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Action of Steroids on H^+ and NH_4^+ Excretion in the Toad Urinary Bladder*

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Summary. This study was done to determine if steroid compounds will stimulate the urinary bladder of the toad to increase its capacity to acidify the urine and excrete NH_4^+. Aldosterone, 17β-estradiol, dexamethasone, pregnenolone, and cholesterol were tested on the bladder. All compounds tested were found to stimulate the rate of acidification by the bladder, above that of a paired control hemibladder. In contrast, only the steroids aldosterone and 17β-estradiol were found to stimulate NH_4^+ excretion in the bladder. Cycloheximide was found to block the action of aldosterone on the NH_4^+ excretion, but did not have a significant effect on the stimulation of acidification by aldosterone. We conclude that steroids stimulate H^+ and NH_4^+ excretion in the toad urinary bladder. In addition, the NH_4^+ excretory system seems to be more specific to this effect than is the H^+ excretory system.

It has been shown by several laboratories that the toad urinary bladder is capable of excreting H^+ and NH_4^+ (Frazier & Vanatta, 1971; Ludens & Fanestil, 1972). In addition, Frazier and Vanatta (1972) have demonstrated that it is possible to stimulate H^+ and NH_4^+ excretion in the toad urinary bladder by using plasma and extracts of plasma from both toads and dogs in metabolic acidosis. This same activity was not present when the animals were in metabolic alkalosis. Ludens and Fanestil (1974) have demonstrated that aldosterone will stimulate the rate of acidification in the isolated urinary bladder.

Studies in humans and other animals suggest that mineralocorticoids play an important role in acid-base balance (Luke & Levitin, 1967; Seldin & Rector, 1972; Welbourne & Francoeur, 1977; Welbourne, 1974). A study by Kurtzman, White and Rogers (1971) demonstrated that aldosterone-deficient animals were unable to acidify urine maximally in response

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to either systemic acidosis or Na_2SO_4 infusion, while bicarbonate reabsorption was not impaired. Their results suggest that systemic acidosis associated with aldosterone deficiency was a consequence of impaired distal H^+ excretion. Since the toad urinary bladder is analogous to the mammalian distal nephron, the present studies were designed to study the effects of steroids on this isolated membrane system without the complexities of interactions among extracellular volume, serum K^+, tubular Na^+ reabsorption, and GFR which may be influenced by steroids.

In our present studies we found that H^+ excretion in the toad urinary bladder was stimulated by all of the steroids tested; aldosterone, 17β-estradiol, dexamethasone, pregnenolone, and cholesterol. NH_4^+ excretion was stimulated by the mineralocorticoid aldosterone and the sex steroid 17β-estradiol. Our results suggest that aldosterone increases the capacity of the bladder to acidify the mucosal solution by a direct effect on the H^+ excretory system. In addition, the NH_4^+ excretory capacity of the bladder is increased by aldosterone. These studies indicate that the H^+ excretory system of the toad bladder is much less specific in its reaction to steroid compounds than is the NH_4^+ excretory system.

Materials and Methods

The toads used in these experiments were *Bufo marinus* of either Colombian or Panamanian origin and were supplied by Charles P. Chase of Miami, Florida. Toads from both Colombia and Panama have been shown to have the ability to acidify the urine in our laboratory. 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, 1971, 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 ammonium was determined colorimetrically by the method of Chaney and Marbach (1962). 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. The Ringer's solution used contained in mM: NaCl, 114.5; KCl, 3.0; $CaCl_2$, 0.9; and sodium phosphate, 1.5; the final pH was 6.80. The aldosterone, 17β-estradiol, pregnenolone, dexamethasone, cholesterol, and cycloheximide were all obtained from Sigma Chemical Co., St. Louis, Mo. All statistics were performed as the mean difference on paired hemibladders using Student's one-tailed *t* test. The one-tailed test was used because the theory being tested was that the steroids stimulate H^+ and NH_4^+ excretion. If the difference were negative, regardless of the magnitude, this would reject the theory.

Paired hemibladders from toads which had been soaked 3–5 days in 120 mM NaCl were used as the in vitro assay system. The hemibladders were mounted between Lucite chambers, each of which held 2 ml. The cross sectional area of each chamber was 1.98 cm^2. In all experiments the bladders were in the open-circuited state. The serosal bath of the

experimental hemibladder contained a phosphate-buffered Ringer's solution to which had been added the respective steroid in a final concentration of 10^{-6}M. In one experiment, cycloheximide 5 mg/ml was added to the serosal solution with the aldosterone. All of the steroid compounds had to first be dissolved in absolute ethyl alcohol and then diluted with the Ringer's solution. The final concentration of the ethyl alcohol in the serosal chamber was 1%. The paired hemibladder contained only the phosphate-buffered Ringer's solution with 1% ethyl alcohol in the serosal chamber.

The mucosal chamber of both experimental and control hemibladders contained a 1.5 mM phosphate-buffered Ringer's solution. The flux period was for 120 min. Ammonium and pH were determined on each mucosal and serosal sample both before and after the flux period. The flux was started after a 30-min equilibration period, during which time the bladder was exposed to the respective steroid or cycloheximide.

In the experiment measuring the time course of the effect of aldosterone on H^+ excretion (Table 4) there was no equilibration period. The hormone was added to the serosal medium at time zero and the mucosal pH and NH_4^+ was determined every 30 min thereafter for a period of 150 min. This was done by draining the mucosal medium at each 30-min interval and replacing with fresh Ringer's solution. H^+ excretion was calculated in the same way as given above.

In a separate group of experiments, plasma levels of aldosterone were determined on three different groups of toads. One group of ten toads was in normal acid-base balance. The second group of ten toads was placed in metabolic acidosis by gavaging with six 8-ml doses of 120 mM NH_4Cl over a 48 hr period. The third group of ten toads was soaked in 120 mM NaCl for five days. Blood specimens were obtained at the time of sacrifice by cardiac puncture. Plasma aldosterone levels were then determined by radioimmunoassay technique according to the method of Chakmakjian, Pryor and Abraham (1974). The 1,2-^{3}H-aldosterone was obtained from New England Nuclear Corp., Boston, Mass.

Results

To determine if aldosterone has an effect on H^+ and NH_4^+ excretion, the bladder was exposed to 10^{-6}M aldosterone. Table 1 shows the results of this experiment. It is clear from this experiment that aldosterone

Table 1. Effect of aldosterone on H^+ and NH_4^+ excretion in toad urinary bladder (excretion in nmol/100 mg bladder × min)

Serosal solution	H^+ excretion[a]	Mean diff. ± SEM	NH_4^+ excretion[a]	Mean diff. ± SEM
PO_4-buffered Ringer	12.33		0.84	
		12.53 ± 3.20 ($P < 0.002$)[b]		0.55 ± 0.22 ($P < 0.025$)[b]
PO_4-Buffered Ringer + 10^{-6}M aldosterone	24.86		1.39	

[a] Average excretion of 12 paired hemibladders.

[b] Calculated from the mean difference.

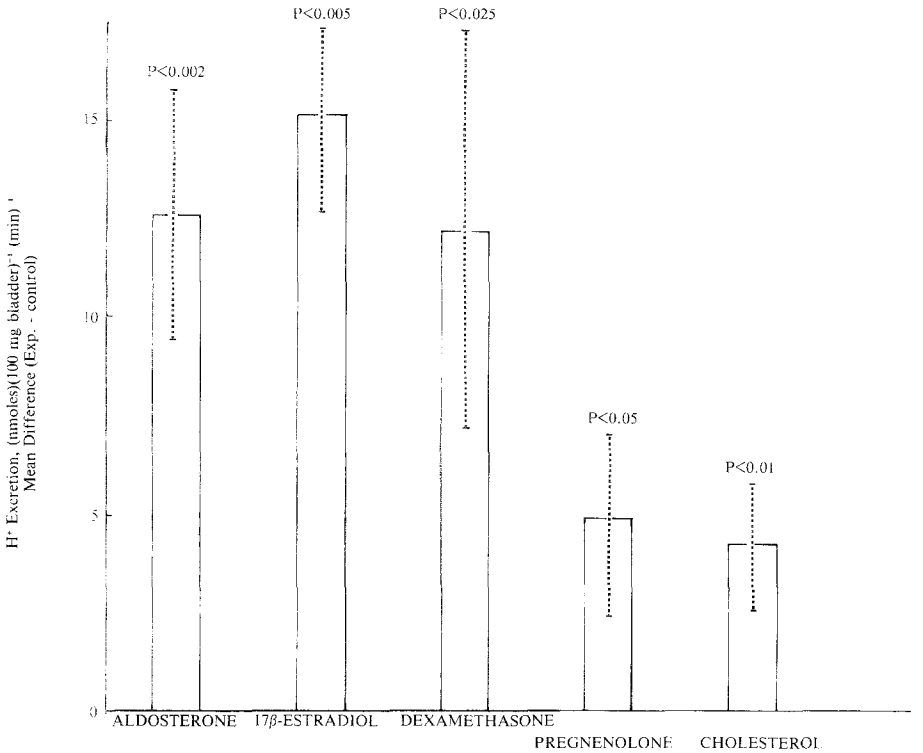

Fig. 1. The effect of steroids on H^+ excretion. The units of excretion are given as the mean difference between paired hemibladders (experimental-control). All experiments were carried out with the bladder stretched between two Lucite chambers, each of which held 2 ml in volume. The cross-sectional area of the bladder exposed in the chamber was 1.98 cm^2. Each of the respective steroids was added to the serosal chamber in a final concentration of 10^{-6} M. Each excretion period was 120 min in duration. The excretion period was started after a 30-min pre-incubation period, during which time the bladder was exposed to the steroid. The vertical bars represent the SEM of the mean difference. $N=12$ for aldosterone and cholesterol experiments. $N=8$ for the estradiol and $N=10$ for the pregnenolone and dexamethasone experiments. P indicates the probability of the mean difference being different from zero

stimulated both H^+ ($P<0.002$) and NH_4^+ ($P<0.025$) excretion. There was a 100% increase in H^+ excretion by aldosterone and approximately a 60% increase in NH_4^+ excretion.

It has been reported that the effect of aldosterone on Na^+ transport in toad bladder is nonspecific for a particular steroid (Porter & Edelman, 1964; Feldman, Funder & Edelman, 1972). We studied four other steroid compounds to determine if this same phenomenon was true for H^+ and NH_4^+ excretion. Shown in Fig. 1 is the mean difference (experimental-control) in H^+ excretion when four other steroids were tested. All of

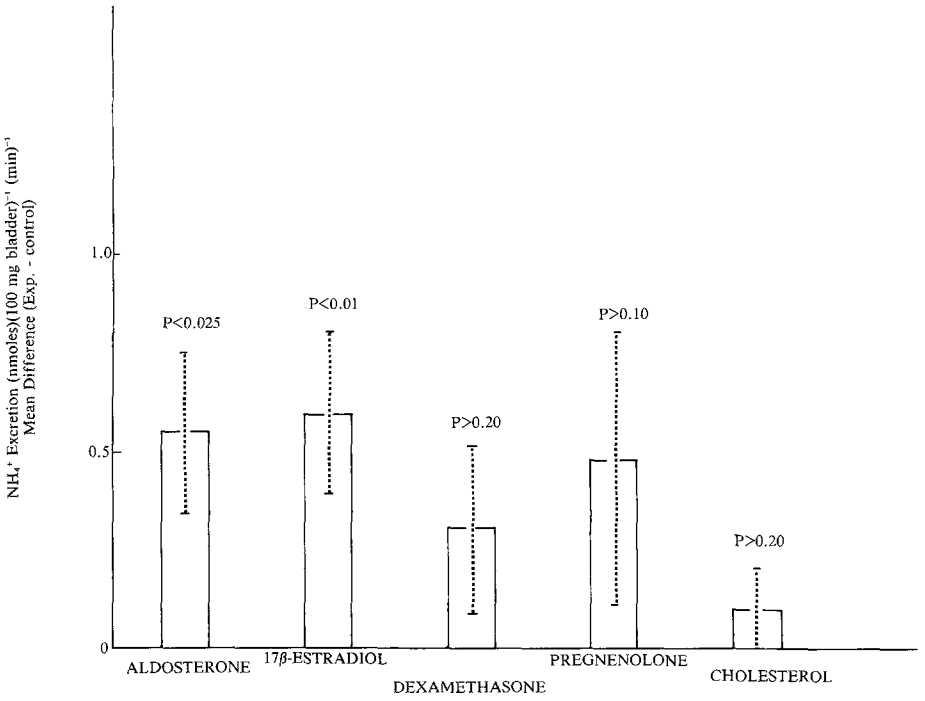

Fig. 2. The effect of steroids on NH_4^+ excretion. Vertical bars represent the SEM difference. *See* legend to Fig. 1 for experimental details

the steroids were effective in stimulating H^+ excretion. Dexamethasone and 17β-estradiol were as effective as aldosterone, while the effect of pregnenolone and cholesterol had less than half the stimulatory effect of aldosterone, but still had a significant effect.

In Fig. 2 the effect of these same four steroids is shown on NH_4^+ excretion. Only 17β-estradiol was effective in stimulating NH_4^+ excretion ($P < 0.01$). These results suggest that the NH_4^+ excretory system is more specific to the mineralocorticoid type response than is the H^+ excretory system.

It seemed important to determine if *de novo* protein synthesis was required for the response observed with aldosterone. It has been previously determined that *de novo* protein synthesis is required for the aldosterone effect on Na^+, metabolism in toad urinary bladder (Sharp & Leaf, 1966). Shown in Table 2 are the results of an experiment using cycloheximide. This compound inhibits amino acid transfer from aminoacyl RNA to the polypeptide chains during protein synthesis. It can be seen that cycloheximide alone has no effect on either H^+ or NH_4^+ excretion. When the cycloheximide was added to the serosal medium with aldoste-

Table 2. Effect of aldosterone on H^+ and NH_4^+ excretion in the presence of cycloheximide (excretion in nmol/100 mg bladder × min)

Serosal solution	H^+ excretion (expt. − control) ± SEM	P^c	NH_4^+ excretion (expt. − control) ± SEM	P^c
Aldosterone[a] (10^{-6}M)	12.53 ± 3.20	< 0.002	0.55 ± 0.22	< 0.025
Cycloheximide[b] (5 μg/ml)	1.12 ± 2.17	> 0.40	−0.07 ± 0.19	> 0.40
Aldosterone + cycloheximide[b]	4.44 ± 1.81	< 0.025	−0.04 ± 0.21	> 0.50

[a] $N = 12$ paired hemibladders.
[b] $N = 10$ paired hemibladders.
[c] Calculated from the mean difference.

Table 3. Dose-response analysis of the effect of aldosterone on H^+ and NH_4^+ excretion (excretion in nmol/100 mg bladder × min)

Aldosterone concentration (serosal solution)	H^+ excretion (expt. − control)	P^a	NH_4^+ excretion (expt. − control)	P
10^{-6}M[b]	12.53 ± 3.20	< 0.002	0.55 ± 0.22	< 0.025
10^{-7}M[c]	8.33 ± 2.95	< 0.025	0.35 ± 0.37	NS
10^{-8}M[c]	3.53 ± 1.96	NS[d]	0.31 ± 0.19	NS
10^{-9}M[c]	−6.28 ± 2.95	NS	0.11 ± 0.04	NS

[a] Calculated from the mean difference.
[b] $N = 12$ paired hemibladders.
[c] $N = 8$ paired hemibladders.
[d] NS = nonsignificance ($P > 0.05$).

rone there was a reduction in the stimulatory response observed with aldosterone. However, there was still a significant stimulation of H^+ excretion observed even in the presence of cycloheximide ($P < 0.025$). In contrast to this, the cycloheximide blocked completely the stimulatory aldosterone response in NH_4^+ excretion ($P < 0.50$).

A dose-response analysis was performed for the aldosterone effect and the results are shown in Table 3. The concentration of aldosterone was varied from 10^{-6} to 10^{-9} M in the serosal media. ΔH^+ and ΔNH_4^+ excretion values represent the mean difference between paired hemibladders, one receiving aldosterone and the paired hemibladder receiving no aldosterone. The P values represent the probability that the ΔH^+ or NH_4^+ excretion is different from zero. The results show for H^+ excretion that (i) there is a significant increase at 10^{-6} and 10^{-7}M; (ii) at 10^{-8}M there is a positive but insignificant effect; (iii) at 10^{-9}M

Table 4. The time course of the effect of aldosterone on H^+ excretion in toad urinary bladder

Time after addition of aldosterone (10^{-6} M) to serosal medium[a]	H^+ excretion[b] (expt. − control) ± SEM	*P* value
30 min	0.52 ± 2.53	NS
60 min	3.13 ± 2.17	NS
90 min	5.27 ± 2.32	< 0.025
120 min	4.78 ± 1.13	< 0.005
150 min	3.75 ± 1.16	< 0.005

[a] Aldosterone was added to the serosal medium at time zero and the mucosal sample collected and pH determined at 30-min intervals thereafter.

[b] Each value represents the mean difference ± SEM of 8 paired hemibladders for that 30-min excretion period.

Table 5. Plasma aldosterone levels of normal, acidotic, and saline-soaked toads

Condition of toad	Plasma aldosterone[a] (μg/100 ml)	*P* value[b]
Normal	0.68 ± 0.09	
Metabolic acidosis (48 hr)	0.79 ± 0.14	> 0.50
Saline soaked (5 days)	0.14 ± 0.04	< 0.001

[a] Values are the average ± SEM of 10 toads.

[b] Probability of that group being different from the normal group (two-tailed Student's *t*-test).

there was no apparent effect. For NH_4^+ excretion there was a significant stimulation only at the 10^{-6} M concentration.

In Table 4 is shown the time course of the effect of aldosterone on H^+ excretion. Aldosterone was added at time zero. The first 30-min period is the same as the equilibration period in all of the previous experiments, and it is clearly evident there was no stimulation of H^+ excretion during this period. The next 30-min period revealed an increase in H^+ excretion but not a significant increase. A significant increase did occur at 90 min ($P < 0.025$), and this appeared to be the peak effect as the 120 and 150-min periods did not increase further and remained essentially the same as the 90-min excretion rate.

If aldosterone is important in the overall acid-base balance of the toad, then one might assume that the blood levels of aldosterone would

be elevated during metabolic acidosis. Plasma aldosterone levels were determined on three groups of toads and are shown in Table 5. Toads in metabolic acidosis had a slightly higher plasma aldosterone level than normal toads, but the increase was not significant ($P > 0.50$). However, the toads which were soaked in saline for four days, like the toads used in our assay for the previous studies, showed a significant reduction in plasma aldosterone levels ($P < 0.001$).

Discussion

Frazier and Vanatta (1971) and Ludens and Fanestil (1972) reported the toad bladder excretes H^+ and NH_4^+, and Frazier and Vanatta (1971, 1972) further reported that the rate of excretion of both of these ions is increased when the animal is in acidosis. By keeping the toads in 120 mM NaCl for 3–5 days, the plasma aldosterone level was reduced, and this allows a greater response to exogenous aldosterone (Crabbé, 1961), and presumably to other steroids. In other studies we were unable to elicit an effect of aldosterone on H^+ and NH_4^+ excretion when the toads were not prepared by soaking in NaCl solution.

As stated above, the toad bladder increased H^+ and NH_4^+ excretion in response to metabolic acidosis. This study was an attempt to determine whether aldosterone might be a contributing factor to causing these observed increases, and if so, if it has a specific effect. We chose not to run the bladders in a short-circuited state for two reasons: (i) we have previously shown (Frazier & Vanatta, 1971) that short-circuiting the bladder increased the rate of H^+ excretion, and we did not feel that this effect should be superimposed on the hormonal response; (ii) this study was designed to determine if the observed increases in H^+ and NH_4^+ excretion could be a result of aldosterone or other steroid hormones and not an attempt to elucidate the biophysical mechanism by which the steroid might increase this excretion.

It might be argued that the effect on H^+ excretion we report is secondary to the stimulating effect on Na^+ transport reported by Sharp and Leaf (1966) and Crabbé (1961). We argue that the effect of steroids on the H^+ and NH_4^+ excretion is a direct effect for the following reasons: (i) it has been shown that there is not a direct link between either the potential difference or Na^+ reabsorption and H^+ excretion in toad bladder (Frazier, 1974); (ii) increasing Na^+ transport in the toad

bladder by administration of vasopressin has no effect on H^+ excretion (Frazier, 1974); (iii) the lag time for an effect on Na^+ metabolism by aldosterone is 60–90 min with the maximal effect after 120 min (Sharp & Leaf, 1966), whereas we found the maximal effect for H^+ excretion between 60–90 min. However, one must exercise caution in comparing lag time of the aldosterone response using different subspecies of toads as revealed in the study by Davies, Martin and Sharp (1968). It should be noted that our study does not eliminate the possibility of changes in intracellular increases in K^+, H^+, or NH_4^+ that may be steroid dependent. Other experiments with more stringent criteria must be performed before this question can be answered.

In the experiments given in Fig. 1 it is apparent that aldosterone is not a specific stimulator of H^+ excretion. Dexamethasone and 17β-estradiol were equally effective, and even the steroids pregnenolone and cholesterol significantly elevated H^+ excretion. On the other hand, the NH_4^+ excretory system responded only to aldosterone and 17β-estradiol (Fig. 2), showing a much greater specificity for steroid action. This low specificity observed for the H^+ excretory system is similar to that observed by Porter and Edelman (1964) and Handler, Preston and Orloff (1969) for the steroid effect on Na^+ transport across the toad bladder. The fact that the NH_4^+ excretory system is more specific to the actions of steroid suggests that this system may be responsive to a different set of receptors than those responsible for stimulation of H^+ excretion. Using the receptor type classification put forth by Funder, Feldman and Edelman (1973a, b) it would appear that the NH_4^+ excretory system is activated by the Type I mineralocorticoid receptor, while the H^+ excretory system is activated by the Type II glucocorticoid receptor. In considering the specificity of the steroids, caution should be exercised. The data on pregnenolone with its large scatter, could be masking a weak effect. More work in this area should be done before a conclusive statement is made.

The evidence presented in this study is consistent with the concept that aldosterone increases the bladder's capacity to excrete H^+. This is similar to findings reported by Ludens and Fanestil (1974) in which they demonstrated stimulation of reverse short-circuit current in the toad bladder by aldosterone. In addition, we have shown that other steroids have this same effect and that NH_4^+ excretion is also stimulated by aldosterone and 17β-estradiol. It should be noted that there are three inconsistencies in our present study with regard to the findings of Ludens and Fanestil (1974). H^+ excretion was not stimulated by 17β-estradiol,

and secondly, it was found that cycloheximide did increase urinary acidification in the report by Ludens and Fanestil (1974). Thirdly, they reported the maximum stimulation by aldosterone at 5 or 6 hr, while our time-course studies showed the maximum effect between 60–90 min. Ludens and Fanestil measure acidification by reversed short-circuit current, while we measure acidification by change in pH. It is possible that we are measuring two different acidification mechanisms, but this is only speculation at the present time. The reason for these apparent inconsistencies is not apparent and will have to await more thorough investigative studies.

In considering the overall relevance of these findings to the physiology of the acid-base balance in the toad requires (i) that the dose-response study for the effect of aldosterone be within the physiological range; and (ii) some degree of hormonal specificity should exist. In the case of H^+ excretion, we found that the minimal effective dose was 10^{-7}M. This is higher than that found by Ludens and Fanestil (1974), who reported 3.5×10^{-8}M as the minimally effective dose. The reason for this discrepancy is not apparent at this time. Aldosterone at 10^{-7}M is nearly an order of magnitude different than the plasma levels observed when the toad was in a metabolic acidosis (approximately 4×10^{-8}M). This indicates that aldosterone may not reach levels during acidosis that would increase H^+ excretion by the bladder. In view of the fact that the H^+ excretory system reacts to all of the steroids tested, it is possible that during metabolic acidosis there is a generalized release of steroids leading to an increase in total circulating steroids, which stimulates H^+ excretion in the toad urinary bladder. This is only speculation at the present time and would have to await more quantitative data on plasma steroid levels in the toad during acidosis. It is of interest to note that a recent report by Quintanilla *et al.* (1978) has shown an increase in both aldosterone and cortisol in dogs during metabolic acidosis.

With regard to NH_4^+ excretion it appears that the minimal effective dose of 10^{-6}M is well below the plasma level observed during metabolic acidosis. It is of interest to note that the sex steroid 17β-estradiol was as active in stimulating NH_4^+ excretion as was aldosterone. We were not able to find any reported data on plasma levels of sex steroids in the toad to determine if 10^{-6}M was in the physiological range. An interesting report by Dumm, Leslie and Ralli (1955) demonstrated that in the rat kidney female sex steroids could stimulate NH_4^+ excretion. It is possible that the sex steroid estradiol could be important in the overall control of NH_4^+ excretion or perhaps be synergistic with the action of aldosterone.

The experiments using cycloheximide show that the stimulation of H^+ excretion by aldosterone was reduced but not eliminated by cycloheximide. This suggests that there might be two mechanisms by which excretion is stimulated, one involving protein synthesis and one which does not. On the other hand, NH_4^+ excretion was blocked completely by cycloheximide. This suggests that NH_4^+ excretion and Na^+ reabsorption might possibly be mediated via similar metabolic pathways.

In the study on the time course of the effect of aldosterone on H^+ excretion, it is clear that the peak effect occurred between 60 and 90 min. The peak effect of aldosterone on Na^+ transport is reported to be 3–4 hr after the addition of aldosterone (Sharp & Leaf, 1966). This would lend support to the concept that the aldosterone action is a direct effect on the H^+ excretory mechanism and not an indirect effect produced by changes in Na^+ metabolism. It would be helpful to know the time course for the effect on NH_4^+ excretion. This was attempted at the same time we measured the effect on H^+ excretion. However, the methodology available for NH_4^+ determination is not sensitive enough to detect with reliability the small differences in the amount of NH_4^+ excretion between the experimental and control in a 30-min excretion period. We therefore did not include the data on the time course of NH_4^+ excretion.

In summary, the present study has demonstrated that aldosterone stimulates H^+ excretion in the toad urinary bladder. This is in agreement with earlier reports by Ludens and Fanestil (1974). In addition, we have shown that aldosterone also stimulates NH_4^+ excretion in the urinary bladder. H^+ excretion was also stimulated by dexamethasone, 17β-estradiol, pregnenolone, and cholesterol, indicating a low specificity to mineralocorticoid type activity for the H^+ excretory system. Conversely NH_4^+ excretion was stimulated only by aldosterone and 17β-estradiol indicating a higher specificity for mineralocorticoid activity. It is not apparent from this study if aldosterone or the other steroids are important in the overall acid-base balance by the toad.

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