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Stimulation of Urinary Acidification by Aldosterone and Inhibitors of RNA and Protein Synthesis

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Summary. Urinary acidification by the urinary bladder of the toad (*Bufo marinus*) was stimulated, relative to control, by the *in vitro* addition of aldosterone (10^{-7} M), actinomycin D (20 μg/ml), puromycin (80 μg/ml) or cycloheximide (5 μg/ml). The action of the inhibitors of RNA or protein synthesis was not additive with that of aldosterone. This is opposite to the situation with Na^+ transport, where the stimulation by aldosterone is abolished by the same concentrations of these inhibitors. That all agents enhanced urinary acidification was verified by: (i) measurement of RSCC (reverse short-circuit current) in the absence of Na^+ transport, (ii) inhibition of RSCC by acetazolamide, an inhibitor of carbonic anhydrase, and (iii) direct measurement of the pH change of the mucosal (urinary) fluid. As in the case of Na^+ transport, spirolactone inhibited the action of aldosterone. Although not a unique model, the apparent paradoxical mimicry of aldosterone's stimulation of urinary acidification may be explained by a model which includes action of aldosterone and the inhibitors via their known effects on RNA and protein synthesis.

Stimulation of the active transport of Na^+ and H^+ by aldosterone has been demonstrated in urinary bladders from both anurans (Crabbé, 1961; Ludens & Fanestil, 1974) and reptiles (LeFevre, 1973; Al-Awqati *et al.*, 1976). A large body of evidence indicates that the stimulation of Na^+ transport is blocked (i) by spirolactones (Porter, 1968; Sakauye & Feldman, 1976), acting as competitive inhibitors of aldosterone binding to the mineralocorticoid receptor (Fanestil, 1968; Marver *et al.*, 1974), (ii) by inhibitors of RNA synthesis, such as actinomycin D (Porter, Bogoroch & Edelman, 1963; Fanestil & Edelman, 1966) and cordycepin (Chu & Edelman, 1972), and (iii) by inhibitors of protein synthesis, such as cycloheximide and puromycin (Porter, Bogoroch & Edelman, 1963; Fanestil & Edelman, 1966). Thus, aldosterone's mechanism of action on

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the active transport of Na^+ resembles the mechanism of action of other steroid hormones. In contrast, the stimulation of the transport of H^+ by aldosterone has not been studied extensively for its dependence upon steroid-receptor interaction or upon RNA and protein synthesis. Indeed, our earlier study (Ludens & Fanestil, 1974) reported an apparent paradox: the inhibitor of protein synthesis, cycloheximide, appeared to mimic rather than block the effect of aldosterone. In that study, we found that both aldosterone and cycloheximide stimulated RSCC (reverse short-circuit current); however, we did not demonstrate that cycloheximide-stimulated RSCC was reflected by enhanced urinary acidification.

The studies reported here were conducted to examine this apparent paradox by determining if aldosterone's stimulation of H^+ transport is: (i) mediated via mineralocorticoid receptors, and (ii) altered by inhibitors of RNA and protein synthesis. Moreover, since we found that all tested inhibitors of RNA or protein synthesis mimicked aldosterone's action on RSCC, we also demonstrated that the inhibitors stimulate urinary acidification by direct measurement of the pH change in the mucosal (urinary) fluid.

Materials and Methods

Toads (*Bufo marinus*) used for these studies originated in Colombia or Nicaragua (obtained from Pet Farm, Inc., Miami, Florida) or in Mexico (obtained from Mogul-Ed, Oshkosh, Wisconsin). Toads were maintained at room temperature on peat moss moistened with tap water. In those studies in which bladders were not preincubated overnight prior to experimentation, the toads were maintained partially submerged for 3–5 days in a 120 mM NaCl solution in order to suppress the effects of endogenous aldosterone (Crabbé, 1962). In other studies, the effects of endogenous aldosterone were suppressed by overnight incubation (preincubation) (Porter & Edelman, 1964) in amphibian-Ringer's solution, which was fortified with 10 mM glucose and contained penicillin sodium (1,400 U/ml) and streptomycin sulfate (1.5 mg/ml). The actinomycin D, antimycin A, cycloheximide, and puromycin were obtained from Sigma Chemical Company, St. Louis, Missouri; D-aldosterone was purchased from Calbiochem, La Jolla, California; acetazolamide from K and K Laboratories, Inc., Hollywood, California. Amiloride was provided by Merck, Sharp and Dohme, West Point, Pennsylvania.

Studies with aldosterone and/or inhibitors of RNA and protein synthesis in preincubated urinary bladders were conducted using a modification of the method of Ussing and Zerahn (1951). Bladders removed from double-pithed toads were mounted as quarterbladders on Lucite chambers with a surface area of either 2.7 or 3.5 cm^2. During the experiment, the incubation solutions (4 ml on each side of the bladder) consisted of a modified Ringer's solution containing (in mM) NaCl, 88; $NaHCO_3$, 24; KCl, 3.6; $CaCl_2$, 2.8; glucose, 5. The pH of this medium, when gassed with 5% CO_2–95% O_2, was 7.25–7.40. Inhibiting Na^+ transport with 0.1 mM amiloride in the mucosal solution (Ludens & Fanestil, 1974) reversed the membrane potential. The reverse short-circuit current (RSCC) required to

null this potential was monitored throughout the experiment. The tissues were short-circuited continuously, except for brief intervals during which the spontaneous potential difference was measured. A stabilization period of 30–60 min after addition of amiloride preceded the simultaneous addition of steroid and/or inhibitors to both the mucosal and serosal bathing media.

Previous studies indicated that the rate of acidification in isolated toad bladder as measured by pH changes of HCO_3^-/CO_2 buffered Ringer's solution was enhanced by a transepithelial potential difference (serosal side of bladder positive) (Ziegler, Fanestil & Ludens, 1976). To compare effects of aldosterone with inhibitors of RNA and protein synthesis on the rate of acidification in the presence of a transepithelial potential difference, bladders were removed from double-pithed toads and mounted as hemibladders on modified Lucite chambers. With the modified chamber, hemibladders (surface area = 5.5 cm^2) were bathed by 2.4 ml of Ringer's solution on the mucosal side and 8 ml on the serosal side. The mucosal and serosal solutions consisted of a Ringer's solution containing (in mM): NaCl, 111; $NaHCO_3$, 2.4; KCl, 3.6; $CaCl_2$, 2.8; glucose, 5. The pH of these solutions when gassed with 1% CO_2–99% O_2 was 6.75 to 7.10. The mucosal solution also contained 0.1 mM amiloride to produce RSCC by elimination of sodium transport. Following a 30-min stabilization period, aldosterone and/or the inhibitors were added to both the mucosal and serosal solution, and RSCC was monitored for the succeeding 5 hr. During this period, all tissues were short-circuited continuously, except for brief intervals during which the spontaneous potential difference was measured. When both steroid and inhibitor were added to the same bladder, they were added simultaneously. Preceding the final RSCC measurement, e.g., 5 hr after additions of steroid and/or inhibitor, mucosal and serosal solutions were removed and replaced with fresh solutions of identical composition. Immediately after the solution change pH measurements (mucosal and serosal) were made, and all bladders were voltage-clamped at 50 mV (serosal side positive) for the ensuing 3 hr, e.g., from 5 to 8 hr after addition of steroid or inhibitor. Following the voltage-clamped period, final pH measurements were made, and in some experiments a final RSCC was measured. In these experiments in which initial HCO_3^- concentration was known and P_{CO_2} remains constant throughout the experiment, acidification of the mucosal solution was assessed by calculating HCO_3 loss (Δ HCO_3) from ΔpH. Acidification rate was expressed as ΔHCO_3^-/100 mg of bladder wet wt/hr. Data were analyzed statistically with the Student t test by the use of paired comparisons. The 0.05 level of probability or less was used as the criterion of significance.

Results

Effect of Puromycin in Pre-Incubated Tissues

As reported previously (Ludens & Fanestil, 1974), pre-incubation for 16–20 hr (overnight) results in the loss of RSCC and hydrogen ion transport. The RSCC of such tissues is stimulatable by aldosterone and by cycloheximide (Ludens & Fanestil, 1974). Figure 1 shows that puromycin also stimulates the RSCC in pre-incubated tissues. The stimulation of RSCC produced by puromycin was not additive with that produced by aldosterone (Fig. 1). Thus, cycloheximide and puromycin do not mere-

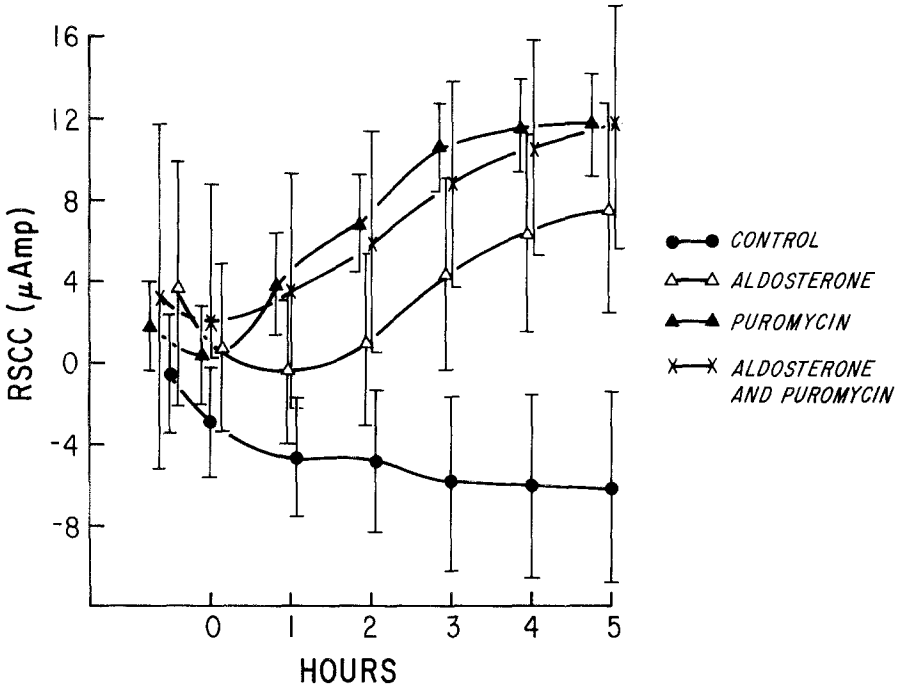

Fig. 1. Effect of puromycin on aldosterone stimulation of RSCC. Bladders from seven animals were pre-incubated overnight as quarter-bladders, provided fresh solutions, and treated with 10^{-4} M amiloride in the mucosal solutions prior to the addition of aldosterone (10^{-7} M), puromycin (80 μg/ml), or the appropriate diluent. The RSCC in the control tissue after 5 hr is significantly different from that in the other three quarter-bladders (paired analysis), while the three treated conditions are not significantly different from one another

ly "stabilize" some component of the RSCC system, but actually stimulate RSCC under appropriate experimental circumstances.

Because the rates of acidification in such incubated tissues are low, it is not possible to measure changes in the rate of acidification with precision. Therefore, all subsequent studies we report here were initiated on hemibladders within an hour after removal from animals which had been submerged in saline to suppress the endogenous production of aldosterone. The rate of acidification of the mucosal solution was also augmented by imposition of a 50-mV transepithelial potential difference (serosa positive). The combination of a larger RSCC in the fresh tissues and the favorable transepithelial potential difference resulted in greater acidification of the mucosal fluid, making measurement of changes in the rate of acidification more precise.

Table 1. Effect of spirolactone on aldosterone stimulation of RSCC

Condition	$RSCC_0$	$RSCC_6$	T_6/T_0
Control	32±7.0	24±3.0	0.82±0.05
Aldosterone (0.5 μM)	32±4.0	31±4.3	0.96±0.03[a]
Spirolactone (50 μM)	31±5.5	23±3.7	0.75±0.04
Aldosterone and spirolactone	32±5.1	23±3.5	0.77±0.05

[a] Significantly different from the other three conditions. Eleven bladders from saline soaked toads were incubated as quarter-bladders for 6 hr with the indicated concentrations of hormone, antagonist, or diluent. The subscripts refer to time in hours, zero being just prior to the additions. Other methods are described in the text. RSCC values are microamperes.

Effect of Spirolactone on RSCC

The spirolactone, SC 14266, at a concentration 100 times that of aldosterone produced complete blockage of the effect of aldosterone on RSCC (Table 1). The finding that SC 14266 in the absence of added aldosterone produced no suppression of the RSCC, when compared with control tissues, suggests that the agent is acting as an antagonist to aldosterone rather than as a nonspecific inhibitor of RSCC.

Effect of an Inhibitor of RNA Synthesis on RSCC

Actinomycin D (20 μg/ml) produced a stimulation of RSCC, relative to control, as shown in Fig. 2. The effect becomes apparent after 4 to 5 hr. The time course of this effect of actinomycin D is similar to that of aldosterone under these conditions (*see* Fig. 2 in Ludens & Fanestil, 1974). As shown in Table 2, the stimulation of RSCC produced by actinomycin D was not additive with that produced by aldosterone.

Effect of Aldosterone and Inhibitors on RSCC and Acidification

Quantitation of the rate of urinary acidification was achieved by measurement of the change in pH of the mucosal fluid in the presence of a favorable 50-mV (serosa positive) transepithelial potential difference, as described in *Materials and Methods*, after hemibladders were incubated

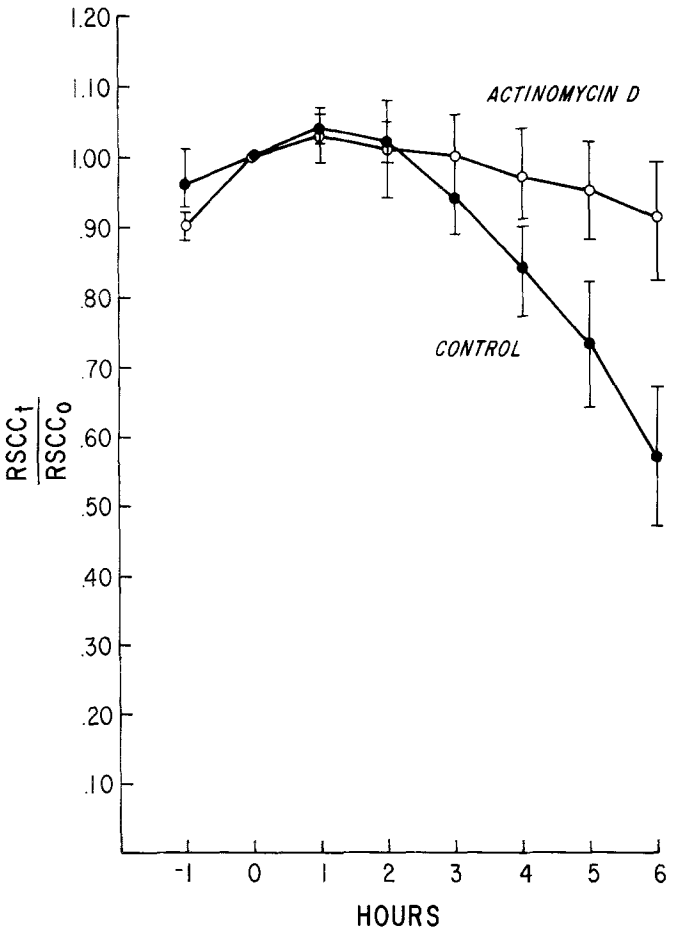

Fig. 2. Effect of actinomycin D on RSCC. The RSCC is expressed as a ratio of the RSCC at any time, *t*, to the RSCC just prior to the addition of actinomycin D (20 μg/ml) or diluent. The absolute RSCC values at zero and 6 hr are shown in Table 2. Other experimental details are in the legend to Table 2

Table 2. Effect of actinomycin D and aldosterone on RSCC

	$RSCC_0$	$RSCC_6$	T_6/T_0	*P*
Control	27±3.8	14±2.4	0.57±0.11	–
Aldosterone	27±3.1	20±2.7	0.78±0.10	0.05
Actinomycin D	27±3.0	23±2.2	0.91±0.08	0.01
Aldosterone plus actinomycin D	26±4.3	21±3.0	0.92±0.12	0.05

Tissues from 12 animals were incubated as quarter-bladders for 6 hr with 10^{-7} M aldosterone, 20 μg/ml actinomycin D or the appropriate diluents. The subscripts refer to time in hours, zero being just prior to the additions. RSCC values are microamperes. The *P* values refer to difference from control.

with the steroid or inhibitors for 5 to 7 hr to allow the effect of the agents on RSCC to develop.

The effect of 10^{-7} M aldosterone on RSCC after 5 and 8 hr and upon the acidification of the mucosal fluid between 5 and 8 hr is shown in Table 3. The hormone's stimulation of RSCC was statistically significant at 8 hr. Moreover, the hormone-treated hemibladders acidified the mucosal solution to a greater extent than did the control hemibladders by 0.52 ± 0.21 μM/hr/100 mg ($P = 0.05$).

The effects of actinomycin D (Table 4), puromycin (Table 5), and cycloheximide (Table 6) were all similar to one another and to aldosterone. In each case, the RSCC was significantly stimulated by the inhibitor, compared with control, and the extent of acidification of the mucosal solution was greater in the inhibitor-treated tissues than in control. Note that in the case of the puromycin-treated tissues (Table 5), the rate of acidification was measured in the presence of the 50-mV potential prior

Table 3. Stimulation of RSCC and urinary acidification by aldosterone

Time	Parameter	Aldosterone	Control	Δ	P
t_0	RSCC	21 ±2.2	21 ±1.8	0	NS
t_5	RSCC	16 ±2.1	12 ±1.8	3.0 ±1.6	NS
t_5/t_0	ratio	0.77 ±0.06	0.61 ±0.07	0.16 ±0.08	NS
t_5 to t_8	Clamp 50 mV serosa positive				
t_5 to t_8	ΔHCO_3^-	1.99 ±0.26	1.47 ±0.26	0.52 ±0.21	0.05
t_8	RSCC	12 ±1.5	6 ±2.4	7 ±1.8	0.01

Bladders from 16 saline-soaked toads were incubated for measurement of mucosal acidification (*see Materials and Methods*). RSCC values are microamperes, ΔHCO_3^- values are micromoles $\times hr^{-1} \times 100\ mg^{-1}$. Subscripts refer to time in hours, zero being just prior to addition of aldosterone (10^{-7} M) or diluent. The P values compare aldosterone to control-treated tissues. NS is not statistically significant.

Table 4. Stimulation of RSCC and urinary acidification by actinomycin D

Time	Parameter	Actinomycin D	Control	Δ	P
t_0	RSCC	31 ±4.9	30 ±5.0	1 ±2.8	NS
t_5	RSCC	25 ±4.2	17 ±6.6	8 ±4.1	NS
t_5/t_0	ratio	0.78 ±0.09	0.59 ±0.11	0.19 ±0.08	0.05
t_5 to t_8	Clamp 50 mV serosa positive				
t_5 to t_8	ΔHCO_3^-	2.03 ±0.36	1.44 ±0.26	0.59 ±0.21	0.025

Bladders from 10 saline-soaked toads were incubated for measurement of mucosal acidification (*see Materials and Methods*). RSCC values are microamperes; ΔHCO_3^- values are μmoles $\times hr^{-1} \times 100\ mg^{-1}$. Subscripts refer to time in hours, zero being just prior to the addition of actinomycin D (20 μg/ml) or ethanol diluent. NS is not statistically significant.

Table 5. Stimulation of RSCC and urinary acidification by puromycin

Time	Parameter	Puromycin	Control	Δ	P
t_{-3}	RSCC	16 ±3.2	17 ±3.0	0.6	NS
t_{-3} to t_0	Clamp at 50 mV serosa positive				
t_{-3} to t_0	ΔHCO_3^-	1.01±0.22	1.02±0.23	0.01	NS
t_0	Addition of puromycin or diluent				
t_4	RSCC	12 ±2.7	0 ±2.7	11 ±3.8	0.01
t_4 to t_7	Clamp at 50 mV serosa positive				
t_4 to t_7	ΔHCO_3^-	0.70±0.21	0.07±0.16	0.63±0.22	0.01

Bladders from nine saline-soaked toads were incubated for measurement of mucosal acidification (*see Materials and Methods*). RSCC values are microamperes $\times 100\ mg^{-1}$. ΔHCO_3^- values are $\mu moles \times 100\ mg^{-1} \times hr^{-1}$. Subscripts refer to time in hours, zero being just prior to addition of puromycin (20 μg/ml) or diluent. NS is not statistically significant. The P values refer to difference from control.

Table 6. Stimulation of RSCC and mucosal acidification by cycloheximide

Time	Parameter	Cycloheximide	Control	Δ	P
t_0	RSCC	21 ±5.4	19 ±3.7	2 ±3.6	NS
t_5	RSCC	18 ±5.9	8 ±2.0	11 ±4.3	0.05
t_5/t_0	ratio	0.83±0.06	0.42±0.08	0.41±0.10	0.01
t_5 to t_8	Clamp at 50 mV serosa positive				
t_5 to t_8	ΔHCO_3^-	1.57±0.21	0.93±0.17	0.64±0.16	0.01
t_8	RSCC	13 ±2.4	1 ±1.8	12 ±2.1	0.005

Bladders from seven saline-soaked toads were incubated for measurement of mucosal acidification (*see Materials and Methods*). RSCC values are microamperes $\times 100\ mg^{-1}$. ΔHCO_3^- values are micromoles $\times 100\ mg^{-1}$. Subscripts refer to time in hours, zero being just prior to addition of cycloheximide (5 μg/ml) or diluent. NS is not statistically significant. The P values refer to difference from control.

to addition of puromycin or diluent. As expected from the identity of RSCC in the two groups of hemibladders, the ΔHCO_3^- was not different in the two groups of tissue prior to addition of the inhibitor. In that same experiment, the RSCC had decayed to near zero in the control tissues by $t=4$ hr. Noteworthy is the observation that there was no measurable urinary acidification in these tissues without RSCC, despite the presence of the favorable transepithelial electrical potential difference.

Effect of Antimycin A and Acetazolamide on RSCC

The dependence of the stimulated RSCC upon metabolic energy derived from oxidative phosphorylation was examined by use of antimycin

Table 7. Effect of antimycin A on RSCC

Treatment	$RSCC_0$	$RSCC_6$	Antimycin A Δ
Aldosterone	35 ± 5	27 ± 5	32 ± 5
Actinomycin D	35 ± 3	41 ± 4[a]	40 ± 3[a]
Puromycin	35 ± 5	36 ± 9	34 ± 7
Cycloheximide	36 ± 3	27 ± 5	34 ± 6

Bladders from six saline-soaked toads were incubated as quarter-bladders for 6 hr with aldosterone (10^{-7} M), actinomycin D (20 μg/ml), puromycin (80 μg/ml), or cycloheximide (5 μg/ml). Subscripts refer to time in hours, time zero being just prior to the above additions. Antimycin A (3.6×10^{-6} M) was added after the 6-hr RSCC reading. The decrease in RSCC one hour later is recorded as "Antimycin A Δ."

[a] Refers to a value statistically significantly different from the corresponding value in aldosterone treated tissues (paired analysis).

Table 8. Effect of acetazolamide on RSCC

Treatment	$RSCC_0$	$RSCC_6$	Acetazolamide Δ
Aldosterone	20 ± 2	17 ± 3	18 ± 3
Actinomycin D	23 ± 6	24 ± 6	22 ± 6
Puromycin	24 ± 5	24 ± 5	21 ± 4
Cycloheximide	22 ± 6	17 ± 4	19 ± 6

Bladders from six saline-soaked toads were incubated as quarter-bladders for 6 hr with aldosterone (10^{-7} M), actinomycin D (20 μg/ml), puromycin (80 μg/ml), or cycloheximide (5 μg/ml). Subscripts refer to time in hours, time zero being just prior to the above additions. Acetazolamide (10^{-3} M) was added after the 6-hr RSCC readings. The decrease in RSCC one hour later is recorded as "Acetazolamide Δ." There are no statistically significant differences.

A, an inhibitor of mitochondrial electron transport. As shown in Table 7, antimycin A virtually eliminated the RSCC present after 6 hr of treatment with aldosterone, actinomycin D, puromycin or cycloheximide.

The results of a similar experiment are shown in Table 8, except that acetazolamide (10^{-3} M) was added after 6 hr of stimulation of RSCC. The inhibitor of carbonic anhydrase reduced the RSCC to near zero in each case. The absolute inhibition produced by acetazolamide (acetazolamide Δ in Table 8) was not statistically significantly different in any of the groups.

Discussion

Our study demonstrates that urinary acidification is stimulated by agents with three different known actions. Aldosterone is believed to

produce its physiologic actions by a steroid-receptor interaction which triggers RNA and protein synthesis. Paradoxically then, an inhibitor of RNA synthesis (actinomycin D) and two inhibitors of protein synthesis (puromycin and cycloheximide) all mimic the action of aldosterone on urinary acidification. The inhibitors of protein and RNA synthesis were used at concentrations which block the action of aldosterone on Na^+ transport (Fanestil & Edelman, 1966). These are concentrations which produce 70 to 90% inhibition of synthesis of RNA or protein in the toad urinary bladder (Fanestil & Edelman, 1966). Thus, there can be no doubt that the inhibitors were exerting their known effects. Similarly, there is little reason to doubt that all agents stimulated a transport process resulting in urinary acidification. The steroid and the inhibitors of RNA and protein synthesis all enhanced RSCC, an accurate measure of urinary acidification in untreated tissues (Ludens & Fanestil, 1972; Ludens & Fanestil, 1974; Ziegler *et al.*, 1976). Moreover, the enhanced RSCC was dependent upon energy from oxidative metabolism and was eliminated by acetazolamide, a known inhibitor of urinary acidification in this tissue (Ziegler, Ludens & Fanestil, 1974). Finally, direct measurement of the amount of acid added to the mucosal fluid under the influence of a favorable electrical potential demonstrated enhanced urinary acidification (Tables 3, 4, 5, and 6).

Two general schemes, which might account for these experimental observations, are outlined in Fig. 3. In scheme *I*, it is proposed that all agents tested exert their known actions on RNA and protein synthesis but, in addition, that all agents have unknown side reactions which stimulate some step(s) in the process of urinary acidification. Scheme *I* cannot be discarded on the basis of our current evidence. However, the fact that a spirolactone blocks the stimulation of acidification by aldosterone (Table 1) may be taken as presumptive evidence that the hormone exerts its effect on acidification via the known receptor-steroid-mediated pathway. This pathway presumably requires RNA and protein synthesis for full expression of the hormonal effect. Therefore, we are attracted to Scheme *II* in Fig. 3 as a possible alternative. In this scheme, both hormone and inhibitors stimulate urinary acidification as a consequence of their known actions on RNA and protein synthesis.

A number of feed-back loops can be devised to meet the requirements of Scheme *II*. The most simple such model is presented in Fig. 4. The H^+ pump is assumed to be a protein with a long half-life (e.g., >18 hr). This H^+ pump protein is a product of a gene which is not regulated by aldosterone. However, the activity of the H^+ pump is under the

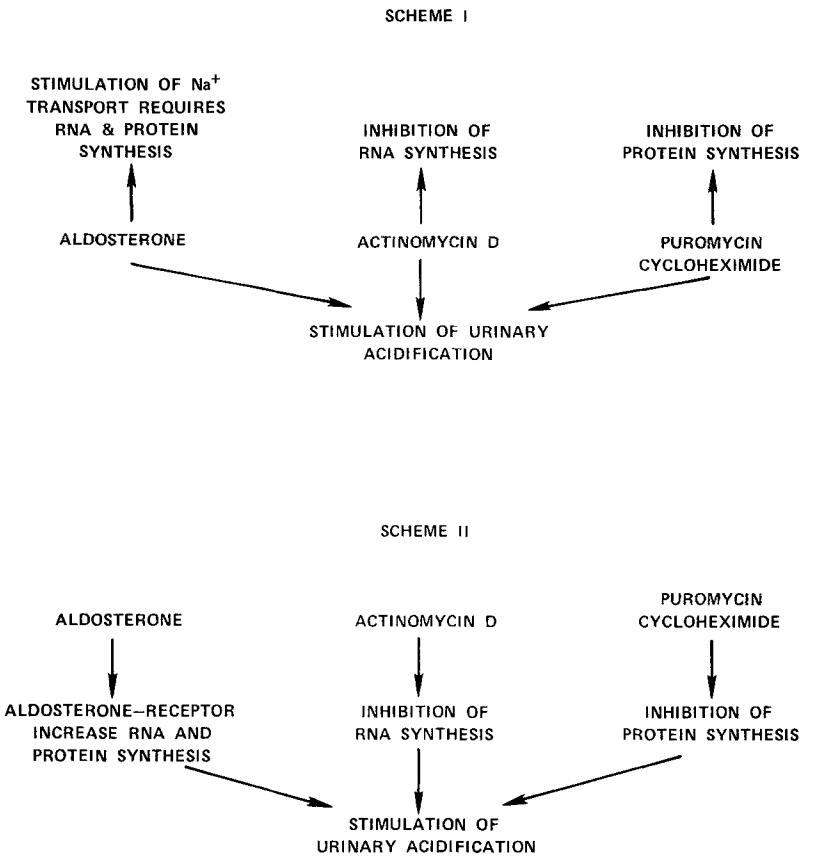

Fig. 3. Two general schemes which account for stimulation of urinary acidification. *See text* for explanation

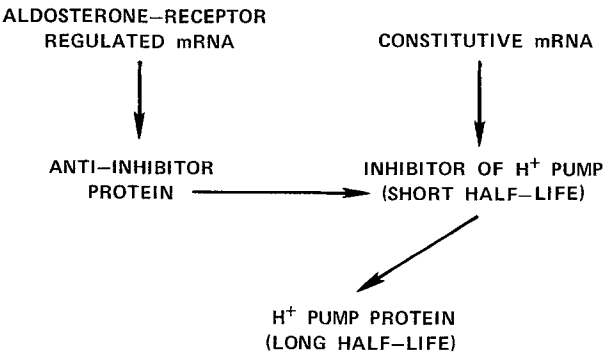

Fig. 4. Model for stimulation of urinary acidification. *See text* for explanation

control of a protein inhibitor, the synthesis of which is also not regulated by aldosterone. This inhibitor of the H^+ pump has a relatively short half-life (e.g., <6 hr) and is, in turn, regulated by an anti-inhibitor. The anti-inhibitor is the product of a gene which is regulated by aldosterone. An example of such an anti-inhibitor would be a specific protease for accelerating the turnover of the inhibitor. Such a model can explain our experimental observations thusly: (i) inhibitors of RNA and protein synthesis abolish the synthesis of the inhibitor of the H^+ pump, thereby releasing the H^+ pump from inhibition, as the existing inhibitor decays; (ii) aldosterone acts via the accepted steroid-receptor mediated RNA and protein synthesis pathway by producing the anti-inhibitor, which negates the action of the inhibitor of the H^+ pump, thereby releasing the H^+ pump from inhibition. Since both aldosterone and inhibitors of RNA and protein synthesis alter acidification by changing the activity of the same inhibitor, the actions of aldosterone and the inhibitors of RNA and protein synthesis on the H^+ pump are not additive. This model is by no means the unique model to explain our findings. Rather, we present this model to demonstrate that our apparent paradoxical finding—that inhibitors of RNA and protein synthesis mimic the action of aldosterone on urinary acidification—can be explained by a model which incorporates the known actions of the hormone and the inhibitors. Testing of such a complicated model may be possible only after the H^+ pump has been defined in molecular terms.

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