# SOME STUDIES ON THE HORMONAL CONTROL OF SODIUM TRANSPORT ACROSS THE TOAD URINARY BLADDER

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TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

Page

## I. INTRODUCTION

The modern approach to the study of epithelial transport can be dated to the measurement by Ussing and Zerahn (1) of the electrical asymmetry in tissues transporting sodium. This work utilized frog skin but was extended by Leaf (2) to the toad urinary bladder. The similarity between the transport processes of this tissue and the distal convoluted tubule and collecting duct was manifested when the sodium transport (3, 4) and hydroosmotic flux (5) were found to be responsive to neurohypophyseal peptides (NHP) and to aldosterone. These hormones had already been implicated as regulators of sodium and water reabsorption in the mammalian distal nephron.

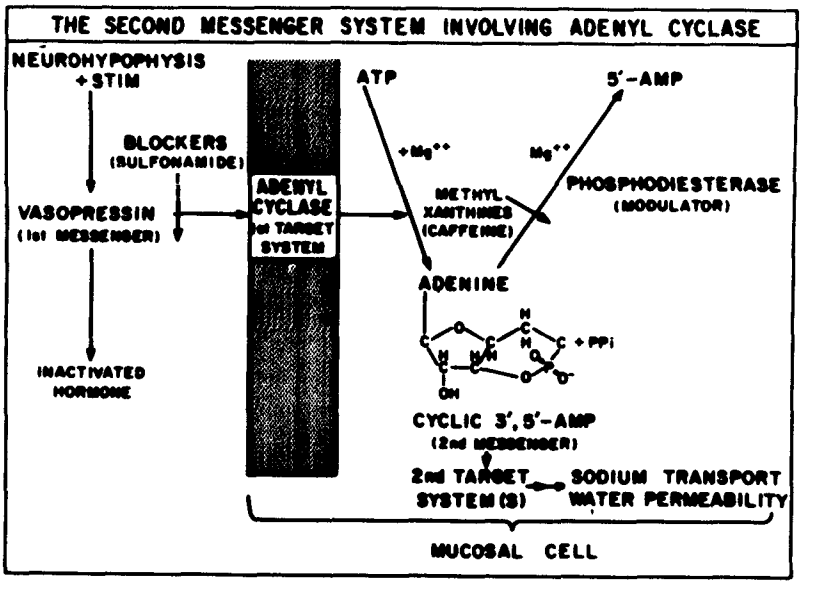
Understanding the mechanisms through which steroid and peptide hormones operate stems from the work of Mueller (6), Jensen (7), Gorski (8) and Sutherland (9).

Steroids have been shown to cause a variety of effects, e.g. sex steroid stimulation of proliferation and differentiation and adrenocorticosteroid control of carbohydrate and amino acid metabolism. Their cellular effects are initiated by binding to soluble cytoplasmic receptors with subsequent translocation of the bound complex to the nucleus (10). It is in the nucleus that the controls are

exerted by directing synthesis of specific messenger RNA with the ensuing physiologic response mediated by the translation of the RNA into proteins (11). On the other hand, the regulatory effects of peptide hormones results from the binding of hormone to cell surface receptors. The primary effect of this interaction is the activation of the membrane bound enzyme, adenylate-cyclase (AC). The resulting increase in cyclic 3',5' adenosine mono-phosphate (cAMP) was found to mediate the physiologic effects associated with the hormone. The original work (9) dealt with the control of glycogen synthesis by glucagon and epinephrine, but the regulatory action of cAMP has been extended for mediation of other peptide hormone effects. Sutherland conceptualized this effect in his "second messenger" hypothesis (12); in this scheme cAMP exerts an allosteric control over rate-limiting processes responsible for the hormone's physiologic effect (Figure 1).

Figure 1

Sutherland: The Second Messenger Hypothesis




Research on the action of NHP and aldosterone on sodium transport in the toad urinary bladder indicates a consistency with the general mechanisms described above. Orloff et al. (13) observed that the effects of vasopressin on hydroosmotic flux could be duplicated by the addition of exogenous cAMP, or by phosphodiesterase inhibitors (the latter act by inhibiting the breakdown of the cAMP, thus allowing its intracellular levels to rise). Evidence

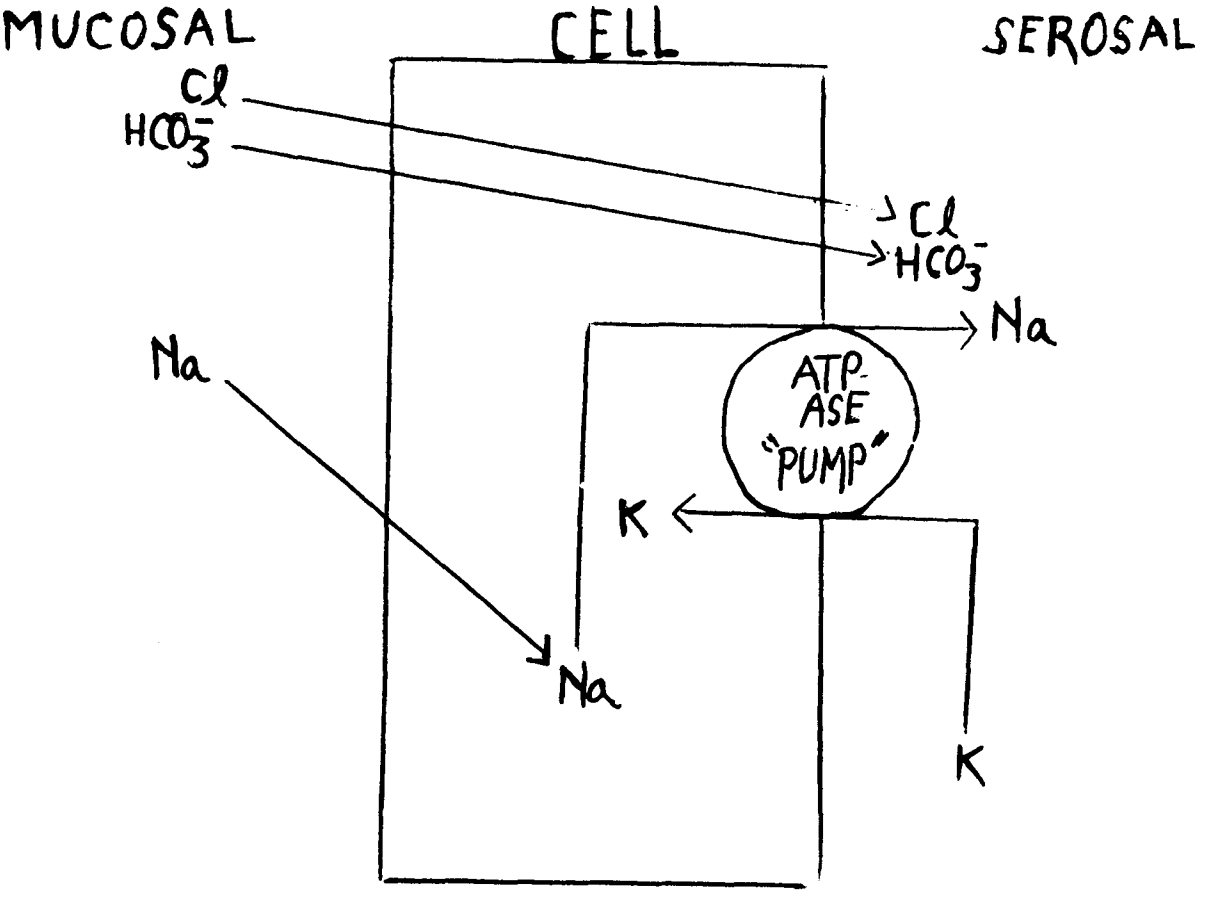
has been presented that in both the toad urinary bladder and rat kidney mineralocorticoids are bound by soluble receptors (14, 15), and become localized in the nuclei (16, 17). A role for RNA and protein synthesis has been indicated (18, 19). Inhibition of RNA or protein synthesis was found to block the effects of aldosterone (20, 21). The physical basis for the transepithelial movement of sodium is not as yet clearly defined. The transcellular flux has been viewed as a two-step process (1) (Figure 2):

(A) Sodium diffuses in from the lumen along its electrochemical gradient and
(B) is pumped against a gradient by an energy-requiring mechanism of the serosal border.

The placement of these influx and efflux steps in the toad urinary bladder is inferred from the changes in the sodium pool after the addition of certain transport inhibitors. The inhibition of Na/K ATPase by ouabain results in an increase in the intracellular sodium pool; amiloride inhibits sodium transport with a concomitant decrease in cell sodium (22,23). The response of the toad urinary bladder to NHP or aldosterone has been measured in terms of sodium pool as well (24). These studies indicated that both hormones increased cell sodium levels and therefore were affecting the presumably passive entry of sodium at the mucosal membrane barrier.

Figure 2

Ussing and Zerahn Model of Epithelial Transport




The action of metabolic inhibitors demonstrated the need for mitochondrial function but the ATPase site surprisingly did not seem to be tightly coupled. These experiments utilized dinitrophenol which causes a rapid

inhibition of sodium transport although results indicate little effect on the efflux associated with the serosal ATPase (26). The transport process has been found to be dependent on $O_2$ (26) but the requirement was not absolute (27), indicating some energy is derived directly from glycolysis. In fact, it was later shown that the glycolytic rate in the toad urinary bladder as well as oxygen consumption were in part determined by the transport load (28). The effects of both NHP and aldosterone on carbohydrate metabolism have been extensively studied (26,31) and were found to be more a result rather than a determinant of transport. An exception is the effect on the oxidation of glucose by the hexose monophosphate shunt which is not sodium dependent (31). A possible effect of the steroid on fatty acid synthesis was suggested (32).

Without knowledge of the final effectors for the actions of cAMP and aldosterone-induced protein, the mediation of hormone action cannot be fully formulated. Additionally, studies of toad bladder cAMP following the addition of hormone have shown only modest increases in the cyclic nucleotide (33). The degree of elevation of tissue cAMP reported was not consonant with the striking physiologic effects evoked by the neurohypophyseal hormones

nor with the large increase in cAMP reported in other instances in which cAMP was presumed to mediate and amplify hormonal stimuli (34). Nor was there any evidence that the physiologic response is temporally dependent upon the precedent increase in tissue cAMP. The purposes of the study described in part B, Section II of this thesis were (a) to examine the time course of the change in tissue cAMP content in response to oxytocin, (b) to relate these changes to the stimulation of sodium transport, and (c) to determine the quantitative relations between the tissue cAMP and sodium transport.

The mucosal epithelium of the toad urinary bladder is heterogeneous in cell population; the epithelium is essentially made up of mitochondria-rich cells (MR) (10-30%) and granular cells (G) (70-85%) (35). Any attempt at determining the contribution of distinct biochemical processes in the transport event should take into account a possible differential contribution from the two cell types. In part C of Section II I will describe results of biochemical studies performed on isolated MR and G cells separated by discontinuous gradient centrifugation. Sections III and IV of this thesis are devoted to work on steroid hormone action. Although the binding of aldosterone by specific receptors had been demonstrated

previously, the dependency of aldosterone's effect upon protein synthesis was based only on the ability to block the steroid effect by inhibitors of protein and RNA synthesis; there had been no successful attempt at isolating an induced protein. I therefore investigated the binding of steroids and the induction of protein in both cell types with the consideration that the homogeneous cell fraction would enable me to detect changes possibly masked by contaminating material from an unresponsive cell type.

## II. THE ROLE OF CYCLIC AMP IN SODIUM TRANSPORT

### A. MATERIALS AND METHODS

The toad used in these studies was Bufo marinus of Dominican (Lemberger Co., Oshkosh, Wisconsin) and Colombian (Tarpon Zoo Co., Tarpon Springs, Florida) origin. The animals were kept at room temperature on moist soil. After the toads were doubly pithed the hemibladders were dissected and rinsed in Ringers solution (NaCl 85 mM; KCl, 4 mM; $NaHCO_3$, 17.5 mM; $KH_2PO_4$, 0.8 mM; $MgSO4$, 0.8 mM; $CaCl_2$, 1.5 mM and dextrose, 10 mM).

In order to monitor the tissue's electrical properties, a hemibladder was mounted in a modified Ussing chamber (1) with the serosal and mucosal surfaces bathed in Ringers solution. The short-circuit current (SCC) was monitored continuously by conventional techniques across 1.5 $cm^2$ of tissue, except for brief intervals in which the spontaneous potential difference was measured. After an equilibration period of 1 hour, oxytocin at concentrations noted in RESULTS was added to the serosal bath and the SCC measured for 30 minutes.

The concentration of intracellular cAMP was measured in the contralateral hemibladder. The paired hemibladder was carefully cut into 12 pieces of approximately equal

size and incubated in a flask containing 5 ml of Ringers solution. The pieces of tissue were treated with the same concentration of oxytocin as the hemibladder in the Lucite chamber and removed at the indicates times following addition of hormone. Cyclic AMP content in the tissues was measured by the radioimmunoassay method of Steiner, Kipnis, Utiger and Parker (36), using commercially prepared materials (Collaborative Research, Cambridge, Mass.). The tissue sections were placed in 1.0 ml of 0.05 M acetate buffer (pH 6.2) at 100°C. After 5 minutes the tissues were disrupted by sonication and centrifuged for 20 minutes at 5,000 rpm, and a 0.3 ml aliquot of the supernatant was used for cAMP determinations. The labeled ligand was succinyl-$^{125}$I-cAMP tyrosine methyl ester (600 Ci/mmol). The cAMP antibody complex was precipitated with goat anti-rabbit IgG using normal rabbit serum as the carrier, collected by filtration (Millipore filter, 0.45 μm), and counted in a Nuclear-Chicago scintillation counter (Nuclear-Chicago Corporation, Des Plaines, Illinois).

Epithelial cells were removed from hemibladders by the following procedure: The neck of each hemibladder was tied to the outlet of a Luer-lock syringe, filled

with and immersed in $Ca^{2+}$-free Ringer's solution containing 2 mM EDTA, and incubated for 40-60 minutes at room temperature. The intraluminal fluid containing the disaggregated mucosal cells was collected and centrifuged in a Sorvall RC2B centrifuge at 3000 x G.

When measuring the response of cells to neurohypophyseal hormones, the sedimented cells were resuspended in Ringer's containing 1.5 mM $CaCl_2$. The cells were incubated in this Ringers for 30 minutes prior to exposure to hormone. The replacement of $Na^+$ by choline or the inhibition of $Na^+$ entry into cells by amiloride is described in RESULTS.

Separation of mucosal cell types by discontinuous gradient centrifugation was performed in cells removed from the bladder and collected as described above.

The sedimented cells were resuspended in 10 mL of EDTA-Ringer solution and layered over a discontinuous gradient of Ficoll in EDTA-Ringer solution prepared in six centrifuge tubes (1 by 3 1/2 inches). The gradient consisted of four solutions of Ficoll having densities of approximately 1.017, 1.035, 1.067, and 1.088 $g/cm^3$ at $4^oC$. The cells were centrifuged at 27,000 rev/min in a Beckman SW-27 rotor for 45 minutes.

The separated cells were handled in several ways. Cells for transmission electron microscopy were prepared by adding the sedimented material to an equal volume of 6 percent glutaraldehyde buffered with 0.2 M cacodylic acid (pH 7.3) for two to six hours. The fixed cells were rinsed in 10 percent sucrose, suspended in a 2 percent aqueous osmium tetroxide solution for two hours, and embedded in Epon 812.

The cells used for further biochemical analysis were washed free of Ficoll by diluting with 30 ml. of EDTA Ringers and centrifuging at 15,000 rpm, and then resuspended and recentrifuged at 3,500 rpm.

Assay of Cytochrome Oxidase:

Horse heart cytochrome c (Sigma Chemical Co., St. Louis, Missouri) was reduced with hydrogen over a palladium catalyst immediately before use. Isolated cells were suspended in 1.0 ml of Ringer solution and sonicated with a Branson sonifier for two 15-second intervals. Samples of this preparation were used to measure the rate of oxidation of cytochrome c in a Gilford absorption spectromdter at 550 nm.

Assay of Carbonic Anhydrase

The sonicate described above was centrifuged at 35,000 x G for 30 minutes. Aliquots were taken and added to Maren buffer (37) equilibrated with $CO_2$. Phenol red was used for end point determination.

Protein was determined using either the method of Lowry (38) or biuret.

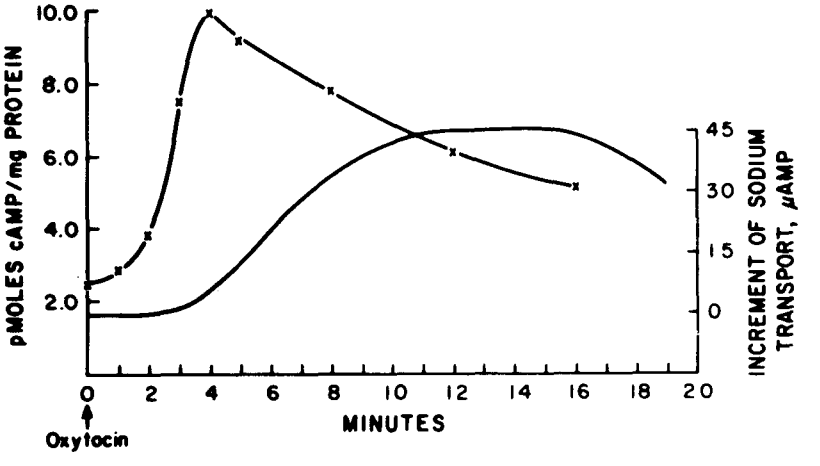
## B. RESULTS

Figure 3 shows a typical experiment. The addition of a submaximal dose of oxytocin ($2 \times 10^{-8}$M) to the bladder was followed by a significant increase in the SCC beginning at approximately 1.5 minutes. The rise in SCC was sustained until a peak level was reached 12 minutes following the addition of hormone. The measurements of tissue cAMP in the matching hemibladder show that the levels of nucleotide were increased by the end of 2 minutes; the peak level of cAMP was attained at 4 minutes, when the SCC was only entering the rising phase. The tissue nucleotide levels then began to fall so that the hormone-induced increment in cAMP had already decayed significantly before the maximum hormonal effect upon

sodium transport was expressed. A similar relationship existed between tissue cAMP concentrations and hormone-induced sodium transport at each supra-threshold concentration of oxytocin studied (Table 1, page 15).

Figure 3

Representative experiment comparing oxytocin-stimulated increases in tissue cAMP and SCC:




A typical experiment showing the response of tissue cyclic AMP (x---x) and SCC (---) to the addition of oxytocin ($2 \times 10^{-8}$M) to the solution bathing the serosal surface of the toad bladder. The bladders were bathed in Ringer's solution (pH 7.8) whose composition was NaCl, 85 mM; $NaHCO_3$, 17.5 mM; KCl, 4 mM; $MgSO_4$, 0.8 mM; $KH_2PO_4$, 0.8 mM; and glucose, 11 mM. The SCC in one hemibladder began to increase at approximately 2.5 minutes, and peaked at 12

TABLE 1

Effects of Oxytocin upon Toad Bladder Cyclic AMP and Short-Circuit Current

| | | Tissue cyclic AMP | | | Increment of sodium transport | |
|---|---|---|---|---|---|---|
| Exp. no. | Oxytocin | Time to doubling of basal conc. | Maximum increment | Time to peak lev. of cAMP | Time to onset | Maximum increment |
| | $Mx10^{-9}$ | min | pmol/mg protein | min | min | $\mu$A |
| 1 | 1.25 | - | 2.94 | 7.0 | - | - |
| 2 | 2.5 | - | 1.87 | 7.0 | - | - |
| 3 | 2.5 | - | 2.41 | 6.5 | - | - |
| 4 | 2.5 | 4.5 | 4.01 | 6.0 | 4.3 | 8 |
| 5 | 2.5 | 5.0 | 3.67 | 6.0 | 4.5 | 6 |
| 6 | 5 | 4.5 | 4.01 | 6.0 | 4.5 | 6 |
| 7 | 5 | 4.5 | 3.51 | 6.0 | 4.5 | 5 |
| 8 | 5 | 2.8 | 6.84 | 4.0 | 3.0 | 32 |
| 9 | 5 | 2.8 | 6.34 | 4.0 | 3.0 | 38 |
| 10-13 | 10 | 3.0 | 6.31±0.43 | 4.5 | 3.5 | 31±3.2 |
| 14-17 | 20 | 2.5 | 9.17±0.73 | 4.0 | 2.5 | 46±4.4 |
| 18-21 | 40 | 1.5 | 14.45±1.0 | 3.0 | 1.5 | 64±4.4 |

The basal cyclic AMP concentration was 3.4±0.18 pmol/mg protein (mean ± SEM; n=20); the basal short-circuit current was 70.0±8.4$\mu$A/1.5 $cm^2$. At those concentrations of oxytocin in which the SCC response was absent or minimal, the results of each experiment are given; in the experiments that were clearly suprathreshold, the results for each concentration of oxytocin are given as the mean ±SEM. The values for time to doubling and time to peak level of cAMP were obtained by interpolation.

minutes. Tissue cAMP in the matching hemibladder, measured at the indicated intervals, began to rise approximately 1.5 minutes after the addition of oxytocin, reached a peak at approximately 4 minutes, and then began to fall.

Inspection of the responses of tissue cAMP and sodium flux to hormone reveal several noteworthy points. There is a direct relationship between the maximum cAMP level attained and the peak increment in sodium flux (Table 1). Doubling of the basal cAMP level seemed to correspond to the onset of the hormone-induced increment in sodium transport. Coincidence of the increment in sodium transport with the doubling of basal cAMP suggests a threshold level to which tissue cAMP must be raised in order to elicit a physiologic response. This conclusion is supported by the effects upon the tissue of 1.25 and 2.5 x $10^{-9}$M oxytocin: tissue cAMP was increased by 65, 81, and 98% (exps. 1, 2, and 3), but there was no evident effect upon sodium transport. A slightly greater increase in the tissue cAMP level, 116, 121, 127 and 131% (exps. 4 - 7), was associated with a small (5-8 $\mu$A/1.5 $cm^2$) increase in the SCC.

Table 1 shows that the time interval between the estimated time of doubling of cAMP and the attainment of peak levels of cAMP is relatively invariant. This suggests

a constant time interval in which hormonal activation of adenylate cyclase is the predominant determinant of tissue cAMP levels, although secondary effects on phosphodiesterase activity cannot be discounted. The differential maximal stimulation elicited by different hormone concentrations is thus reflected in the rate of increase of tissue cAMP, not necessarily an increased period during which hormone is effective. The possibility of a relatively constant period of time in which hormone stimulates adenylate cyclase is supported by the observation that the cAMP levels do not plateau but decay immediately from their peak values at rates seemingly independent of hormone concentration. I also observed that the greater the peak cAMP concentration attained, the longer the SCC plateau was sustained. This may be due to the greater time required for cAMP levels to fall below the apparent threshold concentration (Figure 3). I would speculate, therefore, that one important parameter for sustaining sodium transport is that period of time during which the tissue cAMP is maintained above the critical threshold level; this time, in turn, is related to the amount of cAMP synthesized during the brief interval in which adenylate cyclase activity is evidently enhanced by

oxytocin. To assure that the changes in tissue cAMP reflected the effects of hormone upon nucleotide levels in the mucosal epithelium, I studied the effects of oxytocin ($2 \times 10^{-8}$M) upon isolated mucosal cells. Figure 4 (page 20) shows the rise in cAMP follows a time course similar to that in the whole tissue.

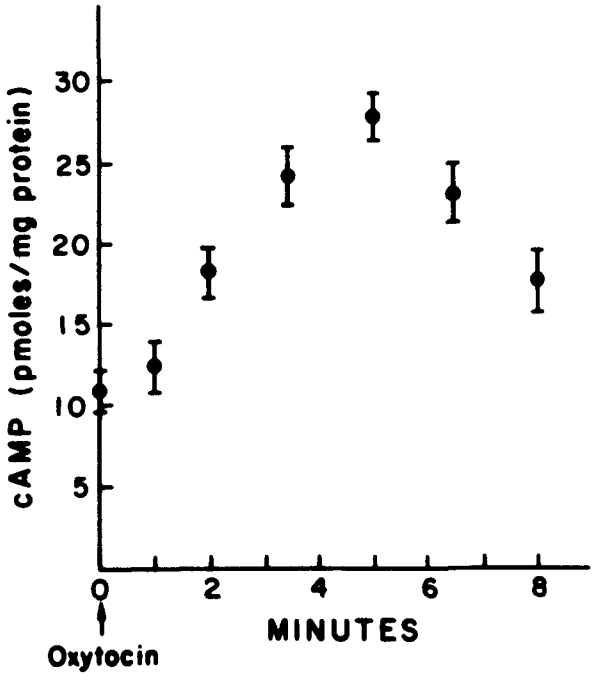
Bar, Hechter, Schwartz, and Walter (39) have shown that the synthesis of cAMP by toad bladder membranes is linear for 20 minutes following stimulation with oxytocin. It appears that the brief synthesis of cAMP we observe in the intact tissue reflects control mechanisms that are lost on fractionation of the tissue. Dousa (40) reported that hormone stimulation of adenylate cyclase in renal medullary membranes is diminished with increasing concentration of sodium chloride.

Our evidence that (a) the termination of hormonal stimulation of adenylate cyclase seems to occur at a fixed time after the cAMP levels are doubled, and (b) the estimated doubling time corresponds closely with the enhancement of SCC (sodium transport), coupled with Dousa's findings, led us to examine the possibility that the activity of the adenylate cyclase may be regulated by the influx of sodium into the mucosal cells.

Tissue cAMP levels were measured in paired hemibladders, one incubated in sodium Ringers as described above, the other in choline Ringers. As shown in Figure 5 (page 21), the cAMP initially increased at similar rates in both preparations, but in the sodium-free tissue the increase was sustained during the period of observation. To rule out the possibility that this effect was due to choline, we examined the same response when the bladder was incubated in sodium Ringers containing amiloride, a potent inhibitor of sodium influx. The results were almost identical to those in choline. I interpret these findings as an indication that intracellular sodium plays a servo-regulatory role in the responsiveness of toad bladder adenylate cyclase to neurohypophyseal hormones.

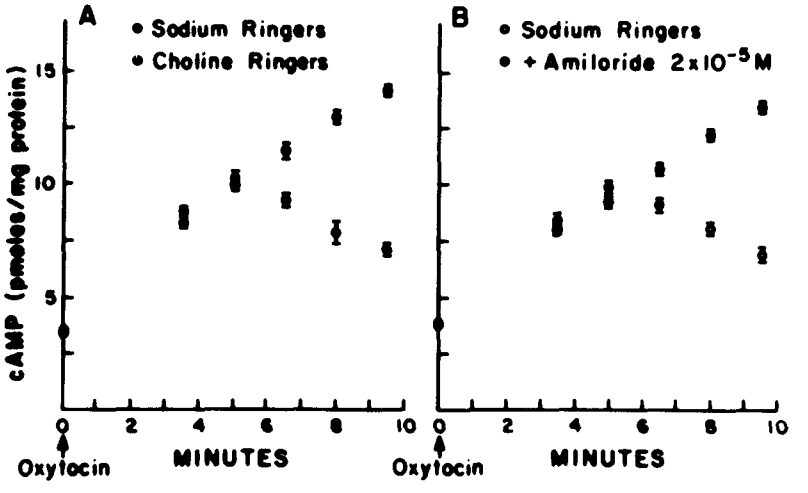
Figure 4

Oxytocin stimulation of cAMP levels in isolated mucosal cells



## Figure 5

Effects of A choline substitution and B amiloride on time course of hormone-stimulated increase in tissue cAMP.




The effects of mucosal sodium upon tissue cAMP. (a) The control hemibladders (0---0) were incubated in Ringer's solution (composition given in Figure 1) and the paired hemibladders (o---o) incubated in Ringer's solution in which the sodium was replaced by choline. Oxytocin ($10^{-8}$M) was added to both sets of hemibladders, and aliquots of tissue were removed at the indicated intervals for measurement of cAMP. The values given are the mean ± SEM of four experiments. (B) Control hemibladders were incubated in Ringer's solution and paired hemibladders in Ringer's solution containing amiloride ($1.3 \times 10^{-5}$M). Oxytocin ($10^{-8}$M) was added to both sets of hemibladders, and aliquots of tissue were removed at the indicated intervals for measurement of cAMP. The values given are the mean ± SEM (n=4).

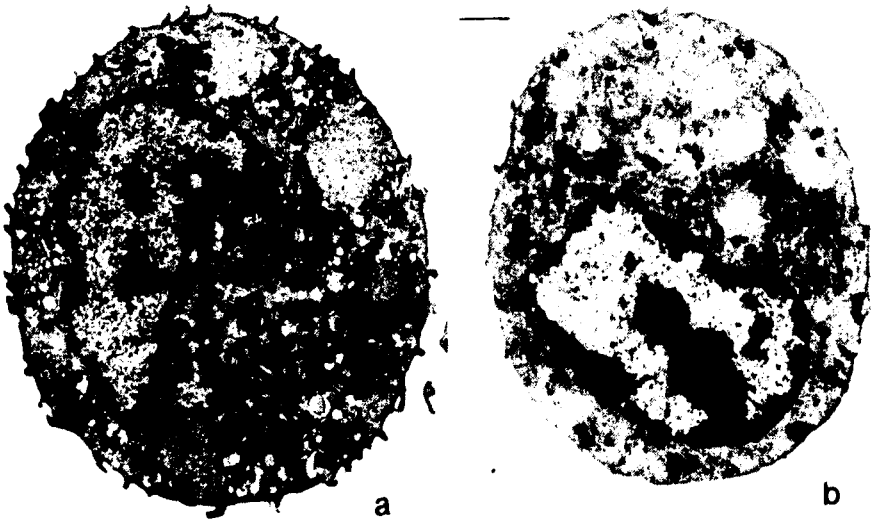
## C. CHARACTERIZATION OF ISOLATED MITOCHONDRIA-RICH AND GRANULAR CELLS OF THE TOAD URINARY BLADDER

After centrifugation of the disaggregated mucosal cells on a Ficoll density gradient as described in part A, four bands of material were evident:

The most buoyant layer, band 1, contained the smallest amount of material. This was composed almost entirely of heterogeneous acellular material and gave a positive periodic acid-Schiff reaction. This may represent the coating of mucus normally found on the luminal surface of the intact bladder. Bands 2 and 3 contained much more material, composed almost entirely of intact epithelial cells. The great majority of cells in band 2 were classified as mitochondria-rich (Figure 6A, page 23). A rare goblet cell was identified, as well as an occasional granular cell. The cells in band 3 were primarily the granular type (Figure 6B, page 28). These cells, which contained large numbers of the characteristic electron-dense granules, also contained significant numbers of mitochondria. This band also contained an occasional small dense cell identified as a "basal" cell. Band 4, the most dense band (1.088 $g/cm^3$) contained cellular debris, as well as some intact epithelial cells, primarily granular cells.

## Figure 6

Electron micrograph of separated A) mitochondria-rich cells and B) granular cells from toad bladder mucosal epithelium



To verify the morphologic evidence that we had separated the mitochondria-rich and granular cell types, we measured the cytochrome oxidase activity in the bands of separated material. We found that the cytochrome oxidase activity was consistently higher in band 2, the mitochondria-rich fraction of cells, than in band 3, which contained primarily the granular cells (Table 2, page 25). It was higher in both bands of mucosal cells prepared from toads of Colombian origin than in toads from the Dominican Republic. The relative amounts of cytochrome oxidase activity in bands 2 and 3 were, however, identical in the toads from the two sources. These data lend support to our morphologic evidence that we had, to a large extent, separated the two major types of toad bladder mucosel cells.

It has been shown that under certain conditions the urinary bladder of the Colombian toad is capable of transporting $H^+$ into the urine (41, 42). Because the acidification is blocked by sulfonamide inhibitors of carbonic anhydrase, the process is presumed to be related to the activity of this enzyme. This enzyme is present in significant amounts in the mucosal epithelial cells and consists of at least three molecular forms. Histochemical studies of the toad urinary bladder, although perhaps lacking in specificity, have indicated that carbonic anhydrase

TABLE 2

Enzyme activities in toad urinary bladder mucosal cells separated by density gradient centrifugation. The data in each row are from one experiment, each using material from six to eight toads; values are per milligram protein. Cytochrome oxidase was assayed spectrophotometrically at 550 nm as the rate of oxidation of reduced cytochrome c. The ratio of cytochrome oxidase activity in the two bands differs from unity at $P < .001$. Carbonic anhydrase activity was measured by the method of Maren et al. (36); the difference in mean values for bands 2 and 3 is significant at $P < .005$. The means given are for both Colombian and Dominican toads.

| Cytochrome oxidase activity (μmole/min) | | | Carbonic anhydrase activity (enzyme units) | | |
|---|---|---|---|---|---|
| Band 2 | Band 3 | Ratio | Band 2 | Band 3 | Ratio |
| | | Colombian toad | | | |
| 142. | 120 | 1.19 | 3.21 | 1.35 | 2.38 |
| 128. | 113 | 1.14 | 1.98 | 0.77 | 2.57 |
| 115 | 96 | 1.20 | 1.68 | 0.52 | 3.23 |
| 157. | 133 | 1.18 | 2.37 | 1.22 | 1.94 |
| | | | 5.86 | 0.91 | 6.44 |
| | | Dominican toad | | | |
| 77.0 | 69.8 | 1.10 | 3.05 | 0.97 | 3.14 |
| 51.7 | 42.7 | 1.21 | 3.01 | 0.96 | 3.14 |
| 45.3 | 39.0 | 1.16 | 1.21 | 0.50 | 2.42 |
| Means | | 1.17 ± 0.02 | 2.80 ±0.50 | 0.90 ± 0.11 | 3.16 ± 0.50 |

is located primarily in the mitochondria-rich cells (43). Material collected by density gradient centrifugation was sonicated and centrifuged at 17,000 rev/min, and carbonic anhydrase activity was assayed in the supernatant solution by the method of Maren (37). Carbonic anhydrase activity was approximately threefold higher in material from band 2 (mitochondria-rich cells) than in material from band 3 (granular cells) (Table 2). The distribution of the enzyme was apparently not related to the origin of the animal. Our data suggest that the sulfonamide-inhibitable transport processes leading to acidification of the urine are located primarily in the mitochondria-rich population of cells; this further corroborates our separation of the two cell types.

To determine the oxytocin responsiveness of the two bands of cells collected by centrifugation, the cells were sedimented and resuspended in sodium Ringer's solution containing 1.5 mM calcium. Portions of cells were added to 0.5M acetate buffer (pH 6.0) at 100°C before and 5 minutes after the addition of oxytocin ($5 \times 10^{-8}$M). The levels of this cyclic nucleotide were determined by the procedure described in METHODS above.

The basal content of cyclic AMP in the two cell

TABLE 3

Comparison of cytochrome oxidase and carbonic anhydrase in band 2 and band 3 cells from both Colombian and Dominican toads

| Cells | Cyclic AMP (pmole) | |
|---|---|---|
| | Band 2 (N=10) | Band 3 (N=16) |
| Basal | $11.13 \pm 0.56$ | $12.69 \pm 0.54$ |
| Oxytocin-treated | $37.87 \pm 2.11$* | $13.58 \pm 0.45$** |

* Significant at $P < .001$.

** Significant at $P > .02$.

fractions was not significantly different (Table 3).

However, following the addition of oxytocin to the cell suspensions, only the cells from band 2 (mitochondria-rich) showed a significant rise in cyclic AMP concentration. This increment in cyclic AMP amounted to 235%, significantly greater than the 145% increase in uncentrifuged mucosal cells under the same conditions.

## SUMMARY AND PARTIAL DISCUSSION OF SECTION II

The contents of Section II may be summarized as follows:

1) The NHP increase in tissue cAMP reaches a level double that in the basal state prior to any increment in the sodium short-circuit current.
2) There exists a quantitative relationship between the cyclic AMP and sodium flux responses.
3) The period of time over which cyclic AMP levels continue to rise after the threshold level has been attained seems invariant with hormone concentration.
4) The rate of increase in cyclic AMP levels during the above-mentioned period increased with hormone concentration.
5) The decline in cAMP after maximal levels are reached can be delayed by inhibiting the passive entry of sodium into the cells.
6) The results indicate that the MR cell is the site of action for NHP stimulation of adenylate cyclase.
7) Per mg. protein, these cells contain three times as much carbonic anhydrase as do the more numerous granular cells.

The findings mentioned in Items 6 and 7 reinforce the need for a cell specific regulatory system. The interdependence of $Na^+$ and $H^+$, e.g. $Na^+/H^+$ exchange during acidification, implies a close proximity of the control systems for these cations. Evidence in support of this hypothesis is the suggestion that carbonic anhydrase activity in the toad bladder epithelium is increased after treatment with aldosterone (41); additionally, aldosterone binding and induction of protein described in the next section seems a property of only the MR cell.

## II. BINDING OF STEROIDS TO TOAD URINARY BLADDER MUCOSAL CELLS

### A. MATERIAL AND METHODS

To measure the specific binding of corticosteroids to soluble receptors, I incubated paired sets of intact hemibladders in separate baths. One bath ("$^3$H-Aldo") contained $^3$H-aldosterone (New England Nuclear, Boston, Mass., 91 Ci/mM) alone in various concentrations, while the paired bath ("Control") contained the same concentrations of $^3$H-aldosterone plus a 1000-fold excess of unlabeled steroid (Sigma Chemical Co., St. Louis, Missouri). Following incubation for 90 minutes, the mucosal cells were separated by density gradient centrifugation and the ammonium-sulfate precipitable $^3$H-aldosterone was measured in each of the two cell types prepared from the "$^3$H-Aldo" and "Control" tissues. The difference in the "$^3$H-Aldo" and "Control" values was assumed to represent specifically-bound or displaceable steroid.

In studies measuring the nuclear uptake of bound steroid, unseparated, washed mucosal cells were disrupted in a Parr pressure homogenizer (Parr Instrument Co., Moline, Illinois) equilibrated with 1350 lb/in$^2$ in $N_2$ for 30 minutes. Intact nuclei were pelleted at 800xg; the resultant supernatant was centrifuged at 100,000xg.

Aliquots of the ammonium sulfate precipitable protein of this supernatant were counted and normalized to protein by the method of Lowry (38).

## B. RESULTS

I found that the amount of displaceable $^{3}$H-aldosterone in MR cell preparations was directly related to the concentration of labeled steroid in the bath; there was no significant binding of displaceable aldosterone by G cell preparations. Table 4 gives values for the displaceable binding of aldosterone in the separated epithelial cells at increasing concentrations of the steroid. Half-maximal binding occurs at approximately 5-6.0 x $10^{-9}$M.

TABLE 4

Binding of $^{3}$H-aldosterone to separated mucosal cells of toad urinary bladder

| | $^{3}$H-Aldo (Mx10$^{-9}$) | Mitochondria-rich cells | | | Granular Cells | | |
|---|---|---|---|---|---|---|---|
| | | $^{3}$H-Aldo | Control | Net | $^{3}$H-Aldo | Control | Net |
| Nov-March | 1.2 | 1.38 | 0.84 | 0.54 | 0.73 | 0.74 | 0 |
| | 2.0 | 1.32 | 0.12 | 1.20 | 0.16 | 0.12 | 0.04 |
| | 2.2 | 2.59 | 1.79 | 0.80 | 1.23 | 0.94 | 0.29 |
| | 2.8 | 3.09 | 1.68 | 1.41 | 0.65 | 0.48 | 0.17 |
| | 5.0 | 3.05 | 0.89 | 2.15 | 1.18 | 0.87 | 0.30 |
| | 20.0 | 8.50 | 4.16 | 4.34 | 4.15 | 5.57 | 0 |
| April-June | 1.0 | 8.70 | 0.07 | 8.6 | 1.50 | 1.86 | 0 |
| | 4.0 | 24.00 | 0 | 24.0 | 4.70 | 6.50 | 0 |
| | 10.0 | 68.80 | 0 | 68.0 | 18.20 | 1.68 | 16.5 |
| | 40.0 | 88.00 | 1.80 | 86.2 | 1.77 | 1.91 | 0 |

The specifically bound steroid in each experiment, the difference between the "$^{3}$H-Aldo" and "Control," is expressed as moles x $10^{-14}$ per mg protein.

TABLE 5

Effects of DOCA and Cortisol upon aldosterone binding in toad bladder MR cells

| Date | $^3$H-Aldo Control | Competing Steroid DOCA | Competing Steroid Cortisol | % Displacement of $^3$H-Aldo by competitor |
|---|---|---|---|---|
| 8/28 | 15.7 | 6.2 | 5 | - | >100% |
| 8/30 | 14.7 | 5.8 | - | 12.3 | 27% |
| 9/10 | 7.4 | 4.5 | - | 6.8 | 21% |
| 9/18 | 3.6 | 1.4 | 1.2 | - | >100% |

Three sets of (8) hemibladders were used: $^3$H-Aldo, hemibladders incubated in 5 x $10^{-9}$M $^3$H-aldosterone; Control, hemibladders incubated in 5 x $10^{-9}$M $^3$H-aldosterone plus 5 x $10^{-6}$M unlabeled aldosterone; and either DOCA or cortisol, hemibladders incubated in 5 x $10^{-9}$M $^3$H-aldosterone plus the competing steroid ($10^{-6}$M). The values given (in moles x $10^{-14}$ $^3$H-aldosterone per mgm ammonium sulfate-precipitable protein) are the amounts of $^3$H-aldosterone bound by the MR cells in each set of hemibladders.

The binding of aldosterone by the MR cell was further investigated by testing a variety of steroids for their ability to compete with aldosterone. The results of these experiments are summarized in Table 5 (page 33). The data in Table 5 indicates that binding of aldosterone in Table 4 was to a mineralocorticoid-specific protein; this is demonstrated by the ability of deoxycorticosterone acetate (DOCA), a potent mineralocorticoid, t compete with aldosterone while a 200-fold excess of cortisol, primarily a glucocorticoid, displaced only 24% of the specifically bound aldosterone.

The subcellular distribution of displaceable $^{3}$H-aldosterone was determined in unseparated mucosal cells. Intact hemibladders were incubated as 10 paired sets of "$^{3}$H-Aldo" and "Control" tissues. Both bathing solutions contained $10^{-8}$M $^{3}$H-aldosterone and the "Control" bath also contained $10^{-5}$M unlabeled aldosterone. Following incubation for 90 minutes, the mucosal cells were removed and the two suspensions of cells were disrupted by pressure homogenization, which leaves the nuclei intact. The homogenates were fractionated by differential centrifugation and ammonium sulfate precipitation. In bladders incubated in $^{3}$H-aldosterone alone, 1222 cpm, representing

approximately 12% of the steroid in the homogenate, sedimented with the nuclei (Table 6, page 36). Receptor-dependent uptake of aldosterone by the nucleus is demonstrated by the large reduction in the amount of labeled steroid sedimenting with the nuclei prepared from control tissue (4.6%). The $^{3}$H-aldosterone bound by the ammonium sulfate precipitate prepared from the supernatant fraction of the cell also demonstrated significant displacement by unlabeled steroid. These data, which confirm earlier studies (16) of subcellular localization of aldosterone, indicate that the steroid is bound by specific receptors in both the nucleus and the cytoplasm of the mucosal cell.

The identification of the MR cell as the aldosterone binding locus in the bladder mucosal epithelium was further evidenced when displaceable steroid was determined in isolated MR cells. Using incubation procedures described earlier, the MR cell nuclei from "$^{3}$H-Aldo" ($10^{-8}$M) and "Control" tissues were isolated. The nuclei from the "$^{3}$H-Aldo" set contained 7650 cpm, which when compared to only 60 cpm in the "Control" nuclei, indicates nuclear accumulation of aldosterone by a specific receptor mechanism in the MR cells. These results, and the demon-

TABLE 6

Subcellular distribution of displaceable $^{3}$H-aldosterone in toad urinary bladder epithelial cells

| Subcellular fraction | "$^{3}$H-Aldo" | "Control" |
|---|---|---|
| Nucleus | 12.0% | 4.6% |
| Post-nuclear pellet | 10.6 | 11.8 |
| Supernatant | 84.1 | 87.5 |
| -Ammonium $SO_4$ Ppt. | 8.4 | 5.1 |

Paired sets of hemibladders were incubated as in Table I; the concentration of $^{3}$H-aldosterone was $10^{-8}$M. After 1.5 hr mucosal cells were removed by incubation in Ringers containing EDTA (2 mM) with no $Ca^{++}$ or aldosterone present. Mucosal cells were disrupted by pressure homogenization; the samples were equilibrated for 30 minutes with nitrogen at 1350 lb/in$^{2}$ before release. Nuclei were sedimented by centrifugation at 600 x g for 20 minutes. The 600 x g supernatant was centrifugated at

(Table 6 continued)

110,000 x g for 30 minutes (post-nuclear pellet) and protein in this supernatant precipitated by ammonium sulfate (50% saturated). Radioactivity was measured by liquid scintillation and the radioactivity in each fraction expressed as the percentage of that in the original homogenate.

stration of aldosterone-induced protein synthesis (see Part B, Section II) suggest a transcriptional effect of aldosterone.

I have also investigated the binding of the glucocorticoid, corticosterone in the two major cell types, MR and G, from the toad urinary bladder. The results of this study are summarized in Table 7 (page 39). The data in this Table indicate that $^{3}H$ corticosterone is displaceable by corticosterone and dexamethasone. It is important to stress that this binding, unlike that observed with aldosterone, is evident in both cell types.

The binding data presented in this Section can be summarized as follows:

1) There exists a specific mineralocorticoid receptor only in the MR cell of the toad urinary bladder. Half-maximal binding of aldosterone occurs at approximately 5-6.0 x $10^{-9}$M. This is in good agreement with the concentration of aldosterone necessary to give a half-maximal physiologic response. Displaceable $^{3}H$-aldosterone was found concentrated in the nuclei of these cells.
2) There exists in both the MR and G cells a corticosterone binding protein.

TABLE 7

Binding of $^{3}H$-corticosterone to separated mitochondria-rich and granular cells of toad bladder mucosal epithelium

| | Steroid Concentr. | Condition | Mitochondria-Rich | | | Granular | | |
|---|---|---|---|---|---|---|---|---|
| | | | A | C | Net | A | C | Net |
| 1) | $10^{-8}$ | Cortico-sterone | 5.00 | 2.23 | 2.77 | 3.44 | 1.68 | 1.76 |
| 2) | $10^{-8}$ | | 4.6 | 1.55 | 3.05 | 4.00 | 1.45 | 2.55 |
| 3) | $.5x10^{-8}$ | | 3.3 | | | 3.66 | | |
| | $+10^{-7}M$ | Dexame-thasone | 1.67 | | | 2.05 | | |
| | $+10^{-7}M$ | Aldo-sterone | 3.87 | | | 3.72 | | |

The specifically bound steroid in each experiment, the difference between the "$^{3}H$-Aldo" and "Control," is expressed as moles x $10^{-14}$ per mg protein.

The mineralo- and glucocorticoid binding and subsequent induced protein synthesis is discussed in Section IV.

# IV. ALDOSTERONE INDUCTION OF PROTEIN

## A. INTRODUCTION

The steroid hormone, aldosterone, increases the reabsorption of sodium from the forming urine by stimulation of the active transepithelial movement of this ion across the distal tubule of the kidney in mammals and the urinary bladder of amphibians. The toad urinary bladder has become a widely used model for the distal portion of the mammalian kidney because of its analogous responses to steroid and neurohypophyseal hormones. The biochemical processes mediating the physiologically important effects of aldosterone has been a focus of controversy. Suggested mechanisms include:

1) Enhancement of the permeability of the apical cell membrane;
2) stimulation of the sodium "pump" on the basal membrane of the mucosal cell;
3) an increase in the metabolic processes coupled to the energy-requiring active transport of sodium.

Because inhibitors of protein and RNA synthesis block the hormonal stimulation of sodium transport it

has been recognized that the induction of protein by aldosterone is integral to the mechanism by which this hormone increases the movement of ions. Therefore, the isolation and characterization of aldosterone-induced proteins is necessary for the elucidation of the actual events leading to increased reabsorption of sodium.

To this end I have undertaken the identification and isolation of soluble and membrane proteins induced by mineralocorticoids in the toad urinary bladder and have generalized these results by extension to the mammalian kidney.

Using a technique previously described for separating the two major morphologic cell types present in the epithelium of the toad bladder, I found (Section III) that the specific binding of aldosterone is limited to the "mitochondria-rich"(MR) epithelial cell. Localization of mineralocorticoid binding in the MR cell, which accounts for only 10-30% of the mucosal cell population, indicates that the initial biochemical events leading to enhanced sodium transport may take place in the MR cell. For this reason I determined the effects of physiologic concentrations of aldosterone on protein synthesis in the separated cell types.

## B. MATERIALS AND METHODS

### Incubation of Isolated Toad Bladders

Toads (Bufo marinus) of Colombian origin (Tarpon Zoo, Tarpon Springs, Florida) were partially immersed in a solution of 0.6% sodium chlorine for 5-8 days before use. Paired hemibladders were incubated at room temperature in two baths of Ringer solution. One bath contained either aldosterone or corticosterone ($2 \times 10^{-8}$M). After one hour [$^3$H]methionine (10 Ci/mM; New England Nuclear Co., Boston, Mass.) was added to one bath, and [$^{35}$S]methionine (100 Ci/mM; New England Nuclear Co., Boston, Mass.) to the other; the final concentration of methionine in each bath was $1-3 \times 10^{-6}$M. Following an additional one-hour incubation, the epithelial cells were removed and separated by centrifugation. The MR cells from the two sets of hemibladders were then mixed, sonicated with a Branson sonifier (2.5 watts) for two 15-second intervals and centrifuged at 105,000 x g for 30 minutes. The two sets of G cells were treated in the same way.

Sephadex chromatography of the cell extracts

The 105,000 x g supernatant fraction of the MR and G cells were each dialyzed against water and lyophilized. The samples were dissolved in a small volume of 0.025M TES (N-tris [hydroxymethyl] methyl-2-aminomethane sulfonic acid).(Sigma Chemical Co., St. Louis, Missouri) and chromatographed on a column (2.5 x 80 cm) of Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with TES, 0.025M (pH 7.4).

Isoelectric focusing of column eluates

Eluates of the Sephadex column were loaded onto an LKB Model 8101 isoelectric focusing column (IEF) in a solution of 2% carrier ampholytes (pH 3-10). The sample was stabilized with a sucrose density gradient and was electrofocused for approximately 72 hours. The initial voltage was 250V and was progressively increased to 600V, with the power never exceeding 2 watts.

Acrylamide gel electrophoresis

Samples from the IEF column were dialyzed for 24 hours against Tris-glycine buffer (pH 8.9) containing 0.1% sodium dodecyl sulfate (SDS). The samples were analyzed by electrophoresis in a discontinuous gradient (4, 10, and

12%) of acrylamide using the same Tris-glycine buffer (pH 8.9) and 0.1% SDS.

Proteolipid methods

In experiments determining the induction of proteolipid amino acid . incorporated into isolated cells, the above procedure was employed. The separated cells were washed free of Ficoll and resuspended in calcium, containing Ringer's. After 20 minutes preincubation in calcium Ringer's, aldosterone or corticosterone in ethanol or ethanol alone was added to the cells. After 45 minutes radioactive amino acid or carbohydrate was added; in double isotope experiments $^{3}H$ methionine was added to the aldosterone-containing cell suspension, and $^{35}S$, to the control. The cells were allowed to incorporate labeled amino acid for 1.25 hours after which time the aldo ($^{3}H$) and non-aldo ($^{35}S$) groups were combined, sonicated and centrifuged in glass Corex tubes at 42,000 x g for 40 minutes in a Sorvall RC2B. The pelleted membrane fraction was drained by inversion and extracted with 5 ml of chloroform-methanol (CM) 1:1. After centrifugation to separate out non-extracted debris, the CM extract was washed by addition of 2M KCl sufficient to give two phases. The lower chloroform phase (proteolipid phase) was chromatographed

on Sephadex LH-20 (Pharmacia) (1.5 cm x 36 cm) equilibrated with CM 1:1 .01 N HCl.

## C. RESULTS

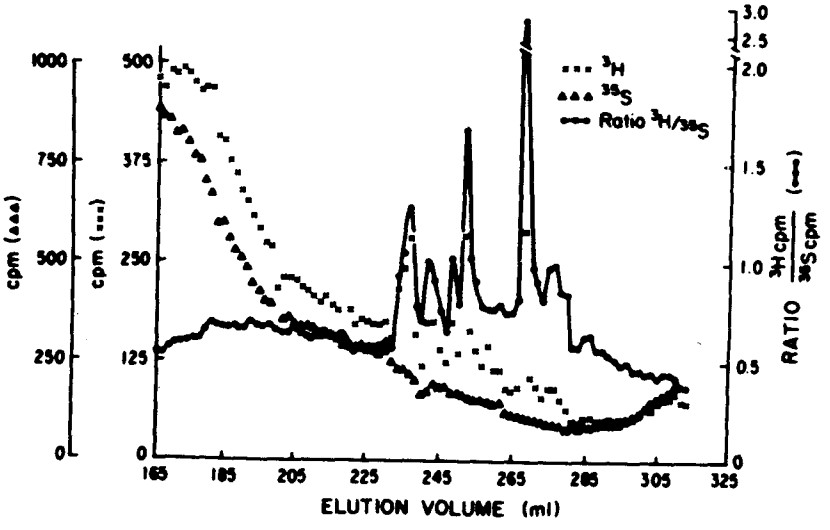
### Exclusion gel chromatography of the supernatant fractions

The MR cells prepared from the two sets of hemibladders ([$^{3}$H] and [$^{35}$H] methionine) were mixed, sonicated, and the "supernatant" fraction prepared by centrifugation. Exclusion gel chromatography of this preparation consistently yielded a series of fractions which contained a relatively greater amount of that radioactive label to which the aldosterone-treated tissue had been exposed. This enhancement by aldosterone of methionine incorporation was demonstrated in twelve experiments and was observed with either nuclide ([$^{3}$H] or [$^{35}$S]) in the aldosterone bath. In the experiment illustrated in Figure 9 (page 47), [$^{3}$H]methionine was added to the aldosterone-containing bath and the baseline ratio ($^{3}$H/$^{35}$S) was 0.55. In those fractions whose elution volumes corresponded to molecular weights ranging from 17,000 to 38,000 daltons, this ratio was increased significantly, reaching a value of 2.7. Characteristically, the elevations of the ratio described

three peaks.

Figure 7

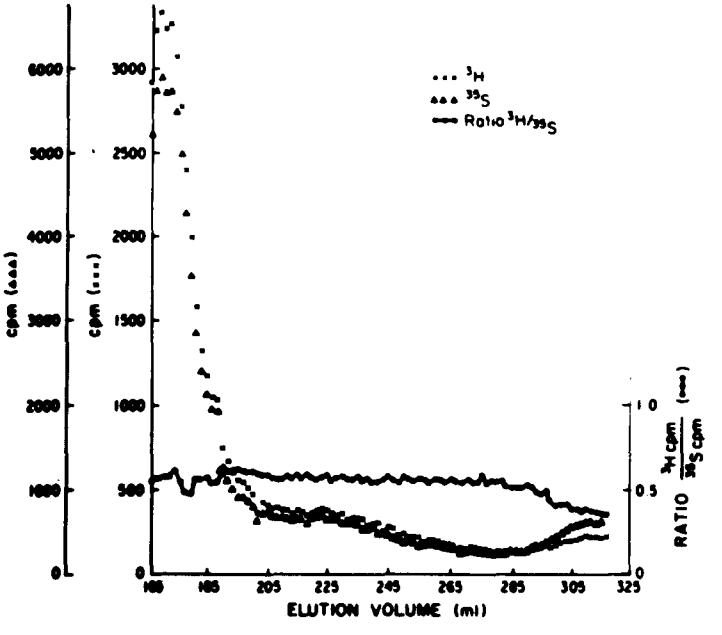
Elution profiles of soluble proteins from aldosterone-treated and control mitochondria-rich cells



The supernatant fraction of the G cells from the same experiment was chromatographed on the same column and the ratio ($^{3}H/^{35}S$) did not increase above baseline values (Figure 10). This indicated that aldosterone did not induce the synthesis of "soluble" proteins in the G cells of the intact bladder, although the large amount of radioactivity appearing in the void volume of the column demonstrates that these cells were actively synthesizing protein.

Figure 8

Elution profiles of soluble proteins from aldosterone-treated and control granular cells

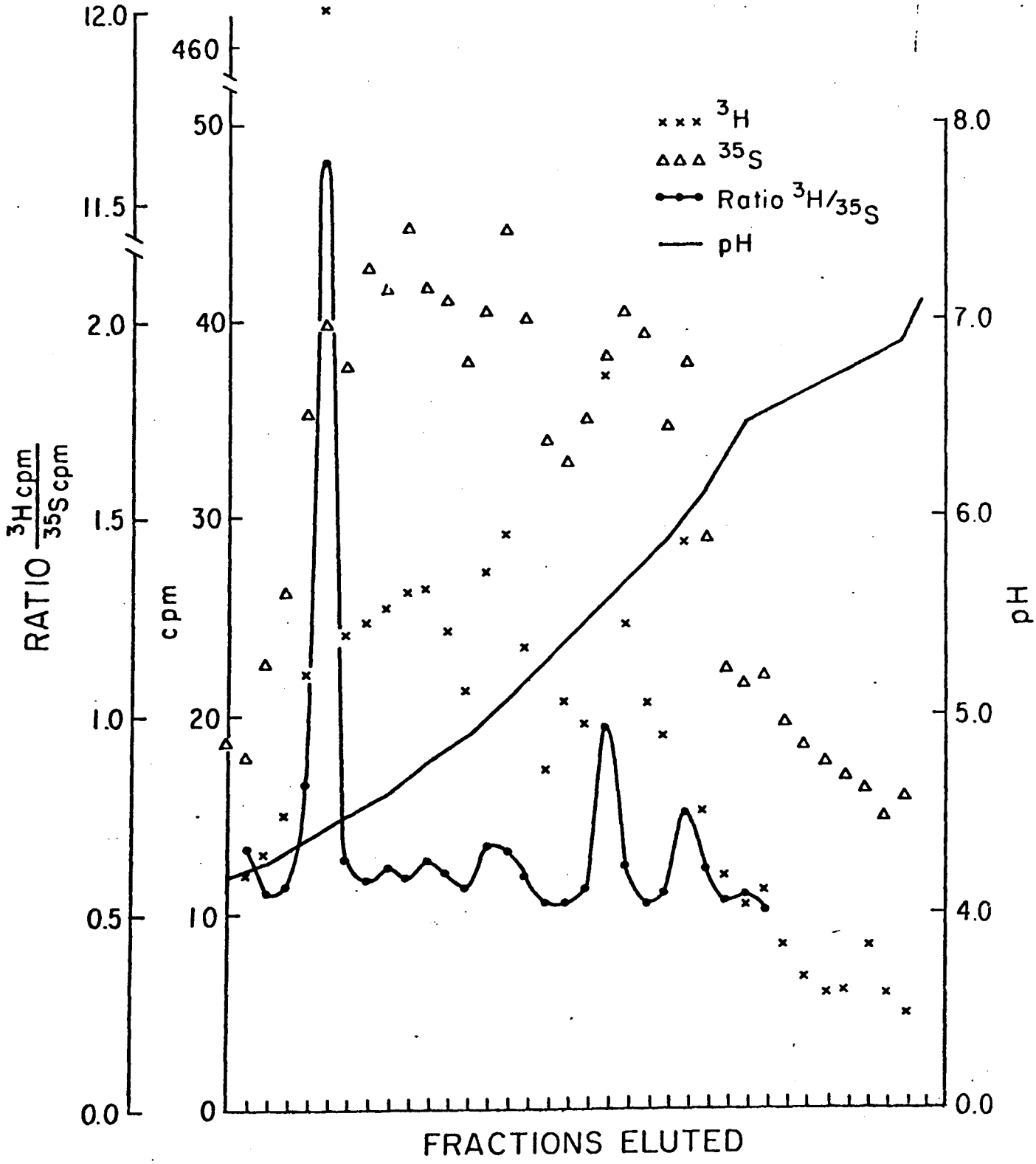


Isoelectric focusing of the Sephadex eluate

Those fractions of the MR cell Sephadex eluate having an elevated ($^{3}H$-$^{35}S$) ratio were pooled for analysis by isoelectric focusing (IEF). In the experiment illustrated in Figure 11, the pooled fractions included the eluate from 240 to 285 ml. Isoelectric focusing of this material yielded several peaks of enhanced ratio, one with a value as high as 11.7 (compared to an initial baseline of 0.55). The peak at pI ≈ 4.5, characteristically the most prominent feature of the IEF analysis of the MR cell preparation, was shown in other experiments to correspond primarily to the higher molecular weight fractions of the pooled Sephadex eluate.

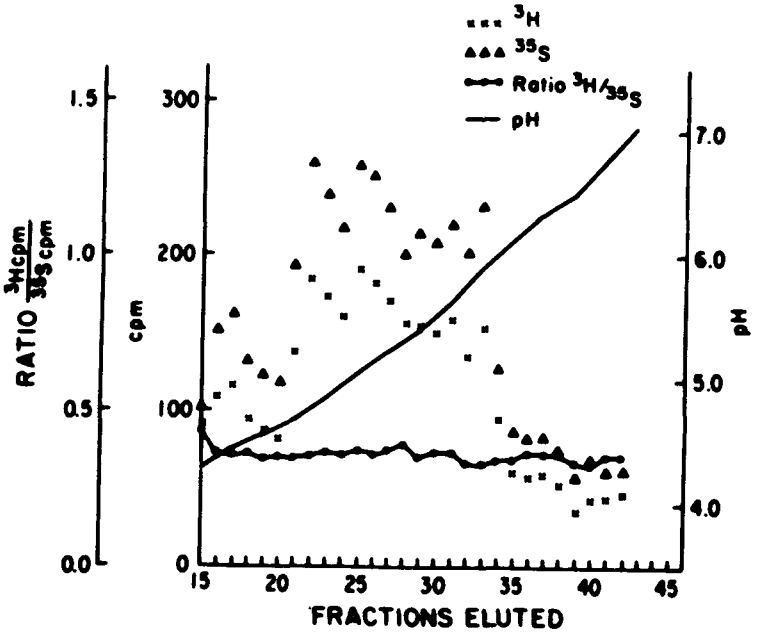
Although the Sephadex eluate of the G cell supernatant fraction showed no apparent increases in the ($^{3}H/^{35}S$) ratio, we pooled those fractions of the G cell Sephadex eluate corresponding to the MR cell fractions with an increased ratio (240 to 285 ml) and analyzed this material by IEF. As shown in Figure 12, the material did not yield any fractions with an increased ($^{3}H/^{35}S$) ratio.

**Figure 9**
Isoelectric focusing of G-75 eluate of soluble protein from aldosterone-treated and control mitochondria-rich cells



## Figure 10

Isoelectric focusing of G-75 eluate of soluble protein from aldosterone-treated and control granular cells



SDS-Acrylamide gel electrophoresis of the IEF eluate

IEF eluate from the peak at pI - 4.5 was analyzed by electrophoresis in an SDS-acrylamide gel system. As shown in Figure 13, the gel contained three bands. The molecular weights of these three proteins are estimated at approximately 12,000, 24,000 and 36,000 daltons.

Figure 11

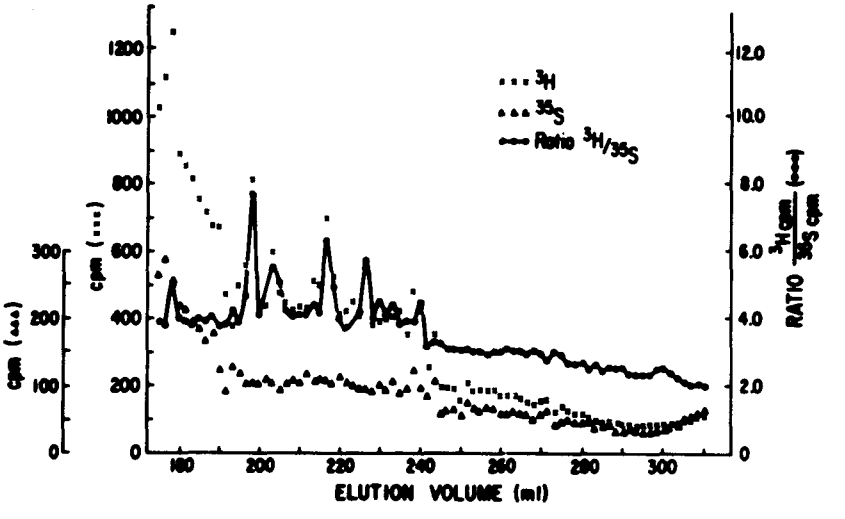
SDS polyacrylamide gel electrophoresis of IEF eluate

Glucocorticoid induction of protein synthesis in mucosal cells

Corticosterone, the natural glucocorticoid of Bufo marinus, has relatively smaller effects upon sodium transport than aldosterone. Using the same number of toads (108) and the same experimental conditions, corticosterone ($5 \times 10^{-8}$M) was added to the hemibladders incubated in [$^3$H]methionine while the [$^{35}$S]methionine hemibladders served as a control. The ratio ($^3$H/$^{35}$S) in the Sephadex eluate of the MR cell preparation is strikingly different from that obtained in the aldosterone-treated tissue--there were no significant increases above baseline in this preparation. On the contrary, the supernatant fraction of the G cells from the corticosterone-treated tissue yielded a series of fractions with elevated ratios (Figure 12). As indicated by their elution volumes, all these fractions in the G cell preparation had molecular weights greater than those proteins induced in the MR cell by aldosterone. The fractions of the Sephadex eluates of the MR cells and the G cells with elution volumes of 240-285 ml were pooled and analyzed by isoelectric focusing. There was no evidence for the induction of any proteins similar to those induced in the MR cell by aldosterone.

## Figure 12

Elution profiles of soluble proteins from corticosterone-treated and control granular cells

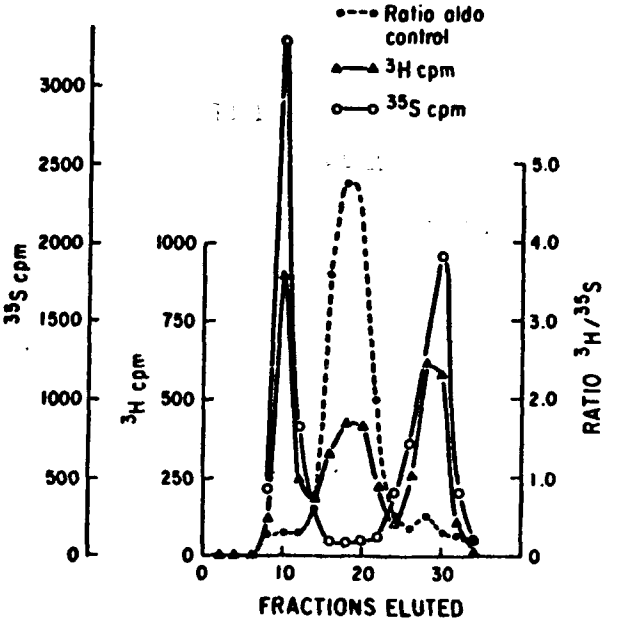


## Proteolipid Results

Fractionation of the MR cell proteolipids by the procedure described earlier yielded the results illustrated in Figure 13. The isotopic ratio ($^{3}H/^{35}S$) corresponding to PLII is 20-fold higher than the ratios of the PLI and PLIII, indicating that the synthesis of this protein was markedly enhanced by aldosterone. In a separate experiment $^{3}H$ leu and $^{14}C$ galactosamine were used and the results are illustrated in Figure 14.

### Figure 13

LH-20 fractionation of aldosterone-treated and control mitochondria-rich cell proteolipid

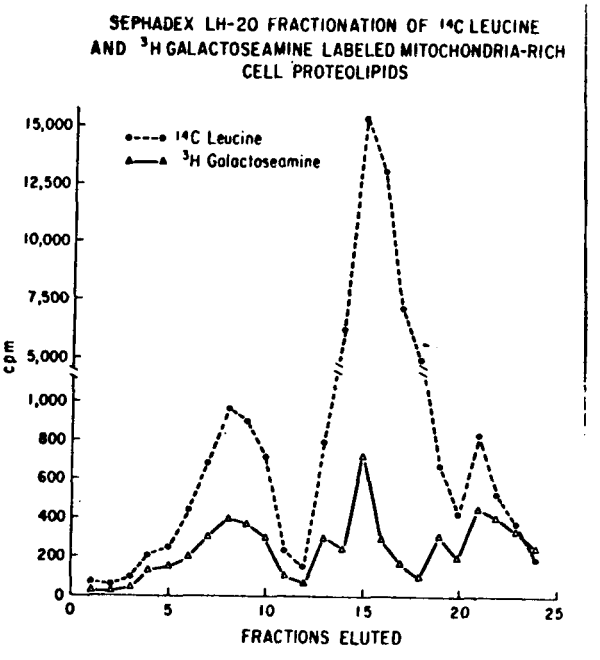


(Figure 13 continued)

Proteolipid extracts from aldo-treated ($_{+}^{3}$H methionine) and control ($_{+}^{35}$S methionine) were prepared as described in Part A of this section. Sephadex LH-20 was swelled in C-M (1:1, v/v). Chromatography was performed on a 1.5 x 36 cm column equilibrated with C-M (1:1, v/v) 0.01N HCl. Flow rate was 10 ml/h. After 8.0 ml elution was in 1.5 ml fractions.

## Figure 14

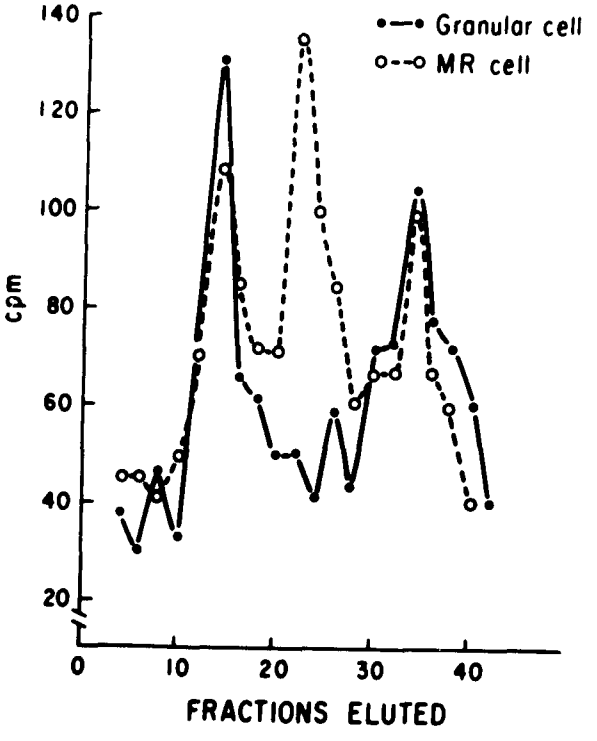
LH-20 fractionation of $^{14}$C-leucine and $^{3}$H-galactosamine labeled mitochondria-rich cell proteolipid




Mitochondria-rich cells were labelled simultaneously with $^{3}$H-galactosamine and $^{14}$C-leucine. The fractionation procedure was as in Figure 13.

Figure 15

Comparison of mitochondria-rich cell and granular cell proteolipid




Proteolipids from MR and G cells were fractionated on separate but identical LH-20 columns. Both cell types were labelled with $^{3}H$ methionine. The fractionation procedure was as described in Figure 13.

Figure 16

LH-20 fractionation of granular cell proteolipids after treatment with corticosterone

Granular cells were labelled and extracted as described in Methods. Fractionation was performed as in Figure 13.

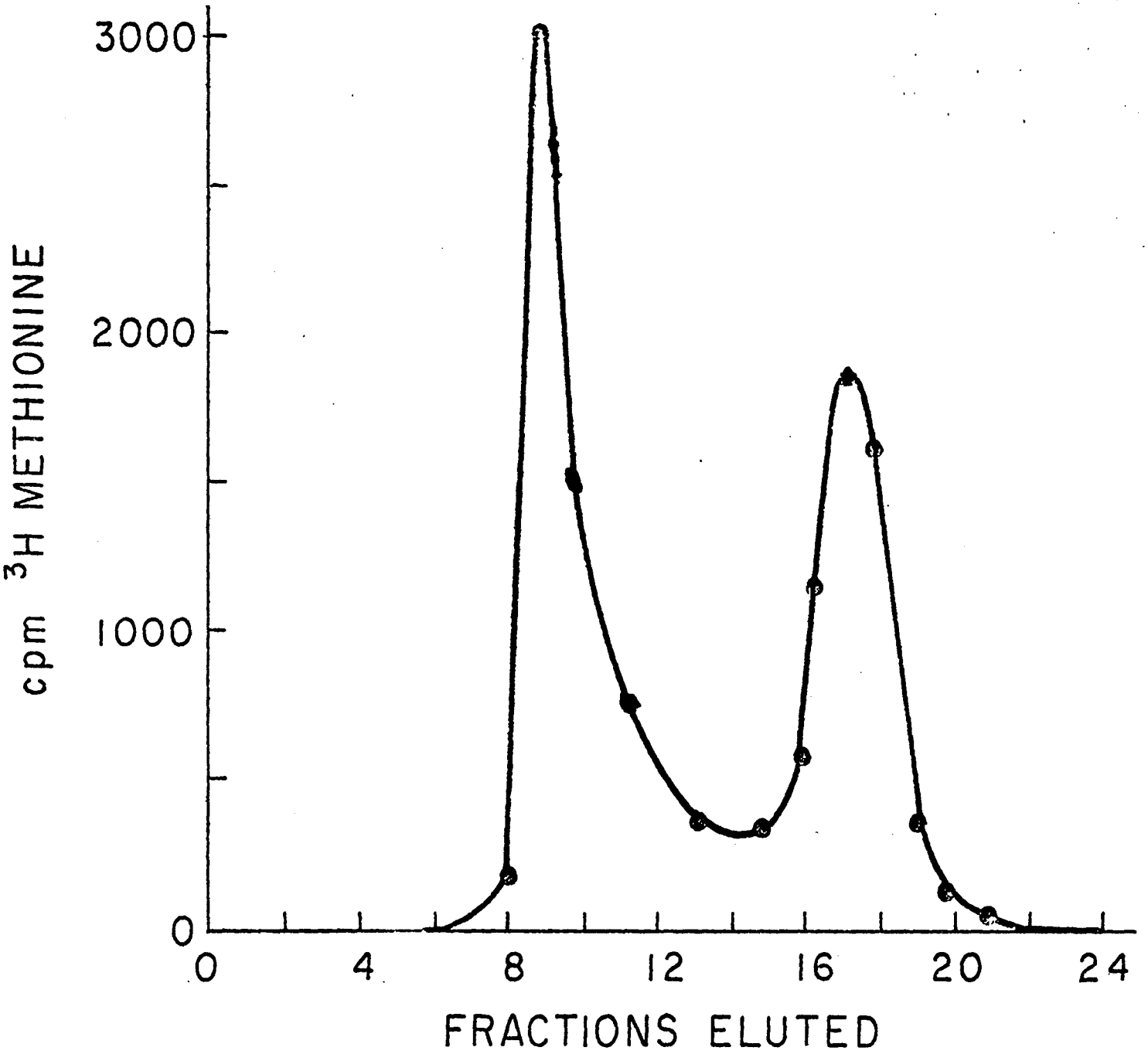
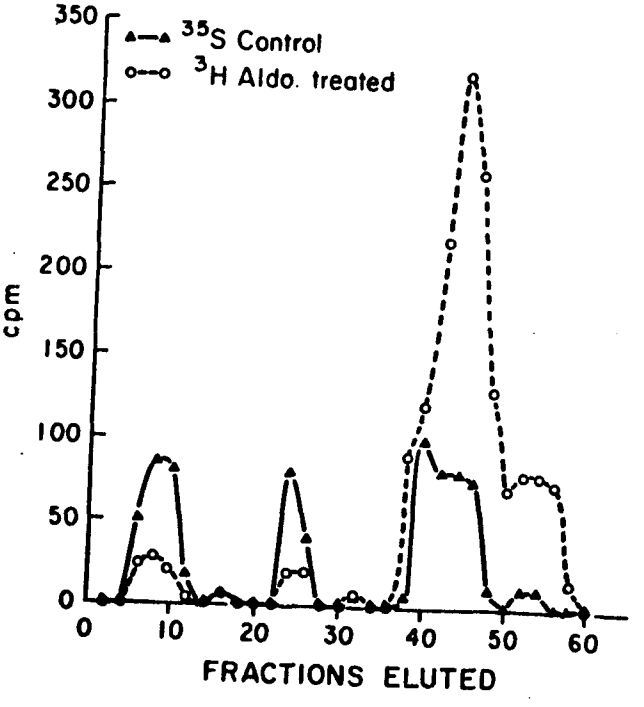

3000
2000
1000
0
cpm $^{3}$H METHIONINE
0
4
8
12
16
20
24
FRACTIONS ELUTED

Figure 17

LH-20 fractionation of proteolipids from aldosterone-treated and control rat kidneys




Fractionation procedure as described in Figure 13.

## DISCUSSION I

The effects of oxytocin upon tissue cAMP and short-circuit current (SCC) were measured in the urinary bladder of the toad, Bufo marinus. Tissue cAMP levels doubled before any increment in SCC was observed, the two hormone responses were quantitatively related, and a threshold level for an effect of cAMP upon sodium transport was demonstrated. The period of time over which cAMP levels continued to rise after the threshold level had been attained seemed invariant with hormone concentration. The rate at which cAMP levels rose increased with hormone concentration yielding hormone concentration-dependent maximal levels. The decay of cAMP levels was delayed when sodium influx was curtailed, suggesting a sodium-regulatory effect upon tissue cAMP levels.

The concept of threshold in NHP action in the toad urinary bladder was first observed by Eggena (44). Briefly, this study demonstrated that certain NHP analogs such as 2- (0-ethyl-tyrosine) oxytocin (OET) could bind to receptors but did not stimulate adenylane cyclase sufficiently to evoke a hydroosmotic response. This conclusion was based on experiments where theophylline was used either alone or with OET; it was found that the

two agents together gave a larger response than the phosphodiesterase inhibitor alone. Amplification of the theophylline response results from an increase in adenylate cyclase by OET that by itself is physiologically ineffective. The result of Section II Part B indicates the threshold corresponds to a doubling of the basal cAMP level. This threshold when obtained starts a series of events eventually producing an increase in sodium transport and/or hydroosmotic flux. My results would also indicate that the increase in sodium flux (specifically the increase in mucosal sodium influx) plays an important regulatory function. The hormone-stimulated increase in cAMP is temporally limited by the influx of sodium. When sodium influx was either blocked by substitution with choline or inhibited by amiloride, the cAMP continued to rise for the period over which it was measured. Previously Dousa (39) showed that vasopressin stimulation of adenylate cyclase of kidney medulla was biphasic with respect to sodium chloride with inhibition manifest at NaCl concentration above 140 mM. Finn (44) also has presented evidence that sodium levels have a regulatory effect on the hormone stimulated sodium short-circuit current. His data

indicate that a decrease in the ambient sodium concentration results in a longer duration and relatively greater magnitude in the vasopressin increment in SCC. He also reported that cAMP stimulation of the SCC varied with the sodium concentration. This effect was qualitatively different in that the initial magnitude was unaffected ($t=20$ min.), but a greater difference was observed after $t=60$ min., due to a slower decay. A point not noted in Finn's discussion is that not only was the maximal $\Delta$SCC (vasopressin) relatively greater in reduced sodium but that it occurred later (20-25 minutes compared to 10-15 minutes). These data may be explained by the results presented in Figures 5A and 5B, in which the cAMP levels continued to rise when sodium entry was blocked; maximal levels were therefore greater and attained at a later time. The increase in duration with lower sodium pools, evident in the second 30 minutes of the vasopressin response, may be an effect of sodium not translated directly through cAMP levels.

I would like to pick up this point of pool in discussing the results of Part C of Section II. In this study I utilized a technique developed in Dr. Walter N. Scott's laboratory for separating the mucosal epithelial

cells from the toad urinary bladder. The cells consist of two major types--granular cells and mitochondria-rich cells. I found that only the mitochondria-rich cells responded to oxytocin with an increase in cAMP. If the intracellular sodium pool derived from the transport of luminal sodium regulates the hormone response, then one would expect the transport pool to be in the proximity of the adenylate cyclase. This strongly suggests that the sodium entry step related to the hormone increment is through the MR cell.

There is data in support of this contention. Finn, Ferguson and MacKnight et al. (45, 46, 23) have published evidence showing the sodium transport "pool" corresponds to approximately 20% of the intracellular sodium. This is in striking correspondence to the proportion of the epithelial cell population made up of MR cells. Dörge (47) demonstrated that the transport pool and the amiloride-sensitive pool are probably the same. This was concluded on the basis of kinetic studies of the re-equilibration of intracellular sodium after the addition of amiloride to the mucosal bathing solution. Cuthbert (48) presents evidence that the amiloride-sensitive pool corresponds to the vasopressin pool (transport pool). The increase in

$^{22}Na$ efflux at the serosa after administration of vasopressin had the same time course as the decrease observed in this flux after treatment of the mucosa with amiloride. The nature of the influx site as described by Ferguson and Smith (46) is through a saturable carrier at luminal sodium concentrations below 40 mM. They estimate the system operates with a KM of 15 mM.

At low sodium concentrations, e.g., when the mucosal solution contains 2 mM sodium, one still measures a sodium short-circuit current, although the intracellular sodium has been measured to be 8-10 mM (22). It therefore seems that sodium influx may be linked to an active process. Other evidence indicating an active step at the mucosa is the rapid decrease in sodium short-circuit current with the addition of dinitrophenol. This drug blocks the mitochondrial oxidative phosphorylation of ADP to form ATP. The effect of DNP on the sodium SCC was concomitant with a drop in the intracellular sodium; the efflux at the serosa continued well after the current decrement had stabilized. This response pattern is what one associates with an effect at the mucosal surface, as seen with amiloride, and certainly not what one would see if the ATPase "pump" was the primary effect, as in the ouabain effect.

Before going on, the points raised in the discussion thus far are summarized below:

1) Sodium influx, whether measured by the SCC or by isotopic sodium entering the transport pool exerts several regulatory functions:

   A) a negative feedback on hormone stimulation of adenylate cyclase;
   B) a truncation with respect to time of the second phase of the NHP increment in SCC.

2) The "sodium effect" on cAMP, 1)a, seems to be within the mitochondria-rich cell, which indicates it may be the site of entry for this sodium pool.
3) The sodium transport pool is the pool effected by NHP and is amiloride-sensitive.
4) Influx into the transport pool is at the mucosal surface and is through a carrier saturable at sodium concentrations above 40 mM.

Aldosterone exerts several well-documented effects on carbohydrate metabolism and oxygen consumption. It has also been observed to increase the turnover of unsaturated fatty acids. As pointed out in the introduction, the effects on oxygen utilization and glycolysis are secondary to the effect on sodium. The early stimulation of $C_1$ glucose oxidation by the pentose cycle as well as the subsequent inhibition (after 1 hour) were shown to be independent of sodium.

None of the above changes in carbohydrate metabolism, sodium independent and sodium dependent, are evidenced when protein synthesis has been blocked. In Section IV of this thesis, evidence was presented for the induction of protein in the soluble fraction of the mitochondria-rich

cell. Identification of this protein as "induced" was based on the increase in isotopic ratio using $^{3}H$ (aldosterone and $^{35}S$ (control). An increased ratio was observed at 3 elution volumes from G 75; this does not necessarily indicate 3 proteins. Benjamin and Singer (49) reported the identification of an aldosterone-induced protein in the toad bladder. Using SDS solubilized protein from mucosal cells and electrophoresis on SDS slab gel, they were able to identify only one protein as induced with a molecular weight of 12,000 Daltons. The molecular weight of the soluble protein described in Section IV was estimated as 16-18,000, 22-25,000 and 32-36,000 Daltons. Using the same isotope technique I isolated an induced proteolipid from the membrane fraction of the MR cell. An induced proteolipid was also identified from the crude mitochondrial fraction from rat kidney. Finding induction in both adrenalectomized rats and toad urinary bladder epithelial cells reinforces the importance of this protein in the action of aldosterone. Proteolipids are usually found as proteins of 30-40,000 daltons, but because they themselves are aggregates (50) they are found to migrate on SDS gels in several forms; the most

commonly observed molecular weights are 28-36,000, 25,000, 18,000 and less commonly a minimum molecular weight of 12,000. I therefore contend that the Benjamin and Singer protein, the soluble proteins I have described, and the proteolipid are one and the same. The solubility of the proteolipid in chloroform methanol does not preclude there being a water-soluble form. Folch-Pi (51) demonstrated that the delipidated apoprotein proteolipid may be converted to a water-soluble form which will still retain solubility in chloroform-methanol.

There are several unique features of the induced proteolipid which easily differentiate it from the others found in the extract. Comparison of the relative incorporation of leu, met and gal-$NH_2$ suggests the inducible proteolipid is the more hydrophobic. Further evidence of relative hydrophobicity is that only this proteolipid migrates on Sephadex LH-20 (CM 1:1 + 0.01N NCl) still bound to a significant amount of lipid. This was determined qualitatively by spotting each fraction and staining with vapor and Rhodamine 6G. This latter point seems to indicate that the proteolipid was situated in a lipid-rich milieu. The finding that the proteolipid

DISCUSSION II

In the second part of the discussion, I would like to discuss possible control mechanisms for sodium transport. I will be trying to limit the number of sites at which an induced protein may be acting.

Cuthbert has proposed a "two-state" model for sodium transport (52). Briefly, he has shown that $Ca^{++}$ has an inhibitory effect on both sodium transport and transport-dependent $0_2$ consumption. The maximal inhibition by $Ca^{++}$ occurs at low concentration; the concentration of $Ca^{++}$ necessary for a given percentage inhibition is increased by the addition of vasopressin. The mechanism of this decrease in $Ca^{++}$ inhibition is correlated with the hormone-stimulated mucosal efflux of this ion (53). The time course of $Ca^{++}$ efflux and the cAMP increase described in Section II Part B correlate very well (54). In several tissues, the binding of $Ca^{++}$ to the surface of mitochondria is associated with an inhibition of $0_2$ (55) consumption; it has also been suggested that $Ca^{++}$ lending to mitochondria is reversed by cAMP. In kidney slices there is evidence that intracellular $Ca^{++}$ binding may also be associated with a stimulation of gluconeogenesis (56). The relevance to the transport system of the toad

bladder is the demonstrated importance (29) of the glycolytic generation of pyruvate. Gluconeogenesis in the kidney and other tissues is associated with a lowering of pyruvate levels and a reversal of glycolysis. This mechanism requires the enzyme Phosphoenolpyruvate carboxykinase (PEPCK) which is activated by $Ca^{++}$ (57). The inhibition of respiration by calcium therefore decreases the oxidative metabolism of pyruvate and inhibits its further generation.

It was proposed that $Ca^{++}$ inhibition of the enzyme pyruvate carboxylase augments the gluconeogenic effect. Pyruvate carboxylase may also be important in that it initiates a cycle which will generate reduced NADP. This is necessary to sustain unsaturated fatty acid synthesis after the inhibition of the cycle by pentose.

Of interest is the ability of pyruvate carboxylase to utilize $HCO_3^-$ as substrate. Orloff et al. (28) noted the stimulation of the sodium short-circuit current by increasing the $HCO_3^-$ at constant pH (7.6) from 2.4 to 25.0 mM. The effect was similar to that of hormone (aldosterone and vasopressin) in that the change in short-circuit current resulted from an observed

decrease in resistance. In the previously mentioned stimulation of gluconeogenesis by $Ca^{++}$ in kidney it was also demonstrated that an increase in $HCO_3^-$ at constant pH (7.4) decreased the $Ca^{++}$ gluconeogenic effect. The interaction between the $HCO_3^-$ sensitive site and the site affected by aldosterone may be inferred by the synergistic nature of these effects at maximal doses of aldosterone $10^{-7}$M. This strongly suggests that these agents may be acting upon identical processes but at different sites.

The ability of aldosterone to stimulate a $HCO_3^-$ - dependent "acidification current" (40) was performed in the absence of sodium transport (amiloride inhibited) but was $O_2$ dependent.

Therefore, an aldosterone effect on transport is mediated through a sodium-independent mechanism.

The last several points may be summarized:

1) Aldosterone stimulation of glycolysis and $O_2$ consumption is sodium-transport dependent.

2) The stimulation of pyruvate levels is the result of increased glycolysis.

3) The sodium "pull" on glycolysis would seem to be related to the utilization of pyruvate. The effect on $O_2$ consumption which shows a similar sodium dependency can be interpreted to be an effect on the oxidation of pyruvate by the Krebs cycle.

4) Sodium transport is itself stimulated by $HCO_3^-$. This effect would seem to be through pyru ate carboxylase. $HCO3^-$ transport is itself stimulated by aldosterone and in addition acts synergistically with aldosterone on sodium transport. This indicates aldosterone effects a transport system to which the movements of sodium and $HCO_3^-$ may be coupled, and which is itself stimulated by $HCO_3^-$. This effect may also have a glycolytic link implemented by the pulling effect of pyruvate carboxylase.

One need go no further than an analogy to $Na^+$, $K^+$ stimulatable ATPase. In such a model the pump would be linked to the sodium influx which, under appropriate conditions (pH and $[HCO_3^-]$ ) would couple to the influx of $HCO_3^-$ or extrusion of $H^+$; thus, in combination with luminal $HCO_3^-$, $CO_2$ is produced. This $CO_2$ can diffuse into the cell where it is converted to $HCO_3^-$.

The coupling to metabolism may be through the utilization of the $CO_2$ or $HCO_3^-$ by pyruvate carboxylase.

The effects of aldosterone are certainly evident at the level of influx or through reactions determining the fate of pyruvate. The proteolipid induced by aldosterone may be acting at one or both of these two loci. The demonstration of an induced proteolipid from rat kidney localized in the crude mitochondrial fraction is suggestive.

More so I have recently obtained preliminary evidence of $Ca^{++}$ and amiloride binding to toad urinary bladder proteolipid. When increasing amounts of $^{14}C$ amiloride or $^{14}C$ amiloride+unlabeled amiloride (control) were incubated with mucosal cells for two minutes in low sodium Ringer's and extracted with chloroform-methanol, displaceable binding was observed (Table 8, page 75).

Table 8

Binding of $^{14}C$ amiloride to chloroform-methanol extracts of toad urinary bladder mucosal cells

| Conc. $^{14}C$ amiloride | cpmx$10^3$ mg protein | | |
|---|---|---|---|
| | A | C | Net |
| $1.0 \times 10^{-7}$ | 24.4 | 11.2 | 13.2 |
| $5.0 \times 10^{-7}$ | 132 | 60.6 | 71.4 |
| $25.0 \times 10^{-7}$ | 403 | 194 | 209 |

The binding of amiloride was measured in washed CM extracts of isolated mucosal epithelial cells. Cells were incubated in $^{14}C$-amiloride at concentrations listed in Table. Control sets of cells were incubated in identical concentrations of $^{14}C$-amiloride with a 400-fold excess of unlabeled amiloride. Protein was determined by the method of Lowry on the washed CM extracts: A=$^{14}C$ amiloride alone, C=$^{14}C$ amiloride + 400x cold amiloride.

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