If the observed increase in pCO_2 in the mucosal fluid during the acidification process was due to the serosal to mucosal CO_2 gradient then the CO_2 increase should have been the same in both periods. One might argue that the treatment with acetazolamide could change the permeability of the mucosal membrane to CO_2, as reported by Enns[13] for the red cell membrane.

In the experiments given in Table III it was shown that there was not any stoichiometry between H^+ excretion and alkalization of the serosal medium These findings could be explained by the process of intracellular hydration of CO_2 and subsequent active transport of H^+ out of the cell. The HCO_3^- would then diffuse out of the cell down an electrochemical gradient into the serosal medium. If the active H^+ system were located at the mucosal side of the cell then the excreted H^+ would be buffered only by the mucosal Ringer solution. While on the other hand the HCO_3^- could be buffered both in the cell and in the serosal fluid leading to a deviation from the one to one stoichiometry. However, this same argument could be made for the situation in which H_2CO_3 is formed in the mucosal medium with subsequent dissociation and the reabsorption of HCO_3^- leaving the H^+ behind in the mucosal medium. It seems unlikely, however, that in our HCO_3^--free system there would be enough HCO_3^- formed in the unstirred mucosal layer to account for all of the acidification observed in our experiments.

The results showing that the H^+ excretion measured by titratable acidity is equivalent to that measured by change in pH demonstrates the absence of any titratable buffer (A^-) being secreted by the bladder into the mucosal medium during the acidification process. We have reported elsewhere[1,6] that the toad urinary bladder is capable of excreting NH_4^+. The values we obtained were always less than 25 % of the total H^+ production and whether they are or are not considered would not change the interpretation of the results presented here.

Ludens and Fanestil[14,15], using amiloride to block the active transport of Na^+ in the toad bladder, were able to demonstrate a reversal in polarity of the potential difference across this tissue They quantitated this observed effect by measuring a reverse short-circuit current. They were further able to demonstrate that this reverse short-circuit current was not due to the transport of Cl^-, that it was dependent on CO_2 in the serosal medium, it was not linked to active Na^+ reabsorption and it could be inhibited by acetazolamide. The conclusion reached from these studies was that there is an active excretion of H^+ into the mucosal medium by the toad urinary bladder. Their findings lend support to the findings we have reported in this communication.

Our earlier reports on the acidification of the mucosal fluid by the toad bladder demonstrated that the process was an active one. This study shows that the acidification process is accomplished by H^+ excretion as opposed to HCO_3^- reabsorption or selective excretion or reabsorption of a phosphate salt. We have further shown that this process is dependent on carbonic anhydrase activity.

ACKNOWLEDGEMENTS

The authors wish to thank Mr Robert C. Lipsey for his technical assistance in this work. This study was supported by a National Science Foundation Grant GB-17610.

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