J. Membrane Biol. 213–220 (1978)

Comparison of Toad Bladder Aldosterone-Induced Proteins and Proteins Synthesized *in Vitro* Using Aldosterone-Induced Messenger RNA as Template

Walter N. Scott, Irwin M. Reich, James A. Brown, Jr., and Chia-Ping H. Yang

Departments of Physiology and Ophthalmology, Mount Sinai School of Medicine, New York, N.Y. 10029

Received 1 February 1978

Summary. 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. Induced proteins have been identified both in the plasma membrane (mol wt = 170,000, 85,000 and 12,000) and the cytosol (mol wt = 36,000, 12,000 and 6,000) fractions of these mucosal cells. We have also shown that aldosterone (Aldo) induces the synthesis of a class of RNA having the properties of messenger RNA (mRNA). mRNA isolated from Aldo-treated mucosal cells was used as template in a cell-free protein-synthesis system prepared from rabbit reticulocytes. Preparations charged with mRNA from Aldo-treated cells synthesized two proteins that were not labeled when mRNA from control tissues was used as template. The electrophoretic mobility of one of these proteins was similar to an Aldo-induced membrane protein (mol wt = 70,000) found in the intact tissue.

Because the stimulation of sodium transport by aldosterone (Aldo) was preceded by a latent period of over an hour, Crabbé suggested in 1961 that the steroid "stimulates the synthesis of a substance involved in active sodium transport." It was subsequently demonstrated that the effects of Aldo upon transport were blocked by inhibitors of protein or RNA synthesis (Edelman, Bogoroch & Porter, 1963; Williamson, 1963; Porter, Bogoroch & Edelman, 1964). While these observations provided strong indirect evidence that the induction of a specific protein was a necessary condition for Aldo's effects upon sodium transport, the direct demonstration of such a protein was elusive. Scott and Sapirstein (1975), using isolated toad urinary bladders incubated in ^{3}H- and ^{35}S-methionine, demonstrated the presence of Aldo-induced proteins in the mitochondria-rich (MR) cells of the toad bladder. Although these data confirmed the hypothesis that the stimulation

0022-2631/78/S040-0213 $01.60
© Springer-Verlag New York Inc. 1978

of transport was related to the synthesis of a specific protein(s), the results did not yield any information about the subcellular localization and/or the nature of the induced proteins.

Characterization of the induced protein(s) is of great interest because it should give us unique insight into the mechanism of active sodium transport and the processes relating to the stimulation of transport by mineralocorticoids. In an effort to characterize the events leading to the synthesis of the Aldo-induced protein, we have combined our techniques for isotopically labeling Aldo-induced RNA and proteins with the use of isolated mRNA as template in an *in vitro* system. Thus we are able to compare the physical properties of proteins synthesized *in vitro* with the Aldo-induced proteins synthesized in the intact mucosal cell.

Mucosal cells were removed from the urinary bladders of forty toads by incubating the tissues in Ringer's solution lacking calcium and containing 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; one bath also contained 7×10^{-8} M aldosterone. After 45 min, amino acids labeled with one isotope (e.g., ^{14}C) were added to the control bath and the same amino acids labeled with another nuclide (e.g., ^{3}H) were added to the aldosterone bath; the combinations used were ^{3}H- *vs.* ^{35}S-methionine, and a mixture of ^{3}H-labeled amino acids *vs.* a mixture of ^{14}C-labeled amino acids. The results obtained were independent of the choice of labeled amino acids for the two baths.

The MR and G (granular) cells were separated by density gradient centrifugation (Scott *et al.*, 1974) and the cells were disrupted by nitrogen cavitation and centrifuged at $5{,}000 \times g$ for 15 min to yield a crude "mitochondrial" pellet. The supernatant was used to prepare a cytosol fraction, and a preparation enriched in plasma membranes was separated from the endoplasmic reticulum using a density gradient technique (Reich *et al.*, 1978)[1] derived from the procedures described by Wallach and Kamat (1966). The subcellular fractions were dissolved in SDS-urea and analyzed by electrophoresis in sodium dodecyl sulfate- (SDS-) acrylamide gels. The gels were sliced, the slices incubated in a tissue solubilizer (at 55 °C) for 2 hr and the ($^{35}S/^{3}H$) or ($^{14}C/^{3}H$) ratio determined.

The results of a typical experiment are shown in Fig. 1. Following an incubation in which ^{35}S-methionine was added to the "Aldo" bath and ^{3}H-methionine to the "control" bath, the plasma membrane fraction

1 Reich, I.M., Gronowicz, G., Slatin, S.L., Masur, S.K., Scott, W.N. 1978. Preparation of a fraction enriched in plasma membranes of toad bladder mucosal cells. (*manuscript submitted)*

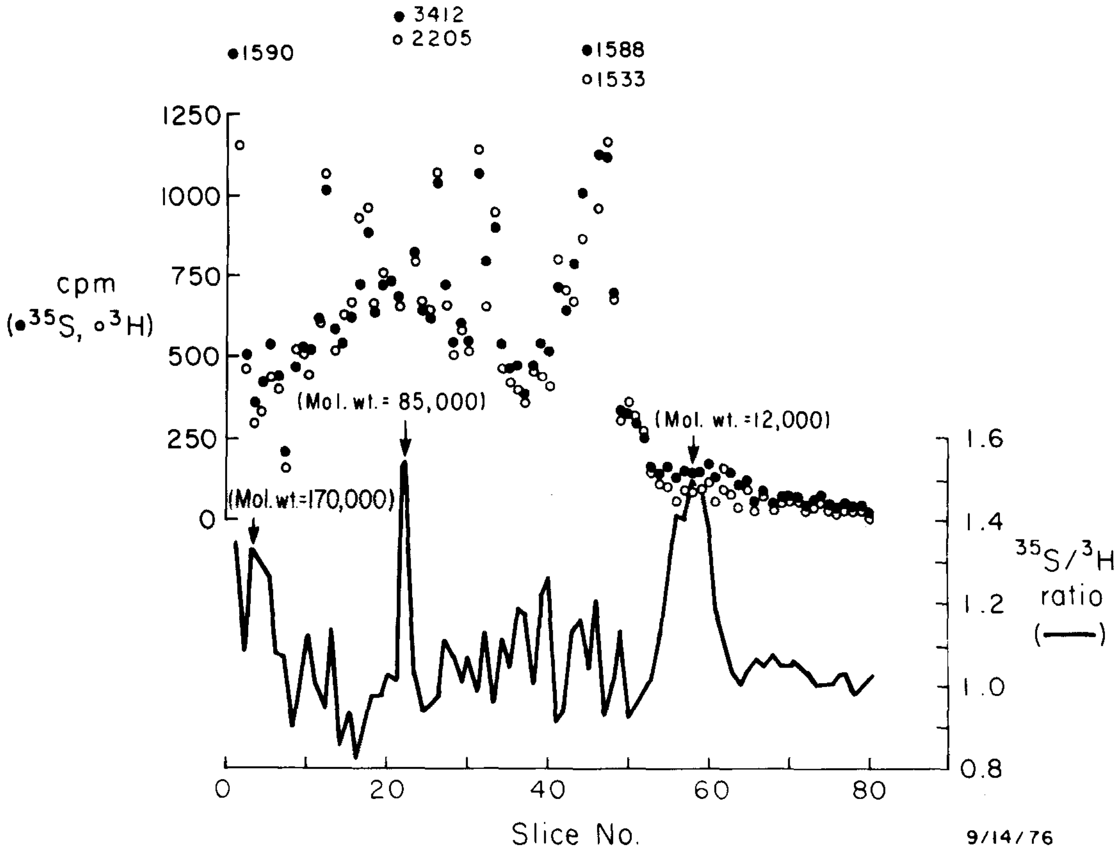

Fig. 1. Aldosterone-induced incorporation of methionine into plasma membranes of "mitochondria-rich" (MR) mucosal cells. Isolated cells were treated with 7×10^{-8} M aldosterone for 45 min, following which (^{35}S)-methionine was added to the bath for an additional 3-hr incubation. Cells from forty paired hemibladders were treated identically except they were not exposed to hormone, and (^{3}H)-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 ^{35}S/^{3}H ratio was measured

was prepared and analyzed by electrophoresis. The preparation from the MR cells (Fig. 1) shows three peaks in the ratio (^{35}S/^{3}H) corresponding to proteins with molecular weights of approximately 170,000, 85,000, and 12,000 daltons. We also analyzed the plasma-membrane fraction from the G cells of the same experiment, and no peak in the ratio was observed.

In our previous studies of the Aldo-induced protein we used exclusion gel chromatography to estimate that the molecular weights of the induced

proteins in the cytosol fraction of the MR cells ranged from approximately 17,000 to 38,000 daltons (Scott&Sapirstein, 1975). In the experiments presented here, analysis of the cytosol by a more accurate method (SDS-acrylamide electrophoresis) indicates there are three aldo-induced proteins (mol wt = 36,000, 12,000 and 6,000 daltons) in this fraction (Fig. 2).

These results encouraged us to attempt to isolate the Aldo-induced messenger-RNA (mRNA) for use as template in a cell-free protein synthesis system. Mucosal epithelial cells were removed from the bladder by incubation in EDTA-Ringer's and the mitochondria-rich (MR) cell fraction and granular (G) cell fraction obtained as described above. The cell preparations were rinsed free of Ficoll and incubated for 30 min at 22 °C in calcium-containing amphibian Ringer's containing 2 mM guanosine, adenosine and cytidine. The MR cells and the G cells were then each divided into three aliquots and 0.06 mCi ^{3}H (5,6) uridine were added to each aliquot of cells. Twenty minutes later, either aldosterone (7×10^{-8} M), aldosterone plus spironolactone (SC-9420 at 200:1 excess), or diluent was added to each preparation. After one hour the cells were pelleted, rinsed, and disrupted by nitrogen cavitation, all at 0–4 °C. Triton X-100 was added to a final concentration of 0.5%, and a pellet and post-mitochondrial supernatant were obtained by centrifugation at $12{,}000\times g$ for 10 min. The supernatants were made 0.5 M in NaCl and chromatographed on oligo-dT-cellulose. The mRNA was protected against hydrolysis by ribonucleases by sterilization of all glassware and by the addition of heparin (50 mg/100 mg) to the solutions. Poly-A-containing, cytoplasmic RNA (mRNA) was eluted from the columns in low salt buffer, heated to 60 °C for 20 min, and layered over 0–28% linear sucrose gradients. After 16 hr of centrifugation at 25,000 rpm, the sucrose gradients were fractionated and the radioactivity determined in each fraction.

We found a large peak of labeled material in the RNA from the Aldo-treated MR cells (Fig. 3). This increment was not found in cells exposed to Aldo *plus* spirolactone nor in control cells. The peak of this material, which had a sedimentation coefficient of approximately 22S, was precipitated with cold ethanol and collected for subsequent use as template in a cell-free protein synthesis system.

The use of cell-free protein synthesis for the translation of heterologous mRNA is an exceptionally versatile tool for the characterization of mRNAs and for the preparation of specific proteins of high specific radioactivity. We have used two systems, the wheat germ preparation of Roberts & Paterson (1973) and the rabbit reticulocyte preparation introduced by Borsook *et al.*, (1952). Our results are similar in the two

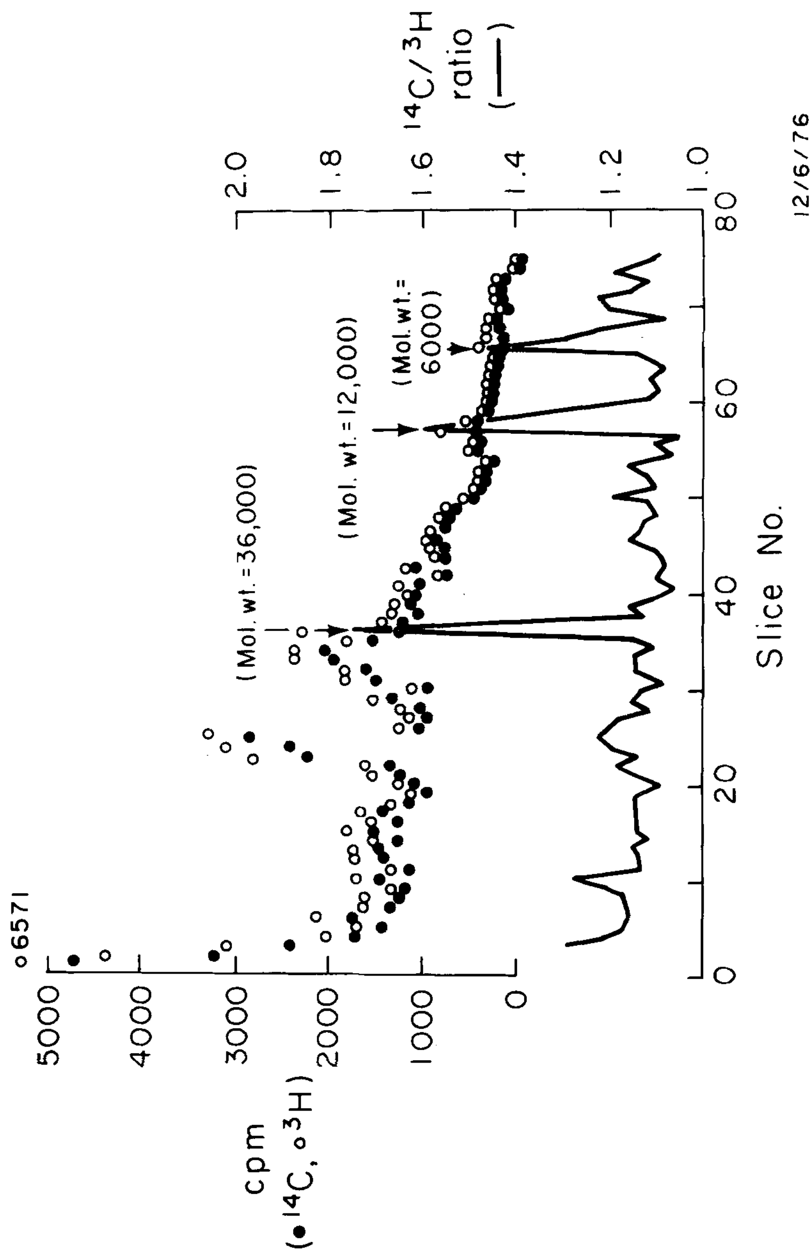

Fig. 2. Aldosterone-induced incorporation of amino acids into cytosol proteins. The proteins were labeled as in Fig. 1 except that the nuclides used were (^{14}C)- and (^{3}H)-labeled amino acids. Intact hemibladders were exposed to 7×10^{-8} M aldosterone for a 45-min incubation. A mixture of either ^{14}C-labeled amino acids (shown here) or ^{3}H-labeled amino acids was then added to the serosal bath. Forty paired hemibladders were treated identically except they received no hormone. After a 3-hr additional incubation period, the two sets of hemibladders were combined, the cells were removed and disrupted by nitrogen-cavitation. The supernatant obtained by centrifuging the homogenate at $195{,}000 \times g$ for 2 hr was analyzed on 8% polyacrylamide-SDS. The radioactivity was determined in the gel slices

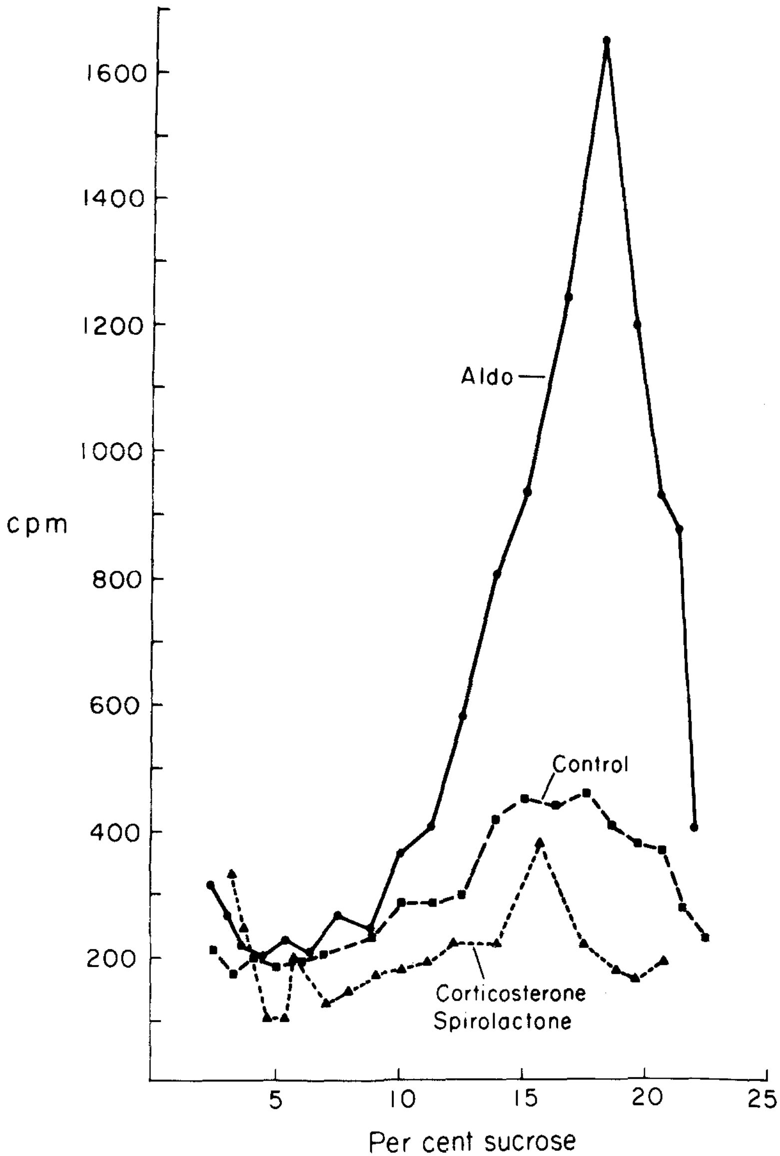

Fig. 3. Sedimentation analysis of mRNA on a linear (0–28%) gradient of sucrose. Following sedimentation for 16 hr in a SW-27.1 rotor (25,000 rpm), the samples were fractionated and the sucrose concentration in each fraction determined by an Abbé refractometer. Radioactivity was measured by liquid scintillation. The fractions containing from 14 to 22% sucrose were pooled and the RNA precipitated for use as template in the reticulocyte lysate system

systems, but because we obtain a higher efficiency of translation in the reticulocyte preparation, we have recently devoted our efforts to this system. Figure 4 shows the results of a typical experiment; mRNA pre-

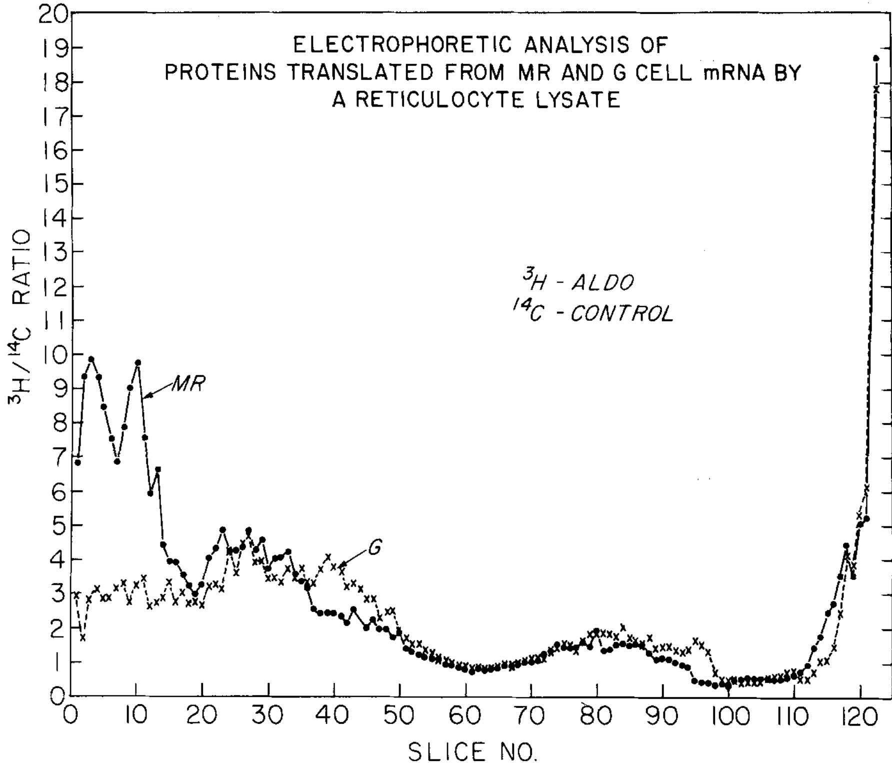

Fig. 4. Electrophoretic analysis of proteins translated from MR and G cell mRNA by a rabbit reticulocyte lysate system. mRNA prepared from MR (or G) cells exposed to Aldo (7×10^{-8} M) was incubated in a lysate system containing (^{3}H)-amino acid mixture, while mRNA prepared from MR (or G) cells treated only with diluent was added to an identical reticulocyte system containing (^{14}C)-amino acid mixture. Following incubation, the proteins from each preparation were dissolved, mixed, and analyzed by 7.5% polyacrylamide-SDS gel electrophoresis

pared from MR cells exposed to Aldo (7×10^{-8} M) was incubated in a mixture of ^{3}H-amino acids and micrococcal nuclease-treated reticulocyte lysate, while mRNA prepared from MR cells treated only with diluent was added to the same reticulocyte system containing ^{14}C-labeled amino acids. Following an incubation of 100 min at 30 °C, the proteins from each preparation were precipitated and mixed. The proteins were dissolved in a buffer containing Tris, mercapto ethanol and SDS, (pH 9.0), dialyzed, and analyzed by electrophoresis in SDS-acrylamide. The gels were sliced and the ratio (^{3}H/^{14}C) determined in each slice. The increase in the nuclide ratio indicates that there are two proteins translated by the system containing mRNA from the Aldo-treated MR cells that are not apparent in the preparation from the control MR cells. The molecular weight (170,000 daltons) of the smaller of these

two peaks is similar to that of the largest peak in the membrane fraction of the intact tissues. There was no evidence for an enhancement of protein synthesis by the mRNA from the Aldo-treated G cells. Although this represents only a preliminary result and positive identification of these proteins as components of the membrane of the mucosal cell awaits the preparation of antibodies against the induced proteins isolated from the intact bladder, we are encouraged that these proteins synthesized in the cell-free system represent the ultimate pathway for the aldosterone-stimulated sodium transport in this tissue.

The sequence of events leading to the physiologic expression of aldosterone's effects upon sodium transport has been the object of much study and analysis. It is known that aldosterone induces the synthesis of mRNA (Rossier, Wilce & Edelman, 1974). We have now identified the Aldo-induced membrane proteins and isolated and translated the mRNA coding for their synthesis. Comparison and characterization of these proteins should provide important insight into the mechanisms regulating the active transport of sodium.

This work was supported by grant #PCM-7602217 from the National Science Foundation and #AM-19130 from the NIH. Walter N. Scott is the Molly Berns Senior Investigator of the New York Heart Association.

References

Borsook, H., Deasy, C.L., Haagen-Smit, A.J., Keighley, G., Lowy, P.H. 1952. Incorporation *in vitro* of labeled amino acids into proteins of rabbit reticulocytes. *J. Biol. Chem.* **196**:669

Crabbé, J. 1961. Stimulation of active sodium transport by the isolated toad bladder with aldosterone *in vitro*. *J. Clin. Invest.* **40**:2103

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

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

Roberts, B.E., Paterson, B.M. 1973. Efficient translation of tobacco mosaic virus RNA and rabbit globin as RNA in a cell-free system from commercial wheat germ. *Proc. Nat. Acad. Sci. USA* **70(8)**:2330

Rossier, B.C., Wilce, P.A., 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

Scott, W.N., Sapirstein, V.S. 1975. Identification of aldosterone-induced proteins in the toad's urinary bladder. *Proc. Nat. Acad. Sci. USA* **72**:4056

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

Wallach, D.F.H., Kamat, V.B. 1966. Preparation of plasma-membrane fragments from mouse ascites tumor cells. *Methods Enzymol.* **8**:164

Williamson, H.E. 1963. Mechanism of the antinatriuretic action of aldosterone. *Biochem. Pharmacol.* **12**:1449