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City University of New York Ph.D. 1979

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CELLULAR EVENTS ASSOCIATED WITH THE STIMULATION OF SODIUM TRANSPORT BY ALDOSTERONE IN THE TOAD URINARY BLADDER

by

IRWIN M. REICH

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the Degree of Doctor of Philosophy, the City University of New York.

1979

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Chairman of Examining Committee

May 27, 1979
date

Executive Officer

Dr. William A. Brodsky, Mt. Sinai

Dr. Lawrence R. Krakoff, Mt. Sinai

Dr. David B.P. Goodman, Yale

Dr. Stanley Ulick, Bronx V.A.
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The City University of New York

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Abstract

Cellular Events Associated with the Stimulation of Sodium Transport
by Aldosterone in the Toad Urinary Bladder

by

Irwin M. Reich

Advisor: Walter N. Scott, M.D.

A technique is described for the preparation from toad urinary bladder mucosal cells of a fraction enriched in plasma membranes. Electron microscopy reveals a membrane preparation composed primarily of vesicles; enzymatic characterization shows a considerable enrichment in putative plasma membrane associated enzymes; and preparation of membranes from bladders whose apical membranes were radio-iodinated (with lactoperoxidase) shows a great enrichment of isotope-labelled proteins.

The active mucosa-to-serosa sodium transport is believed to be via a transcellular (rather than paracellular) pathway, and indirect evidence points to an increase in the mucosal cell apical plasma membrane Na conductance as being the predominant mediator of the increased short circuit current (SCC) observed in response to aldosterone (Aldo); another possibility is increased Na-K ATPase activity in the serosal membrane. Direct evidence for Aldo-induced proteins (AIP) has recently been found, but has not been well-localized to subcellular organelles. The present study uses the method for preparing plasma membranes in order to investigate whether exposure to mineralocorticoids results in biochemical changes in this fraction.

Using double-labeled isotope techniques, it can be shown that aldosterone induces the synthesis of several proteins in the mitochondria-rich (MR) cells of the toad's urinary bladder. Incubation with hormone proceeded for 3 1/4-4 hours, following which induced proteins were identified in both the plasma membrane (mol wt = 170,000, 85,000, and 12,000) and the cytosol (mol wt = 36,000, 12,000, and 6,000). Since the incubation period corresponds to the time for the hormone to cause a maximal rise in SCC, the proteins appear to be related, at least temporally, with steroid-controlled sodium transport. Both the hormone-induced rise in SCC and the appearance of specifically synthesized proteins are blocked by cycloheximide.

2-methyl-2-[p-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid (TPIA), an acetyl CoA carboxylase inhibitor, blocks the Aldo-induced increase in transepithelial sodium transport. To examine the requirement for ongoing fatty acid synthesis and/or elongation in the Aldo-induced alteration of cellular protein metabolism, the effect of TPIA has been examined in double-labeled amino acid incorporation experiments. TPIA itself is shown to have no effect on the pattern of protein labeling in either the soluble or the plasma membrane-enriched fraction. However, inhibition of fatty acid synthesis selectively inhibits the Aldo-induced incorporation of amino acids into membrane proteins without altering the labeling of soluble cell protein. These membrane proteins have molecular weights very similar to the AIP's described above. These results indicate that ongoing fatty acid synthesis is required for the hormone-induced changes in plasma membrane protein metabolism related to sodium transport.

I. Literature Review

A. The Amphibian Urinary Bladder as a Model for the Mammalian Distal Nephron.

It was first demonstrated in the 1950's that toads (Bufo regularis) and bull frogs (Rana catesbeiana) reabsorb fluid from their urinary bladders in response to neurohypophyseal hormones released during dehydration (Ewer, 1952; Sawyer and Schisgall, 1956). Leaf, Anderson, and Page (1958) demonstrated that the urinary bladders isolated from Bufo bufo and B. marinus actively transport sodium from the mucosal (urinary) side to the serosal (blood) side, and that this transport is stimulated by neurohypophyseal hormones. Simultaneously, Bentley (1958) demonstrated that exposure of the serosal side of the bladders of B. marinus to antidiuretic hormone (ADH) resulted in the almost immediate stimulation of the osmotic movement of water across the tissue, thus establishing the bladder as a research tool for studying the transport properties of the mammalian distal tubule.

Leaf and his colleagues concluded that the baseline transport of Na by the bladder is active after observing that radioisotopes of Na move more rapidly from the mucosa to the serosa than vice versa, and that this occurs when the Na concentrations are equal on both sides of the tissue (Leaf et al, 1958; Leaf and Dempsey, 1960). Transport is thus against the electrochemical gradient, and is reduced by metabolic inhibitors or inhibitors of Na-K ATPase. In the process, a potential difference (PD) is established across the membrane due to the difference in Na concentrations between the two sides. The serosal side is electropositive by as much as

120 mV under baseline conditions. Electrical current can be applied across the membrane, and when this current is such that the spontaneously generated PD is reduced to zero, it is termed the short-circuit current (SCC) (Ussing and Zerahn, 1951). Leaf's group determined the SCC to be equal to the net rate of Na transport under a variety of conditions, establishing the SCC as a relatively simple means of measuring Na transport in the toad bladder, obviating the necessity of using sodium isotopes.

Another landmark advance was made by Crabbe (1961a, b) who demonstrated the stimulation of sodium transport in the toad bladder in response to aldosterone (Aldo). As opposed to the prompt response observed with ADH, this response was characterized by a latent period of 90 minutes, and the passage of 3 hours before maximal Na transport occurred. The stimulation of Na transport was observed at physiologic concentrations of hormone (10-8 to 10-9 M) and persisted for 6-8 hours. This work provided the foundation for subsequent studies designed to elucidate the mechanism of action of Aldo (c.f., Edelman, Bogoroch and Porter, 1963; Sharp and Leaf, 1964).

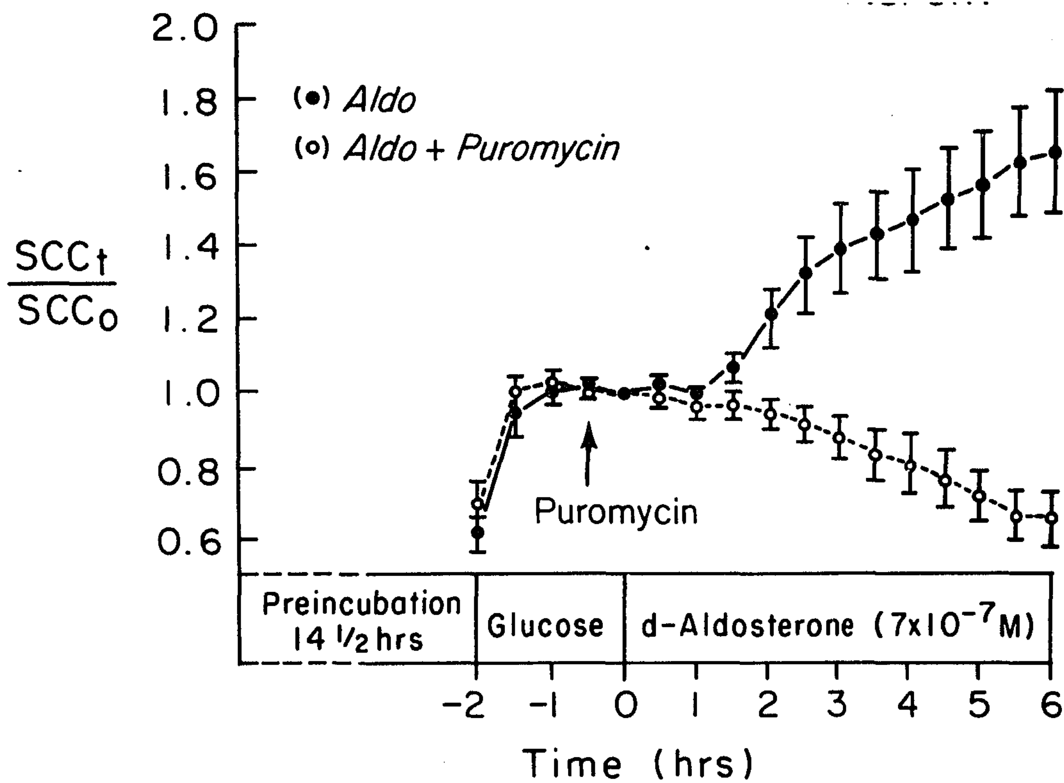
The toad bladder consists of a single layer of mucosal cells supported by connective tissue and smooth muscle. The epithelial cells can be classified according to their morphology into four types, of which the "granular" (G) cells and "mitochrondria-rich" (MR) cells account for approximately 80% and 15%, respectively (Choi, 1963) in Dominican toads. There are also a small number of mucous secreting cells and basal cells. Both the MR and G cell traverse the epithelial membrane, and are thus in contact with both the urinary surface and basement membrane of the tissue (DiBona, Civan, and Leaf, 1969a). Each MR cell is adjacent to and

surrounded by an array of granular cells. It is of interest that the arrangement of mucosal cells is somewhat different in the bullfrog bladder, which is physiologically similar to the toad bladder with respect to hormone responsiveness. In this tissue the basal cells "cup" under the granular cells in such a way that the granular cells are never in contact with the serosal surface of the basement membrane (Strum and Danon, 1974). A unifying theory of the mechanisms of hormonal responsiveness in these tissues should attempt to take both of these anatomic configurations into account.

B. The Proposed Mechanisms of Aldo-Induced Stimulation of Sodium Transport

Aldosterone is the most potent of the corticosteroids in its effects on transepithelial sodium and potassium fluxes. Its primary site of action in mammals is the kidney distal tubule (Vander et al, 1958), where Na+ reabsorption is effected while H+ and K+ secretion are enhanced. The hormone also affects ion transport in the colon, sweat glands, and salivary glands.

The major hypotheses concerning the mode of stimulation of Na transport by Aldo are based on the presumption that the hormone causes the appearance of new cellular proteins. This is an outgrowth of the demonstration that there is a latent period of 90 minutes before the Aldo-induced increase in SCC is oserved, suggesting that transcription and translation must first occur (Crabbe, 1961b). It was subsequently confirmed that the hormone-induced increase in Na transport could be blocked by either puromycin or actinomycin D (Edelman, Bogoroch and Porter, 1963; Williamson, 1963; Porter, Bogoroch, and Edelman, 1964) (Fig. 1). Three possible functional roles for the Aldo-induced protein(s) (AIP) are generally considered: (1) the AIP may be a plasma membrane component on the luminal (urine) side acting as a "sodium permease", lowering the resistance to Na entry into the cell (2) the AIP may be the Na-K ATPase, or a component of this enzyme, on the serosal (blood) side of the tissue, thus increasing the active extrusion of Na from the cell (3) the AIP may be a mitochondrial enzyme(s) which results in the formation of ATP feeding into the Na-K serosal pump, thus indirectly resulting in the increased extrusion of Na



From Edelman, Bogoroch and Porter, PNAS 50:1169.1963

Figure 1. Stimulation of Na transport across the toad urinary bladder by aldosterone. Following a pre-incubation of 14 1/2 hours, bladders were incubated in aerated amphibian Ringer's solution containing glucose for 2 hours. A significant increase in SCC (Na transport) was observed, suggesting an energy-requiring process. Aldosterone was then added to the serosal bath resulting in a marked increase in SCC after 1 1/2 hours, although paired tissues incubated with puromycin as well exhibited a decrease in SCC. From Edelman, Bogoroch, and Porter, 1963.

from the cell (Fig. 2). It is quite possible that none of these mechanisms operates exclusively, but that some combination of these enzymes is under the control of the steroid.

In considering these three mechanisms, it is apparent that they can be viewed as offering two fundamental alternatives, i.e., either the hormone acts by facilitating the entry of Na into the cell from the urinary fluid, and/or it acts primarily by enhancing extrusion of Na serosally. The first process involves passive ion transfer down an electrochemical gradient, but is assumed to be dependent on the presence of specific low-resistance sites on the luminal plasma membrane. The second process implies active transport since the ion involvement is against an electrochemical gradient, and the active Na pump is believed to be located on the serosal side. Situated between the luminal entry sites and the serosal pump is considered to be the "Na pool" awaiting transport, which may also serve to functionally coordinate the mucosal and serosal transporting mechanisms. For example, it is possible that an increase in the Na pool may stimulate the Na- pump, or that an independent increase in the activity of the Na-pump may result in an increased rate of Na entry into the cell from the urine side. Thus the mucosal and serosal transport processes may not be functioning independently of each other. In order to gain further insight into this question, measurements have been made of the size of the intermediate "Na transport pool"; however, it has proven difficult to draw reliable conclusions from these data.

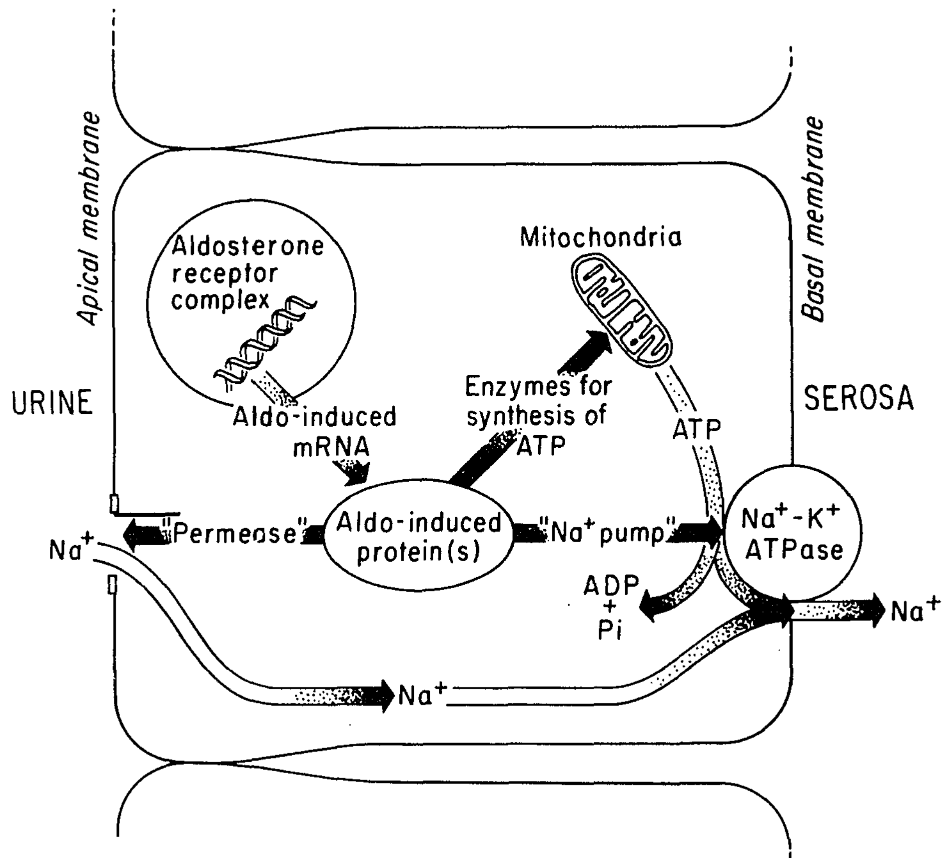


Figure 2. Proposed mechanisms for the stimulation of sodium transport by aldosterone. The aldosterone-receptor complex in the nucleus unmasks transcription, and the mRNA codes for aldosterone-induced protein(s). These may enhance active sodium extrusion from the cell either directly ("Na pump") or indirectly (mitochondrial enzymes leading to increased ATP/ADP), or the protein may act on the apical side as a sodium "permease".

Among the problems in interpretation of Na pool measurements is controversy over whether the observed electrochemical gradient is adequate for the occurrence of diffusional entry of Na across the mucosa; if it is not, it may be necessary to alter the model to include an active Na pump located apically. A second problem is that of the measured "Na pool", i.e. the Na supposedly awaiting transport; some of the sodium may have already crossed the Na-pump and would therefore be moving out of the cell by diffusion rather than by active transport (Zerahn, 1969; Finn, 1976). The technical methods that have been used to measure the Na pool experimentally have been prey to this criticism (Leaf and MacKnight, 1972). These techniques include measurement of total Na content by flame photometry or equilibration of a sodium isotope in the mucosal fluid bathing solution with intracellular Na (c.f. Sharp and Leaf, 1964a; Crabbe and DeWeer, 1969). Measurements of the Na transport pool have also been made using isolated epithelial cells rather than intact tissues (Lipton and Edelman, 1971; Handler, Preston, and Orloff, 1972). Objections may also be raised to this approach, however, because the post Na-pump transport pool could be intracellular, and because studies involving isolated cells may be "less physiologic" than experiments using intact tissues.

Crabbe' (1974) attempted to measure the size of the Na transport pool kinetically by exposing the mucosal side of the bladder to a solution containing a sodium radioisotope, quickly removing this solution (time zero), and measuring the rate of efflux of isotope into the serosal solution. The efflux appeared to have a rapid component (pool A)

considered to be the transport pool, and a slow component (pool B) considered to be inaccessible to the transport processes. By extrapolation of the rate of Na efflux to time zero, the size of the Na transport pool (pool A) was estimated. It appeared that exposure to Aldo did not affect the size of the Na transport pool, which suggested to Crabbe' that the hormone increases SCC by a mechanism other than stimulation of the serosal Na pump. However, the author conceded that even with these kinetic measurements it could not be assured that all of the sodium in transport pool is proximal to the Na-pump.

Another approach has been used by MacKnight, Civan, and Leaf (1975 a), who scraped mucosal cells from the bladder and measured the amount of intracellular sodium radioisotope after either the mucosal or serosal surface of the tissue had been exposed to radioactive sodium. Approximately 20% of the intracellular pool equilibrated with the isotope when applied to the mucosal surface, and approximately 80% equilibrated with the isotope when applied to the serosal surface. In a follow-up study the intracellular sodium pool was found to be increased in size after the tissue was exposed to ouabain, and the increment in sodium apparently originated from the mucosal surface (MacKnight, Civan, and Leaf, 1975b). These data support the concept of passive diffusion of sodium at the luminal surface with the presence of an Na-K pump at the serosal bladder.

C. Cellular Events Associated with the Aldosterone Response

In 1963 Williamson observed that by inhibiting the transcription of DNA with Actinomycin D, the antinatriuretic action of Aldo could be blocked (Williamson, 1963). (Interestingly, the kaliuretic effect was uninfluenced by this protocol). Also in that year, Edelman, Bogoroch and Porter demonstrated that puromycin blocked the Aldo-induced increase in SCC (which reflects Na transport) across the toad bladder (Edelman, Bogoroch, and Porter, 1963). Thus, indirect evidence began to accumulate that the Na transport effect of Aldo in mammalian kidney and toad bladder, if not the K transport effect, was dependent on protein synthesis beginning at the transcriptional level (Karlson, 1963).

Data supporting this evidence for Aldo-induced unmasking of transcription was obtained by Castles and Williamson who demonstrated in the rat kidney the synthesis of RNA in response to Aldo (Castles and Williamson, 1965; 1967). Fimognari, Fanestil, and Edelman, also working with rat kidney, demonstrated increased synthesis of new proteins in response to the hormone, and also showed that Actinomycin D blocks the Aldo-regulated antinatriuresis (Fimognari, Fanestil, and Edelman, 1967). The latter effect was subsequently confirmed in dogs (Lifschitz, Schrier, and Edelman, 1973).

The picture thus emerging suggested that aldosterone had a mechanism of action similar to that which had been worked out for other steroid hormones, e.g. estradiol, dihydrotestosterone, and progesterone (Fig 3). As formulated by O'Malley (1971), steroid hormones enter the target cell and combine with specific charged receptor proteins in the cytosol. The

penetration of the hormone through the plasma membrane is postulated to be unrelated to cárrier molecules. After binding, the receptor proteins undergo a transformation in which their sedimentation coefficient is significantly altered. This transformation is followed by the migration of the receptor-hormone complex to the nucleus where it is bound by acidic chromatin. Transcription of messenger RNA ensues, which results in the translation of specific cellular proteins. Other RNA species often appear as well. The newly-synthesized proteins are the ultimate mediators of the hormone's effects.

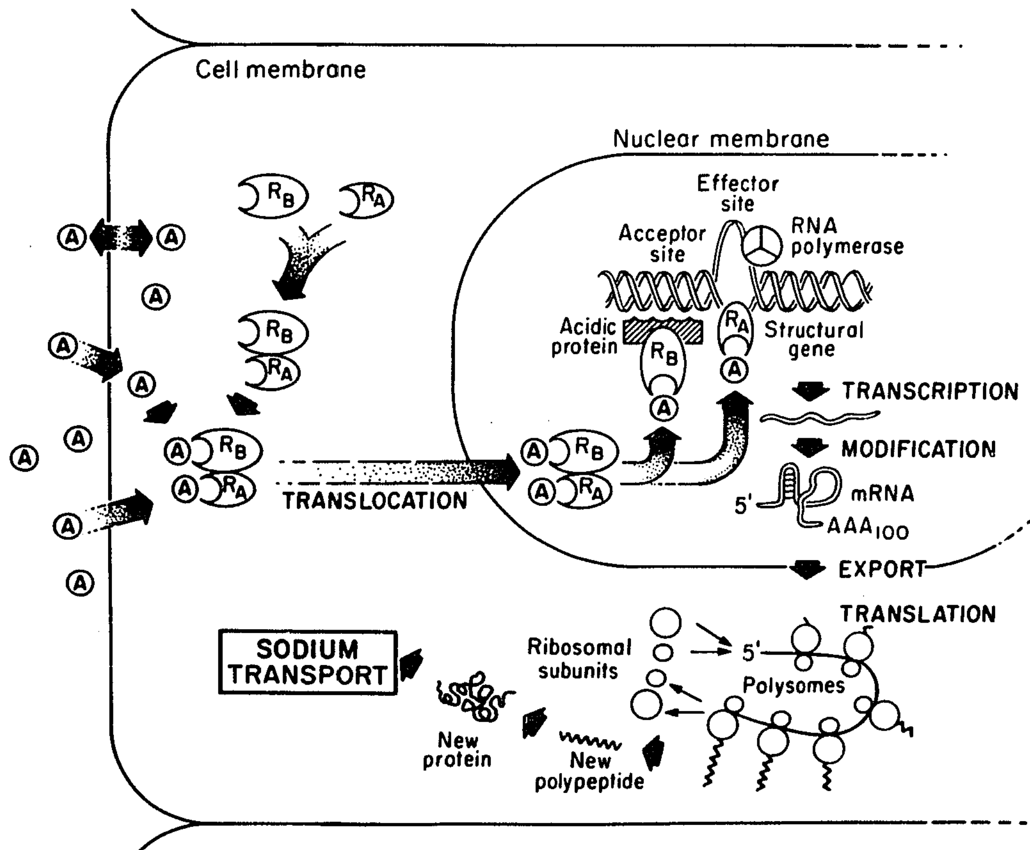


Figure 3. O'Malley's general scheme for steroid cellular physiology. Steroids permeate the cell membrane and combine with cytosol receptors. The hormone- receptor complex is "transformed" and migrates to the nucleus where one subunit binds to acidic chromatin and the second subunit binds directly to DNA. Transcription of mRNA follows, which is exported to polysomes and translation of effector proteins occurs. In the case of aldosterone, the result (by unknown mechanisms) is increased sodium transport.

I. The Aldosterone Receptors

Pursuing the postulated mechanism for Aldo, Edelman and his colleagues performed experiments designed to elucidate the nature of the Aldo receptor proteins. They showed that a steady-state level of Aldo was achieved in the toad bladder 30 minutes after exposure to hormone, but that there was a 90 minute latent period before a hormone-stimulated increase in SCC was noted (Edelman, Bogoroch, and Porter, 1963). Interestingly, removal of the hormone after the initial exposure did not alter either the kinetics or the magnitude of the response. This suggested that an early event in the Aldo target cell is high affiniy binding by a receptor. Sharp, Komack, and Leaf (1966) subsequently found two saturable binding sites, one of which was high affinity (Kd=6.7x10-11M), the other characterized by a lower affiniy (Kd=10-10M). However, the high affiniy sites were so few (9 x 10-14/g tissue) that they were saturated at concentrations of Aldo considered to be sub-physiological. The number of the lower-affinity sites was almost two orders of magnitude greater. The authors also examined the effects of various inhibitors on the Aldo-binding, and found both sites to be "specific", i.e., 3H-Aldo was not displaced by non-steroids, and the steroid with which the greatest degree of displacement was obtained was unlabeled Aldo.

Snart (1967), using methods similar to those of Sharp, Komack and Leaf, found three binding sites. Site A, (with a Kd=1.9x10-11, n=9x10-11

moles/kg tissue) was similar to the lower affinity site of Sharp, Komack, and Leaf. Site B had a very low affinity (Kd=4.84x10-7) and was considered to be a cortisol binding site. Site C, with even less affinity (Kd=10-4), was considered to be a non-specific binding site.

In an effort to localize the Aldo-binding within the toad bladder target cells, Ausiello and Sharp (1968) disrupted the nuclei of the bladder epithelial cells with detergents and osmotic shock. They found binding sites numbering 3x10-12/g tissue which were 60% saturated at an Aldo concentration of 10-7M. Alberti and Sharp (1969) continued these efforts, isolating three Aldo-binding macromolecular complexes from toad bladder nuclei. Bound 3H-Aldo was dissociated from the complexes by pronase, but not by DNAase or RNAase, thus identifying the macromolecules as proteins. One of these complexes dissociated rapidly and was found to have the same number of binding sites, as well as similar mineralocorticoid specificity, as the lower affinity site previously described by Sharp, Komack, and Leaf. The remaining two macromolecular complexes were unsaturable and apparently non-specific. Attempts to isolate the Aldo-binding complex were unsuccessful. In a search for other Aldo-binding sites, epithelial cell homogenates were purified by differential sucrose gradient centrifugation, and cytosol binding greater than nuclear binding was found. However, this binding was considered to be due to "leakage" of nuclear binding complexes into the cytoplasm in view of the lack of cytosol binding seen when extractions were performed in cold Tris buffer or 0.4M KCl.

To further characterize the Aldo-binding in the toad bladder nuclei, Alberti and Sharp (1970) compared this binding to that in the tissues in which increases in SCC are obtained in response to Aldo. The Aldo-binding in the toad bladder nuclei was three times greater than that in the epithelial nuclei of human colon, five times greater than that in toad colon, and ten times greater than that in rat colon. Half the Aldo-binding in the toad bladder dissociated in two hours. The binding in the rat colon nuclei appeared to be less mineralocorticoid specific than that of the other tissues.

Fanestil and Edelman (1966) extended these studies to mammalian kidney. Adrenalectomized rats were injected with 3H-Aldo, subsequently nephrectomized, and the renal cells were fractionated into subcellular components. It was found that Aldo was specifically bound in the nucleus, a conclusion based on the saturability of the binding in the nucleus and on the ability of 9α-fluorocortisol, another potent mineralocorticoid, to displace the Aldo. Non-mineralocorticoid steroids, e.g., cortisol or estradiol, could not be substituted for the 9α-fluorocortisol. The nuclear receptors were half-saturated at an Aldo concentration of 6x10-9M and were identified as proteins. Scatchard plot analysis of the data failed to yield a straight line, suggesting that more than one binding site moiety was involved. Since steroid binding in the nucleus follows cytoplasmic binding in the proposed mechanism, Edelman's group sought to demonstrate Aldo-binding in the cytosol as well (Funder, Feldman and Edelman, 1972). They successfully showed that in rat kidney cells Aldo is specifically bound to soluble receptors in the cytosol.

Marver et al (1972) also demonstrated cytoplasmic receptors in rat kidney, and their analysis of the hormone-receptor complex sedimentation in density gradients suggested a "transformation" of the charged cytoplasmic receptor. This follows the proposed general scheme for steroid receptors as stated by O'Malley (1971). Subsequently, Marver and her colleagues were able to show that the Aldo-cytosol receptor complex formation in the kidney is competitively inhibited by spironolactones (Marver et al, 1974). The ability of various spironolactones to block the Aldo-receptor binding directly correlated with the potency of each compound in blocking the effects of aldosterone on urinary Na:K ratios. Furthermore, the labeled spironolactone bound by the kidneys was quantitatively similar to the binding capacity for labeled aldosterone, and it appeared that the same classes of sites were involved.

In a study designed to further localize the initial cellular events in response to Aldo, Sapirstein and Scott (1975) incubated intact toad bladders with 3H-Aldo, followed by preparative separation of the mitochondria-rich (MR) and granular (G) cells on Ficoll gradients (Scott, Sapirstein, and Yoder, 1974). The cells were sonicated and the quantity of labeled steroid bound by ammonium sulfate-precipitable protein was measured. The results indicated that specific 3H-Aldo binding was limited to the MR cell. The 3H-Aldo binding was extensively displaced by DOCA, a potent mineralocorticoid in the toad bladder, but cortisol displaced the aldosterone by only 24%. The granular cells appeared to exhibit specific 3H-corticosterone binding. When the nuclei were recovered intact

for study, it was found that the MR cell nuclei contained over twice as much displaceable Aldo as the cytoplasmic fraction; high concentrations of unlabeled hormone displaced virtually all the nuclear 3H-Aldo. Interestingly, the MR cell Aldo-binding was significantly greater in the summer months, which may be related to the often-observed seasonal variations in responsiveness of the total bladder to mineralocorticoids.

Recently, Agarwal (1975) has reported isolating Aldo receptors from rat kidney. The mineralocorticoid receptors were separable from the glucocorticoid receptors, exhibited a monomeric molecular weight of 67,000 daltons, but primarily existed as a high-molecular weight aggregate (Agarwal, 1976).

II. Aldosterone-Induced Transcription of RNA

As noted previously, actinomycin D blocks the effects of Aldo on sodium excretion in adrenalectomized rats (Williamson, 1963) and inhibits the hormone's effects on SCC in the in vitro toad bladder (Edelman, Bogroch, and Porter, 1963). These studies were extended by Crabbe' and DeWeer (1964) who showed that the drug blocked the Aldo effect on Na transport in both the toad's skin and urinary bladder whether administered in vivo or in vitro. The first direct evidence for Aldo-directed transcription was recorded by Edelman, Bogoroch and Porter (1964) who demonstrated a modest increase in the incorporation of 3H-uridine into toad bladder RNA before and during the period of increased SCC. However, these results were complicated by the use of an Aldo concentration approaching the glucocorticoid range, and by the finding that progesterone also increased RNA synthesis after 3 hours. Edelman's group also observed that significant increases in the labeling of RNA could be found biochemically only after 90 minutes, corresponding to the latent period for the Aldo-induced stimulation of SCC (Porter, Bogoroch and Edelman, 1964).

Rousseau and Crabbe' (1968) incubated toad bladders in the presence of Aldo and 3H-uridine, and found that as compared to control tissues, the hormone effected an increase in incorporation of uridine into rapidly labeled RNA, and that most of the rapidly labeled RNA was in the region of heterodisperse nuclear RNA (HnRNA). Simultaneously, the same group found increased 3H-uridine incorporation into RNA in response to Aldo even when

sodium was lacking from the mucosal bath, suggesting that the hormone-directed RNA synthesis may be independent of sodium transport (DeWeer and Crabbe', 1968). They estimated the half-lives of the actinomycin D-blocked intermediates and the puromycin-blocked intermediates at 150 minutes and 90 minutes, respectively. These experiments were marred, however, principally by the use of glucocorticoid concentrations of Aldo (5x10-6M). Nevertheless, the mineralocorticoid specificity of the hormone-induced RNA synthesis was confirmed by Hutchinson and Porter (1970) who showed that after incubating tissues in the presence of Aldo and 3H-uridine, the Aldo-directed increase in RNA over control tissues was blocked by spironolactone (500:1). The hormone-influenced increases in labeled RNA obtained from the nuclear and post-mitochondrial fractions were 10% and 14%, respectively.

Edelman's group subsequently extended these studies to the rat kidney, showing increased incorporation of the uridine precursor orotic acid into RNA obtained from kidney homogenates from adrenalectomized rats that had been injected with Aldo. In these experiments the greatest amount of labeled RNA was found in the nucleus, and the labeling was unaffected by estradiol (Fimognari, Fanestil, and Edelman, 1967). Also using rat kidney, Castles and Williamson (1964) found an Aldo-directed increase in the incorporation of labeled uridine into RNA, although the total amount of RNA was also increased.

It should be mentioned that the efforts of some research groups to demonstrate a stimulation of RNA synthesis in response to Aldo met with failure. Using both mineralocorticoid and glucocorticoid concentrations of the hormone, Sharp and Komack could not demonstrate an increase in 3H-RNA over control until after 4 hours of incubation with 10-5 M Aldo. Baseline RNA synthesis in the toad bladder was examined by Vancura, Sharp and Malt (1971) who found that 18S and 28S rRNA were not labeled until 8 hours after 3H-uridine was added to the incubation medium. Exposure to Aldo (5x10-7M) was not observed to produce a significant change in 3H-uridine incorporation in any fraction of RNA studied. The authors suggested that the modest increases in RNA labeling reported by others may have been due to bacterial contamination of the tissues and/or the use of glucocorticoid concentrations of hormone. Rousseau and Crabbe' (1972) found that Aldo (5x10-6M) increased total RNA synthesis after 1 and 3 hours (although not at 2 hours). However, the hormone had no effect on the relative sucrose gradient profiles of phenol-extracted whole cell RNA, and polysomal RNA synthesis was unchanged after 150 minutes of incubation. Furthermore, no effect of Aldo was observed regarding the monosome/polysome ratio or the uptake of labeled amino acids into the ribosomes. It was suggested that observed increases in RNA-labeling in response to Aldo may have been due to increased uptake by the mucosal cells of the 3H-uridine.

This last objection was specifically addressed by Hutchinson and Porter (1975) who showed that the increase in nuclear RNA specific radioactivity in epithelial cells scraped from toad bladders after brief

treatment with Aldo (7x10-7M) resulted from the contribution of endogenous, rather than exogenous, precursor. Their conclusion was reached on the basis of estimates of the relative contributions of exogenous and endogenous precursor after comparing the rates of incorporation of 3H-uridine into nuclear RNA at two different exogenous concentrations. The authors supported their conclusion of Aldo-induced accelerated RNA turnover by demonstrating a significant decrease in 3H-uridine concentration of the precursor pool of prelabeled cells following unlabeled uridine chase. Specifically, they found no evidence for Aldo increasing the uptake of labeled uridine into the acid-soluble pool. This study was therefore particularly noteworthy because it ruled out the possibility that the enhanced incorporation of label into RNA was due to steroid-induced increased cellular uptake of uridine.

Other recent studies have improved on the previous reports by using larger amounts of 3H-uridine and by the utilization of affinity chromatography for collecting RNA. Rossier, Wilce, and Edelman (1974) were able to demonstrate a direct correlation between the fractional change in SCC after 180 minutes exposure to Aldo and the fractional change of the specific activity of the cytoplasmic 9-12S RNA (an arbitrary mean RNA fraction). Aldo increased the incorporation of labeled uridine into 9-18S nonmethylated RNA, characteristic of mRNA. These observations were supported by analysis of poly(A)-rich RNA isolated by oligo(dT)-cellulose chromatography, and the labeling of these RNA species was decreased by the addition of spironolactone (SC-9420) to the Aldo-containing bath. These

data supported the inference that the induction of mRNA synthesis mediates the action of Aldo on Na transport. Scott and Sapirstein (1974) confirmed that the Aldo-induced labeled RNA exhibits an affinity for poly(dT) and poly(dU), presumably due to the presence of poly(dA) in the terminus of the mRNA molecule. This group subsequently combined this approach with their method of preparatively separating the mitochondria-rich (MR) cells and the granular (G) cells on Ficoll gradients, and found that the increased labeling of mRNA following Aldo was limited to the MR cells (Scott et al, 1978). The labeling of RNA peaked at approximately 90 minutes after treatment with hormone, following which the mRNA was rapidly degraded into smaller fragments.

Most recently, studies have been carried out to further assess the roles of poly(A)(+) and poly(A)(-) in RNA in the response to Aldo (Rossier, Gaggeler, and Rossier, 1978). 3'-deoxyadenosine inhibited up to 80% of the incorporation of 3H-uridine into poly(A)(+) mRNA of toad bladder mucosal cells, while the drug only partially inhibited the Aldo-induced stimulation of SCC. Actinomycin D had similar effects on 3H-uridine incorporation, but also totally blocked the hormone stimulated increase in Na transport. The two drugs differed in that 3'-deoxyadenosine, in contrast to Actinomycin D, failed to inhibit poly(A)(-) RNA sedimenting between 4S and 18S. This RNA species is likely to be poly(A)(-) mRNA (Jelinek et al, 1973). The authors therefore concluded that an intact poly (A)(-) mRNA pathway is necessary in order to obtain a complete mineralocorticoid effect.

In summary, direct as well as indirect evidence has accumulated that Aldo induces the synthesis of mRNA, apparently an early required event in the stimulation of Na transport by the hormone. The toad bladder has proven to be an invaluable aid for studying this effect. However, many of the studies have been hampered by possible bacterial contamination of the tissue's mucosa, by the small number of cells that the bladder's single epithelial layer can provide for analysis, and by the lack of recognition that only mineralocorticoid concentrations of hormone should be used. Lastly, there is emerging evidence that only the MR cells, a limited population, initiate the response to the steroid with mRNA induction.

III. Aldosterone-Induced Proteins

A. Demonstration of hormone-induced proteins

The evidence for aldosterone-induced mRNA implies the presence of Aldo-induced protein(s) (AIP), but the direct demonstration of these proteins has proven to be quite difficult. The first report of aldosterone-induced incorporation of amino acids into a protein of toad bladder mucosal cells (mol. wt.=12,000) did not appear until 1974 (Benjamin and Singer, 1974), but this study was open to criticism on at least 3 points: (1) the synthesis of the protein could not be induced by dexamethasone, a potent mineralocorticoid in the toad bladder (2) glucocorticoid concentrations of Aldo were used (10-6M) (3) the synthesis of the observed protein may not have been mineralocorticoid-specific, since insulin was similarly effective in inducing the incorporation of amino acids into the putative AIP. No attempt was made to determine the subcellular localization of the AIP which was found upon analysis of a sample obtained after solubilization of entire mucosal cells.

Using a double-label protocol, Scott and Sapirstein (1975a) demonstrated the presence of several AIP's. Hemibladders were incubated in the presence of 35S-methionine and physiologic concentrations of Aldo (2x10-8M) for 2 hours, while paired hemibladders were incubated in the presence of 3H-methionine. The two sets of tissues were then mixed, the MR and G cells were removed and separated (Scott, Sapirstein, and Yoder, 1974), the cells

were sonicated and the "soluble" proteins were analyzed by exclusion gel chromatography and isoelectric focusing. Upon analysis of the 35S/3H ratio in each eluted fraction, steroid-induced proteins having molecular weights ranging from 17,000 to 38,000, were identified exclusively in the MR cells. When the cells were extracted with methanol-chloroform solvent to obtain the membrane proteolipid fraction (Soto et al, 1969), proteolipids were also found to have been specifically synthesized in response to Aldo exclusively in the MR cells (Scott and Sapirstein, 1975b). Furthermore, this fraction bound radioactive amiloride, suggesting that the AIP(s) may be the sodium "permease" alluded to earlier, controlling the luminal influx of the ion into the cells. The authors concluded that the toad bladder MR cells respond to aldosterone with the synthesis of proteins detectable in the "soluble" and proteolipid fractions, and that the membrane proteins may represent sodium ion channels.

Recently, Law and Edelman (1978a) applied the double-label technique to examine the Aldo-responsiveness of rat kidneys. One hour after adrenalectomized rats were injected with hormone, the animals were killed and nephrectomized, and the renal medullary, cortical and papillary slices were incubated in the presence of either (3H) or (35S)-methionine for 2 hours. Following incubation, hormone-exposed and control tissues were combined, and the cells were homogenized and subcellularly fractionated by differential centrifugation. Polyacrylamide gel analysis of the trichloroacetic acid-precipitable proteins from the medullary cytosol revealed that Aldo significantly increased methionine incorporation into at

least one protein wih a molecular weight of approximately 31,000. This effect was inhibited by the pretreatment of the rats with either actinomycin D or spirolactone (SC-26304). These results were considered to be supportive of the results obtained by Scott and Saperstein (1975a) in the toad bladder.

Using a similar experimental approach, Law and Edelman (1978b) examined the possibility that an important enzyme synthesized in response to Aldo may be mitochondrial citrate synthase (E.C. 4.1.3.7). The renal enzyme was isolated by ATP-sepharose column chromatography and immunoprecipitation with specific antiserum, or by polyacrylamide gel electrophoresis (PAGE). Methionine incorporation into renal citrate synthase in response to the hormone was increased by 55% in the immunoprecipitates, and by 43% in the enzyme peak resolved by PAGE. No similar efffect was found in response to an equimolar dose of dexamethasone, and the response (as determined by PAGE analysis) was inhibited by either actinomycin D or spirolactone (SC-26304). When the experimental protocol was modified so that labeled methionine was injected in vivo simultaneously with the hormone, Aldo was found to augment methionine incorporation into renal citrate synthase by 55%. Amino acid incorporation into the hepatic enzyme was unchanged. These observations of increased renal citrate synthase activity were postulated as reflecting a hormone-controlled modulation of the "available" ATP/ADP which could alter both sodium conductance and active sodium transport (Lipton and Edelman, 1971).

B. Function of the aldosterone-induced proteins

Although the AIP is usually considered to be a sodium "permease", a Na-K ATPase component, or a rate-limiting mitochondrial enzyme, the possibility that AIP's exist which are not directly involved in these mechanisms cannot be discounted. There is also no reason to suspect that the AIP has exclusively one function.

1. The sodium "permease" theory is supported by the data from the measurements of the "Na transport pool". This evidence has been reviewed in a previous section. However, interpretation of these experiments is hazardous for the reasons referred to, and it is therefore difficult to make an unequivocal conclusion that the results demonstrate the validity of the "permease" theory.

One approach to demonstrating the presence of a sodium "permease" utilizes amiloride, an acylguanidine diuretic whose action is to inhibit the influx of sodium through the apical membrane (Bentley, 1968; Cuthbert, 1974). The toad bladder epithelium binds amiloride reversibly and in a saturable process which apparently directly correlates with the blockade of sodium influx into the cell. It has also been shown that suppressing endogenous Aldo secretion by immersing toads in saline reduces the number of amiloride binding sites in the excised bladders by one-third, whereas the number of binding sites doubles after these tissues are exposed to hormone. These steroid effects are abolished by either cycloheximide or

actinomycin D, and no similar changes are observed in response to vasopressin. The data provide strong evidence that an important action of Aldo is to stimulate sodium influx from the luminal side, although they do not prove that the AIP is the permease itself, rather than a membrane component which alters the membrane conformation in a manner such that the actual permease is more accessible to the apical surface. Demonstration of identity of the AIP with an Na permease awaits isolation and preparation of the AIP, with the subsequent observation that insertion of the AIP into an artificial membrane results in a sodium-specific augmentation of ion permeation.

2. While it may be theoretically simple and attractive to ascribe a role for an AIP as an Na-K ATPase component, attempts at demonstrating such an effect have been unsuccessful. Bonting and Canady (1964) were not able to reveal any effect of Aldo on Na-K ATPase activity while monitoring the characteristic rise in SCC across the toad bladder. Aldo (10-7M) causes a 35% increase in oxygen consumption in the toad bladder, but no correlating increase in the amount of Na-K ATPase in the tissue homogenates can be found when measured at hourly intervals after exposure to hormone, up to the time of maximal SCC (Dalton and Snart, 1970). In an attempt to determine whether Aldo effects a change in the characteristics of this enzyme, rather than the total amount, Hill, Cortas, and Walser (1973) developed a more sensitive ATPase assay than those previously available. These investigators examined a variety of conditions, including pre-treatment of the toads with either water or saline, or overnight

incubation of the bladders in saline, and measured the subsequent response to Aldo. No significant changes in either the total amount of Na-K ATPase or in the kinetics of the reaction were observed under any set of conditions. When the enzyme activities at low sodium concentrations in the control and hormone-treated tissues were compared by Hill plot analysis they were found to be identical. It remains possible that the assay of enzyme activity in the intact epithelium may not correlate with that in tissue homogenates; on balance, however, these data constitute good suggestive evidence that the induction of an Na-K ATPase component is not a significant mediating event in the action of Aldo.

3. Direct evidence that Aldo may induce the synthesis of a tricarboxylic acid (TCA) cycle intermediate has been obtained recently (Law and Edelman, 1978a, b), and has been reviewed in a previous section. The basis for these experiments was the observation by Kirsten et al (1968) that Aldo (10-7M) increased the citrate synthase activity in the toad bladder by 25% two hours after treatment, and that the enzyme activities of isocitrate dehydrogenase, glutamate dehydrogenase, glutamate-oxaloacetyl transaminase, and malate dehydrogenase were also increased. These changes were not similarly observed in enzymes not involved in the TCA cycle, including lactic dehydrogenase, and the effects were inhibited by actinomycin D or puromycin. The increase in citrate synthase activity was not a secondary result of Na transport, as indicated by the independence of enzyme activity from the presence of sodium in the mucosal bath; furthermore, the effect of Aldo on citrate synthase activity correlated well with the hormone's stimulation of SCC.

Indirect support for this concept of Aldo-mediated stimulation of the TCA cycle reactions was obtained by Kirchberger et al (1968) who used the rate of generation of $^{14}CO_2$ from glucose labeled at either C-1 or C-6 to show that Aldo decreased the activity of the pentose shunt pathway in the toad bladder while simultaneously increasing the oxidation of glucose by the TCA cycle. This effect was sensitive to actinomycin D, although the drug itself did not affect the pentose phosphate pathway. These experiments corroborated the results of Kirsten et al (1968) in that the hormonally-controlled metabolic changes exhibited a similar time course and dose-dependency as the steroid's stimulation of SCC, and the effects were unchanged when sodium was removed from the mucosal bath.

The possibility of an Aldo effect in toad bladder mucosal cells upon the amount of succinic dehydrogenase has also been investigated (Dalton and Snart, 1970). After 3 hours of exposure to hormone, no increase in enzyme levels was detected, despite a 35% increase in the oxygen consumption of the tissue.

4. The urinary bladders of Colombian toads in vivo and in vitro can respond to Aldo with an increase in the rate of acidification of the urine (Scott et al, 1970), although toads obtained from the Dominican Republic do not appear to share this property. This interesting function is generally ascribed to carbonic anhydrase activity in the tissue, as significant amounts of this enzyme have been found in the toad bladder epithelium and the effect is inhibited by acetazolamide (Scott et al, 1970). There are

three forms of carbonic anhydrase in the bladder mucosal cells, each with a distinctive isoelectric point, and the enzyme appears to be identical to its toad erythrocyte counterpart although it is possible that a membrane-bound form of the enzyme also exists (Scott and Skipski, 1979). While actinomycin D does not block the effects of Aldo on urinary acidification in the mammalian kidney (Lifschitz, Schrier, and Edelman, 1973), this response in the toad bladder is sensitive to cycloheximide (Ludens and Fanestil, 1974). The implication is that Aldo may stimulate the synthesis of carbonic anhydrase, a process which may either be independent of the hormone's stimulation of SCC, or which may be related indirectly, e.g., acidification of the micro-environment of the luminal aspect of the membrane may modify the permeability of the membrane to sodium. However, attempts to directly demonstrate the stimulation of carbonic anhydrase synthesis in response to Aldo have been unsuccessful (Reich, Skipski, and Scott, 1979).

5. Because Aldo and vasopressin often act in concert on the distal tubule in vivo, their combined actions have also been studied using the in vitro toad bladder. Both the sodium transport response to ADH in this tissue (Fanestil, Porter and Edelman, 1967) and the hydro-osmotic response (Handler, Preston, and Orloff, 1969) have been reputed to be augmented by Aldo. Extended studies of the hydro-osmotic effect also showed that an increased response is noted when Aldo-treated bladders are exposed to exogenous cyclic AMP (cAMP), which suggested a common mediating step distal to the stimulation of adenylate cyclase. Stoff, Handler, and Orloff (1972)

determined that bladders stimulated by ADH responded with greater increases in tissue cAMP when pre-treated with Aldo, as compared to controls. The effects of Aldo on cAMP phosphodiesterase were subsequently found by Stoff et al (1973) to be 20% decreased in toad bladder homogenates. However, while this was an attempt to determine whether the cAMP increase in response to the steroid was secondary to increased synthesis or decreased degradation, the cAMP phosphodiesterase levels were measured only after 24 hours of exposure to Aldo, whereas the maximal hormonal effect on sodium transport occurs at approximately 3 hours after addition of steroid.

More recent data casts some doubt on the synergestic role that Aldo may play with vasopressin with respect to sodium transport. The SCC response of the steroid-treated tissues was the same as the controls when the data was expressed as percentage increase over baseline values after addition of peptide (Debnam, Hewitt, and Snart, 1975). In fact, when the original data of Fanestil, Porter, and Edelman (1967) are analyzed in this manner, their conclusion that Aldo augments the ADH-induced increase in sodium transport was not confirmed. Debnam et al also determined that the in vitro hydro-osmotic synergism between Aldo and neurohypophyseal hormones is only observed at low concentrations of peptide, not at high doses.

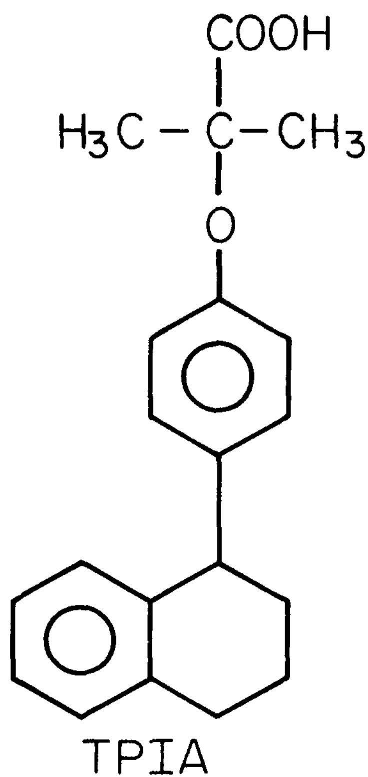
6. While it is widely accepted that the effects of ADH are mediated by cyclic AMP (Orloff and Handler, 1962), the function of this "second messenger" remains unclarified. Although there have been various reports that cAMP stimulates protein kinase activity in amphibian bladders (Jard

and Bastide, 1970; Kirchberger, et al, 1972) and mammalian kidney (Dousa et al, 1972), DeLorenzo et al (1973) have found that cAMP decreases the net extent to which a specific toad bladder membrane protein (protein D) is phosphorylated, an effect secondary to an increased rate of dephosphorylation (DeLorenzo and Greengard, 1973). Liu and Greengard (1974) investigated the effects of Aldo on phosphatase activity in the toad bladder and found it to be increased. Since this response was inhibited by actinomycin D and cycloheximide (as well as by spironolactone), they hypothesized that Aldo induces the synthesis of a phosphatase enzyme which acts with cAMP to control the degree of phosphorylation of protein D, and thereby regulates the rate of sodium transport. Other data, however, showing that vasopressin similarly decreases membrane protein phosphorylation (Ferguson and Twite, 1974), suggests that the dephosphorylated protein is involved in the regulation of the hormonally-controlled hydro-osmotic flux rather than the natriferic response.

7. Goodman et al (1971) demonstrated that, in the toad bladder, Aldo increases the conversion of 14C-pyruvate into fatty acids, enhances the incorporation of newly synthesized lipid into the 2-position of phospholipids, and increases the weight percentage of several long-chain polyunsaturated fatty acids in the phospholipid fraction of the tissue lipids. This study constituted the first direct evidence that a fundamental action of the hormone may be to alter the fatty acid metabolism of membrane phospholipids. It was subsequently demonstrated that Aldo

specifically enhances both the elongation and desaturation of oleic acid and the recycling of 14C-acetyl-CoA derived from 14C-oleic acid into membrane phospholipid fatty acids in toad bladder exposed to hormone for four hours (Goodman, Wong and Rasmussen, 1975). In support of these data, Lien et al showed that 2-methyl-2(p-[1,2,3,4,-tetrahydro-1-naphthyl] phenoxy)-propionic acid (TPIA, an inhibitor of acetyl-CoA carboxylase, (Fig. 4) blocked Aldo's stimulation of sodium transport in the toad bladder (without effect on basal sodium transport) when added to the mucosal side. The drug also inhibited the hormone-induced stimulation of lipid synthesis and the coincident increase in weight percentage of phospholipid long-chain polyunsaturated fatty acids. TPIA itself had no effect on protein or RNA synthesis. Subsequently it was shown that inhibitors of protein and RNA synthesis block the steroid induced changes in membrane fatty acid metabolism.

Phospholipase A2 has been shown to shorten the latent period that is characteristic of the stimulation of SCC by Aldo (Goodman et al, 1971), and phospholipase C simulates the effects of Aldo upon sodium transport in the frog skin (Yorio and Bentley, 1976). Thus, sodium transport in amphibian epithelia appears to be dependent on the fatty acid composition of membrane phospholipids, suggesting that the Aldo-induced proteins may be enzymes involved in regulating the composition and turnover of these phospholipids. This function of the AIP(s) may or may not be exclusive of a direct role of hormone-induced proteins acting directly in the membrane transport process.



2-methyl-2-(p-[1,2,3,4-tetrahydro-1-naphthyl] phenoxy)-propionic acid

Figure 4

C. Localization of the aldosterone response

Since the granular cells of the toad bladder mucosal epithelium account for approximately 80% of the cells, while the mitochondria-rich cells comprise only approximately 20%, the former have generally been assumed to be the cells directly involved in the hormone responses of the tissue. After exposure of the bladder to vasopressin, the granular cells appear swollen and the apparent density of the granular cells decreases if the mucosal solution is hypotonic to the serosal solution, correlating with the hormone-induced flux of hypotonic solution through the tissue (DiBona, Civan, and Leaf, 1969b). With respect to sodium transport, however, other investigators have found the MR cells to be more sensitive than the G cells to amphotericin B-induced changes in sodium uptake and active sodium transport (Saladino, Bentley, and Trump, 1969), which was associated with condensation of mitochondrial matrices. Depending on whether the sodium isotope was initially placed on the mucosal or serosal surface, disparate %results of labeling of intracellular sodium are obtained (MacKnight, Civan, and Leaf, 1975a). Two pathways for sodium transport have been proposed to explain the data. These observations may also be explained on the basis of partition of function of the various cell types.

Voute, Hanni, and Ammann (1972) found that in both the frog skin and toad bladder epithelia, only the MR cell responded in a characteristic fashion to variations in sodium load and to stimulation by parenteral aldosterone. In sodium-rich medium the MR cells became flasklike in shape,

and the cytoplasm became polychromatic. In aldosterone-stimulated tissues, the MR cell cytoplasm became engorged with mitochondria, ribosomes became very abundant, and the luminal pole of the cells was characterized by a large number of small vesicles and the formation of microvilli extending into the lumen of the bladder.

Further studies on cellular function were made possible by the development of a technique to preparatively separate the MR and G cells. Hays et al (1965) had shown that if bladders were incubated in a calcium-free medium, free intact epithelial cells would detach from their supportive basement membrane. These cells were functional, as measured by their increase in oxygen consumption in response to vasopressin. Sapirstein and Scott (1973) confirmed the viabiliy of these cells by demonstrating an ADH-induced increase in intracellular cAMP levels. Using this method to obtain functional, disaggregated mucosal cells, Scott, Sapirstein and Yoder (1974) separated the cells by centrifugation in discontinuous Ficoll density gradients. Bands of material sedimenting at different levels of the gradient were differentially enriched in MR and G cells, as determined by electron microscopy. The validity of the tehcnique was subsequently confirmed by Handler and Preston (1976).

The first application of this approach was the demonstration that although the cAMP levels in the separated cells were similar to those in the unseparated disaggregated cells, vasopressin caused an increase in the cAMP levels of the MR cells exclusively (Scott, Sapirstein, and Yoder,

1974). These results suggested that the initial step in the ADH transport effect was localized to the MR cells. Supporting this conclusion was the observation by Strum and Danon (1974) that in the intact frog urinary bladder (a tissue similarly responsive to ADH and aldosterone), the granular cells are not directly accessible to the basement membrane. However, Handler and Preston (1976) separated the MR and G cells from the toad bladder and found that ADH had an inconsistent effect upon the intracellular cAMP levels, and that homogenates of both the MR and G cells exhibited increased adenylate cyclase activity in response to ADH. These results raised the possibility that significant cell damage may be occurring during the course of cell separation, although the process of homogenization grossly alters the properties of the ADH-stimulated adenylate cyclase (Bar et al, 1970).

Since the cellular events in the toad bladder response to ADH appeared to be segregated according to cellular morphology, it seemed reasonable to investigate the possibility of cellular partition of function in the tissue's response to Aldo as well. Sapirstein and Scott (1975) exposed the intact tissue to 3H-Aldo, separated the MR and G cells, and found the displaceable labeled Aldo to be present only in the MR cells. These data suggested that although the nature of the cellular events in the stimulation of sodium transport by vasopressin and Aldo appear to be fundamentally different, in both cases the initial step was apparently localized to the MR cells. This finding was again consistent with the morphology of the hormone-responsive bullfrog bladder (Strum and Danon, 1974).

The problem remained, however, to localize the subsequent steps mediating the effects of the hormones. A distinct possibility is that at least some of the hormone-induced changes associated with either sodium or hydro-osmotic flux are in the G cells, since these cells comprise 80% of the mucosal epithelium. Davis et al (1974) showed that in the intact bladder the great majority of G cells are contiguous with an MR cell in forming a stellate array of G cells around a central MR cell. These observations were interpreted as supporting the hypothesis that the cell types may be functioning in a cooperative manner. Goodman et al (1975) attempted to localize intracellular cAMP with immunoflourescent techniques and were unable to demonstrate that cAMP was limited to the MR cells. One interpretation offered for the appearance of cAMP in G cells following ADH was that it was due to the migration of vasopressin-induced cAMP from the MR cells to the G cells, thus providing a means of intercellular communication.

In an extension of their Aldo studies, Brown and Scott (1976) have found evidence that the Aldo-induced mRNA appears exclusively in the MR cells, and the same laboratory has shown that Aldo-induced cytosol proteins similarly are found only in the MR cells (Scott and Sapirstein, 1975a; see above). Furthermore, there is evidence that new membrane proteolipids are synthesized in response to Aldo, and that these cellular components also can only be isolated from MR cell extracts (Scott and Sapirstein, 1975b; see above). Thus there appears to be ample reason to believe that there is significant partitioning of cellular function in the toad bladder response to Aldo as well as to vasopressin.

II. Introduction

The evidence that exposure of the toad bladder to aldosterone results in the synthesis of new cytosol and membrane proteins has been reviewed (preceding sections). Indirect evidence favors the theories that the Aldo-induced proteins (AIP's) may be either mitochondrial enzymes or a plasma membrane component, although other possibilities exist. Direct evidence for the existence of these proteins has been very difficult to obtain, although recently a report has appeared demonstrating the synthesis of citrate synthase in a rat kidney mitochondrial preparation in response to Aldo (Law and Edelman, 1978). If an AIP is a plasma membrane component, it is likely to be a "sodium permease" on the luminal aspect of the cell, or possibly an Na-K ATPase component on the serosal side. Since attempts at demonstration of such proteins from whole cell or whole tissue homogenates have been unsuccessful, and since these approaches would regardless yield no information concerning subcellular localization of the AIP, the first objective of the present work was to obtain a tissue fraction enriched in plasma membranes. This purified fraction could then be used to attempt to demonstrate and localize on AIP.

Although Hays and Barland (1966) briefly described a technique for the preparation of plasma membranes from toad urinary bladder, the procedures available at that time placed limitations on the purity and yield of membranes. The tissue fraction they obtained was enriched only 3.9 fold in Na-K ATPase, and no measurements of marker enzymes for possible

contaminating organelles were reported. The technique of subcellular fractionation has been considerably refined and developed in recent years (Wallach and Schmidt-Ullrich, 1977) and the present work represents an effort to use these newer approaches to obtain a fraction of toad bladder mucosal cells that is significantly more enriched in plasma membranes.

Once a satisfactory technique for the isolation of plasma membranes was achieved, it became possible to attempt to identify an AIP in this tissue fraction. For this purpose a double-label experimental approach was used; thus, hemibladders or disaggregated cells were incubated in the presence of Aldo (8x10-8M) and either (35S)-methionine or a mixture of (14C)-labeled amino acids, while control tissues were incubated with diluent and either (3H)-methionine or (3H)-amino acids. The isotopes used in the control and experimental tissues were reversed at regular intervals. In several experiments, control tissues were exposed to Aldo plus cycloheximide, rather than diluent. Tissues were then combined, MR and G cells were separated, and the cells were used to prepare plasma membranes which were solubilized, electrophoresed, and analyzed for the presence of peaks in the (14C/3H) or (35S/3H), which would be indicative of stimulation of specific protein synthesis.

Similar experiments were carried out using TPIA, an inhibitor of fatty acid synthesis, to further examine the inhibition of Aldo's sodium transport effects by this agent (Goodman, Wong, and Rasmussen, 1975). This drug could afect the incorporation of the AIP into the plasma membrane, if

the AIP were a proteolipid or lipoprotein or if a specific membrane lipid milieu is required. Tissues were incubed in the presence of hormone, TPIA, and (14C)-amino acids, while control bladders were exposed to steroid alone and (3H)-amino acids. Tissues were combined and plasma membranes were prepared and examined for the possible suppression of the appearance of AIP(s) by TPIA.

III. Materials and Methods

A. Preparation of a Tissue Fraction Enriched in Plasma Membranes

I. Preparation of toad bladder musocal cells: Urinary hemibladders from fifty female toads (Bufo marinus) of Dominican origin were mounted as sacs on Luer-lock syringes and were filled with and immersed in Ringer's solution (pH=7.2-7.6) whose composition (in mM) was: NaCl, 85; KCl, 4; NaHCO3, 17.5; KH2PO4, 0.8; EDTA, 1; and glucose, 10. ("EDTA-Ringer's" solution). Following incubation for 45 minutes at room temperature the disaggregated mucosal cells were removed by gently expressing the mucosal fluid from the sacs into a syringe, and sedimented at 800xg for 15 min. The cells were suspended in 8 ml of buffer containing 5mM HEPES (pH=7.2-7.4), 0.25mM MgCl2, and 250mM sucrose (buffer A).

ii. Isolation of a plasma membrane-enriched fraction: This procedure, a modification of the approach of Wallach and Kamat (1964), is outlined in Figure 5. The suspension of mucosal cells was equilibrated for 30 minutes with nitrogen at 1500 psi in a Parr "bomb" (Parr Instruments, Moline, Ill.) and disrupted by nitrogen cavitation. All subsequent steps were performed at 0° C. EDTA was added to the homogenate to a final concentration of 1 mM. The homogenate was centrifuged at 5,000xg for 15 minutes in a Sorvall RC2-B centrifuge to yield a pellet containing nuclei, granules, lysosomes, and mitochondria ("mitochondrial pellet"). The supernatant was centrifuged at 195,000xg for 2 hr. in a Beckman 42.1 rotor (Beckman Instruments, Inc.,

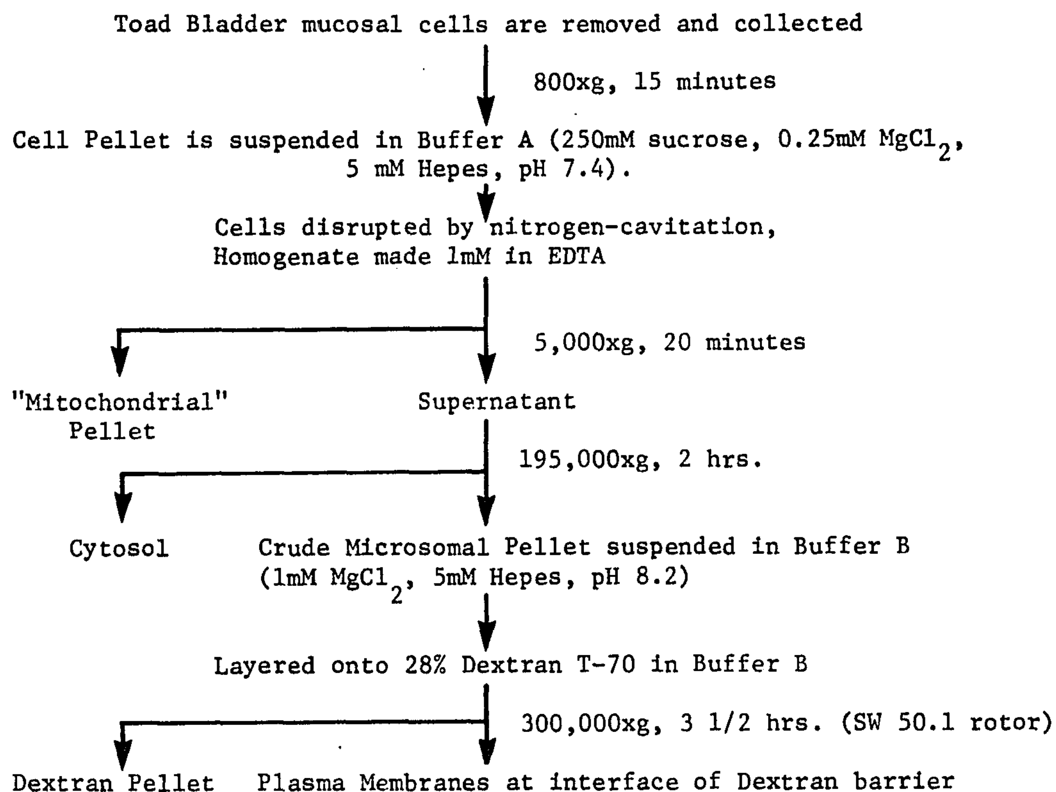


Figure 5. Flow-diagram for the preparation of plasma membranes from toad urinary bladder mucosal cells. See text for details.

Fullerton, Ca.) to obtain a cytosol fraction and a crude microsomal pellet; the latter was suspended in 1 ml of buffer B (5 mm HEPES, 1 mm MgC12 pH=8.2) and was layered over 28.5% dextran T-70 (Pharmacia, Inc.) in buffer B (density = 1.134 g/cm3). After centrifugation in an SW-50.1 rotor at 50,000 rpm for 3 1/2 hr., (g max = 300,000xg) two fractions were collected: a bilayered (but non-separable) interface at the buffer-dextran barrier ultimately designated as the plasma membrane fraction, and a pellet. Each fraction was washed twice in buffer B, pelleted by centrifugation at 195,000xg for 90 minutes, and then stored in buffer B at -28oC prior to enzyme determinations. Preparations used for electron microscopy were fixed immediately in modified Karnovsky's solution (2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1M sodium cacodylate, pH=7.2).

iii. Enzyme determinations: 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) was measured according to the method of Widnell (1969). Final concentrations in the incubation mixture were 3 mM MgC12, 2.5 mM 5'-AMP, 20 mM glycine (pH=8.5) and the tissue sample contained 25-100 ug protein. The total reaction volume was 0.5 ml and reactions were continued at 25oC for one hour before termination by the addition of an equal volume of 20% trichloroacetic acid and chilling of the sample on ice. The mixture was centrifuged at 1000xg for 5 minutes and inorganic phosphate was measured in the supernatant by the method of Chen, Toribara and Warner (1956).

Glucose-6-phophatase (EC 3.1.3.9) was measured by the method of Nordlie and Arion (1966). Each assay tube contained 0.3 ml of 100 mM cacodylate (pH=6.5), 0.1 ml of 150 mM sodium glucose 6-phosphate, 25-100ug protein and water to a final volume of 0.5 ml.

Cytochome c oxidase (EC 1.9.3.1) was measured as described by Smith (1955). The reaction vessel (cuvette) contained 25-100 ug protein, 50 ul freshly reduced cytochrome c, and 50 mm KH2PO4 (pH 7.3) in a total volume of 1 ml. Absorbance at 550 nm was recorded on a Gilford model 220 recording spectrophotometer.

Protein was determined according to Lowry, Rosebrough, Farr, and Randall (1951).

iv. Preparation of samples for electron microscopy: After fixation of the sample in Karnovsky's solution at room temperatue for one hour, the samples were post-fixed in 1% OsO4 in 0.1 m Na2HPO4 (pH=7.4) for 1 hour at 0oC (Palade 1952), dehydrated in a graded series of ethanol and embedded in epon (Luft, 1961). Silver to gray sections were cut with a diamond knife on a Porter-Blum MT2B microtome and examined in a Phillips 201 electron microscope.

v. Lactoperoxidase-125I labelling of mucosal cells: Sixty hemibladders were filled with Ringer's solution and bathed in Ringer's solution containing 10-8M vasopressin for 20 minutes. To label the apical membranes

of intact hemibladders, labeling solution was prepared just prior to use; Enzymes were weighed dry. Lactoperoxidase concentrations used ranged from .02 mg/ml to .01 mg/ml. Glucose oxidase was one half to one third of the lactoperoxidase by weight. 125I was purchased in 20 mCi lots as Na125I dissolved in about 40 ul of 0.1 NaOH. Upon breaking the seal the volume was brought up to 200 ul with deionized water and overlayed with benzene. A trace of the dye phenol red was generally added to the 125I solution as a safety measure. Three ml of the labeling solution was added to each hemibladder, which was incubated with aeration in the presence of 10-8M vasopressin (serosal bath) for 15-20 minutes. The mucosal solution contained glucose oxidase, 0.03 mg/ml; lactoperoxidase, 0.09 mg/ml; and Na(125I) (New England Nuclear, 17 Ci/mg), 10-7M. The hot solution was removed and the bladder filled with 3mM cold NaI, usually in EDTA-Ringers for one minute. They were next rinsed with EDTA-Ringers and then filled with and immersed in EDTA-Ringers to remove the cells. Following a 45 minute incubation, the disaggregated cells were collected and plasma membranes were prepared as described above.

vi. Resolution of proteins: Membrane pellets were solubilized in 2% sodium dodecyl sulfate (SDS), 2% mercaptoethanol, and 8M urea, and were sonicated for 10 seconds at ice temperature in a Branson Model L sonicator (Branson Sonic Power Co., Danbury, Ct.), followed by heating at 100oC for 5 minutes. After cooling to room temperature, these samples, and aliquots of cytosol were layered upon 8.0% polyacrylamide gels containing 0.16% SDS. The gels were then electrophoresed for 6 hours at 5 mA/tube, stained with Amido Black overnight, followed by destaining in methanol/acetic acid/water

(50:8:50). After destaining, the gels were optically scanned in a Canalco Model J gel scanner. In experiments where the plasma membranes were labeled with 125I, the radioactivities of whole subcellular fractions or aliquots thereof were measured in a Searle Gamma Counter Model 1185 prior to electrophoresis of the sample, obtaining a counting efficiency of 66%.

B. Identification of Aldosterone-Induced Proteins

i. Incubation of Isolated Mucosal Cells (Aldosterone vs. Control): Fifty female toads (Bufo marinus) of Dominican origin were partially immersed in a solution of 0.6% NaCl for 3-5 days to suppress the secretion of endogenous Aldo. Paired hemibladders were dissected from pithed toads and mounted as sacs on Luer-lock syringes. Mucosal cells were removed from the urinary bladders by incubating the tissues in Ringer's solution lacking calcium and containing 85 mM NaCl, 4 mM KCl, 17.5 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgSO_4$, 10 mM glucose, and 1 mM EDTA. The mucosal cells were divided into two aliquots and incubated in 5 ml of Ringer's solution containing 1.5 mM $CaCl_2$ and lacking EDTA ("Calcium-Ringers"); one bath also contained 7×10^{-8}M Aldo. After 45 minutes, amino acids labeled with one isotope (e.g., ^{35}S 250-400 uCi) were added to the control bath and the same amino acids labeled with another nuclide (e.g., ^{3}H, 250-400 uCi) were added to the Aldo bath; the combinations used were ^{3}H- vs. ^{35}S-methionine or a mixture of (^{3}H)-amino acids vs. a mixture of (^{14}C)-amino acids. The results obtained were independent of the choice of labeled amino acids for the two baths. The labeled amino acids obtained from New England Nuclear Co., Boston, Mass, were: (^{3}H)-labeled L-amino acids (NET-250) and (^{14}C)-labeled amino acids (NEC-445) or (^{3}H)-methionine (NET-061X) and (^{35}S)-methionine (NEG-009T). Incubation proceeded a total of 3 1/2 hours, following which the disaggregated mucosal cells from each set of bladders were collected, combined, and sedimented at 800xg for 15 minutes. In some experiments, the cells were suspended in 24 ml EDTA-Ringer's, and MR and G cells were separated on gradients of Ficoll as described below.

ii. Separation of Mitochondria-Rich and Granular Cells: Two ml of the cell suspension from the two sets of hemibladders were layered separately over each of twelve discontinuous gradients of Ficoll-400 (Pharmacia, Piscataway, N.J.) and centrifuged at 27,000 RPM for 45 minutes using Beckman SW-27 rotors at 4oC as previously described (Scott, Sapirstein, and Yoder, 1974), except that 100 mM EGTA was substituted for EDTA in the Ringer's solution used in the preparation of the Ficoll. The bands of material containing MR cells (gradient density = 1.035 g/cm3) and G cells (1.067 g/cm3) were removed separately, diluted with Ringer's solution containing 1.5 mM calcium and lacking EDTA or EGTA (calcium-Ringer's), and sedimented.

iii. Subcellular Fractionation: Each set of cells was disrupted by nitrogen cavitation and centrifuged at 10,000xg for 15 min to yield a crude "mitochondrial" pellet. The supernatant was used to prepare a cytosol fraction and a microsomal pellet. The latter was used for the preparation of a fraction enriched in plasma membranes, separated from endoplasmic reticulum as described in Section IIA.

iv. Solubilization and Electrophoresis of Membranes: Membrane pellets were solubilized in 2% SDS, 2% mercaptoethanol, and 8M urea, as described in Section IIIA-VI. These samples and cytosol aliquots were similarly electrophoresed as previously described. After optical scanning, the gels were cut into approximately eighty 1 mm slices, and each slice was

incubated overnight at 50oC in vials containing 0.5 ml of 90% Beckman Tissue Solubilizer. Upon returning to room temperature, 10 ml of a toluene-based scintillation fluid was added to each vial, and the radioactivity was determined in a Beckman LS-9000 Liquid Scintillation counter. This technique afforded uniform counting effeciencies of 38-42% for (3H) and 84-85% for (14C) or (35S).

v. Incubation of Intact Hemibladders (Aldo vs. Aldo plus Cycloheximide, and Aldo + Cycloheximide vs. Control): Fifty female Bufo marinus were partially immersed in a solution of 0.6% NaCl for 3-5 days to suppress the secretion of endogenous aldosterone. Paired hemibladders were dissected from pithed toads, mounted as sacs on Luer-lock syringes, and the tissues were filled with and immersed in calcium-Ringer's solution. Aldosterone ("Aldo vs. Aldo + Cycloheximide") at a concentration of 7 x 10-8M, or diluent ("Control vs. Aldo + Cycloheximide") was added to the serosal bath and 15 minutes later a mixture of amino acids labeled with (3H) (250-400 uCi) was added. Paired hemibladders used as controls were incubated in the presence of a similar concentration of Aldo and (3H)-labeled amino acids, after a 15 minute pre-incubation with cycloheximide (1 ug/1ml bath). The labeled amino acids obtained from New England Nuclear Co., Boston, Mass, were (3H)-labeled L-amino acids (NET-250) and (14C)-labeled L-amino acids (NEC-445). Incubation with hormone proceeded for 3 1/2 hours, after which the tissues were rinsed in Ringer's solution, followed by immersion in a bath of EDTA-Ringer's solution. The mucosal solutions were then also changed to EDTA-Ringer's.

Following incubation for 45 minutes at room temperature the disaggregated mucosal cells from the two sets of bladders were collected and combined, sedimented at 800xg for 15 minutes, suspended in 24 ml EDTA-Ringer's, and separated on gradients of Ficoll as described above. Subcellular fractionation, solubilization and electrophoresis of membrane pellets were performed as described in the previous sections.

A similar procedure was used for Control vs Control experiments, except that neither set of hemibladders was exposed to hormone.

vi. Incubation of Intact Hemibladders (Aldo + TPIA vs. Aldo, TPIA vs. Control, and TPIA + Aldo vs. Control): Forty intact hemibladders were filled with approximately 3 ml Calcium-Ringer's solution containing 2 mM TPIA (final concentration) in 0.3% v/v Me2SO, while paired tissues were filled with Calcium-Ringer's containing Me2SO diluent. Both sets of tissues were incubated in baths of Calcium-Ringer's. After 2 hours, Aldo (7 x 10-8M) was added to both serosal baths, and 45 minutes later, a mixture of 3H-labeled amino acids was added to one bath, and a similar mixture of 14C-labeled amino acids to the other bath ("Aldo + TPIA vs Aldo"). Following three additional hours of incubation, the bladders were rinsed, immersed in EDTA-Ringer's and the mucosal cells removed. The mucosal cells from the two sets of tissues were mixed, disrupted, and fractionated as described above. The subcellular components were analyzed by SDS-acrylamide electrophoresis.

A similar protocol was followed for the control experiments. In one control experiment ("TPIA vs Control"), one set of hemibladders was exposed to 2 mM TPIA (in Me2SO) in the mucosal bath for 2 hours. After 45 minutes,

3H-amino acids were added to the serosal bath and incubation proceeded for an additional 3 hours. Paired hemibladders were exposed to mucosal Me2SO diluent for 2 hours, followed by addition of 14C-amino acids 45 minutes after the serosal bath was changed. Three hours later all bladders were rinsed, immersed in EDTA-Ringer's, and the mucosal cells from both sets were removed and combined. Subcellular fractionation and analysis by electrophoresis proceeded as described above. In the second control experiment ("TPIA + Aldo vs Control") an identical procedure was carried out, except that the fresh serosal bath of the TPIA-exposed hemibladders contained 7 x 10-8M Aldo.

IV. Results

A. Preparation of a Plasma Membrane Enriched Fraction

i. Recovery of Total Cell Protein: The distribution of protein within the subcellular fractions is given in Table 1. Approximately half the total cell protein was recovered in the 195,000xg supernatant, while the 5,000xg "mitochondrial" pellet" represents the largest amount of protein in a membranous fraction. The material recovered from the dextran interface, which is enriched in plasma membranes, accounted for approximately 1.5% of the total protein in the initial homogenate.

ii. Enzyme Activities of Subcellular Fractions: Table 2 presents the data from the enzyme assays of the various fractions. The plasma membrane fraction is enriched 11-fold in 5'-nucleotidase activity relative to the initial homogenate. The plasma membranes contain only 1% of the total glucose-6-phosphatase and 0.3% of the total cytochrome oxidase activity while over 16% of the total recovered 5'-nucleotidase is in the plasma membrane fraction.

iii. Electron Microscopy of Subcellular Fractions: Figures 6A-C show the 5,000xg pellet, dextran interface (plasma membranes), and dextran pellet, respectively, obtained from a preparation of mucosal cells. Photomicrographs of the 5,000xg pellet demonstrate intact nuclei, granules,

PROFILE OF SUBCELLULAR FRACTIONS*

Fraction	Protein (ug. Total)	% of Total
Homogenate	56,538	
Mitochondrial Pellet	20,325	36.2
Microsomal Pellet	5,825	10.3
Plasma Membrane	810	1.5
Supernatant	28,388	50.1
Recovery		98.1

*Values represent the means from 4 experiments.

Table 1.

TABLE 2

ENZYMATIC PROFILE OF SUBCELLULAR FRACTIONS OF TOAD BLADDER MUCOSAL CELLS

Fraction	Glucose-6-Pase[1]		Cytochrome Oxidase[1]		5'-Nucleotidase[1]	
	Sp. Act.[2,3]	Tot. Act.[4]	Sp. Act.[2,3]	Tot. Act.	Sp. Act.[2,3]	Tot. Act.
Homogenate	42.6	2410.1	2.44	136.3	6.4	361.8
Mitochondrial Pellet	43.7	889.2	6.26	127.1	7.4	150.4
Microsomal Pellet	50.9	296.5	3.66	15.2	20.1	117.1
Plasma Membrane	31.7	25.7	0.49	0.4	72.2	58.5
Supernatant	39.0	1107.6	0	0	1.0	28.4
Recovery (%)		96.2		104.9		97.9
Enrichment[5]	0.74		0.20		11.3	

1--All values represent the means from 4 experiments.
2--Specific Activity = ug Pi/mg protein/hour
3--Values are given as Sp. Act. x 10^3
4--Total Activity = (Sp. Act.)(Total protein in that fraction)
5--Enrichment = (Plasma Membrane Sp. Act.)/(Homogenate Sp. Act.)

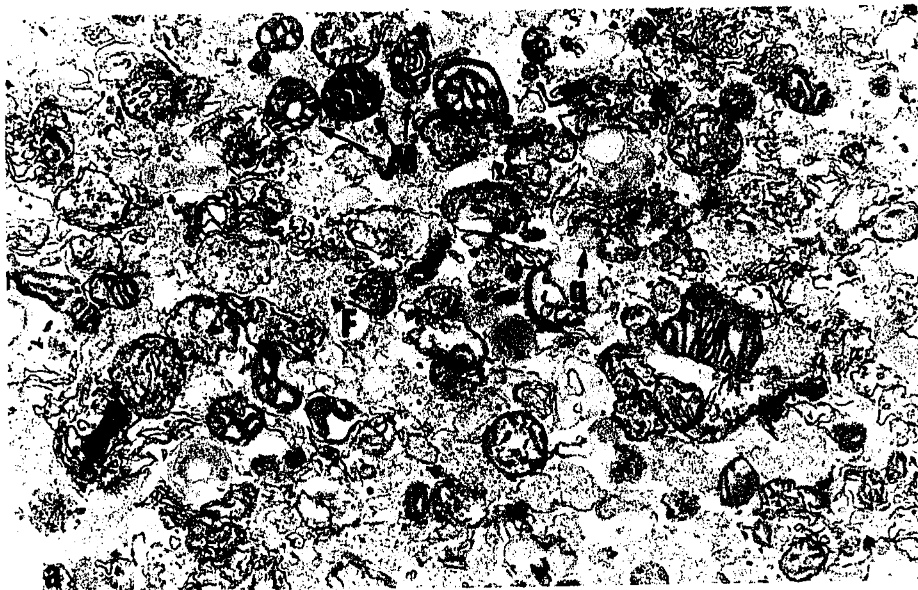
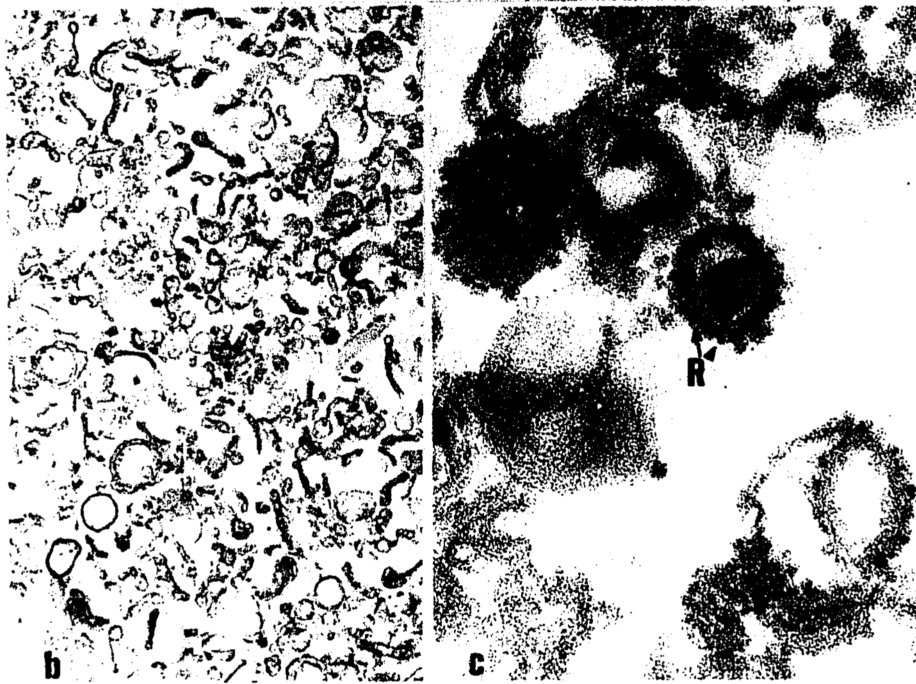


Figure 6. Electron micrographs of the subcellular fractions obtained in the preparation of plasma membranes. A. 10,000xg pellet B. Dextran interface. C. Dextran pellet.


b
c
R

lysosomes and mitochondria. The pellet from the sedimentation in Dextran T-70 consists of membranous material with rough microsomes as the predominant identifiable species. The plasma membrane fraction, obtained from the Dextran interface, yielded membrane fragments including vesicles, tubules, and vacuoles free of any recognizable organelles. The photomicrographs suggest that homogenization by nitrogen-cavitation disrupts the cell membrane but leaves most intracellular organelles intact. This increases the likelihood that the isolated membrane vesicles are derived primarily from the plasma membrane.

iv. 125I-Radioiodinated plasma membranes were prepared from mucosal cells whose apical surfaces had been catalytically radioiodinated in the intact bladder (Figure 7). The results of a representative experiment are shown in Table 3. The specific activity of (125I) in the plasma membrane fraction is 20 times greater than that in the 5,000xg pellet, and is increased 5-fold over the dextran pellet. Over 27% of the total membrane-bound radioactivity was recovered in the plasma membrane preparation.

v. Resolution of Proteins: Figure 8 shows the patterns obtained by optical density scanning of the material obtained from the "mitochondrial pellet", the dextran interface, the dextran pellet, and the cytosol. Twenty to thirty distinct proteins were resolved for each subcellular fraction, and the unique patterns obtained for each fraction supports the validity of the fractionation technique. The patterns obtained exhibited a high degree of reproducibility.

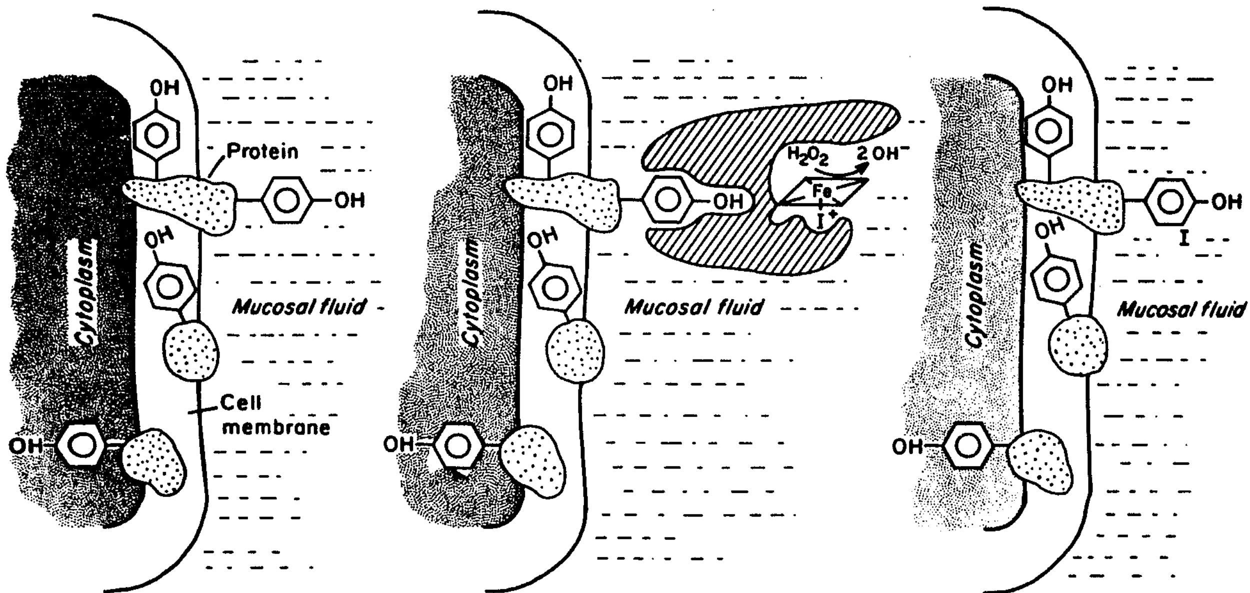


Figure 7. Iodination of plasma membrane proteins. The exposed tyrosine residues of exterior membrane proteins combine with a catalytic site on the lactoperoxidase enzyme. Via the reduction of hydrogen peroxide to hydroxyl ions, iodide is simultaneously oxidized and the tyrosine aromatic ring is oxidized at a carbon adjacent to the hydroxyl group.

TABLE 3. LACTOPEROXIDASE-^{125}I LABELING OF TOAD BLADDER MUCOSAL CELLS*

Fraction	CPM	% of Membrane-Bound Radioactivity	Sp. Act. (CPM/μg Protein)
"Plasma Membranes"	7,067,150	27.2	8818.3
"Mitochondrial Pellet"	9,018,500	34.7	447.3
Dextran Pellet	9,886,950	38.1	1716.7

*In this experiment 84.9% of the radioactivity in the initial homogenate was recovered.

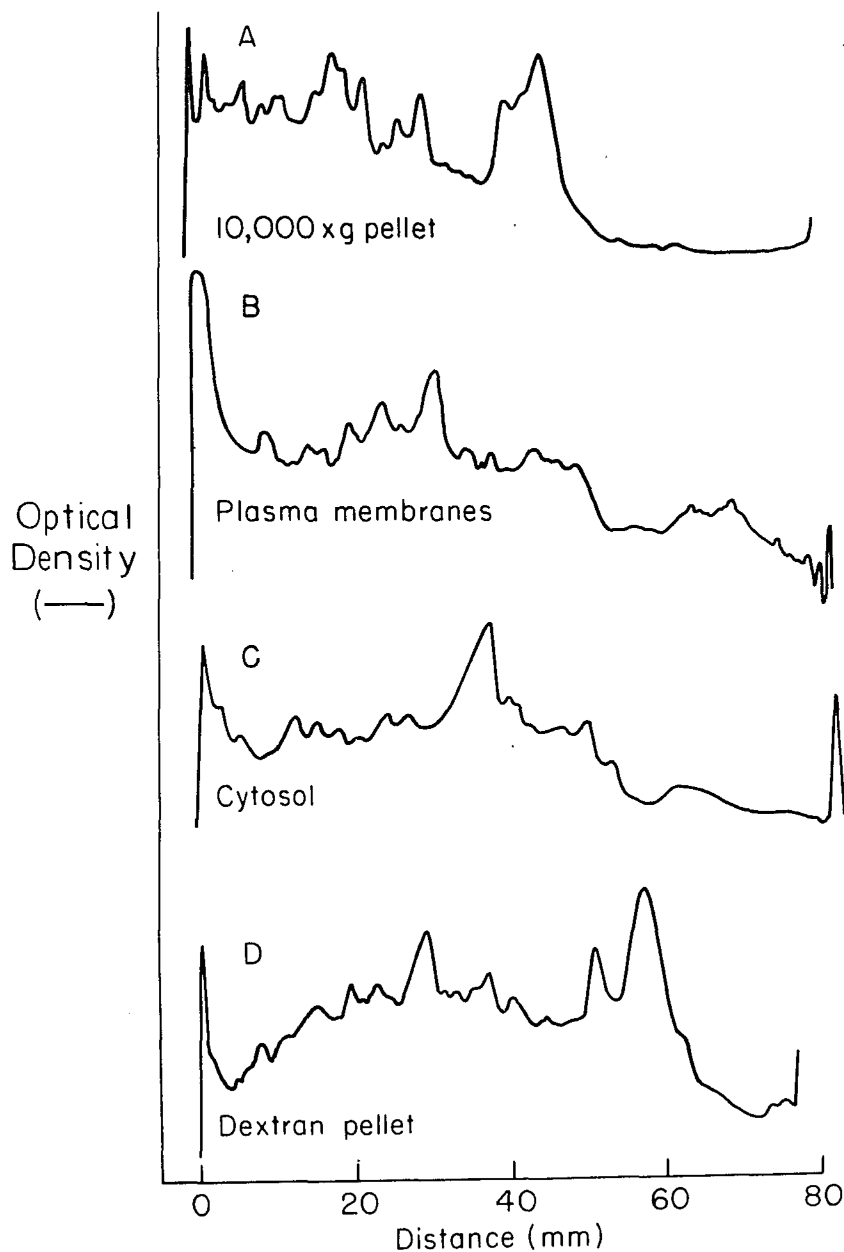


Figure 8. Resolution of proteins by polyacrylamide gel electrophoresis (PAGE). Membrane pellets were suspended in 2% SDS, 2% mercaptoethanol, and 8M urea, sonicated, and heated in a water bath at 100oC for 5 min. After returning to room temperature, 150ug protein of these samples and a similar aliquot of cytosol were layered over 8% SDS-polyacrylamide gels and electrophoresed for 6 hours at 5mA/gel. The gels were then stained with 1% Amido Black, destained wih 7% acetic acid in 50% methanol, and optically scanned at 550 nm. A. 10,000xg pellet. B. Plasma membranes. C. Cytosol D. Dextran pellet.

B. Identification of Aldosterone-Induced Proteins

i. Aldosterone vs. Control: The results of a typical experiment are shown in Figures 9-13. Following an incubation in which 35S-methionine was added to the "Aldo" bath, the plasma membrane fraction was prepared and analyzed by electrophoresis. The preparation from MR cells (Fig. 9-_shows three peaks in the ratio (35S/3H) corresponding to proteins with molecular weights of approximately 170,000, 85,000, and 12,000 daltons. Figure 10 illustrates the electrophoretic analysis of the plasma-membrane fraction from the G cells of the same experiment, and no similar peak in the ratio is observed. The excellent resolution of membrane proteins obtained in these experiments is shown in Figures 11 and 12. Figure 11 illustrates the pattern obtained for the MR cell preparation, and demonstrates that the ratio peaks at 170,000 and 85,000 daltons correspond to portions of the gels containing significant amounts of protein, although the low molecular weight peak corresponds to a gel section with relatively little protein. A similar, although not identical pattern, was obtained upon optical densiy scanning of the plasma membrane gel derived from the G cells of the same experiments (Fig. 12).

Previous demonstration of the Aldo-induced protein in the MR cell indicated the existence in the soluble fraction of induced proteins with molecular weights ranging from approximately 17,000 to 38,000 (4). In the experiment described here, preparation of the subcellular fractions and

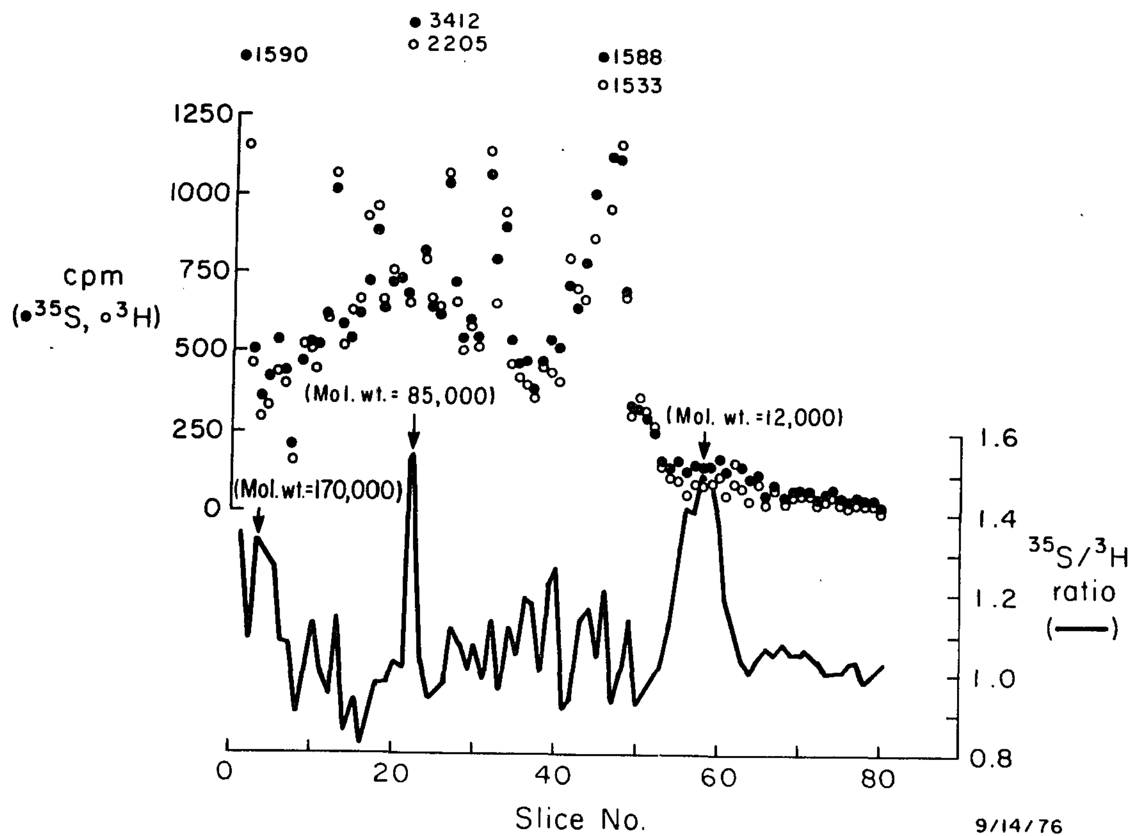


Figure 9. Aldosterone-induced incorporation of methionine into plasma membranes of mitochondria-rich (MR) mucosal cells. Isolated cells were treated with 7x10-8M aldosterone for 45 min., following which (35S)-methionine was added to the bath for an additional 3-hr. incubation. Cells from forty paired hemibladders were treated identically except they were not exposed to hromone, and (3H)-methionine was added to the bath. The "Aldo" and control cells were combined and MR and G cells were separated on Ficoll gradients. Plasma membranes were prepared from the MR cells, solubilized, and analyzed on 8% polyacrylamide- SDS gels which were sliced and the 35S/3H ratio was measured in each slice.

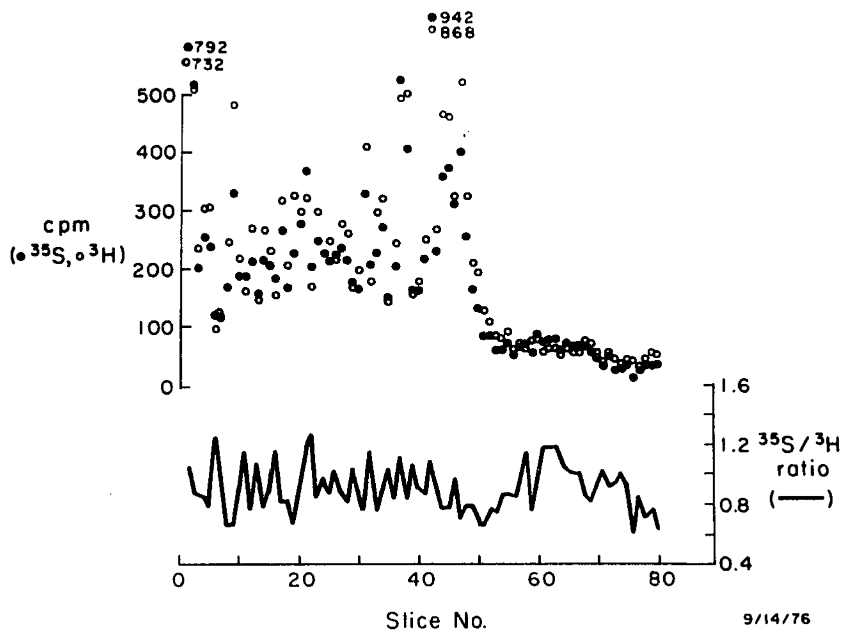
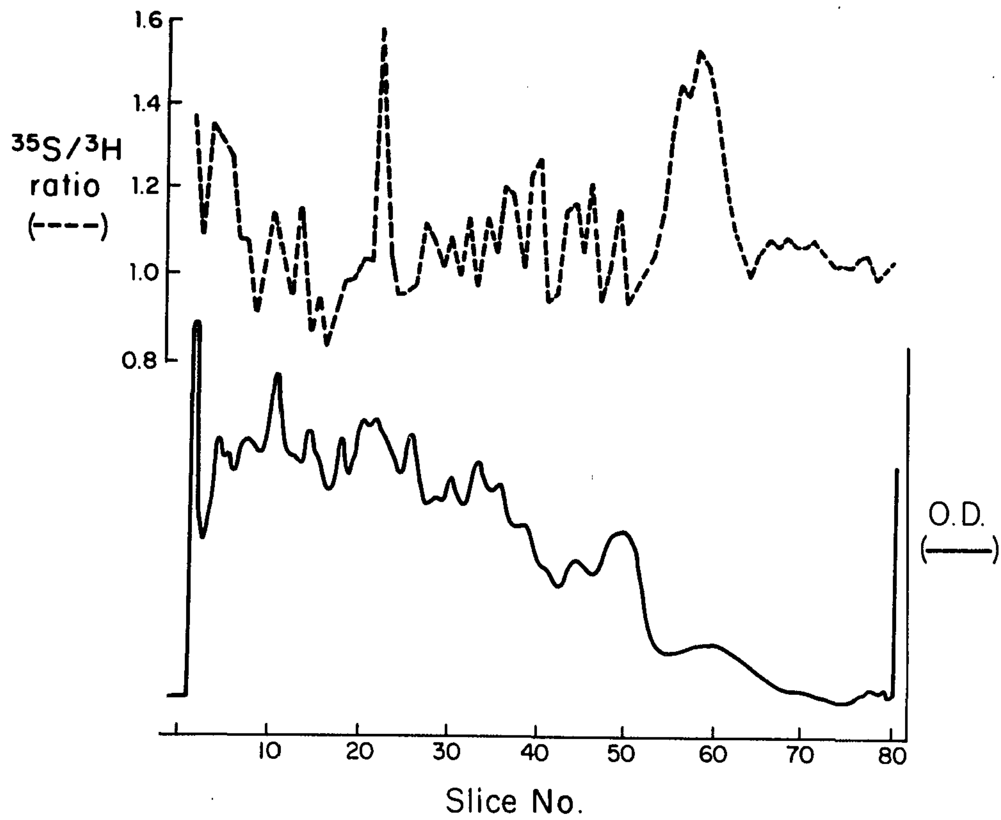


Figure 10. Aldosterone-induced incorporation of methionine into plasma membranes of granular (G) mucosal cells. Results shown are for the plasma membranes obtained from the G cells of the same experiment described in Figure 8. This subcellular fraction was handled in a manner identical to that of the MR cell preparation.



Figuure 11. Analysis of the plasma membranes obtained from MR cells ("Aldo vs. Control"). The 35S/3H ratio as measured in each gel slice by radioassay (top scale) is aligned with the optical density scan of the same gel before it was cut (bottom scale).

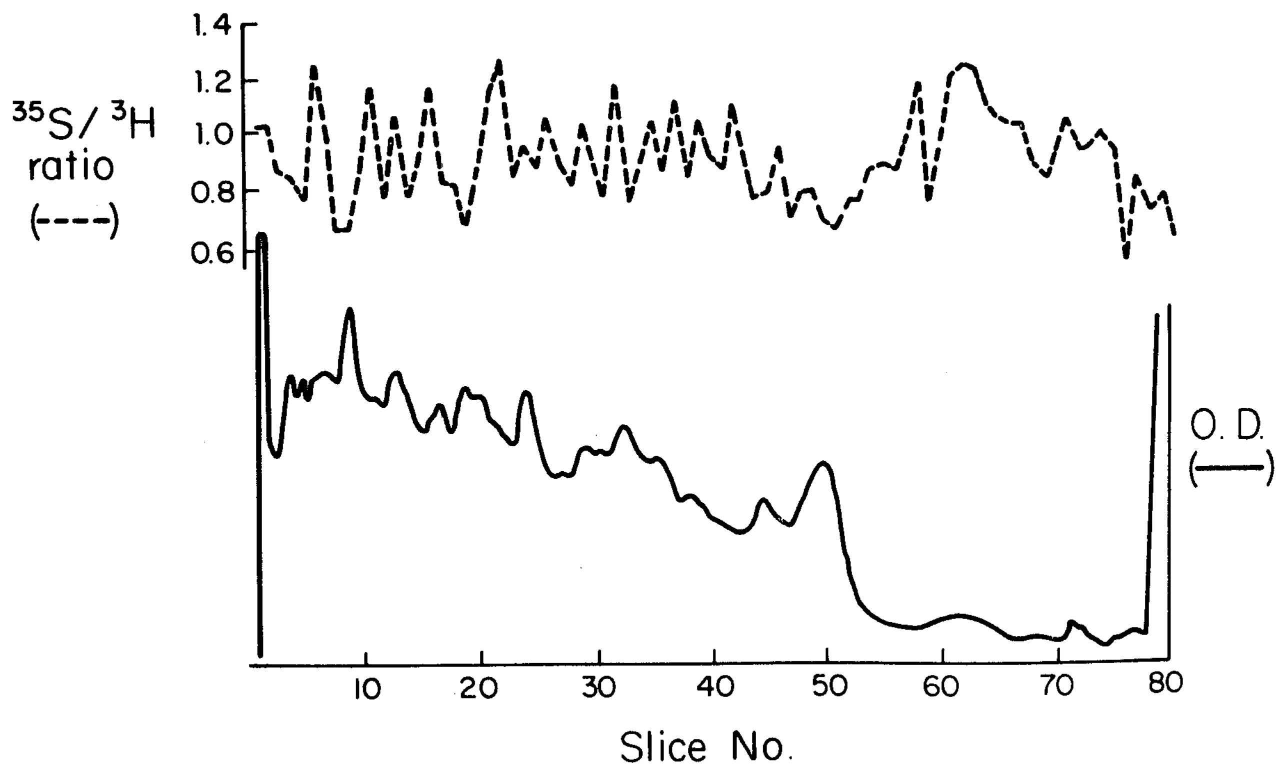


Figure 12. Analysis of the plasma membranes obtained from the G cells ("Aldo vs. Control"). Shown are the results from 35S/3H radioassay of individual gel slices (top scale) and optical density scanning prior to gel slicing (bottom scale) of the same gel. These data were obtained upon analysis of the plasma membrane preparation from the G cells of the same experiment illustrated in Figures 8-10.

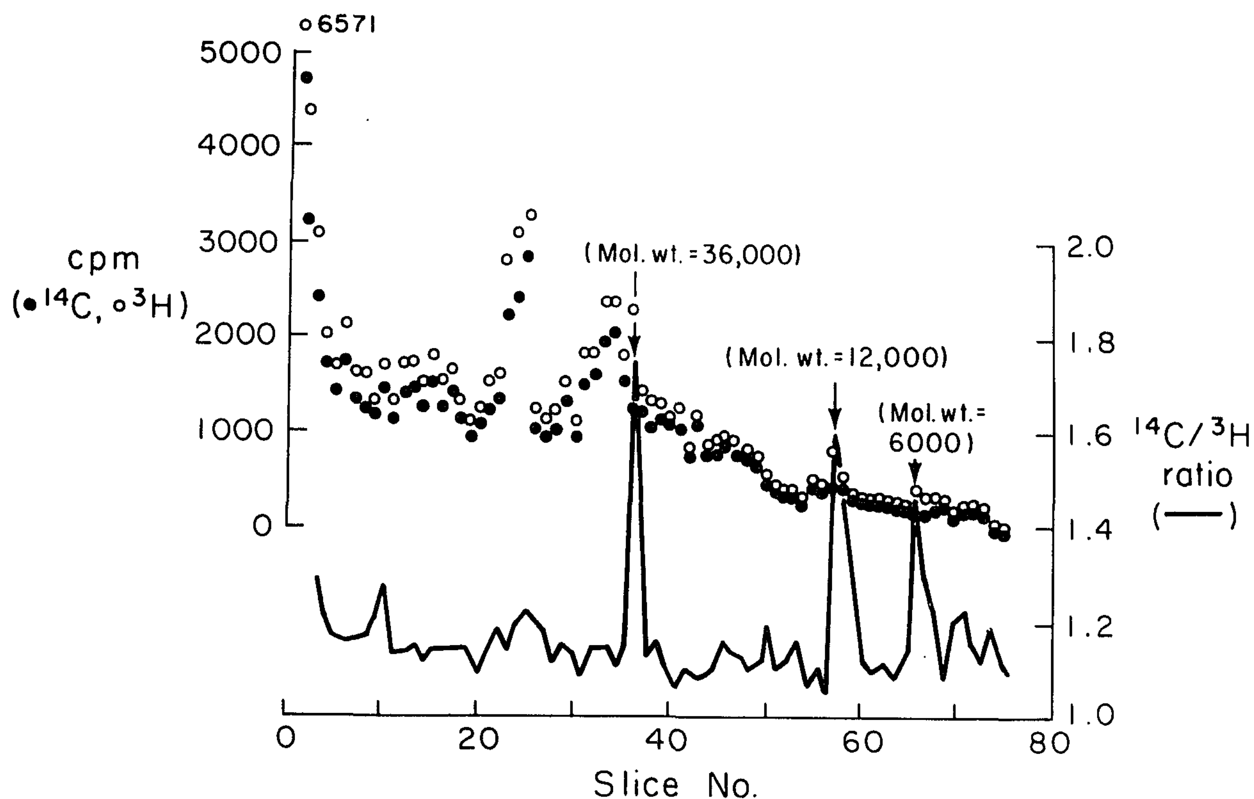


Figure 13. Aldosterone-induced incorporation of amino acids into cytosol proteins. Intact hemibladders were exposed to 7x10-8M aldosterone for a 45-min. incubation, followed by the addition of 500uCi of a mixture of (14C)-labeled amino acids. Forty paired tissues were simultaneously incubated with diluent for 45 min., followed by the addition of 250uCi of (3H)-amino acids. After a 3-hr additional incubation period, the two sets of hemibladders were combined, and the cells were removed and disrupted by nitrogen cavitation. The supernatant obtained by centrifuging the homogenate at 195,000xg for 2 hrs. was analyzed on 8% polyacrylamide-SDS The radioactivity was then determined in each gel slice.

analysis of the cytosol by a more sensitive method (SDS-acrylamide electrophoresis), indicated there were three Aldo-induced proteins (MW=36,000, and 12,000 and 6,000 daltons) in this fraction (Figure 13).

ii. Aldo vs. Aldo + Cycloheximide: Figure 14 shows the results of a typical experiment. In this particular experiment (3H)-amino acids were added to the Aldo-containing bath, but similar results are obtained when the nuclides are reversed. The major ratio peaks correspond to molecular weights of 170,000, 110,000, and 85,000 daltons.

The plasma membrane fraction from the G cells is shown in Fig. 15. This preparation contains more incorporated radioactivity than the MR cell preparation, indicating that the cells were actively synthesizing protein. However, the baseline nuclide ratio is similar to that for the MR cells, suggesting a similar basal level of protein synthesis, and the high measures of radioactivity appearing in the protein- containing slices demonstrate that both cell types were actively synthesizing proteins. However, in no discrete gel section did the nuclide ratio increase significantly above the baseline. These results indicate that Aldo did not specifically induce the synthesis of plasma membrane proteins in the disaggregated G cells.

Analysis of the "mitochondrial" fraction of the MR cell showed the presence of an induced protein of large molecular weight, and a protein with a molecular weight of approximately 85,000 daltons (Fig. 16). Because enzymatic analysis of this fraction showed it was contaminated with plasma membrane fragments, it is possible that this increase in nuclide ratio reflects contamination of this 10,000xg pellet with plasma membranes

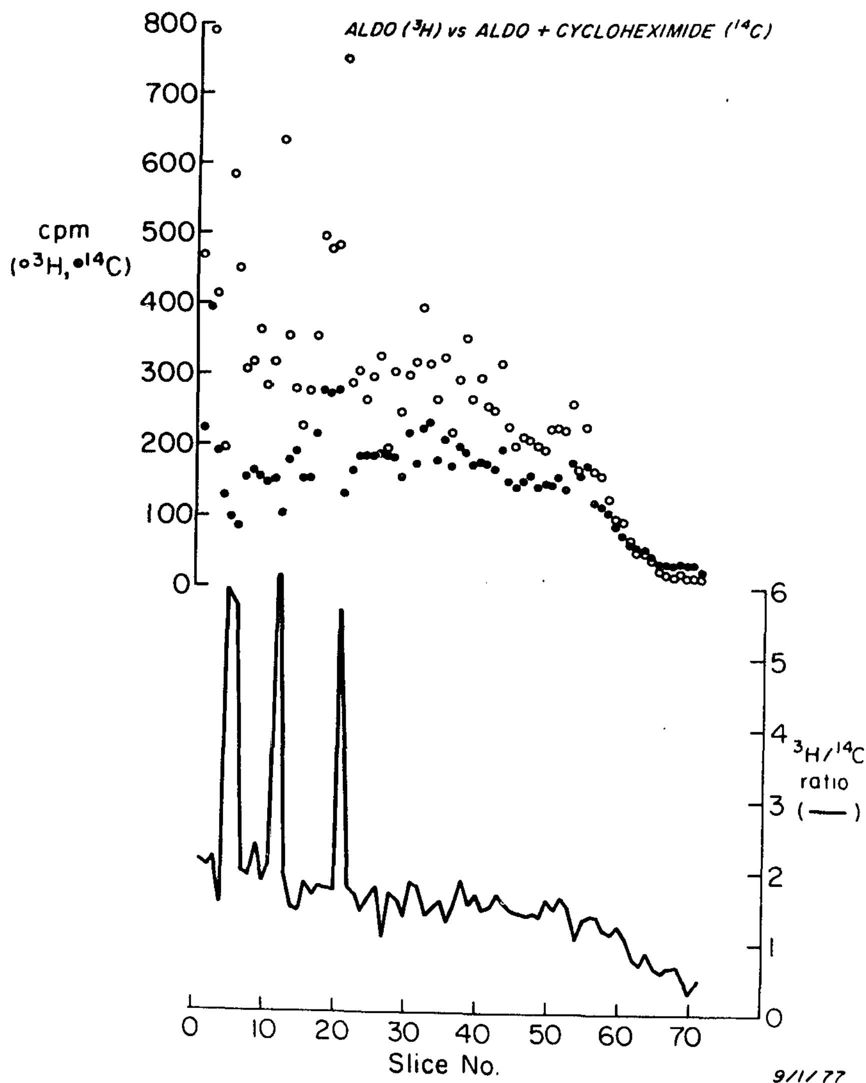


Figure 14. Aldosterone-induced incorporation of amino acids into MR cell plasma membranes. Intact hemibladders were treated with 7×10^{-8}M aldosterone for 15 min., following which (3H)-amino acids were added to the bath for a total incubation period of 3 1/2 hrs. Fifty paired tissues were pre-incubated for 15 minutes with cycloheximide (1 ug/ml) and otherwise treated identically except for incubation with (14C)-amino acids. After incubation, the two sets of tissues were combined, and MR and G cells were separated on Ficoll gradients. Plasma membranes were prepared from each cell type, solubilized, and analyzed on 8% polyacrylamide gels which were sliced and the (3H/14C) ratio was measured.

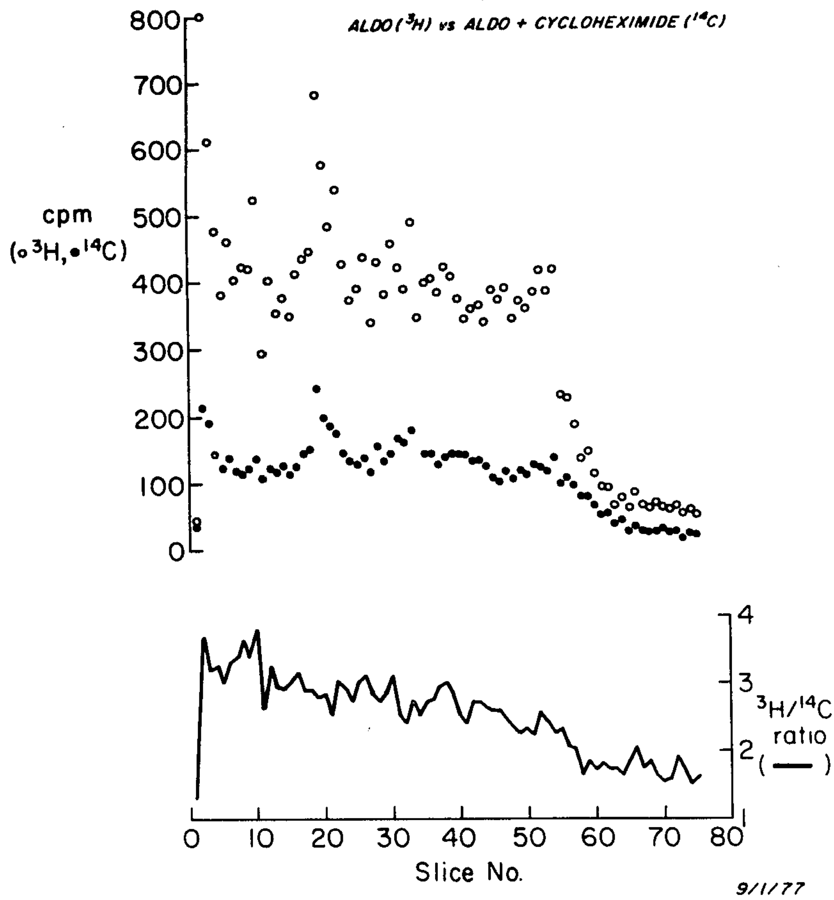


Figure 15. Aldosterone-induced incorporation of amino acids into G cell plasma membranes. Illustrated is the analysis of the plasma membrane fraction obtained from the G cells of the same experiment described in Figure 13.

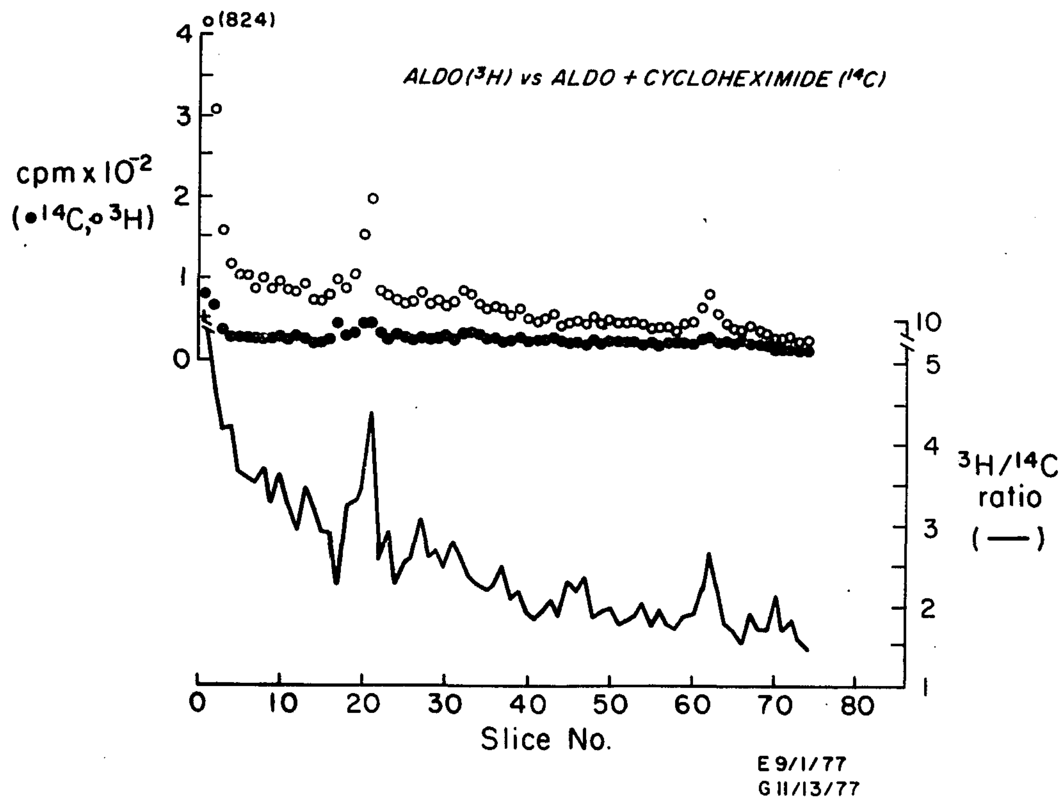


Figure 16. Aldosterone-induced incorporation of amino acids into proteins of the 10,000xg pellet of the MR cells. These results were obtained for the same experiment described in Figure 13. In the preparation of the MR cell plasma membranes, the pellet obtained by centrifuging the homogenate at 10,000xg for 15 min. was analyzed by the same PAGE system. This tissue fraction, consisting primarily of mitochondria, is also known to contain contaminating plasma membrane fragments as well as nuclei, granules, and other large organelles.

containing induced protein. The corresponding 10,000xg pellet from the G cells showed no similar peaks in nuclide ratio (Fig. 17). In addition, analysis of the dextran pellet, or "rough ER" fraction, obtained from the MR cells shows an increase in the nuclide ratio corresponding to proteins with estimated molecular weights of 170,000 and 85,000 (Fig. 18). This fraction is also known by enzymatic data to contain plasma membrane fragments (Section IVA), and therefore may be reflecting the presence of induced proteins in the plasma membranes. Again the corresponding "rough ER" fraction from the G cells showed no similar peaks in nuclide ratio (Fig. 19), supporting the conclusion that no specifically hormone-induced proteins are present in these cells.

Similar analysis of the cytosol fraction of the MR cells demonstrated the presence of three induced proteins, with molecular weights of approximately 36,000, 12,000 and 6,000 daltons (Fig. 20). The G cell preparation showed no similar peaks in the nuclide ratio (Fig. 21).

Figure 22 illustrates the results obtained upon double-isotope analysis of the cytosol and plasma membrane fractions from the control experiments, "Aldo + Cycloheximide vs. Control." None of these gels exhibited a region of clear departure from the baseline similar in magnitude to that observed for the "Aldo vs. Aldo + Cycloheximide" experiment. This experiment was performed to eliminate in part the possibility that cycloheximide may have resulted in the ratio peaks observed in Figs. 14 and 20.

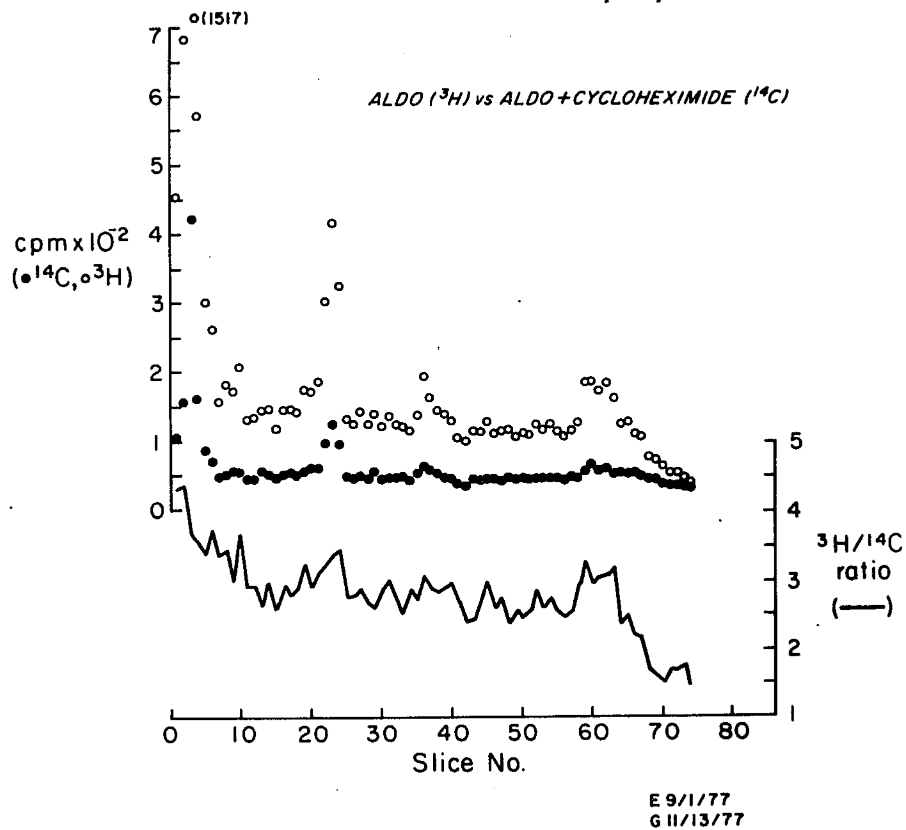


Figure 17. Aldosterone-induced incorporation of amino acids into proteins of the 10,000xg pellet of the G cells. The results shown are for the same experiment described in Fig. 13. Shown is the PAGE analysis of the membrane pellet obtained by centrifuging the G cell homogenate at 10,000xg for 15 min.

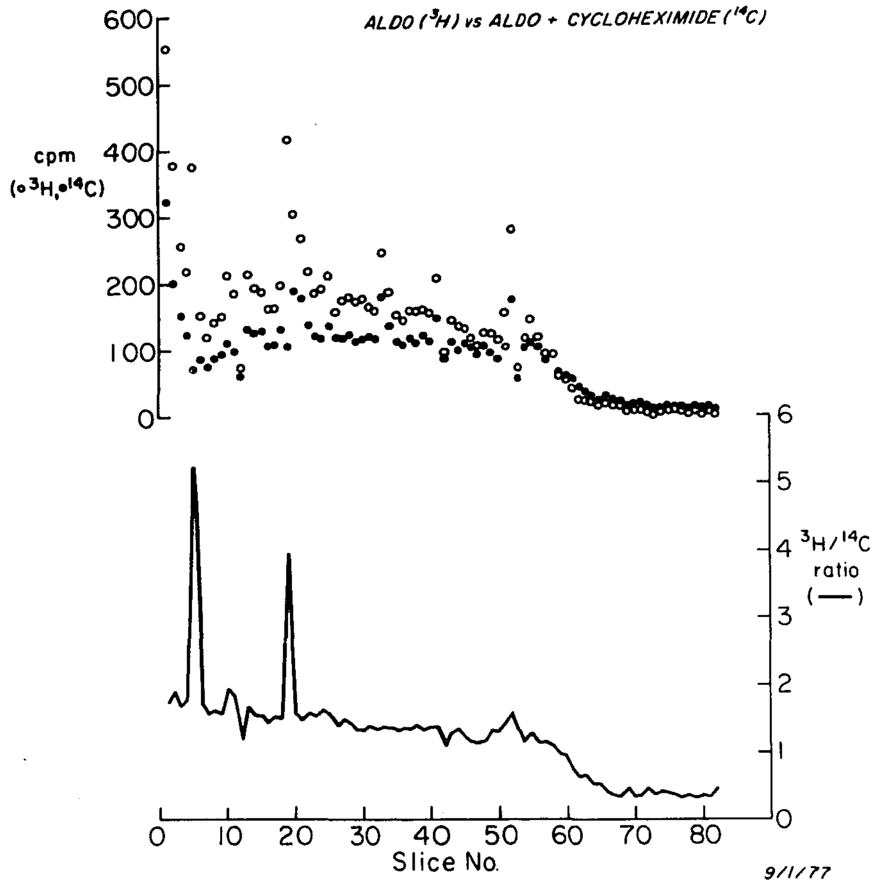


Figure 18. Aldosterone-induced incorporation of amino acids into proteins of the MR cell dextran pellet. Shown are the results obtained upon PAGE analysis of the pellet obtained by centrifuging the suspended microsomal pellet on a 28.5% Dextran solution at 300,000xg for 3 1/2 hrs ("rough ER" fraction). Although this fraction consists primarily of endoplasmic reticulum vesicles and Golgi membranes, it is known to contain some contaminating plasma membrane fragments.

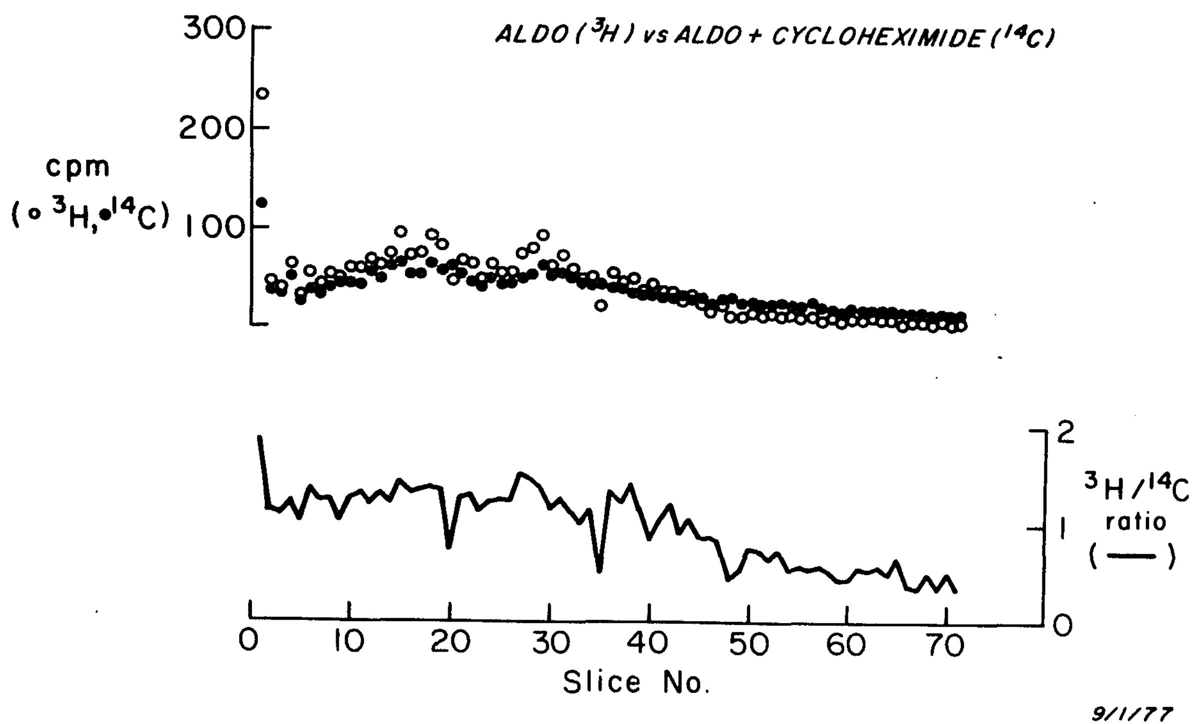


Figure 19. Aldosterone-induced incorporation of amino acids into proteins of the G cell dextran pellet. Illustrated are the results of PAGE analysis of the dextran pellet obtained from the G cells of the same experiment described in Figure 13.

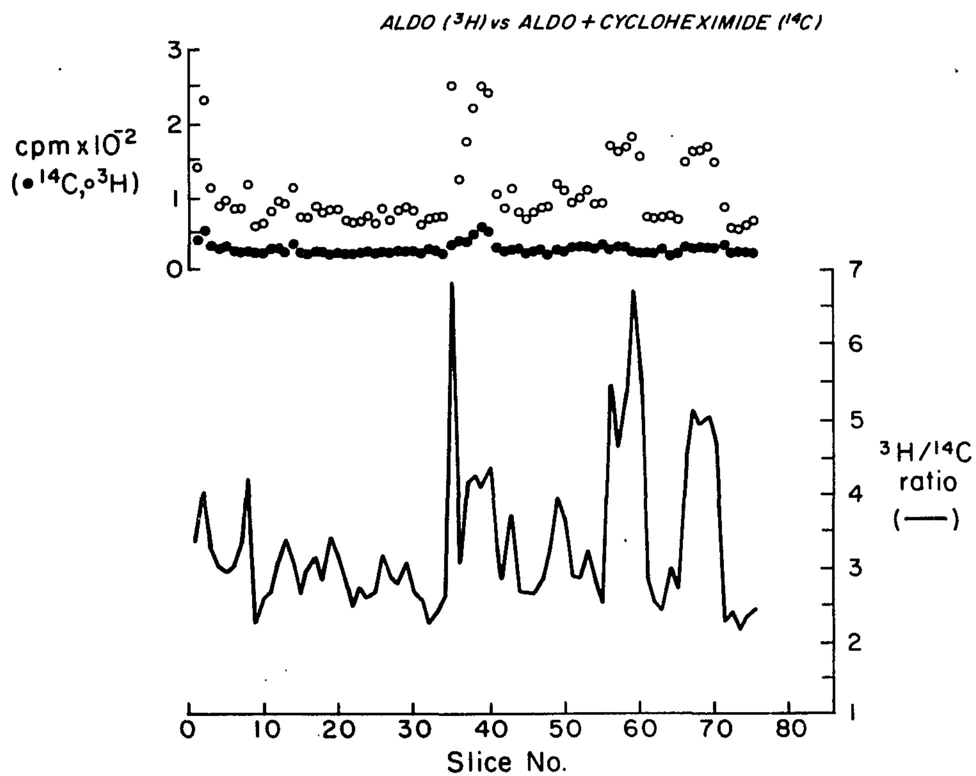


Figure 20. Aldosterone-induced incorporation of amino acids into cytosol proteins of the MR cells. These results were obtained upon PAGE analysis of the 105,000xg supernatant of the MR cells from the same experiment described in Figure 13.

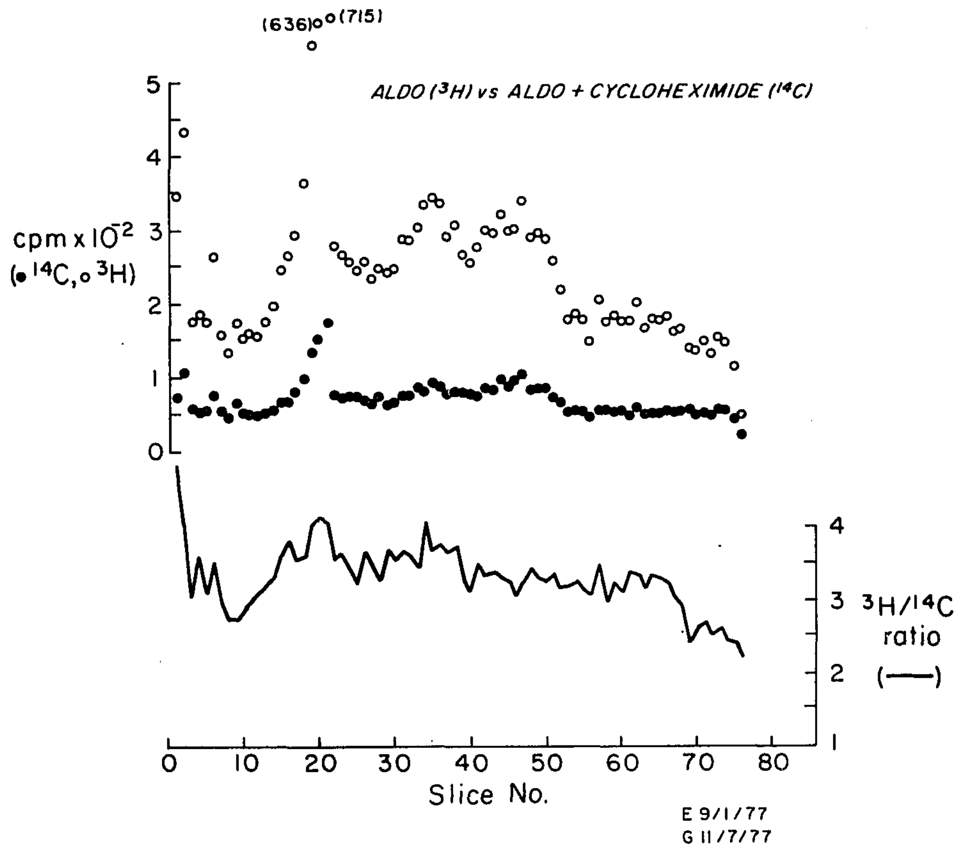


Figure 21. Aldosterone-induced incorporation of amino acids into cytosol proteins of G cells. Shown are the results obtained upon PAGE analysis of the 195,000xg supernatant of the G cells from the same experiment described in Figure 13.

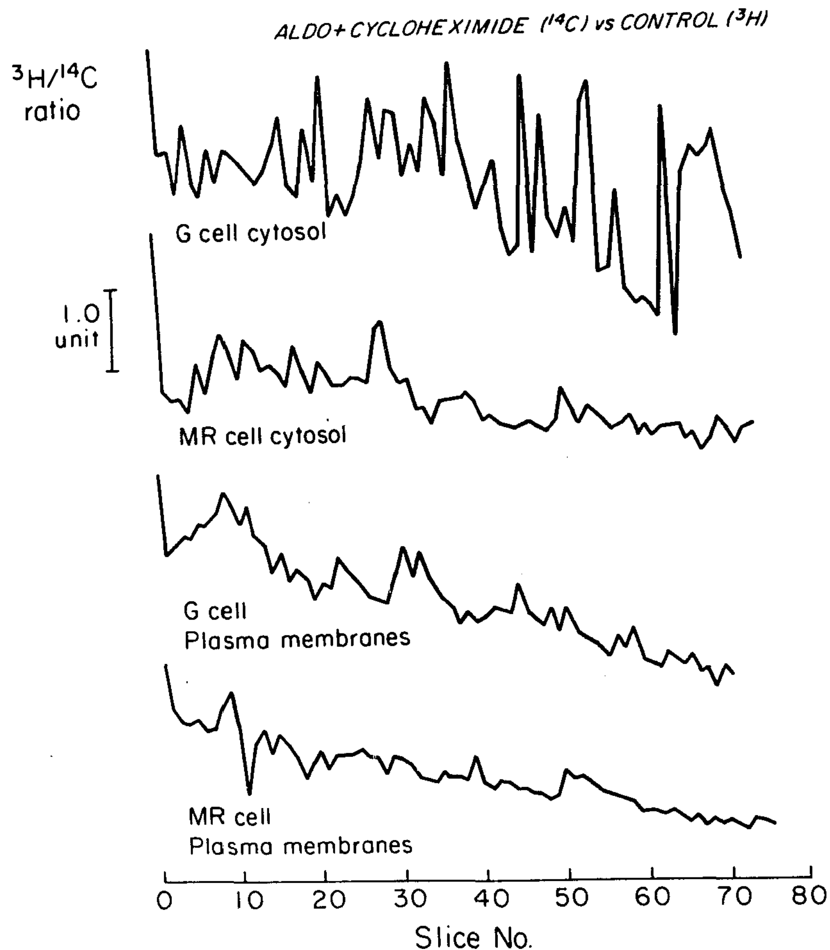


Figure 22. Effects of aldosterone + cycloheximide on the incorporation of amino acids into cytosol and plasma membrane proteins. Intact hemibladders were pre-incubated for 15 minutes with cycloheximide (1 ug/ml) followed by the addition of aldosterone (7x10-8M) to the serosal bath. Fifteen minutes later, (14C)-amino acids were added to the bath. Paired tissues were incubated with diluent rather than hormone or drug, followed by the addition of (3H)-labeled amino acids. 3 1/2 hours after the addition of hormone the cells from all bladders were harvested, MR and G cells were separated on Ficoll gradients, plasma membranes were prepared and a cytosol fraction was obtained. These fractions were analyzed on 8% polyacrylamide gels which were sliced for radioassay.

The results of the "Control vs. Control" experiments are illustrated in Figures 23-26. To eliminate the possibility that the previously observed ratio peaks may have been due to differences inherent in the commercially available amino acid mixtures, tissues were incubated in the presence of amino acid mixtures above; neither hormone nor cycloheximide was added. Analysis of the plasma membrane and cytosol fractions from the MR and G cells revealed no significant deviations in the $^3H/^{14}C$ ratio from the baseline.

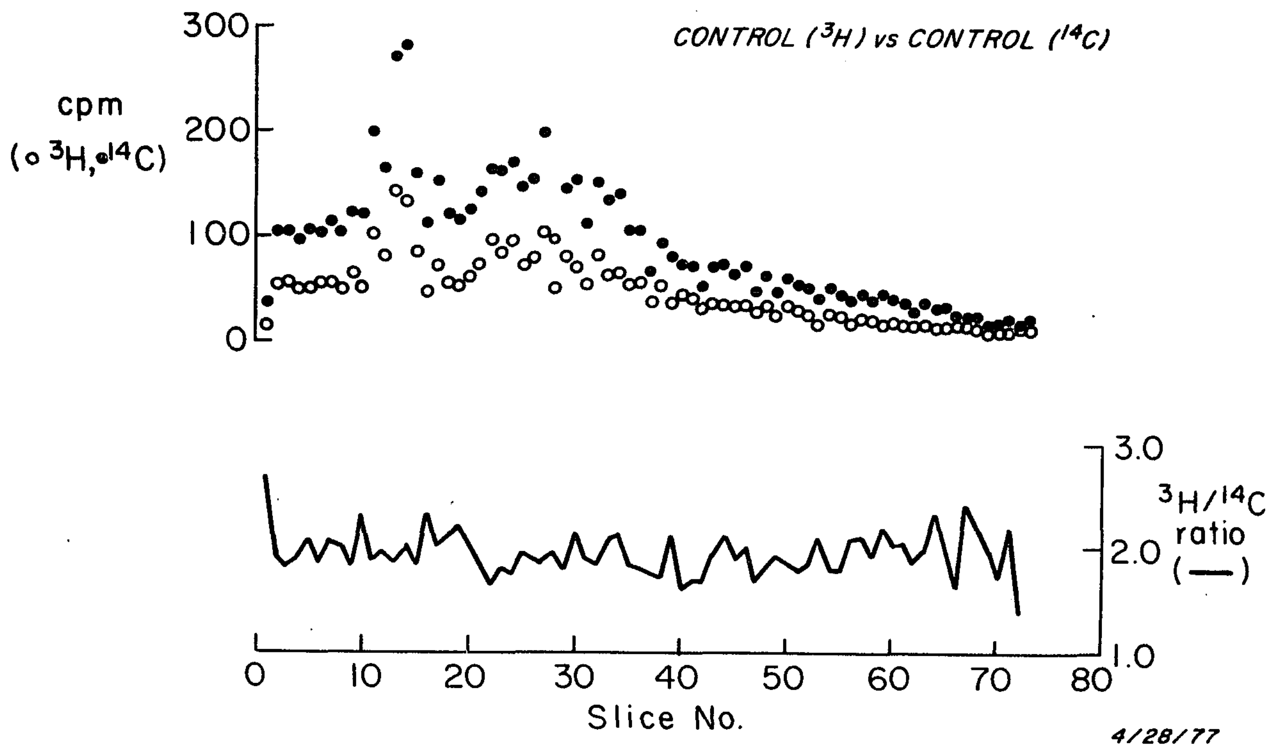


Figure 23. Incorporation of amino acids into MR cell plasma membrane proteins. Tissues were incubated with diluent (to simulate cycloheximide) for 15 min., followed by the addition to both these and paired tissues of a second aliquot of diluent (to simulate Aldo). Fifteen minutes later, (^3H)-amino acids were added to half the serosal baths, and (^{14}C)-amino acids were added to the baths of the matched hemibladders. After an additional incubation of 3 1/4 hours, the cells were harvested, combined, MR and G cells were separated, and plasma membranes were prepared. These were solubilized, analyzed on 8% polyscylamide gels, and each gel was sliced for radiosassay.

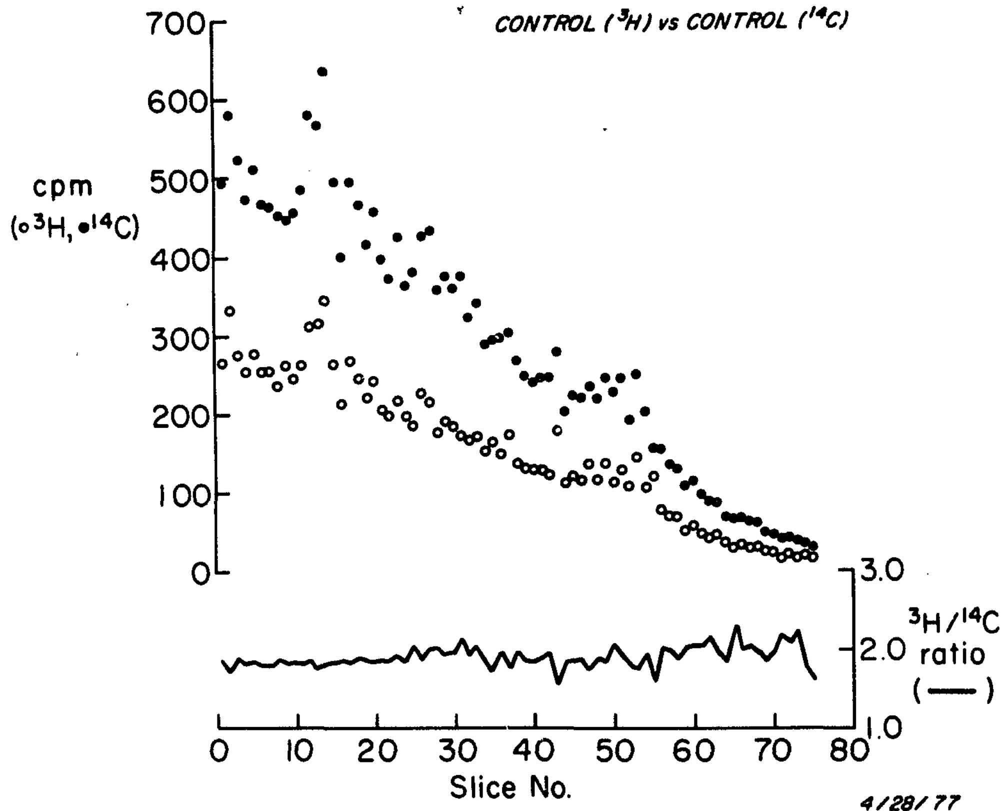


Figure 24. Incorporation of amino acids into G cell plasma membrane proteins. These results were obtained upon PAGE analysis of the G cells from the same experiment described in the preceding figure.

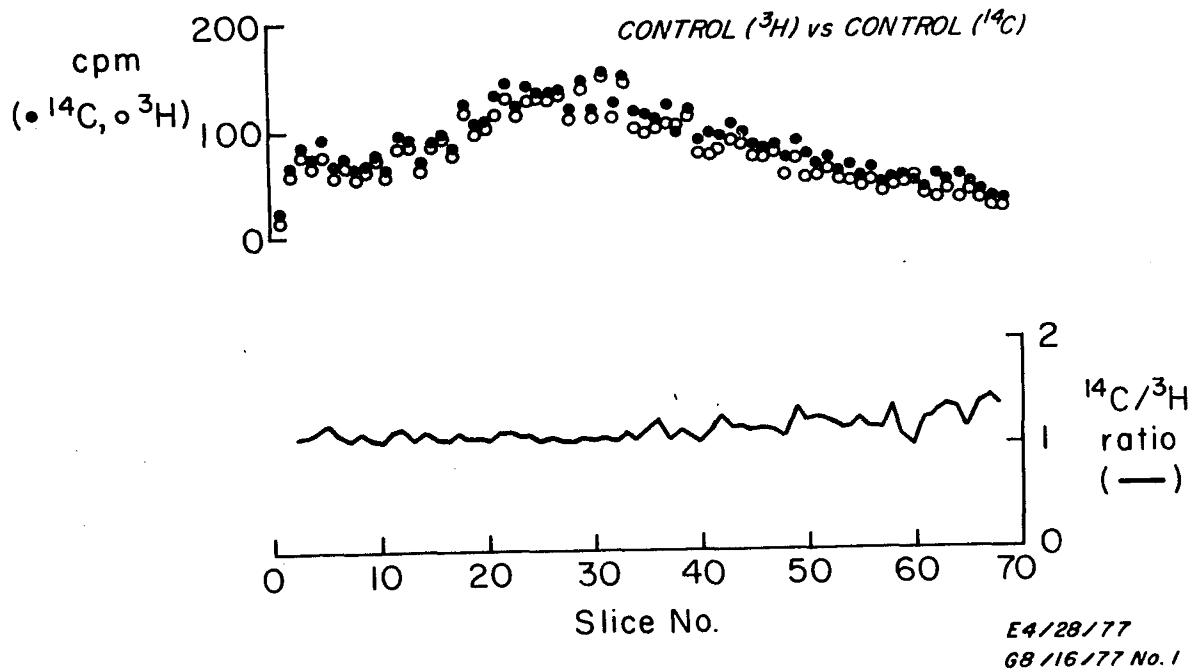


Figure 25. Incorporation of amino aicds into MR cell cytosol proteins. The results shown are for the PAGE analysis of the 195,000xg supernatant obtained from the MR cells of the same experiment described in Figure 23.

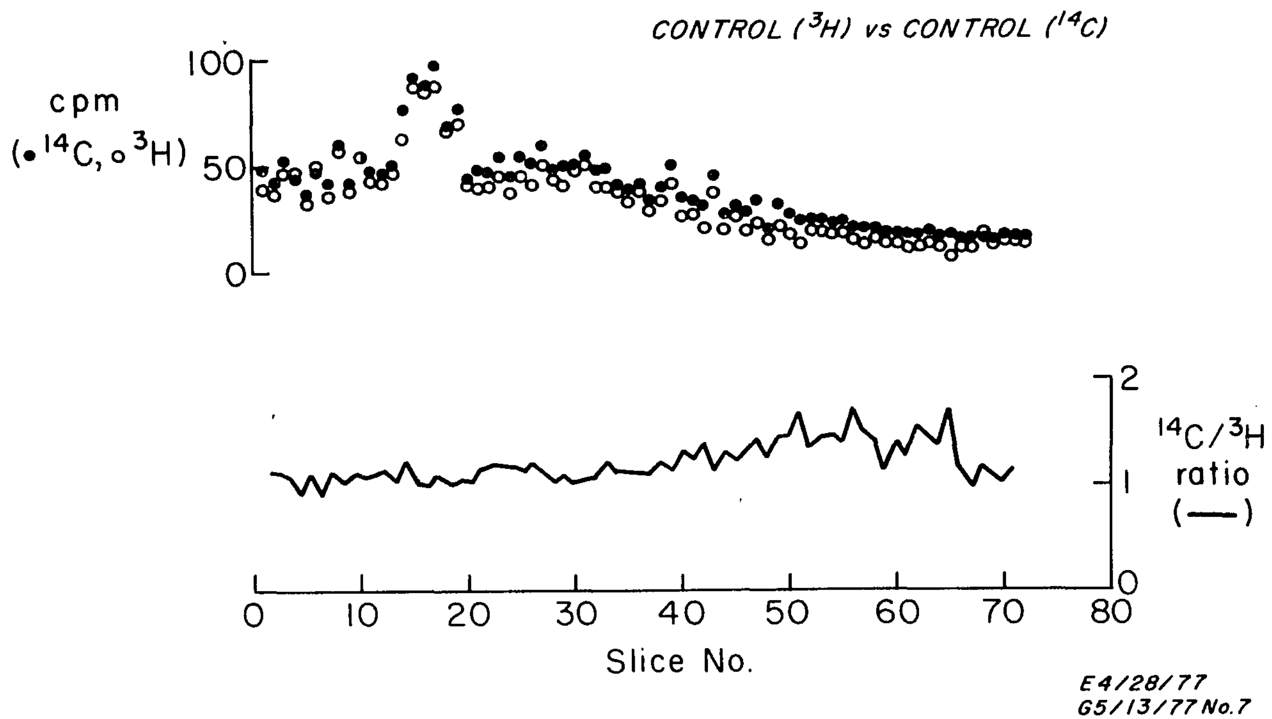


Figure 26. Incorporation of amino acids into G cell cytosol proteins. Illustrated are the data obtained upon PAGE analysis of the 195,000xg supernatant from the G cells of the same experiment described in Figure 23.

iii. Aldosterone vs. Aldosterone + TPIA: In the specific experiment described here, the hemibladders exposed to TPIA and aldosterone were incubated in the presence of (14C)-amino acids, while those treated with hormone alone were exposed to (3H)-amino acids. Thus, if TPIA blocked the appearance of hormone-induced proteins in a given fraction, peaks would be noted in the 3H/14C ratio.

As shown in Figure 27, TPIA caused four pronounced changes in the nuclide ratio, indicating a marked reduction in the Aldo-induced incorporation into the plasma membrane of radioactively labeled proteins. Three of the affected proteins had molecular weights corresponding well to the Aldo-induced protein illustrated in Figs. 9 and 13 (mol. wts. = 170,000, 85,000, and 6,000 daltons). In addition, there is a peak in the 3H/14C ratio corresponding to a protein of molecular weight of 32,000 daltons. Optical density scanning of this gel prior to slicing demonstrates that the ratio peaks occur in gel portions with significant protein content (Fig. 28). Upon analysis of the "mitochondrial pellet", the appearance of one protein with a molecular weight of approximately 95,000 was affected by TPIA (Fig. 29). However, analysis of the "cytosol" fraction (Fig. 30) of these mucosal cells revealed no significant shifts in the nuclide ratio, indicating that TPIA did not noticeably alter the Aldo-induced incorporation of amino acids into cytosol proteins.

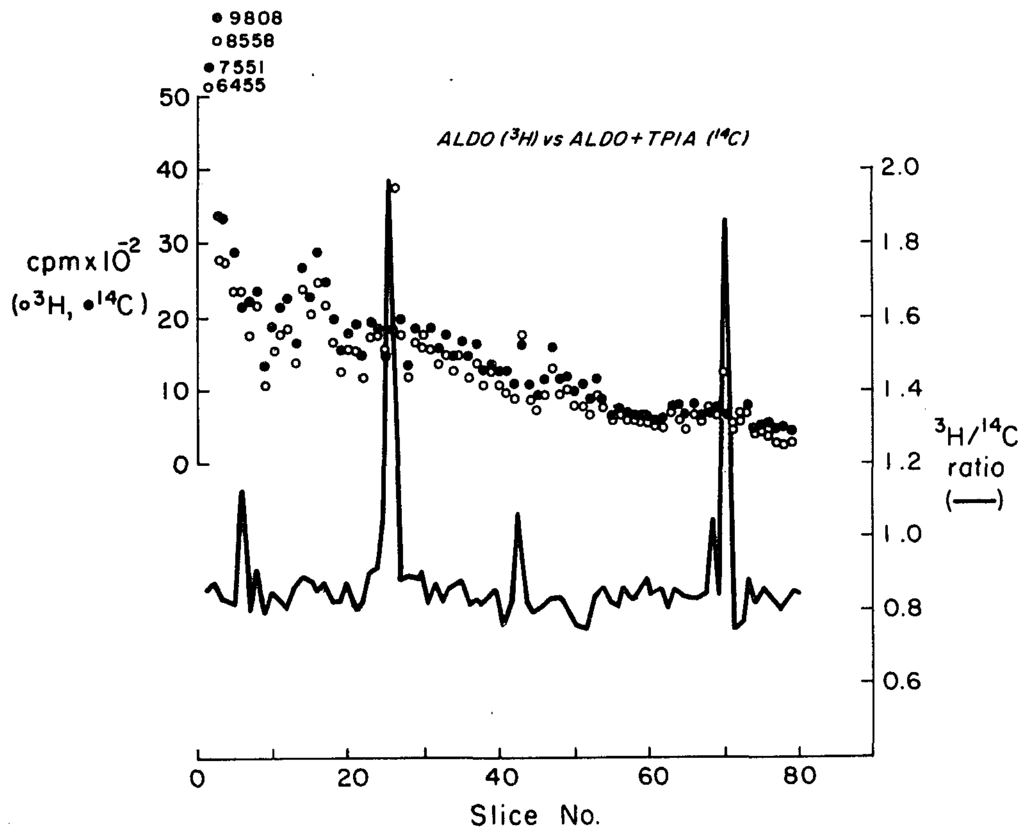


Figure 27. Effects of TPIA on the aldosterone-influenced incorporation of amino acids into plasma membrane proteins. Forty hemibladders were incubated in Ringer's solution for 2 hours, to mucosal solution containing 2 mM TPIA in 0.3% (v/v) DMSO, while the mucosal bath of paired tissues contained DMSO diluent alone. Aldo (7×10^{-8}M) was then added to all serosal baths. Forty-five minutes later, (^{14}C)-amino acids were added to the serosal baths of the TPIA-treated tissues, and their matched pairs were incubated with (^{3}H)-amino acids. After an additional 3 hours of incubation, cells from the two sets of tissues were collected, mixed, disrupted by nitrogen cavitation, and plasma membranes were prepared. The membrane pellet was solubilized, analyzed by PAGE, the gel was sliced and the ^{3}H/^{14}C ratio was measured.

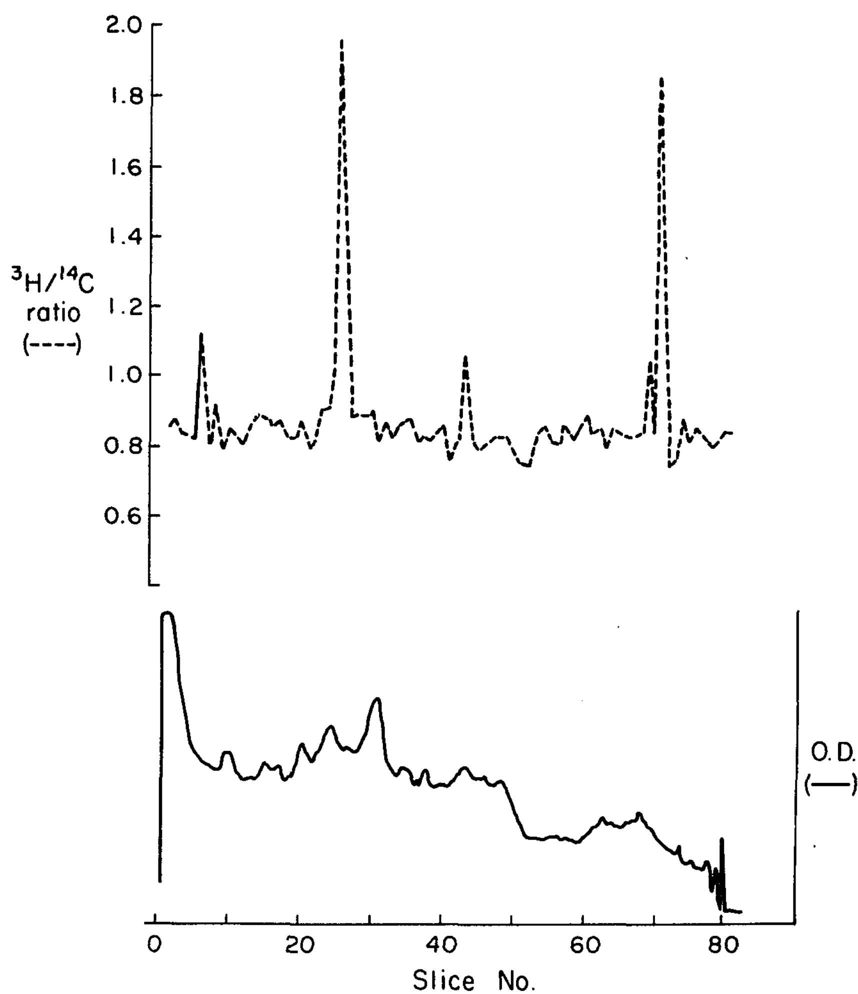


Figure 23. Effects of TPIA on aldosterone-induced plasma membrane proteins. The 3H/14C ratio as measured in each slice by radioassay (top scale) is compared with the optical density scan of the same gel before it was sliced (bottom scale).

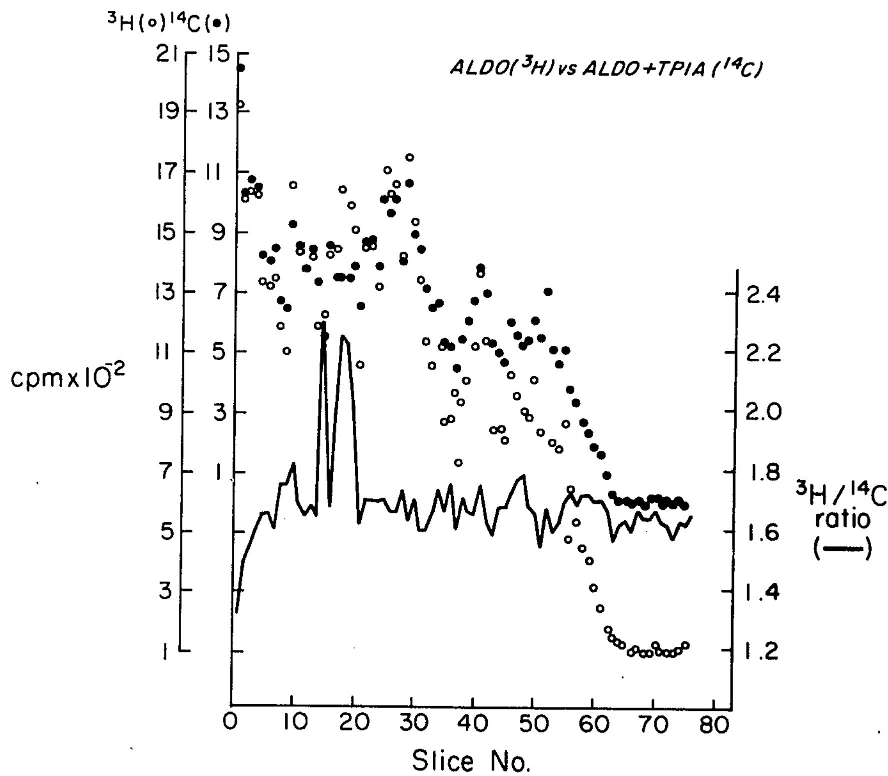


Figure 29. Effects of TPIA on the aldosterone-influenced incorporation of amino acids into proteins of the 10,000xg pellet. These results are from the same experiment described in Figure 23. Illustrated is the PAGE analysis of the membrane pellet obtained by centrifuging the homogenate at 10,000xg for 15 min.

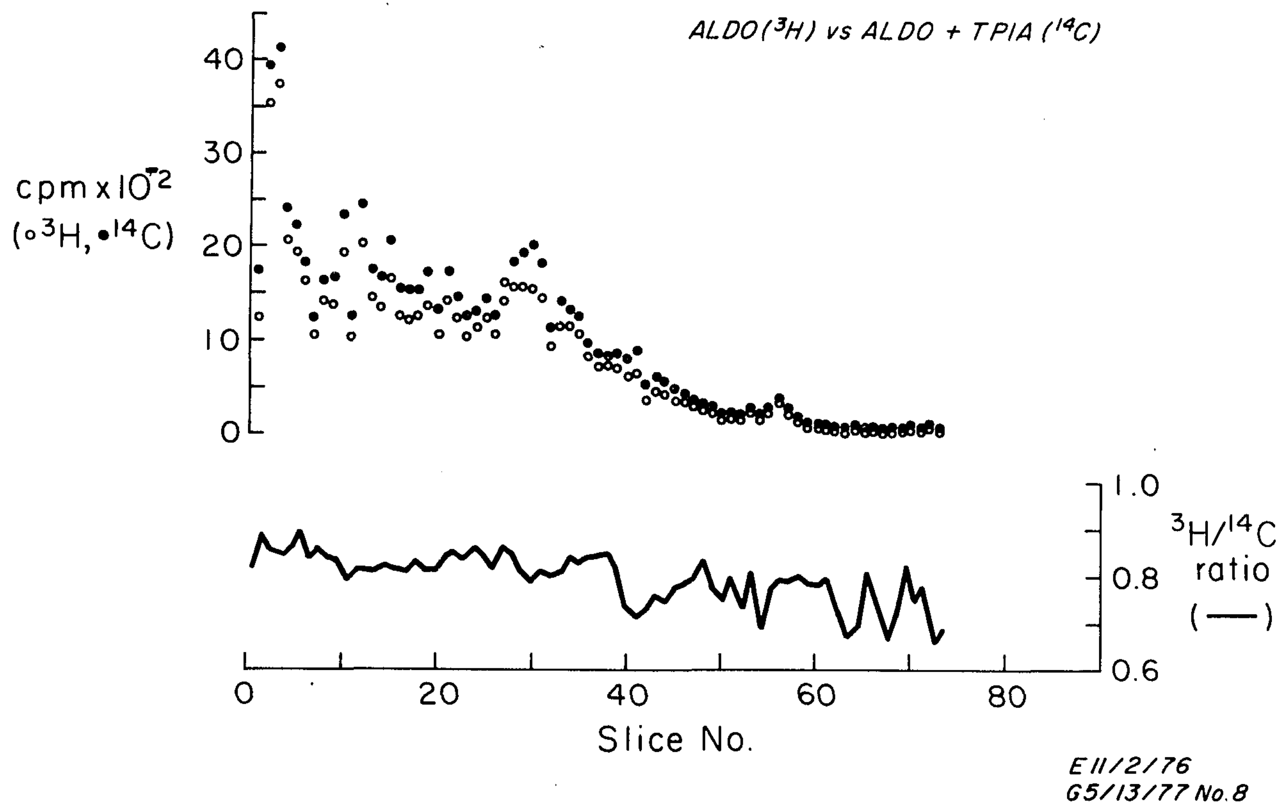


Figure 30. Effects of TPIA on the aldosterone-influenced incorporation of amino acids into cytosol proteins. The results shown were obtained upon PAGE analysis of the 105,000xg supernatant from the same experiment described in Figure 23.

iv. TPIA vs. Control; TPIA + Aldo vs. Control: Fractions analyzed were the plasma membranes, the "mitochondrial pellet", and the cytosol proteins (Figs. 31-36). In neither of these control experiments did any of the analyzed fractions show a clear shift in nuclide ratio from the baseline, indicating that the effects of TPIA per se cannot account for the peaks observed in Figs. 27-29.

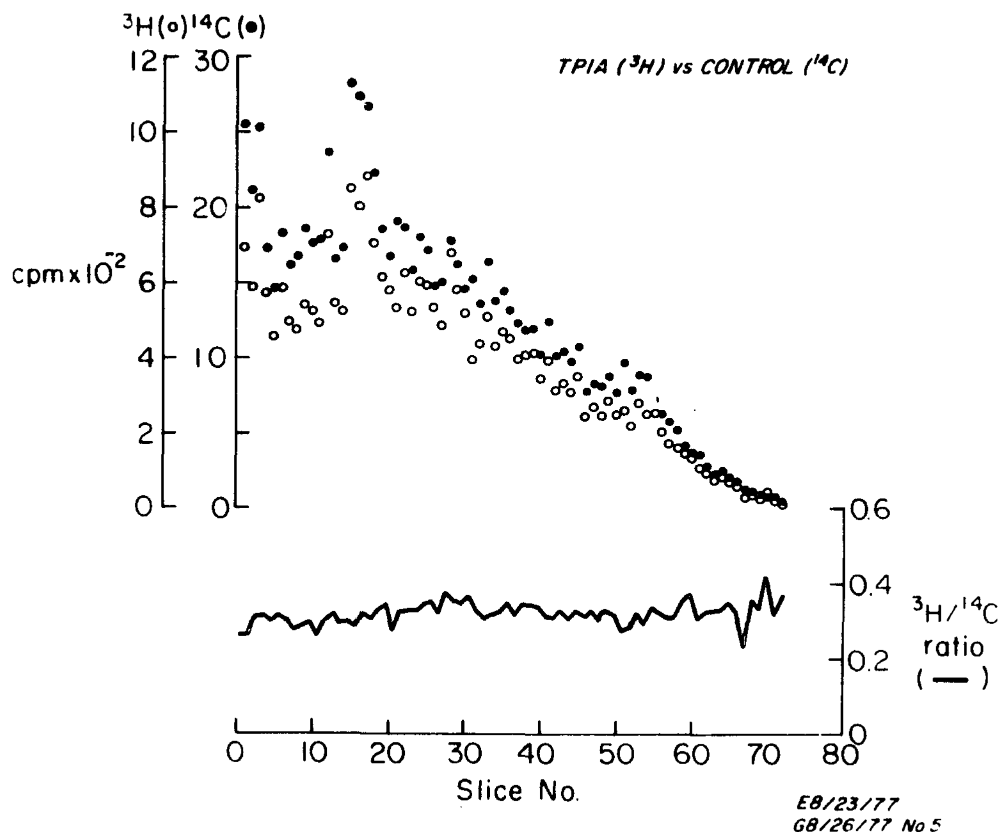


Figure 31. Effects of TPIA on the incorporation of amino acids into plasma membrane proteins. A procedure identical to that described in Figure 27 was used, except that DMSO diluent rather than %hormone was added to all serosal baths 2 hours after the initial incubation with TPIA had commenced. (3H)-amino acids were added to the serosal bath of the TPIA-treated tissues, and (14C)-amino acids were added to the serosal bath of the matched pairs. After a total incubation of 5.75 hours, cells from the two sets of tissues were removed, combined, disrupted by nitrogen cavitation, and plasma membranes were prepared. The membrane pellet was analyzed by PAGE, the gel was sliced, and the 3H/14C ratio was measured.

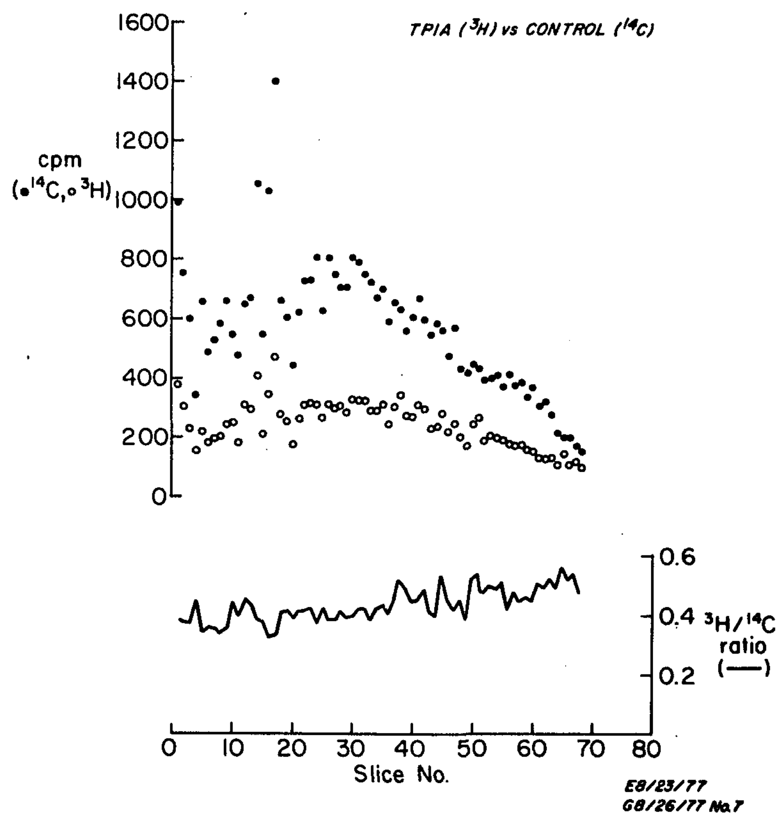


Figure 32. Effects of TPIA on the incorporation of amino acids into "mitochondrial pellet" proteins. These data were obtained upon PAGE analysis of the 10,000xg pellet from the same experiment described in the preceding figure.

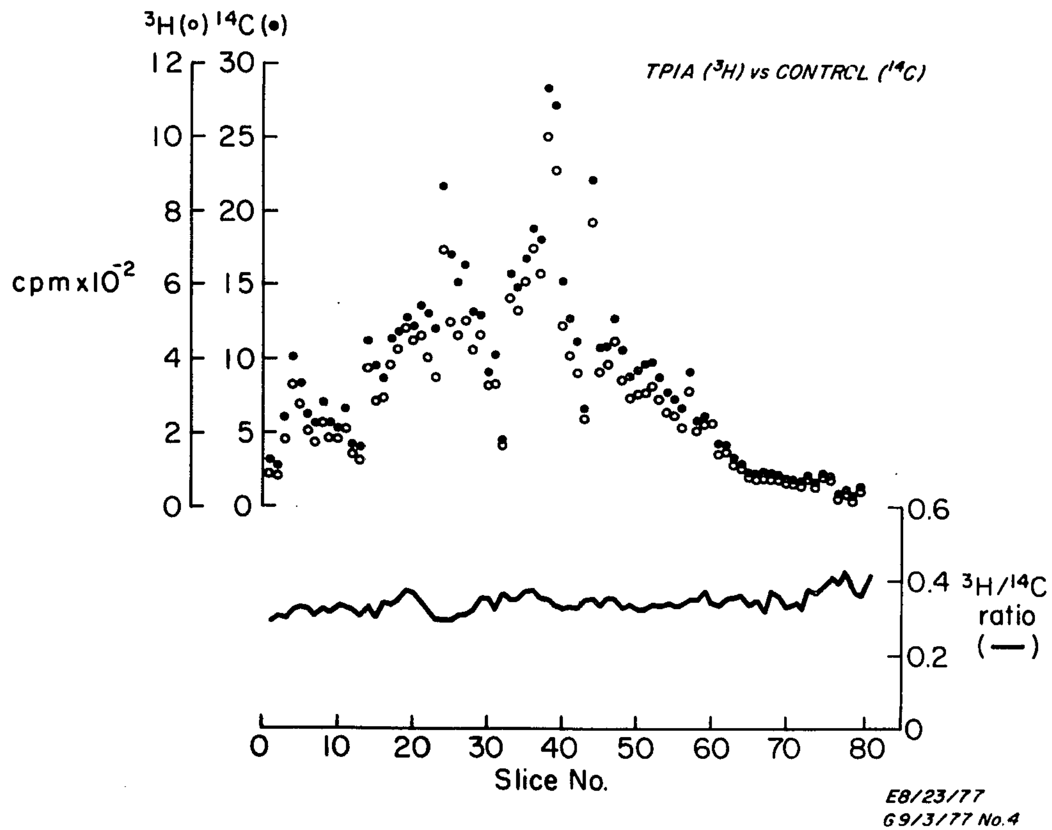


Figure 33. Effects of TPIA on the incorporation of amino acids into cytosol proteins. Illustrated are the results obtained upon PAGE analysis of the 105,000xg supernatant from the same experiment described in Figure 31.

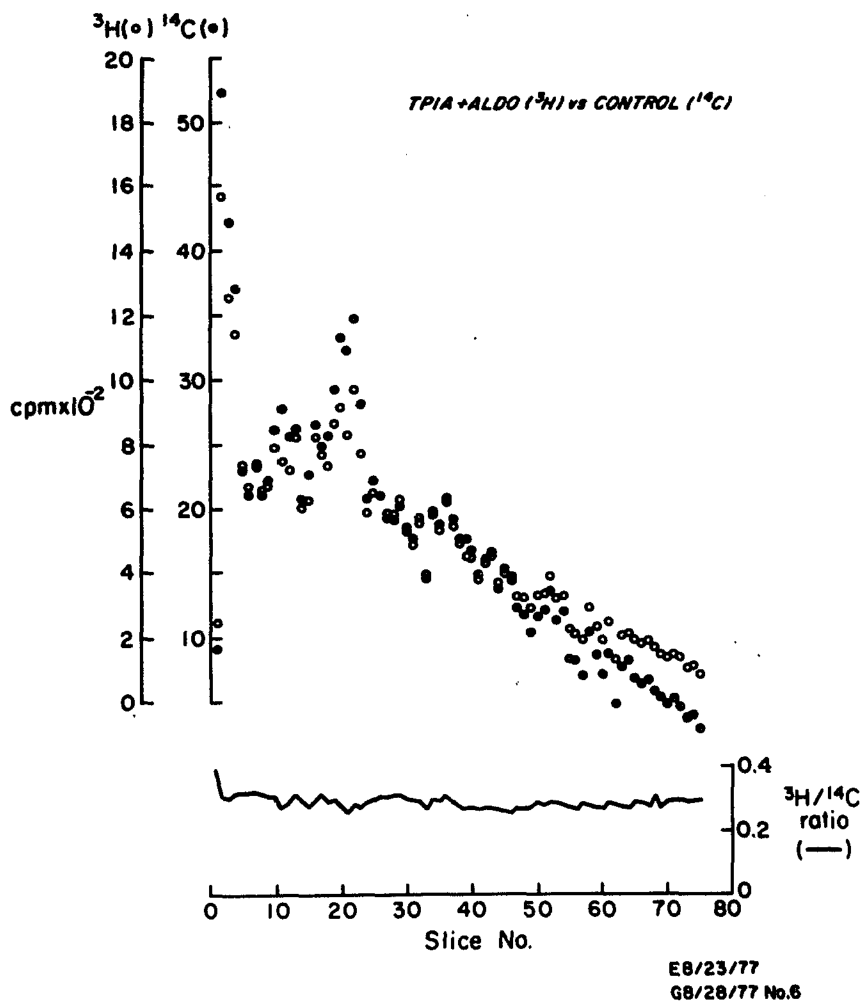


Figure 34. Effects of TPIA + Aldosterone on the incorporation of amino acids into plasma membrane proteins. The experimental protocol was identical to the described in Figure 31, except that while one set of tissues was incubated with TPIA, aldo, and (3H)-amino acids, the matched pairs were exposed only to diluent and (14C)-amino acids at the appropriate times. Incubation proceeded 5.75 hours, following which cells from all bladders were harvested, mixed, disrupted by nitrogen cavitation, and plasma membranes were prepared. The membrane pellet was solubilized and analyzed on an 8% polyacrylamide gel which was cut and the 3H/14C ratio was measured in each slice.

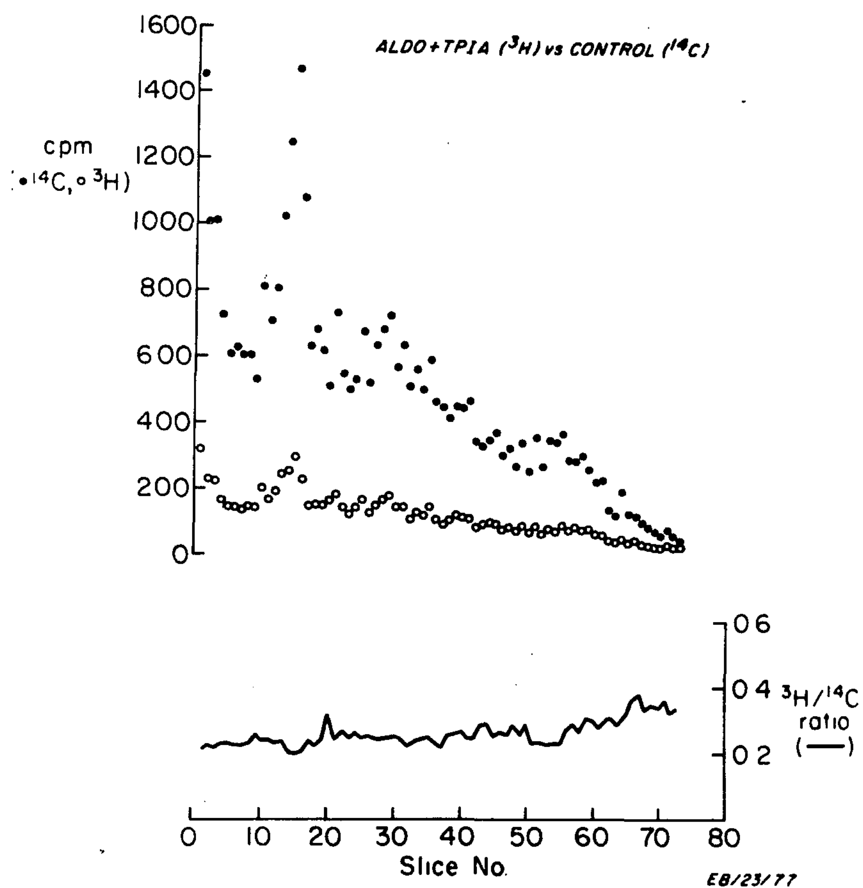


Figure 35. Effects of TPIA + Aldosterone on the incorporation of amino acids into proteins of the "mitochondrial pellet." The data shown were obtained upon PAGE analysis of the 10,000xg pellet from the same experiment described in Figure 34.

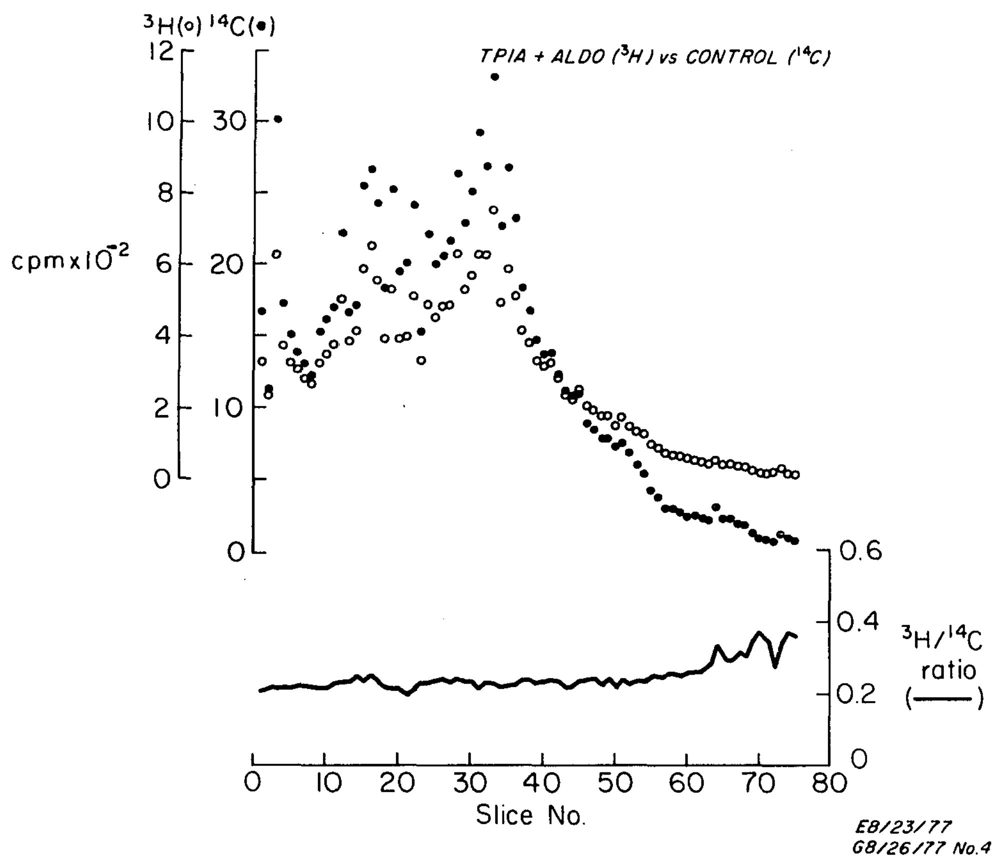


Figure 36. Effects of TPIA + Aldosterone on the incorporation of amino acids into cytosol proteins. These results were obtained upon PAGE analysis of the 105,000xg supernatant from the experiment described in Figure 34.

V. Discussion

A. In 1966, Hays and Barland published a method for the preparation of a plasma-membrane enriched fraction from toad bladder mucosal cells. However, more recent techniques have afforded the means of both enhancing the fraction purification and decreasing the variabiliy in the results obtained from experiment to experiment. For example, the current work replaces Dounce homogenization with the more reliable method of nitrogen cavitation. A second major difference is the use of the "microsomal" fraction rather than the "mitochondrial" fraction as the crude pellet from which the plasma membranes are obtained. It also became necessary to devise a technique suitable for cells obtained by disaggregation with EDTA and suspension as opposed to mucosal cells simply scraped from the bladder. Furthermore, the distributions of certain "marker" enzymes and 125I-labeled apical membranes amongst the various fractions have now been documented.

The approach of Wallach and Kamat (1964), originally applied to Ehrlich ascites tumor cells, has since been successfully expanded to obtain plasma membranes from a variety of cell suspensions as well as solid tissues, including pig lymphocytes (Ferber, Resch, Wallach and Imm, 1972), calf thyroid (Stanbury and Lafferty, 1968), rat liver (Graham, Higgins and Green, 1968) and rat adipocytes (Avruch and Wallach, 1971). The disruption of cells by nitrogen-cavitation in isotonic sucrose containing 0.25 mM MgCl2 apparently stabilizes internal membranes (Wallach and Schmidt-Ullrich, 1977). Nitrogen cavitation is a highly reproducible method of

disrupting cells because 1) the forces acting to disrupt the cells are identical for each cell in the suspension, 2) the milieu is isosomotic, and 3) there is no danger of local heating, such as may occur in abrasive techniques. And as shown in Figure 6A, the larger intracellular organelles remain largely intact and may be recovered by differential centrifugation in the "mitochondrial" fraction. The plasma membrane, on the other hand, is fragmented into vesicles which sediment primarily in the "microsomal" fraction. This fraction can then be separated into its component plasma membrane vesicles and endoplasmic reticulum vesicles in a dextran solution, provided the solution is carefully titrated to obtain precise conditions of pH, ionic strength, and density (Steck, Strauss and Wallach, 1970).

Among the problems arising in the preparation of plasma membranes from toad bladder mucosal cells is the paucity of cells available for study. Fifty toads yield only approximately 10^8 cells, two orders of magnitude less than most authors use in isolation of plasma membranes, regardless of the method employed (cf. Ferber et al., 1972). This results in a yield of only 810 ug protein, on the average, in the plasma membranes prepared. Hays and Barland (1966) reported that 1% of the total homogenate protein was recovered in the plasma membrane fraction. This value is similar to that obtained in preparations from other tissues (cf. Marx et al., 1972, Ferber et al., 1972, and Neville, 1974) and to the present figure of 1.5%.

Both glucose-6-phosphatase and cytochrome c oxidase activity were reduced in the plasma membrane fraction. The marked reduction in cytochrome c oxidase activity is consistent with the electron micrographic evidence that the mitochondria were left largely intact by nitrogen cavitation and that there were no recognizable mitochondria present in the plasma membrane fraction. Although the reduction in the specific activity of glucose-6-phosphatase was not as great, the activity is less than one-half that of the 5'-nucleotidase specific activity and represents a reduction relative to the initial homogenate; the specific activity of 5'-nucleotidase, on the other hand, is significantly increased over that in the homogenate. It is possible that some glucose-6-phosphatase activity may be endogenous to the in plasma membrane, or that glucose-6-phosphate may serve as a substrate to 5'-nucleotidase. This problem has been reviewed by Neville (1974).

Lactoperoxidase-catalyzed iodination has been shown to be an effective technique for the labeling of membrane proteins exposed to the aqueous milieu (Phillips and Morrison, 1970). Radioautographic evidence has been presented (Strum and Edelman, 1973) showing that no interior cell proteins in the toad bladder mucosal cell are labelled by this method. The same study demonstrated that proteins in the apical potion of the toad bladder mucosal cells can be radioiodinated without loss of transport function. Furthermore, this iodination is quantitatively increased in bladders exposed to an antidiuretic hormone (Scott and Slatin, 1979). This approach was therefore utilized to covalently label the plasma membrane proteins and quantitate their recovery. The results support the data obtained by enzymatic assays, showing recovery of over 27% of the plasma membranes in a significantly enriched fraction.

In summary, a technique has been devised for isolating plasma membranes from toad bladder mucosal cells, which gives highly enriched preparations of plasma membranes, as evidenced by enzymatic and morphologic assays.

B. Scott and Sapirstein (1975) used intact bladders to demonstrate Aldo-induced incorporation of amino acids into mucosal cell proteins. The effect was limited to a small fraction of cells enriched in MR cells . In the initial experiments described in this section the concentration of labeled amino acid (and the amount of isotope incorporated into protein) is increased by using the disaggregated mucosal cells in a small volume of incubation medium for the labeling experiments. The isotope was thus increased by approximately 100-fold, giving greater sensitivity to the entire procedure, and the results showed that aldosterone induces the synthesis of at least two membrane proteins, which can be localized to a tissue fraction of the MR cells enriched in plasma membranes.

The data described in the subsequent section show that the hormone-induced proteins can be identified in the plasma membranes when intact bladders are incubated in the presence of a mixture of labeled amino acids, and that the appearance of these proteins is blocked by either cycloheximide or TPIA. The latter is an inhibitor of fatty acid synthesis. These studies also show that when intact bladders are incubated with Aldo, the specific proteins synthesized may be further localized to the plasma membranes of the MR cells, and that this effect is cycloheximide-sensitive.

The fraction of MR cells enriched in plasma membranes contained three proteins whose synthesis was apparently induced by Aldo. The labeling of these proteins was independent of the combination of isotopes used and was inhibited by cycloheximide. It is possible that the 85,000 and 170,000 dalton peaks may represent a monomer-dimer relationship.

In contrast to the Aldo vs. Control and TPIA studies, the Aldosterone vs. Aldosterone + Cycloheximide experiment also shows an isotope ratio peak in a protein of 110,000 daltons. While it is possible that this may represent an Aldo-induced protein hitherto unidentified, analysis of the tissue fraction enriched in endoplasmic reticulum (but known by enzymatic assays to additionally contain some plasma membrane vesicles) showed that this ratio peak was absent while the two at 170,000 and 85,000 remained prominent. Therefore, it is more likely that this peak represents a proteolysis fragment of the larger hormone-induced protein. Secondly, the data failed to show a significant isotope ratio increase at MW = 6,000 daltons or 12,000 daltons, where such peaks were previously noted under different experimental conditions. In the earlier studies, however, these peaks were variable and were always noted in gel slices whose content of protein and labeled amino acids, in optical density and in CPM, were low. The present data therefore largely substantiate the previous results in identifying major Aldo-induced proteins of mol. wts. 170,000 and 85,000 in the plasma membranes of the MR cells.

Analysis of the cytosol proteins showed the presence of three induced proteins, with molecular weights of approximately 36,000, 12,000 and 6,000 daltons. These proteins apparently correspond to the proteins described by Scott and Sapirstein (1975) as having moleculr weights ranging from approximately 38,000 to 17,000 daltons. The techniques of tissue labeling and analysis here are considerably improved and give a more accurate resolution of the labeled proteins.

C. Brown (1978) has partially characterized the Aldo-induced mRNA in the toad bladder and has estimated the molecular weights to be expected for the translated proteins. For RNA species sedimenting at 25S, 18S, 12S and 7S, the corresponding proteins would be 162,500 daltons, 84,500 daltons, 26,000 daltons, and 6,500 daltons. These rough estimates are based on certain assumptions: (1) each mRNA contains a poly(A) segment of 150 residues (50,000 daltons), (2) the average nucleotide molecular weight is 333 daltons (1000 per codon), and (3) the mean molecular weight of the amino acid residues is 130g/mole. The molecular weights of the Aldo-induced proteins in the present work correspond remarkably well to these predictions, clearly within the limits of measurement and estimation errors. Using a rabbit reticulocyte cell-free translation system, Scott et al (1978) have characterized proteins coded for by partially purified Aldo-induced mRNA. The major translation product had a molecular weight of 168,000 daltons, again correlating well with Brown's predictions and with the proteins presently described.

The Aldo-induced membrane proteins appear to be lipoproteins or proteolipids. Sapirstein (1975) characterized low molecular weight AIP's in the toad bladder, and was able to demonstrate an induced proteolipid from the membrane fraction of the MR cells. He suggested that this protein may be identical with a soluble Aldo-induced protein that he characterized with molecular weight 32-36,000 daltons since proteolipids are usually found as proteins of 30-40,000 daltons, and it is possible that a proteolipid may exist in a water-soluble form. A delipidated proteolipid may be converted

to a water-soluble specie which still retains solubility in chloroform-methanol solvent (Folch-Pi, 1972). The present work demonstrates that the appearance of various AIP's in the toad bladder mucosal cell plasma membrane can be inhibited with TPIA, an acetyl CoA carboxylase inhibitor. This drug has been shown to prevent the hormone-induced alterations in both sodium transport and membrane lipid structure (Lien, Goodman, and Rasmussen, 1975). TPIA had been previously shown to have no effect on the incorporation of labeled amino acids into protein (ibid), which has now been confirmed. When considered together, these data indicate that ongoing fatty acid synthesis and/or elongation is required for the steroid-induced alteration in plasma membrane protein metabolism to occur.

Inhibition of fatty acid synthesis and elongation resulted in diminished labeling of four plasma membrane proteins (170,000, 85,000, 32,000, and 6,000 daltons). While TPIA blocks both the Aldo-induced stimulation of Na transport and the steroid's effects on membrane lipid structure, incubation of tissues with amiloride results only in a blockage of the hormone-induced increase in SCC, presumably by preventing sodium entry at the luminal membrane; the latter drug does not affect the Aldo-induced changes in membrane lipids. It therefore appears that the Aldo-induced changes in membrane lipid structure are not secondary to increased sodium transport, but are instead a primary change in metabolism which may well be involved in mediating the steroid's effects on transcellular sodium transport.

However, the precise relationship between the Aldo-induced changes in membrane lipd metabolism and the steroid's effects on protein turnover remain unresolved. It is possible, for example, that Aldo is inducing the synthesis of lipoproteins, i.e., that AIP's are synthesized at the level of the endoplasmic reticulum, complex with specific boundary lipid, and are transported and inserted into the plasma membrane. Alternatively, a primary alteration in membrane lipid milieu could affect the rate of entry of AIP's (which are not inherently bound to lipids) into the plasma membrane.

The biochemistry of the AIP's is quite likely reflecting the specific mechanisms by which Aldo increases transcellular sodium transport. Theories concerning these mechanisms may ultimately be divided into two basic proposals: Aldo increases membrane sodium permeability and/or the activity of the "sodium pump". Thus, the hormone may act by inducing the synthesis of proteins which serve either as membrane sodium channels and/or as components of the serosal sodium-potassium ATPase. Both of these mechanisms are clearly compatible with the present findings that Aldo induces the synthesis of proteins which are incorporated into the plasma membrane.

Since membrane proteins functioning as ion channels would be integrated into the luminal aspect of the membrane while proteins that were components of an Na-K pump would be located on the serosal side, it might be possible to resolve this issue by free-flow electrophoresis. This approach could

be used to separate plasma membrane veicles into those derived from the apical membrane and those derived from the serosal membrane. However, the technique is expensive and not widely available. An indirect solution to this question may be sought by employing the 125I-lactoperoxidase labeling technique described in the Methods section for labeling the apical plasma membrane proteins. If labeling were performed after the tissues were exposed to Aldo, increased 125I-labeling of the specific plasma membrane proteins whose synthesis is induced by Aldo would suggest that the AIP's were luminal plasma membrane proteins, thus presumably acting as Na "pores". However, negative results would not resolve the issue since there could be at least three possible alternatives: (1) the AIP's are serosal proteins and 125I-labeling is therefore not increased, (2) the AIP's are apical plasma membrane proteins but are "interior" proteins and are not exposed to the lumen, (3) the AIP's may be "exterior" apical proteins but lacking sufficient exposed tyrosine residues to exhibit a quantitatively significant difference in 125I-labeling from controls. Although this approach has been tried (Reich and Slatin, unpublished data), the results were, unfortunately, inconclusive. A third approach to the question of whether the AIP's are luminal or serosal proteins may lie in the development of a cell-free translation system from which quantitative amounts of AIP's could be prepared. It might then be possible to determine the characteristics of amiloride-binding to these proteins. Since amiloride appears to act by blocking Na entry into luminal ion channels, a low dissociation constant for amiloride-AIP binding would suggest that the proteins are these apical Na conduits. Additionally, it might be possible

to incorporate AIP's into artificial vesicles and measure the change in Na flux across the membrane.

Evidence that an AIP may be an Na-K ATPase has been reviewed in section IB. Most investigators have not been able to demonstrate an increase in either the total amount or the activity of the enzyme following treatment with Aldo. In contrast, Schmidt et al (1975) found a prompt increase in Na-K ATPase activity in the thick ascending limb of adrenalectomized rat kidneys following in vivo injection with Aldo; this rapid enzyme activation was completely inhibited by actinomycin D and cycloheximide. However, it is not clear why their data revealed a return to baseline enzyme activity after only one hour post-injection, which would not be expected for a typical Aldo response.

Much of the data from measurements of the "Na transport pool" tends to support the concept of the AIP functioning as a sodium ion channel (see section IB). This is supported by experiments in which amiloride has been shown to bind to one-third fewer sites in bladders from salt-immersed (Aldo-suppressed) toads, consistent with the drug's effects on diminishing the Aldo-induced stimulation of short circuit current. In these experiments in Cuthbert's laboratory, Aldo increased the number of amiloride binding sites in isolated toad bladder mucosal cells by 115%, an effect which was prevented by actinomycin D and cycloheximide (Cuthbert and Shum, 1975). The average increment in (14C)amiloride labeling of the cells in the presence of hormone was 44%, similar to the increase in Na transport

(52%) observed with same concentration of Aldo (5x10-7M). It was concluded that Aldo either stimulates the de novo synthesis of sodium ion translocating sites, or it induces a protein which acts to expose latent channels already in the apical plasma membrane. Unfortunately, the proportionality between the number of mucosal entry sites and transepithelial transport could not be determined; if this were known, it would allow one to estimate whether the induction of such sites sufficed to account for Aldo's stimulation of SCC, or whether another mechanism (increased serosal active Na pumping, whether directly or indirectly) must be implicated.

The intriguing finding that phospholipase C, when placed on the corium side of frog skin, increases transmembrane SCC tends to support the "Na pore" theory (Yorio and Bentley, 1976). This amphibian epithelium increases Na transport in response to Aldo in a manner similar to the toad bladder, while the enzyme specifically cleaves phosphatidylcholine at its ester linkage. When unidirectional Na fluxes were studied using 22Na, only influx was found to have increased. Since the effects of phospholipase C and Aldo on SCC were additive, it appeared that their precise sites of action may be different, although the authors suggested that the mechanisms may be quite similar.

A highly purified preparation of Na-K ATPase from toad kidney has recently been obtained (Geering and Rossier, 1979). While reports had previously appeared characterizing the enzyme activity from amphibian

tissues, including toad bladder, under various conditions, none had achieved as a high a degree of specific activity nor been able to estimate the molecular weight of the enzyme subunits (c.f. Corntos and Walser, 1971; Park and Hong, 1976). Upon SDS-polyacrylamide gel electrophoresis of the enzyme, Geering and Rossier observed three bands of protein, at 116,000, 62,000 and 26,000 daltons. The 116,000 dalton protein was the most prominent, and was identified as the catalytic subunit by phosphorylation with γ-[32P]ATP in the presence of sodium. The less prominent 62,000 dalton protein stained for glycoproteins. It appeared that the 3 bands may have been primary aggregates formed by the subunits of Na-K ATPase, suggesting an intact enzyme molecular weight of approximately 200,000 daltons. However, none of these molecular weights corresponds to any of the membrane proteins in the present study that were found to be specifically synthesized in response to Aldo. (An exception is the 110,000 dalton protein found in the "Aldo vs. Aldo + Cycloheximide" experiment plasma membrane fraction section IVB; however, as discussed earlier, in no other experiment was the protein found to have a differentially increased incorporation of labeled amino acids in response to hormone.) Taken together, the data therefore indicate that the direct synthesis of Na-K ATPase is probably not a significant mediating step in the effects of Aldo on transepithelial Na transport.

However, there is evidence from several laboratories that the activity of the serosal sodium pump affects the entry of sodium at the apical surface. Inhibition of the pump with ouabain decreases the sodium uptake

at the mucosal surface of frog skin, toad skin, and toad bladder (Biber, 1971; Larsen, 1973; Finn, 1975). Reuss and Finn (1975) also showed that a decrease in the serosal transmembrane potential follows that of the mucosal transmembrane potential within less than 25 milliseconds after the addition of amiloride to, or the removal of sodium from, the mucosal surface. These data led the authors to conclude that the mucosal and serosal borders may well "signal" each other in some manner. Cuthbert and Shum (1977) extended these studies, using frog skin, to demonstrate that as the intracellular sodium concentration is raised following ouabain treatment of the serosa, sites for sodium entry became increasingly unavailable as measured by (14C)amiloride binding. Ouabain had no significant effect on the affinity of amiloride binding. This differed from the effects of lowering mucosal bath Na concentration to 5% of normal, which was associated with a fall in SCC to 17% of normal, and an apparent fivefold increase in the affinity of amiloride. In either case, however, there appeared to be regulation by positive feedback, i.e., Na influx is reduced when the intracellular concentration is increased. Clearly, the extent to which these regulating mechanisms are affected by Aldo is a critical and as yet unresolved question.

The function of the Aldo-induced cytosol proteins is unclear. Not surprisingly, their synthesis was not inhibited by TPIA, suggesting that they have few inherent lipid components (section IVB). Although it is nevertheless still possible that they are soluble forms of proteolipids, as discussed above, this explanation does not appear likely. Alternatively,

they may represent hydrophilic membrane proteins whose hydrophobic lipid moieties have not yet been attached. Another explanation is that they may be soluble mitochondrial enzymes weich thus contribute to ATP production, indirectly contributing to the Na-K ATPase pump. Of interest is recent evidence concerning calmodulin, a cytoplasmic protein of human red blood cells that appears to regulate a membrane bound Ca-Mg ATPase pump. This protein has a molecular weight of approximately 17,000 daltons and is readily soluble. It appears to interact with the cytoplasmic face of the plasma membrane to modulate active calcium transport (Larsen and Vincenzi, 1979). The Aldo-induced cytosol proteins may be functioning in a similar fashion.

D. The precise locus of action of the Aldo-induced proteins is still unsettled, although it is believed that sodium traverses the epithelium (mucosa-to-serosa) via a transcellular pathway (MacKnight, Leaf, and Civan, 1971). Leaf (1965) suggested that serosa-to-mucosa fluxes of Na, K, and Cl are via channels separate from active Na transport in the opposite direction. Evidence has since accumulated that little, if any, sodium enters the cells from the serosal side, and that in the serosa-to-mucosa direction Na, K, and Cl all traverse a common, paracellular pathway (Saito, Lief, and Essig, 1974; Beauwens and Al-Awqati, 1976). This pathway appears to be the space between the mucosal cells at the level of the apical junction (DiBona and Civan, 1973); in fact, the authors were inclined to suggest that the terms "tight junction" and "zonula occludens" be replaced by the term "limiting junction" to more accurately portray the physiologic function (as opposed to purely anatomic configuration) of this structure. Since the recycling of sodium through the active transport pathway has since been shown to be negligible (Beauwens and Al-Awqati, 1976), the above findings that serosa-to-mucos Na flux was via a paracellular pathway and that this channel was different from the active Na transport pathway all supported the conclusion that transepithelial active Na transport (mucosa-to-serosa) in the toad bladder is via a transcellular pathway. This constituted an important basis for the approach used in the current work, i.e., attempting to characterize changes in the mucosal cell plasma membranes which might well prove to be a crucial limiting barrier in active Na transport.

There appears to be a degree of partitioning of physiologic function among the various morphologic cell types of the bladder. For example, it is generally agreed that urinary acidification in Colombian toads is mediated via the MR cells (Frazier, 1978; Rosen, Oliver, and Steinmetz, 1974). An important remaining question is which cell type constitutes the primary pathway for Na conductance. Evidence has been reviewed that the MR cell appears to mediate at least the initial events in the epithelium's response to Aldo (section IC). Briefly, Saladino, Bentley, and Trump (1969) first suggested that the MR cells might be involved in the Aldo response after they found that amphotericin B increased SCC across the toad bladder in a manner that appeared to be temporarily compatible with observed striking changes in MR cell morphology. Both morphologic and biochemical studies have since supported the conclusion. Thus, Voute, Hanni, and Amman (1972) using bladders and skins from both frogs and toads have found changes in the morphology of the MR cell as well as a quantitative increase in their number after hormone treatment. Nerve endings in the epithelium proper of frog skin are found in the vicinity of MR cells (Whitear, 1974). Sapirstein and Scott (1975) have shown that cytosol receptors for Aldo are found exclusively in the MR cells, and that Aldo-induced proteins may be identified exclusively in a tissue fraction enriched in MR cells (Scott and Sapirstein, 1975a). These results were supported by the isolation and characterization of Aldo-induced mRNA obtained from MR cells (Scott et al, 1978). In addition, there is suggestive biophysical evidence as well. The SCC measured across the urinary bladder of the aquatic urodele Amphiuma is

approximately 20uAmp/cm2 as compared to the baseline SCC across the toad bladder of approximately 80-150 uAmp/cm2 (Mullen, et al, 1976). It may be significant that only 5% of the mucosal epithelium of the urodele bladder is composed of MR cells, as compared to 20% of the mucosal cells of the toad bladder. Thus it is possible that a quantitative relationship exists between the proportion of MR cells in an epithelium and the measured Na transport.

Some debate still exists, however, over the basic histologic structure of the toad bladder. In an early transmission electron microscope study, Choi (1963) concluded that both the MR and G cells must be considered as candidates for mediation of active Na transport. A subsequent scanning EM investigation revealed the G cells to be in "close contact" with the MR cells in a stellate array, usually in a ratio of 3 or 4 G cells for each MR cell (Danon, Strum, and Edelman, 1974). Rarely was an MR cell in contact with only 2 G cells. This arrangement suggested a possible physiologic functional "communication" between the different cell types. Further evidence for the possibility of intercellular cooperation was offered by a study of the bullfrog bladder, in which it was observed that the granular cells were never in contact with the basement membrane, although MR cells were occasionally seen touching this structure. In addition, Whitear's finding that nerve terminals just beneath the basement membrane were in close proximity only to the MR cells was confirmed (Strum and Damon, 1974). However, a recent report by DiBona (1978) employing differential interference-contrast EM takes issue with some of these prevous observations. He found that the "rosette" arrangement of G cells around MR

cells was inconstant, and that 35% of the granular cells from bladders of Dominican toads made no contact with a mitochondria-rich cell. Clearly these are important points which must be resolved before the full nature of both basal and hormonally-influenced Na transport in this tissue can be completely understood.

The current work supports the evidence in favor of the mitochondria-rich cell playing an important role in the response of the toad bladder to Aldo. Incubation of tissues wih steroid was terminated after 3 1/4-4 hrs., the time of maximal SCC, in order to investigate the presence of biochemical changes which might correlate (at least temporally) with sodium transport. Analysis of the plasma membranes derived from the epithelium consistently revealed that increased amino acid incorporation into specific membrane proteins was limited to the MR cell. Additionally, aldo appeared to induce the synthesis of lower molecular weight cytosol proteins, again only in the MR cell. These data extend those of Sapirstein and Scott (1975) and Scott et al (1978) in demonstrating that major biochemical changes in RNA and protein metabolism occur in the MR cell in response to mineralocorticoid. It is possible that these events constitute initiating steps in the Na transport process which is ultimately mediated through the granular cells via intercellular cooperation, or else that hormonally-induced active Na further study.

References

Agarwal, M.K. (1975). Chromatographic demonstration of mineralocorticoid-specific receptors in rat kidney. Nature (Lond.) 254: 623-625.

Agarwal, M.K., (1976) Identification and properties of renal mineralocorticoid receptors in relation to glucocorticoid binding in rat liver and kidney. Biochem. J. 154: 567.

Alberti, K.B.M.M. and Sharp, G.W.G. (1969). Macromolecular binding of aldosterone in the toad bladder. Biochim. Biophys. Acta 192: 335-346.

Ausiello, D.A. and Sharp, G.W.G. (1968). Localization of physiological receptor sites for aldosterone in the bladder of the toad. Endocrinology 82: 1463-1469.

Avruch, J., and Wallach, D.F.H. (1971). Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. Biochim. et Biophys. Acta. 233: 334.

Bar, H.-P., Hechter, O., Schwartz, I.L., and Walter, R. (1970). Neurohypophyseal hormone-sensitive adenyl cyclase of toad urinary bladder. Proc. Nat. Acad. Sci. (USA) 67: 7.

Beauwens, R. and Al-Awqati, Q. (1976). Further studies on coupling between sodium transport and respiration in the toad urinary bladder. Am. J. Phys. 231: 222.

Benjamin, W.B. and Singer, I. (1974): Aldosterone-induced protein in toad urinary bladder. Science 186: 269-272.

Bentley, P.J. (1958). The effects of neurohypophyseal extracts on water transfer across the wall of the isolated urinary bladder of the toad Bufo marinus. J. Endocr. 17: 201-209.

Bentley, P.J. (1968). Amiloride: a potent inhibitor of sodium transport across the toad bladder. J. Physiol. 195: 317.

Bentley, P.J. and W.N. Scott (1977). The action of aldosterone. In I. Chester Jones and L.W. Henderson. General, Comparative, and Clinical Endocrinology of the Adrenal Cortex. Academic Press, London, Volume 2. pp. 497-564.

Biber, T.U.L. (1971). Effect of changes in transepithelial transport on the uptake of sodium across the outer surface of the frog skin. J. Gen. Phys. 58: 131.

Bonting, S.L. and Canady, M.R. (1964). Na-K activated adenosine triphosphatase and sodium transport in toad bladder. Am. J. Phys. 207: 1005.

Brown, J.A., Jr., and Scott, W.N. (1976). Aldosterone induces the synthesis of messenger RNA in mucosal cells of the toad's urinary bladder. The Physiologist 19: 141.

Brown, J.A., Jr. (1978). Biosynthetic processes related to the stimulation of sodium transport by aldosterone and insulin in the toad's urinary bladder. Thesis, Department of Physiology, Mt. Sinai School of Medicine. (CUNY).

Castles, T.R. and Williamson, H.E. (1965). Stimulation in vivo of renal RNA-synthesis by aldosterone. Proc. Soc. Exp. Biol. Mod. 119: 308-311.

Castles, T.R. and Williamson, H.E. (1967). Mediation of aldosterone induced anti-natriuresis via RNA-synthesis de novo. Proc. Soc. Exp. Biol. Med. 124: 717-719.

Chen, P.S., Toribara, T.Y., and Warner, H. (1956). Microdetermination of phosphorous. Analyt. Chem. 28: 1756.

Choi, J.K. (1963). The fine structure of the urinary bladder of the toad, Bufo marinus. J. Cell Biol. 16: 53-72.

Crabbe', J. (1961a). Stimulation of active sodium transport across the isolated toad bladder after injection of aldosterone to the animal. Endocrin. 69: 673-682.

Crabbe', J. (1961b). Stimulation of active sodium transport by the isolated toad bladder with aldosterone in vitro. J. Clin. Invest. 40: 2103-2110.

Crabbe', J. (1974). Influence of aldosterone on active sodium transport by the toad bladder: A kinetic approach. Acta Physiol. Scand. 90: 417-426.

Crabbe', J., and DeWeer, P. (1969). Relevance of sodium transport pool measurements in toad bladder tissue for the elucidation of the mechanism whereby hormones stimulate active sodium transport. Pflugers Arch. 313: 197.

Cuthbert, A.W. (1974). Interactions of sodium channels in transporting epithelia: a two state model. Molec. Pharm. 10: 892-903.

Cuthbert, A.W. and Shum, W.K. (1975). Effects of vasopressin and aldosterone on amiloride binding in toad bladder epithelial cells. Proc. Roy. Soc. Lond. B. 189: 543-575.

Cuthbert, A.W. and Shum, W.K. (1977). Does intracellular sodium modify membrane permeability to sodium ions? Nature (Lond.) 266: 468-469.

Dalton, T. and Snart, R.S. (1970). Respiratory and enzymic changes in response to steroid hormones. J. Endocr. 47: 159-166.

Davis, W.L. et al., (1974). Vasopressin-induced changes in the toad urinary bladder epithelial surface. J. Cell Biol. 61: 544.

Debnam, E.S., Hewitt, S. and Snart, R.S. (1975). Effect of steroid depletion on the response of toad bladder to vasopressin. J. Physiol., Lond. 251: 371-382.

DeLorenzo, R.J. and Greengard, P. (1973). Activation of adenosine 3':5'-monophosphate of a membrane-bound phosphoprotein phosphatase from toad bladder. Proc. Natl. Acad. Sci., U.S.A. 71: 3869.

DeLorenzo, R.J., Walton, K.G., Curran, P.F., and Greengard, P. (1973). Regulation of phosphorylation of a specific protein in toad bladder membrane by antidiuretic hormone and cyclic AMP, and its possible relationship to membrane permeability changes. Proc. Nat. Acad. Sci. U.S.A. 70: 880.

De Weer, P., and Crabbe', J. (1968). The role of nucleic acids in the sodium retaining action of aldosterone. Biochim. Biophys. Acta. 155: 280-289.

DiBona, D.R., Civan, M.M. and Leaf, A. (1969). The anatomic site of the transepithelial permeability barriers of toad bladder. J. Cell Biol. 40: 1-7.

DiBona, D.R., and Civan, M.M. (1973). Pathways for movement of ions and water across toad urinary bladder: 1. Anatomic site of transepithelial shunt pathways. J. Membrane Biol. 12: 101.

Dousa, T.P., Sands, A., and Hechter, O. (1972). Cyclic AMP-dependent reversible phosphorylation of renal medullary plasma membrane protein. Endocrinology. 91: 757.

Edelman, I.S., Bogoroch, R., and Porter, G.A. (1963): On the mechanism of of aldosterone on sodium transport: The role of protein synthesis. Proc. Nat. Acad. Sci. USA, 50: 1169-1177.

Ewer, R.F. (1952). J. Exp. Biol. 29: 173.

Fanestil, D.D. and Edelman, I.S. (1966). Characteristics of the renal nuclear receptors for aldosterone. Proc. Nat. Acad. Sci. 56: 872-879.

Fanestil, D.D., Porter, G.A., and Edelman, I.S. (1967). Aldosterone stimulation of sodium transport. Biochim. Biophys. Acta. 135: 74-88.

Ferber, E., Resch, K., Wallach, D.F.H., and Imm, W. 1972. Isolation and characterization of lymphocyte plasma membranes. Biochim. et Biophys. Acta 266: 494.

Ferguson, D.R. and Twite, B.R. (1974). Effects of vasopressin on toad bladder membrane proteins. Relationship to transport of sodium and water. J. Endocr. 61: 501-507.

Fimognari, G.M., Fanestil, D.D. and Edelman, I.S. (1967): Induction of RNA and protein synthesis in the action of aldosterone in the rat. Am. J. Phsyiol. 213: 954-963.

Finn, A.L. (1975). The action of ouabain on sodium transport in toad urinary bladder. Evidence for two pathways for sodium entry. J. Gen. Phys. 65: 503.

Finn, A.L. (1976). Changing concepts of transepithelial sodium transport. Physiol. Rev. 56: 453-464.

Folch-Pi, J., Stoffyn, P.K. (1972). Proteolipids from membrane systems. Ann. N.Y. Acad. Sci. 195: 86.

Frazier, L.W. (1978). Cellular changes in the toad urinary bladder in response to metabolic cidosis. J. Memb. Biol. 40: 165.

Funder, J.W., Feldman, D. and Edelman, I.S. (1972). Specific aldosterone binding in rat kidney and parotid. J. Steroid Biochem. 3: 209-218.

Geering, K. and Rossier, B.L. (1979). Purification and characterization of (Na+ + K+)-ATPase from toad kidney. Biochem. Biophys. Acta 566: 157.

Goodman, D.B.P., Allen, J.E., and Rasmussen, H. (1971): Studies on the mechanism of action of aldosterone: Hormone-induced changes in lipid metabolism. Biochemistry 10: 3825-3831.

Goodman, D.B.P., Bloom, F.E., Battenberg, E.R., Rasmussen, H. and Davis, W.D. (1975). Immunofluorescent localization of cyclic AMP in toad urinary bladder: possible intercellular transfer. Science. 188: 1023-1025.

Goodman, D.B.P., Wang, M., and Rasmussen, H. (1975): Aldosterone induced membrane phospholipid fatty acid metabolism in the toad urinary bladder. Biochemistry 14: 2803-2809.

Graham, J.M. (1975) Cellular membrane fractionation. In New Technques in Biophysics and Cell Biology. R.H. Pain and B.J. Smith, editors. Vol. II, pp. 1-42. John Wiley & Sons, New York.

Graham, J.M., Higgins, J.A., Green, C. (1968). The isolation of rat liver plasma membrane fragments. Biochim. Biophys. Acta. 150: 303.

Green, H.H., Harrington, A.R., and Valtin, H. (1970). On the role of antidiuretic hormone in the inhibition of acute water diuresis in adrenal insufficiency and the effects of gluco- and mineralocorticoids in reversing the inhibition. J. Clin. Invest. 49: 1724-1736.

Handler, J.S., Preston, A.S., and Orloff, J. (1969): Effect of adrenal steroid hormones on the response of the toad's urinary bladder to vasopressin. J. Clin. Invest. 52: 185-189.

Handler, J.S. and Preston, A.S. (1976). Study of enzymes regulating vasopressin-stimulated cyclic AMP metabolism in separated mitochondria-rich and granular epithelial cells of toad urinary bladder. J. Memb. Biol. 26: 43.

Hays, R.M., Singer, B., and Malamud, S. (1965). The effect of calcium withdrawal on the structure and function of the toad bladder. J. Cell. Biol. 25: 195.

Hays, R.M., and Barland, P. (1966). The isolation of the membrane of the toad bladder epithelial cell. J. Cell. Biol. 31: 209.

Hill, J.H., Cortas, N., and Walser, M. (1973). Aldosterone action and sodium- and potassium-activated adenosine triphosphatase in toad bladder. J. Clin. Invest. 52: 185-189.

Hutchinson, J.H. and Porter, G.A. (1970). Spirolactone inhibition of aldosterone induced nuclear RNA synthesis in toad urinary bladder. Res. Comm. Chem. Pathol. Pharmacol. 1: 363-369.

Hutchinson, J.H. and Porter, G.A. (1975). Accelerated RNA turnover in toad urinary bladder epithelial cells during early aldosterone action. Physiol. Chem. Physics. 7: 453-464.

Jard, S. and Bastide, F. (1970). A cyclic AMP-dependent protein kinase from frog bladder epithelial cells. Biochem. Biophys. Res. Comm. 39: 559.

Jelinek, W., Adensik, M., Salditt, M., Sheiness, D., Wall, R., Molloy, G., Phillipson, L., and Darnell, J.E. (1973). Further evidence on the nuclear origin and transfer to the cytoplasm of polyadenylic acid sequences in mammalian cell RNA. J. Mol. Biol. 75: 515-528.

Karlson, P. (1963). New concepts on the mode of action of hormones. Persp. Biol. Med. 6: 203-214.

Kirchberger, M.A., Martin, D.G., Leaf, A. and Sharp, G.W.G. (1968). The effect of aldosterone on glucose metabolism in toad bladder. Biochim. Biophys. Acta 165: 22-31.

Kirchberger, M.A., Schwartz, I.L., and Walter, R. (1972). Cyclic 3',5'-AMP-dependent protein kinase activity in toad bladder epithelium. Proc. Soc. Exp. Biol. Med. 140: 657.

Kirsten, E., Kirsten, R., Leaf, A., and Sharp, G.W.G. (1968): Increased activity of enzymes of the tricarboxylic acid cycle in response to aldosterone in the toad bladder. Pflugers Archiv. ges. Physiol. 300: 213-235.

Larsen, F.L. and Vincenzi, F.F. (1979). Calcium transport across the plasma membrane: stimulation by calmodulin. Science 204: 306.

Larson, E.H. (1973). Effect of amiloride, cyanide and ouabain on the active transport pathway in toad skin. In Transport Mechanisms in Epithelia. H.H. Ussing and N.A. Thorn, eds. Munksgaard, Copenhagen.

Law, P.Y., and Edelman, I.S. (1978a). Effect of aldosterone on incorporation of amino acids into renal medullary proteins. J. Memb. Biol. 41: 15-40.

Law, P.Y. and Edelman, I.S. (1978b): Induction of citrate synthase by aldosterone in the rat kidney. J. Membrane Biol. 41: 41-64.

Leaf, A. and Dempsey, E. (1960). Some effects of mammalian neurohypophyseal hormones on metabolism and active transport of sodium by the isolated toad bladder. J. Biol. Chem. 235: 2160-2163.

Leaf, A. and MacKnight, A.D.C. (1972). The site of the aldosterone induced stimulation of sodium transport. J. Steroid Biochem. 3: 237-245.

Leaf, A., Anderson, J. and Page, L.B. (1958). Active sodium transport by the isolated toad bladder. J. Gen. Physiol. 41: 657-668.

Lien, E. L., Goodman, D. B. P., and Rasmussen, H. (1975). Effects of an acetyl-coenzyme A carboxylase inhibitor and a sodium-sparing diuretic on aldosterone-stimulated sodium transport, lipid synthesis, and phospholipid fatty acid composition in the toad urinary bladder. Biochem. 14: 2749-2754.

MacKnight, A.D.C., Civan, M.M. and Leaf, A. (1975b). Some effects of ouabain ions and water in epithelial cells of toad urinary bladder. J. Membrane Biol. 20: 387-401.

Maragoudakis, M.E. (1969). Inhibition of hepatic acetyl coenzyme A carboxylase by hypolipidemic agents. J. Biol. Chem. 244: 5005-5013.

Maragoudakis, M.E. (1970). On the mode of action of lipid-lowering agents. II. In vitro inhibition of acetyl coenzyme A carboxylase by a hypolipidemic drug. Biochemistry 9: 413.

Marver, D., Goodman, D. and Edelman, I.S. (1972). Relationships between renal cytoplasmic and nuclear aldosterone receptors. Kidney Int. 1, 210-223.

Marver, D., Stewart, J., Funder, J.W., Feldman, D. and Edelman, I.S. (1974). Renal aldosterone receptors: studies with 3H-aldosterone and the anti-mineralocorticoid 3H-spirolactone (SC-26304). Proc. Nat. Acad. Sci., U.S.A. 71: 1431-1435.

Marx, S.J., Fedak, S.A., and Aurbach, G.D. (1972). Preparation and characterization of a hormone-responsive renal plasma membrane fraction. J. Biol. Chem. 247: 6913.

Mullent, T.L. et al (1976). Ion transport and structure of urinary bladder epithelium of Amphiuma. Am. J. Physiol. 231: 501.

Neville, D.M. (1974). Problems in quantitating surface membrane components in normal and pathologic cells and tissues. In Cell Surfaces and Malignancy. P.T. Mora, ed. pp. 5-16. DHEW Publication No. (NIH) 75-796, Washington, D.C.

Nordlie, R.C., and Arion, W.J. (1966). Glucose-6-phosphatase. In Methods in Enzymology, W.A., ed. Vol. IX 619-625. Academic Press, New York.

Lien, E.L., Goodman, D.B.P. and Rasmussen, H. (1976). Effects of inhibitors of protein and RNA synthesis on aldosterone-stimulated changes in phospholipid fatty acid metabolism in the toad urinary bladder. Biochim. Biophys. Acta. 421: 210-217.

Lifschitz, M.D., Schrier, R.W. and Edelmen, I.S. (1973). Effect of actinomycin D on aldosterone-mediated changes in electrolyte excretion. Am. J. Physiol. 224: 376-380.

Lipton, P. and Edelman, I.S. (1971). Effects of aldosterone and vasopressin on electrolytes of toad bladder epithelial cells. Am. J. Physiol. 221: 733-741.

Liu, A.Y., and Greengard, P. (1974). Aldosterone-induced increase in protein phosphatase activity of toad bladder. Proc. Nat. Acad. Sci. U.S.A. 71: 3869-3873.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265.

Ludens, J.H. and Fanestil, D.D. (1974). Aldosterone stimulation of acidification of urine by isolated urinary bladder of the colombian toad. Am. J. Physiol. 226: 1321-1326.

Luft, J.M. (1961). Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9: 409.

Macknight, A.D.C., Leaf, A., Civan, M.M. (1971). Effects of vasopressin on the water and ionic composition of toad bladder epithelial cells. J. Memb. Biol. 6: 127, 1971.

MacKnight, A.D.C., Civan, M.M. and Leaf, A. (1975a). The sodium transport pool in toad urinary bladder epithelial cells. J. Membrane Biol. 20: 365-386.

O'Malley, B.W. (1971). Unified hypothesis for early biochemical sequence of events in steroid hormone action. Metabolism 20: 981-988.

Orloff, J. and Handler, J.S. (1962). The similarity of effects of vasopressin, adenosine 3',5'-phosphate (cyclic AMP) and theophylline on the toad bladder. J. Clinical Invest. 47: 702.

Palade, G.L. (1962). A study of fixation for electron microscopy. J. Exp. Med. 95: 283.

Park, Y.S. and Hong, S.K. (1976). Properties of toad skin Na-K-ATPase with special reference to effect of temperature. Am. J. Physiol. 231: 1356.

Phillips, D.R., Morrison, M. (1970). The arrangement of proteins in the human erythrocyte membrane. Biochem. Biophys. Res. Commun. 40: 284.

Porter, G.A., Bogoroch, R., and Edelman, I.S. (1964): On the mechanism of of action of aldosterone on sodium transport: The role of RNA synthesis. Proc. Nat. Acad. Sci. USA 52: 1326-1333.

Reuss, L. and Finn, A.L. (1975). Dependence of serosal membrane potential on mucosal potential in toad urinary bladder. Biophys. J. 15: 71.

Reynolds, E.W. (1963). The use of lead citrate as an electron-opaque stain in electron membrane. Biochem. Biophys. Res. Commun. 40: 284.

Rogers, P.W., Flynn, J.J., III, and Kurtzman, N.A. (1975). The effect of mineralocorticoid deficiency on renal concentrating and diluting capacity. Proc. Soc. Exp. Biol. Med. 148: 847-853.

Rossier, B.C., Gaggeler, H.P., and Rossier, M. (1978). Effects of 3'deoxyadenosine and actinomycin. J. Memb. Biol. 41: 149-166.

Rossier, B.C., Wilce, P.A., and Edelman, I.S. (1974): Kinetics of RNA labeling in toad bladder epithelium: Effects of aldosterone and related steroids. Proc. Nat. Acad. Sci. USA 71: 3101-3105.

Rossier, B.C., Wilce, P.A., and Edelman, I.S. (1977): Spironolactone antagonism of aldosterone action on Na+ transport and RNA metabolism in toad bladder epithelium. J. Membrane Biol. 32: 177-194.

Rousseau, G. and Crabbe', J. (1968). Stimulation by aldosterone of a rapidly labelled RNA fraction in toad bladder tissue. Biochim. Biophys. Acta. 157: 25-32.

Saito, T., Lief, P.D., and Essing, A. (1974). Conductance of active and passive pathways in the toad bladder. Am. J. Physiol. 226: 126.

Saladino, A.J., Bentley, P.J., and Trump, B.F. (1969). Ion movements in cell injury: effect of amphotericin B on the ultrastructure and function of the epithelial cells of the toad bladder. Am. J. Pathology. 54: 421.

Sapirstein, V.S. (1975). Some studies on the hormonal control of sodium transport across the toad urinary bladder. Thesis, Department of Physiology. Mt. Sinai School of Medicine (CUNY).

Sapirstein, V.S. and Scott, W.N. (1973): Cyclic AMP and sodium transport: Quantitative and temporal relationships in toad urinary bladder. J. Clin. Invest. 52: 2379-2382.

Sapirstein, V.S. and Scott, W.N. (1975). Binding of aldosterone by mitochondria-rich cells of the toad urinary bladder. Nature. 257: 241.

Sawyer, W.H. and Schisgall, R.M. (1956). Increased permeability of the frog bladder to water in response to dehydration and neurohypophyseal extracts. Am. J. Phsyiol. 187: 312.

Schmidt, U., Schmid, J., Schmid, H., and Dubock, U.C. (1975). Sodium-and potassium-activated ATPase, a possible target of aldosterone. J. Clin. Invest. 55: 655, 1975.

Scott, W.N. (1973). Isolation and partial characterization of carbonic anhydrase of the toad, Bufo marinus. The Physiologist. 16: 445.

Scott, W.N., Reich, I.M., Brown, J.A., Jr., and Yang, Chia-Ping H. (1978). Comparison of toad bladder aldosterone-induced proteins and protein synthesized in vitro using aldosterone-induced messenger RNA as template. J. Membrane Biol. 40: (Special Issue), 213-220.

Scott, W.N. and Sapirstein, V.S. (1974). Localization of the action of aldosterone in the urinary bladder of the toad. Fed. Proc., 33: 1572.

Scott, W.N. and Sapirstein, V.S. (1975). Identification of aldosterone-induced proteins in the toad's urinary bladder. Proc. Nat. Acad. Sci. U.S.A. 72: 4056-4060.

Scott, W.N., Sapirstein, V.S. and Yoder, M.J. (1974): Partition of tissue functions in epithelia: Localization of enzymes in "mitochondria- rich" cells of toad urinary bladder. Science 184: 797-800

Scott, W.N., Shamoo, Y.A. and Brodsky, W.A. (1970). Carbonic anhydrase content of turtle urinary bladder mucosal Cell. Biochim. Biophys. Acta, 219: 248-250.

Scott, W.N. and Skipski, I.A. (1979). Isolation and characterization of carbonic anhydrase of the toad. Comp. Biochem. Physiol. 62B: 500.

Scott, W.N., and Slatin, S.L. (1979). Vasopressin-induced changes in the distribution of proteins in the luminal membrane of the toad's urinary bladder. J. Clinical Invest., submitted.

Sharp, G.W.G., and Leaf, A. (1964). Biological action of aldosterone in vitro. Nature (London). 202: 1185.

Sharp, G.W.G. and Leaf, A. (1966). Mechanism of action of aldosterone. Physiol. Rev. 46: 593-633.

Sharp, G.W.G., Komack, G.I. and Leaf, A. (1966). Studies on the binding of aldosterone in the toad bladder. J. Clin. Invest. 45: 450-459.

Smith, L. (1955). Spectrophotometric assay of cytochrome c oxidase. In Methods of Biochemical Analysis. D. Glick, ed. Vol. II. pp. 427-434. Interscience Publishers, Inc. New York.

Snart, R.S. (1967). Binding of aldosterone in the toad bladder. Biochem. Biophys. Acta. 135: 1056.

Stanbury, J.B., and Lafferty, M.A. (1968). The membranes of the thyroid cell. In Thyroid Neoplasia. S. Young and D.R. Inman, eds. pp. 425-439. Academic Press, New York.

Steck, T.L., Strauss, J.H., and Wallach, D.F.H. (1970). A model for the behavior of vesicles in density gradients: implications for fractionation. Biochim. et Biophys. Acta. 203: 385.

Stoff, J.S., Handler, J.S., and Orloff, J. (1972). The effect of aldosterone on the accumulation of adenosine 3',5'-cyclic monophosphate in toad bladder epithelial cells in response to vasopressin and theophylline. Proc. Nat. Acad. Sci. (U.S.A.) 69: 805.

Stoff, J.S., Handler, J.S., Preston, A.S., and Orloff, J. (1973). The effect of aldosterone on cyclic nucleotide phosphodiesterase activity in toad urinary bladder. Life Sci. 13: 545.

Strum, J.M. and Danon, D. (1974). Fine structure of the urinary bladder of the bullfrog (Roma catesbiana). Anat. Rec. 178: 15.

Strum, J.M. and Edelman, I.S. (1973). Iodination (125I) of the apical plasma membrane of toad bladder epithelium; electron-microscopic autoradiography and physiological effects. J. Memb. Biol. 14: 17.

Suzaki, S., Ogawa, E., and Inoue, Y. (1976). Effects of aldosterone, actinomycin D, puromycin and cycloheximide on RNA synthesis, carbonic anhydrase and ATPase activities of the kidney and on urinary excretion of sodium in adrenalectomized mice. J. Steroid Biochem. 7: 429-438.

Ussing, H.H and Zerahn, K. (1951). Active transport of sodium as the source of electric current in the short-circuited frog skin. Acta Physiol. Scand. 23: 110-127.

Vancura, P., Sharp, G.W.G., and Malt, R.A. (1971). Kinetic of RNA synthesis in toad bladder epithelium: action of aldosterone during the latent period. J. Clin. Invest. 50: 543.

Vander, A.J., Malvin, R.L., Wilde, W.S., Lapides, J., Sullivan, L.P. and McMurray, V.M. (1958). Effects of adrenalectomy and aldosterone on proximal and tubular sodium reabsorption. Proc. Soc. Exp. Biol. Med. 99: 323-325.

Voute, C.I., Hanni, S., and Amman, E.(1972). Aldosterone-induced morphological changes in amphibian epithelia in vivo. J. Steroid Biochem. 3: 161-165.

Voute, C.L., Thummel, J., and Brenner, M. (1975). Aldosterone effect in the epithelium of the frog skin - a new story about an old enzyme. J. Steroid Biochem. 6: 1175-1179.

Wallach, D.F.H., and Schmidt-Ullrich, R. (1977). Isolation of plasma membrane fragments from Ehrlich ascites carcinoma. Biochem. 5: 721.

Wallach, D.F.H., and Schmidt-Ullrich, R. (1977). Isolation of plasma membrane vesicles from animal cells. In Methods in Cell Biology. D.M. Prescott, ed., Vol. XV. pp. 235-276. Academic Press, New York.

Watson, M.L. (1958). Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochemn Cytol. 4: 475.

Whitear, M. (1974). The nerve in frog skin. J. Zool. 172: 503.

Widnell, C.C. (1969). Purification of rat liver 5'-nucleotidase as a complex with sphingomyelin. In Methods in Enzymology. Vol. XXXIV. pp. 368-369. Academic Press, New York.

Williamson, H.E. (1963). Mechanism of the antinatriuretic action of aldosterone. Biochem. Pharmacol. 12: 1449-1450.

Wu, J.Y., and Yang, J.T. (1970). Physiocochemical characterization of citrate synthase and its subunits. J. Biol. Chem. 245: 212-218.

Yorio, T. and Bentley, P.J. (1976). Phospholipase A and the mechanism of action of aldosterone. Nature (Lond.) 261: 722-723.

Zerahn, K. (1969). Nature and localization of the sodium pool during active transport in the isolated frog skin. Arch. Scand. 77: 272-281.