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BIOSYNTHETIC PROCESSES RELATED TO THE STIMULATION OF SODIUM TRANSPORT BY ALDOSTERONE AND INSULIN IN THE TOAD'S URINARY BLADDER

by

JAMES A. BROWN,JR.

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

1978

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

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ABSTRACT

Biosynthetic Processes Related to the Stimulation of Sodium Transport by Aldosterone and Insulin in the Toad's Urinary Bladder

by

James A. Brown, Jr.

Adviser: Dr. Walter N. Scott

Physiological doses of aldosterone affect RNA metabolism in the mitochondria-rich (MR) cells of the mucosal epithelium of the toad's urinary bladder in a manner similar to the effects of other steroid hormones on their respective target tissues. Aldosterone causes the apparent synthesis of several short-lived, cytoplasmic RNA species which contain poly-adenylated segments (mRNA). The mRNA was isolated from cytosol fractions of cells which had been disaggregated from the bladder wall in EDTA-Ringer's, centrifuged in discontinuous Ficoll gradients to separate the two major cell types, the MR from the granular or G cells, incubated as control or hormone-treated, and disrupted. G cells did not respond to aldosterone with any detectable change in RNA metabolism, while in MR cells aldosterone increased the incorporation of ^{3}H-uridine into the 12,000g pellet (nuclei, mitochondria), tRNA, and mRNA which, in sucrose gradient centrifugation, sedemented as 25S, 18S, 12S, and 7S peaks. Aldosterone's effects on RNA metabolism in the MR cell precede its effects on sodium transport (as measured by the short-circuit (SCC) technique) by some forty to sixty minutes both in onset and disappearance of the effect. Aldosterone's

effects on RNA metabolism in the MR cell are mineralocorticoid specific since they were blocked by prior addition of the specific mineralocorticoid blocking agent, spironolactone (SC-9420). 17 -Estradiol also increased in MR cells only, the incorporation of ^{3}H-uridine into a single peak of mRNA which migrated at about 7S on sucrose gradient centrifugation, but the significance of this effect is unclear since the hormone had no effect on SCC for up to 20 hours in vitro. Neither progesterone nor glucocorticoids had any effect on RNA metabolism in either cell type.

Insulin increases in SCC are apparent within 5 minutes after addition of the hormone. The rate of rise in the SCC is rapid for the next 40-60 minutes, coming to a 50% increase over control levels, and then slowly rising another 30-50% over the next 20 hours. The initial, rapid rise in SCC is not blocked by compounds which interfere with RNA and protein synthesis (i.e., actinomycin D and cyclohexamide) while the secondary, slower rise in SCC is blocked by these inhibitors. Insulin also increases the uptake of ^{14}C-amino acids and ^{3}H-uridine into thecytoplasm of both MR and G cells. The uptake of ^{14}C-amino acids plateaus in 60 minutes in both insulin-treated and control cells, while the uptake of ^{3}H-uridine in insulin-treated cells continues to rise for three hours. Insulin stimulates, in a linear fashion, the incorporation of these precursors into ^{3}H-RNA and ^{14}C-proteins in all fractions tested in both MR and G cells. The apparent insulin-induced synthesis of both new ribosomes and a 7S mRNA species in both cell types is an especially interesting finding.

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STATEMENT OF THE PROBLEM

Aldosterone, a steroid hormone, and insulin, a peptide hormone, both increase the reabsorption of sodium in the distal nephron of the mammalian kidney and in its model system, the urinary bladder of the toads. The metabolism of RNA was studied in the mucosal epithelial cells of the bladder, the site of the active transport of sodium, and the effects of the two different hormones on RNA metabolism was determined in an effort to elucidate the molecular basis of their physiological action.

The mucosal epithelium has two major cell types, the granular (G) cell, which comprises 70-80% of the cell population, and the mitochondria-rich (MR) cells, 15-20% of the cell population. The roles of each of these cell types in mediating the responses of these hormones was also investigated.

INTRODUCTION

I. Steroid Hormone Mechanisms

The steroid hormones, though diverse in function and target tissues, all seem to share a common mechanism of action. Studies on the action of the female reproductive hormones, 17β-estradiol and progesterone, have been most fruitful in providing insight to the chain of biochemical events initialed by steroid hormones in target cells. One reason for the success of studies of these hormones is that the target cells in the immature chick oviduct are not active until the addition of estrogen or of estrogen plus progesterone thus eliminating most background "noise" in RNA and protein synthesis. Secondly, these steroids cause the synthesis of large amounts of proteins relative to the mass of the tissue. Thus, the laboratories of Gorski and Jensen studying the action of estrogen, and O'Malley's group, studying the action of progestins, were the first to obtain direct evidence of the biochemical events of steroid hormone stimulation. Gorski, et al, in a 1968 review (1), summarized the data then known about estrogen. "Target tissues" are characterized by the uptake and retention of radioactively labelled hormone (estrogen). It is proposed that a specific hormone 'receptor' which is present only in target cells of the hormone in question. Since steroids function at extremely low blood concentration (10^{-10} to 10^{-7} M), the receptor must have a high affinity for the hormone (KD = 10^{-9} M), The response of the tissue to the hormone shows saturation, therefore at least one step in the series of events is rate limiting. 17β-Estradiol is not metabolized during uptake experiments in vivo at physiologic doses in the uterus. Tritiated estrogen was bound by both nuclei free of cell debris and by chromatin. Trypsin, but not nucleases (RNase, DNase), released bound estrogen from

those nuclei indicating that the binding site is protein in nature. Bound estrogen from the nuclei migrated as a 5S complex in continuous sucrose gradient ultracentrifugation studies. Only steroids which had estrogenic activity competed with H^3-17β-estradiol uptake into nuclei. Estrogens were also bound by a cytoplasmic complex digestable by proteases which migrated at 8S in sucrose gradients. The binding was specific for estrogens as tested by competition studies using radioactive 17β-estradiol versus excess cold estrogens. The dissociation constants for the cytosol receptor showed higher affinity for the more potent 17β-estradiol (KD = 7×10^{-10} M) compared to the less potent estriol (KD = 2×10^{-9} M). Specific cytosol binding of estrogens was not temperature dependent. Estrogens were not bound by isolated pure nuclei until the cytosol fraction was added and the temperature was raised to 25°C. Therefore, two steps were required for nuclear binding of estrogens: first, binding to a 8S cytosol protein, and second, a temperature dependent transformation of the 8S protein into a nuclear 5S complex.

O'Malley, in 1971 (2), published a unified hypothesis for the action of all steroid hormones, summarizing his data from studies of progesterone's effect on the chick oviduct as well as data from studies on other steroid hormones. The first common denominator in steroid hormone action was that inhibitors of transcription of DNA (actinomycin D) and of translation of mRNA (puromycin, cyclohexamide) were known to block most effects of steroid hormones. This, coupled with the fact that radioactively-labelled steroid hormones accumulate in the nucleus in autoradiographic and specific binding studies, pointed to the action of steroid hormones involving the unmasking of some gene or genes and

the subsequent synthesis of new proteins. In the specific case of progestins in the (estrogen pre-treated) chick oviduct, the new protein was avidin, the protein of hen's eggs which binds vitamin B_6.

O'Malley studied the cytosol progesterone receptor and found it to be 4S in 0.3MKCl and 8S in 5M KCl. The molecular weight of the 4S was about 10,000 daltons and the Kdiss was 8×10^{-10}M for progesterone. All of these figures are similar to those of the estrogen receptor, the dihydrotestosterone receptor of the rat prostate, and the glucocorticoid receptor of liver cells and of HTC hepatoma cells. A temperature-dependent, "two-step" transfer of cytosol bound steroid to the nucleus was found for progesterone, dihydrotestosterone, estrogens and glucocorticoids. A competitive inhibitor of androgens, cysroterone, displaced androgenic steroids from the binding protein, and the most potent androgens showed the most avid binding constant for the receptor. This parallel in binding affinity pototency was also seen in estrogens and progestins. Finally, the androgen binding protein of rat prostate, and the progestin receptor of chick oviduct were differentiated from cortisol binding globulin (CBG), a contaminant of the cytosol fraction from the blood plasma, by agarose gel chromatography, discontinuous polyacrylamide gel electrophoresis, isoelectric focusing, and other techniques.

In summary, steroid hormones seem to act by the following sequence of events; permeation of the cell membrane via their lipid nature; binding of steroid to a specific, non-dialyzable, heat sensitive, cytoplasmic protein receptor; transfer of the receptor-steroid complex to the nucleus by a temperature-dependent mechanism; binding of the receptor-steroid complex to specific sites on the chromatin-DNA; synthesis of new RNA molecules; and the subsequent synthesis of a protein(s) in the cytoplasm.

II. The Toad Bladder as a Model for the Mammalian Distal Nephron.

The mammalian kidney contains a large number of cell types, including those of the proximal tubule, the distal tubule, the collecting duct, the juxtaglomerular apparatus, connective tissue firboblasts, smooth muscle cells, capillary endothelium and the cells of the glomerulus. It is difficult therefore to study the cellular mechanisms of hormones which may only affect a few of these cell types. The urinary bladder of the marine toad, Bufo marinus, is often used as a model for the mammalian distal nehphron since both tissues respond to ADH by increasing water and urea permeability, and to aldosterone by increasing Na+ retention. The time course and mechanism of action of each hormone is similar in each tissue. The great advantage of the toad bladder lies in the fact that it is a simple epithelium on a connective tissue sub-layer. This allows for use of the tissue either in Ussing chambers to measure short-circuit current as a measure of Na+ transport or mounted as bags and weighed to measure water permeability. It, therefore, is a most convenient model for the distal tubule and is widely used.

III. History of Mineralocorticoids.

Seventy years after Addison discovered in 1855 that the adrenal gland had a vital function, Lucas noted that hypochloremia, uremia and hemoconcentration followed adrenalectomy in the dog. Baumann & Kurland (1927) observed that adrenalectomy lowered plasma sodium and chloride while raising plasma potassium and magnesium concentrations in the cat. Loeb, et al (1933), concluded that the primary target of mineralocorticoid action was the kidney, which lost NaCl after adrenalectomy. The first steroids with mineralocorticoid action to be isolated and characterized were corticosterone by de Fremey, et al (1937), and deoxycorticosterone by Steiger & Reichstein (1937). Aldosterone, the principal and most potent mineralocorticoid, was not discovered and synthesized until the 1950's (Grunchy et al, 1952; Simpson et al, 1954; Schmidtin et al 1957). Although aldosterone is the major mineralocorticoid of vertebrates, cortisol and corticosterone also have significant effects on sodium transport in anurans and mammals(3).

IV. Binding of Aldosterone in the Toad Bladder

Sharp, Komack and Leaf (18) in 1966 studied the binding of steroids and inhibitors of mineralocorticoid action in the isolated toad bladder. Bladders were incubated in H^3-aldosterone for one hour before the addition of a competitive inhibitor. They found that H^3-aldosterone binding was freely reversible with a half-life of 30 minutes if placed in an aldosterone-free bath, but one wonders whether the addition of unlabelled steroids one hour after H^3-aldosterone might not yield an adequate profile of specific binding in the tissue. Sharp and co-workers described two saturatable aldosterone binding sites: the site with high affinity (KD = 6.7×10^{-11}M) had so few binding sites (9×10^{-14}/g tissue) that it would be saturated at physiologic levels of aldosterone concentration, placing its physiological role in doubt. The other site bound H^3-aldosterone with less affinity (KD = 10^{-10}M) and the maximum number of sites (3×10^{-12}/g tissue) made it a likely candidate for the physiologic mineralocorticoid receptor. Both sites were "specific" in that H^3-aldosterone binding was inhibited by the following hierarchy of compounds: aldosterone > DOC > Cortisol > progesterone = spirolactone. H^3-aldosterone was not displaced from this site by cholesterol, ouabain, puromycin, or actinomycin D. No information was obtained as to the intracellular location of these sites.

Snart (5) studied aldosterone binding in the toad bladder using the same methods as Sharp, Komack and Leaf. He found three binding sites: site A (KD = 1.9×10^{-11}, n = 9×10^{-11}moles/Kg tissue), site B (KD = 4.8×10^{-7}, n = 4×10^{-9}moles/Kg tissue) and site C (KD = 10^{-4}; n = 10^{-4}moles/Kg tissue). Site A is similar to Sharp, Komack and Leaf's low affinity site. Site B was presumed to be a plasma cortisol binding

globulin and site C a non-specific binding site.

Ausiello and Sharp (6) found specific mineralocorticoid binding sites in nuclei of epithelial cells of the toad bladder using two detergents and osmotic shock at 0-4 °C to disrupt the nuclei. They found 60% saturation of the binding sites (which numbered 3×10^{-12}/g tissue or 12.8×10^{-14}/100mg DNA) at aldosterone concentrations of 10^{-7}M.

Alberti and Sharp (7) isolated from toad bladder nuclei three macromolecular complexes which bound aldosterone. These macromolecules were identified as protein because pronase, but not DNase or RNase, dissociated bound H^3-aldosterone. One complex dissociated rapidly and had the same mineralocorticoid specificity and the same number of binding sites (3×10^{-12}/g tissue) as the large pool described by Sharp, Komack and Leaf (4). Binding became irreversible during isolation of the nuclear complex. The two other aldosterone-macromolecular complexes were stable, unsaturable, and non-specific. Alberti and Sharp failed to isolate the mineralocorticoid specific macromolecular complex on a Sepharose 4B column. Sulfhydryl protecting agents did not stabilize aldosterone binding to the macro-molecular complex. Glycerol, which stabilized aldosterone binding in rat kidney and estrogen binding in rat uterus did not stabilize in this system. Cytosol binding was not seen when extractions were carried out in cold Tris buffer or 0.4 M KCl (no radioactivity was found in the supernatant of nuclear suspensions). Cytosol binding greater than nuclear binding was found when epithelial homogenates were purified by differential sucrose centrifugation in the cold (0.-4°C). However, the authors considered this cytosol binding to be "leakage" of nuclear binding complexes into the cytoplasm on homogenization.

Alberti and Sharp (8) later demonstrated a separation of the binding affinity and hormone potency which they described as due to "multiple points of attachment" of steroid to receptor. They define four types of steroids by their interaction with mineralocorticoid receptors in the toad bladder:

1) steroids which bind to receptors and are capable of producing the full biological effect (e.g., aldosterone, DOC, DOCA, 9α-fluocortisol, cortisol)

2) steroids which do not bind and therefore give no effect and do not inhibit aldosterone (e.g., 17-isoaldosterone, cholesterol, dihydrotestosterone, 17β-estradiol)

3) steroids which are not agonists but which competitively inhibit aldosterone (e.g., progesterone, cortisone, spirolactone)

4) steroids which do bind and give a partial response (partial agonist) and can inhibit aldosterone (e.g., cortexolone).

Alberti and Sharp (9) compared the amounts of specific mineralocorticoid binding in the nuclei of toad bladder epithelial cells to the specific binding in the colon of the toad, rat, and man, all of which respond to aldosterone by increases in their short circuit current (SCC). The toad bladder had three to ten times as much aldosterone binding capacity as colonic epithelium. The order of binding capacity of epithelial nuclei in the colon was: man toad rat. The aldosterone binding of toad bladder was easily reversible with half dissociating in two hours. Most of the total binding was mineralocorticoid specific in the toad and human colon but not in the rat colon.

Sapirstein and Scott (99) studied H^3-aldosterone binding in toad bladder epithelial cells. H^3-aldosterone or H^3-aldosterone plus 1000-

fold excess unlabelled aldosterone were added to paired hemibladders and incubated 90 minutes. Epithelial cells were removed by placing the bladders in Ca++-free amphibian ringers containing 2mM EDTA for 45 minutes. The disaggregated mucosal cells were collected and the two major cell types, the mitochondria-rich (MR) and the granular (G) cells, were separated by centrifugation on gradients of Ficoll. Specific H^3-aldosterone binding was limited to the MR cell. DOCA, a potent mineralocorticoid in the toad bladder, completely displaced H^3-aldosterone binding in MR cells while cortisol, a weak mineralocorticoid, displaced H^3-aldosterone only by 24%. Corticosterone, the natural glucocorticoid of amphibians, and/or H^3-corticosterone were given to paired hemibladders as in the aldosterone binding study. Specific H^3-corticosterone binding was localized in the G cells. After 90 minutes incubation with 10^{-5} M aldosterone and/or 10^{-8} M H^3-aldosterone mucosal cells were removed, separated and homogenized. The homogenates were fractionated by centrifugation to a nuclear pellet, a post-nuclear pellet, and a supernatant fraction. More H^3-aldosterone binding was seen in the nuclei prepared from tissues exposed to H^3-aldosterone than in nuclei from tissues exposed to both H^3- and cold aldosterone, thus demonstrating the specificity of the binding site in the nucleus.

In summary, while no specific cytosol binding protein has been isolated, evidence for four binding sites in the toad bladder nuclei has been demonstrated. Two sites show show saturability, high affinity, and specificity for mineralocorticoids, but one is saturated below the minimum effective dose of aldosterone, and the other dissociates from H^3-aldosterone so quickly that it cannot be isolated in agarose chromatography. The sedimentation coefficients of the nuclear mineralocor-

ticoid binding sites have not been determined. Two other sites have been demonstrated using agarose chromatography, but have not been demonstrated to have any requirements of a mineralocorticoid receptor.

V. RNA and Protein Synthesis in the Toad Bladder

In 1963, Edelman, Bogoroch, and Porter found that puromycin (10), an inhibitor of translation, and actinomycin D (11), an inhibitor of transcription, each blocked the effects of aldosterone upon sodium transport by the toad bladder. These agents had no effect on the response of the toad bladder to ADH. Edelman's group also emonstrated, by autoradiography, that H^3-aldosterone was preferentially localized in the nuclei of toad bladder epithelial cells, while H^3-progesterone was evenly distributed between cytoplasm and nuclei. Aldosterone (7×10^{-7}M) was added to the baths and the SCCs were measured. At the end of the latent period, some of the bladders were washed several times. No reduction in the SCC was obtained by rinsing the bladders in aldosterone-free medium at the end of the latent period. This observation indicated that after aldosterone had initiated a certain sequence of biochemical events, its presence in the solution bathing the tissue was no longer necessary to obtain the physiologic response. It was found, however, that these "washed" bladders still contained 10% of the dose of aldosterone. Aldosterone binding thus is not freely reversible since a significant amount of aldosterone remained in the tissue after the washing.

In 1964, Edelman, Bogoroch and Porter (11) compared the effects of aldosterone and progesterone at 7×10^{-7}M on the SCC and on the incorporation of H^3-uridine into RNA in the toad bladder. Aldosterone increased the amount of label incorporated into RNA before and during the period of increased SCC. Progesterone had no effect on the SCC

but did increase RNA synthesis after 3 hours. This was not considered a mineralocorticoid effect since progesterone does not competitively inhibit aldosterone nor increase Na^+ transport in toad bladders. The effect of progesterone on RNA synthesis clouds the aldosterone results by implying that the increase in RNA synthesis could have been a non-specific effect. Also, they again used 70 times the maximum effective dose of aldosterone in the toad bladder (12) and aldosterone does have a glucocorticoid action at high dose.

Porter, Bogoroch and Edelman (13) further studied H^3-uridine incorporation into toad bladder epithelium RNA, again using 7×10^{-7}M aldosterone. H^3-uridine and either aldosterone or progesterone were added to the bathing medium. One, two and four hours after the addition of steroid, the epithelial cells were scraped from the bladder and the radioactivity measured. Significant increases in labeling of RNA were seen in the aldosterone-treated tissue at two and four hours but not one hour following the addition of steroid (within the latent period). When H^3-uridine was added only in the last 30 minutes of incubation with steroid, significant increases in H^3-RNA could be demonstrated 1.5, 3, and 6 hours after aldosterone. Autoradiography of toad bladders given H^3-aldosterone was used in an attempt to localize the site of the aldosterone-dependent RNA synthesis. More label was seen in the nuclei of aldosterone-treated bladder epithelial cells than in control tissue after 30 minutes. Progesterone had no effect on the SCC in this study nor did it compete with aldosterone's effect on the SCC when each were present at 7×10^{-7}M. Progesterone did however, increase the H^3-uridine incorporation into RNA after three

hours of incubation.

Fanestil and Edelman (14) studied the effect of inhibition of RNA and protein synthesis on aldosterone-stimulated sodium transport in the toad bladder. Actinomycin D (5 µg/ml), puromycin (10 µg/ml) and cycloheximide (50 µg/ml) did not affect baseline SCC but did depress the aldosterone-stimulated SCC. At higher doses these drugs blocked the aldosterone-stimulated sodium transport completely, but also depressed the baseline SCC. Several other inhibitors of proteins synthesis were used in an effort to block the aldosterone-stimulated SCC. Both fluorophenylalanine and l-canavarine abolished the aldosterone-induced sodium transport, but also caused an accelerated decay in the baseline SCC. Phenylalanine overcame the inhibition of fluorophenylalanine. The aminonucleotide of puromycin, 6-azauracil, methyl-serine, and βthienyl-serine did not affect either baseline or aldosterone-induced SCC.

Rousseau and Crabbe´ (15) incubated toad hemi-bladders in H^3-uridine for 150 minutes. At time zero, aldosterone (5×10^{-6}M) was added to half the hemi-bladders and after 135 minutes, unlabeled uridine was added. The tissue was quick-frozen in liquid N_2 and homogenized. The nucleic acids were extracted and separated either by chromatography on columns of MAK-methylated bovine serum albumin followed by sucrose density centrifugation, or by sucrose density centrifugation alone. Most of the rapidly labeled RNA was in the region of heterodisperse nuclear RNA (HnRNA). Aldosterone caused an increase in the incorporation of uridine into the rapidly-labeled RNA pool. The

interpretation of the results is difficult because of the high concentration of aldosterone used in these experiments.

De Weer and Crabbe´ (16) confirmed the inhibition by actinomycin D and puromycin of aldosterone's effect on the toad bladder. Using the very high dose of 10^{-5}M aldosterone, they reported increased H^3-uridine incorporation into RNA whether or not sodium was present in the mucosal medium. Because removal of sodium from the mucosal medium abolishes the SCC, their data indicates that the effect of aldosterone on RNA synthesis is independent of sodium transport. The effect on RNA synthesis may not have been mineralocorticoid-specific because aldosterone is known to have glucocorticoid effects at these concentrations. The experimental design also lacked an attempt to block the RNA synthesis with spironolactone and a control for the glucocorticoid effects of aldosterone. They estimated the half life of the intermediates of aldosterone's effect by adding, at the beginning of the aldosterone-stimulated SCC, puromycin or actinomycin D. By timing the course of the inhibition, they calculated the half life of the actinomycin-blocked intermediates was 150 minutes and 90 minutes for those blocked by puromycin.

Lehav, Herman and Edelman (17) administered cycloheximide to aldosterone-treated bladders for the first 90 minutes of the latent period, then washed it out. Thirty minutes later, they observed the expected increase in SCC, but the slope of the aldosterone-induced SCC appeared greater than normal. From these data, they concluded that:

1) aldosterone-induced mRNA accumulated during cyclohexamide inhibition; 2) only 30 minutes of the latent period can be assigned to translation of the aldosterone-induced RNA; 3) aldosterone acts directly only at the transcriptional level; and 4) the steroid does not have a stabilizing effect on any aldosterone-induced protein(s).

Lehav and Dietz (18) used a double label technique to study the effect of aldosterone (10^{-8}M) on the incorporation of leucine into problems of the toad bladder. The tissue was given a one-hour pulse of H^3-leucine, then aldosterone, and 4 hours later, a one hour pulse of C^{14}-leucine. After 4 to 5 hours, aldosterone increased the C^{14}/H^3 ratio by 24% from control values. No effect could be demonstrated during the latent period. The effect after 4 hours was independent of Na^+ in mucosal medium and was abolished by the presence of spirolactone. Administration of 17β-estradiol resulted in a modest decrease in C^{14}-leucine incorporation. There was no control for possible changes in the size of the intracellular pool of leucine.

Hutchinson and Porter (19-22) studied the effect of 7×10^{-7}M aldosterone on paired toad hemibladders, one hemibladder receiving only H^3-uridine and the other, aldosterone plus H^3-uridine. Two hours after the aldosterone was added, the cells were harvested, homogenized and centrifuged. Aldosterone increased the labelled RNA in the purified nuclear and post-mitochondrial fractions 10 ± 5% and 14 ± 6% respectively. No indication of statistical significance was mentioned. Spirolactone (500:1) was given to one hemibladder; H^3-uridine and aldosterone were added to both hemibladders one half hour later, and 3 hours after that the cells were harvested. Labelled RNA was 20 ± 5% less in the hemibladder given spirolactone, demonstrating that the effect of aldosterone on RNA was mineralocorticoid specific.

Sharp and Komack (23) used H^3-uridine pulse labelling in the presence of 5 x 10^{-7}M and 10^{-5}M aldosterone in the toad bladder and failed to demonstrate any increase in H^3-RNA over control until after 4 hours of incubation at the higher concentration of steroid.

Vančura, Sharp, and Malt (24) studied the incorporation of H^3-uridine into toad bladders *in vitro* and *in vivo*. RNA synthesis was found to be quite slow compared to mammalian tissues. Incorporation of H^3-uridine into HnRNA and 7S RNA was seen in 30 minutes, into 40S and 4S RNAs in 60 minutes, but not into mature 18S and 28S rRNA until 8 hours after H^3-uridine was added. Aldosterone (5 x 10^7M) did not produce any evident changes in H^3-uridine incorporation in any fraction of RNA examined. They criticised previous studies dealing with aldosterone's effect on RNA synthesis in the toad bladder for using supramaximal doses (greater than 10^{-7}M) of aldosterone, and for not establishing that an increase uptake of label into RNA was effect on synthesis.

Hutchinson and Porter (25) in 1972, further examined H^3-uridine incorporation into RNA in the toad bladder. Studies of temperature dependence showed that maximum incorporation occurred in this tissue at 24°C. Aldosterone (7 x 10^{-7}M) did not affect RNA/DNA weight, nor did it affect $HClO_4$-insoluble RNA specific activity after 1.5,3, or 20 hours of incubation with H^3-uridine. Aldosterone did increase the specific activity of the "$HClO_4$-soluble RNA" by 74% after 3 hours and by 200% after 20 hours. No effect was seen on whole cell RNA at any time. Aldosterone caused an increased incorporation of low specific activity H^3-uridine into RNA in their purified nuclear fraction after 1.5 hours, but not in any other fraction at any time. When high specific activity H^3-uridine was used, no effect was found, even in the

1.5 hour purified nuclear fraction. These authors (26) had previously shown that overnight incubations in Kanamycin caused a 50% decrease in H^3-uridine incorporation into RNA. They were able, in similarly treated bladders, to demonstrate a mild simulation of SCC induced by aldosterone. The fact that the authors used overnight incubations in Kanamycin prior to aldosterone and H^3-uridine treatment probably explains the poor results of this (39) paper.

Rousseau and Crabbe´ (27), using 5×10^{-6}M aldosterone, found that this supramaximal dose increased total RNA synthesis at 1 and 3 hours after aldosterone (but not at 2 hours). Ten of 30 experiments, in which no change in RNA synthesis was seen, were discarded. Aldosterone had no effect, even at this dose, on the relative sucrose gradient profiles of phenol-extracted whole cell RNA. Polysomal RNA synthesis did not respond to 5×10^{-6}M aldosterone at 150 minutes. No effect on monosome/polysome ratio or the uptake of labelled amino acids into the ribosomes was seen from incubations in this large dose of aldosterone.

In two publications (28,29), Greengard and co-workers established that a 49,000 dalton membrane-bound protein of toad bladder epithelial cell was de-phosphorylated at an increased rate upon the addition of monobutyryl cyclic AMP, vasopression (ADH), theophylline, adenine, prostaglandin E, and $MnCl_2$. The dephosphorylation of protein D preceded the changes in potential difference across the tissue. No consistent relationship between the level of phosphorylation of protein D and osmotic water permeability was seen. Thus, protein D was implicated in the effect of ADH on sodium flux. Liu

and Greengard (30) found that preincubation of toad bladders with 10^{-6}M aldosterone or 9α-fluorocortisol for 10 hours increased the dephosphorylation of protein D. This effect was a result of an increase in protein D phosphatase activity and the effects of aldosterone, but not that of cAMP, was prevented by actinomycin D (10 µg/ml), cycloheximide (2 µg/ml), puromycin (10 µg/ml), and spironolactone (0.5 mM). Preincubations with androgens produced no effect. A dose response curve indicates that aldosterone is effective from 10^{-9} to 10^{-7}M, the latter also producing a maximal effect. The effects of cAMP and a 10 hour preincubation in 10^{-6}M aldosterone were additive on the dephosphorylation of protein D. This is especially interesting because aldosterone increases the responsiveness of the toad bladder to ADH. Liu and Greengard also noted a 37,000 dalton protein in the cell sap whose de-phosphorylation was affected by both aldosterone and cAMP. The significance of this dephosphorylation was not understood.

Scott and Sapirstein (102), using a double label technique with either H^3- or S^{35}-methionine found on separated epithelial cells of the toad bladder that only MR cells synthesized proteins in response to aldosterone stimulation and that only G cells sythesized protein(s) in response to corticosterone stimulation. The aldosterone-induced proteins obtained from the supernatant fraction of sonicated MR cells had an isoelectric point of 4.5 and molecular weights ranging from 16,000 to 38,000 daltons,

Rossier, Wilce, and Edelman (31) measured SCC in one hemibladder and the incorporation of H^3-uridine and methyl[C^{14}]methionine into RNA in the paried hemibladder in response to 30, 60, 120 or 180

minute incubations with 7×10^{-8}M aldosterone. They found an increased incorporation of H^3-uridine into 9-18 S nonmethylated RNA. Cytoplasmic RNA was chromatographed on an oligo-dT cellulose column, which binds poly(A)-containing RNA, to reveal a heterogeneous sedimentation pattern from 6S to 35S with a plateau at 13-26S. This result was not attributed by the authors to the presence of aldosterone. The acid-soluble pool of H^3-uridine was found to be only slightly increased by the addition of aldosterone and the TNase activity was unchanged. Spironolactone (SC-9420) reduced the effects of aldosterone on SCC and on the incorporation of H^3-uridine into RNA. Cortisol and 17α-isoaldosterone had no

effect on either transport or labeling of RNA. Pulse labeling with H^3-uridine 150 minutes after the administration of hormone resulted in lesser incorporation of H^3-uridine into 9S-12S RNA than a similar pulse during the first 30 minutes of exposure to the steroid. This study was the first to demonstrate that aldosteron's effects on RNA labeling are not due to an increase in the size of the H^3-uridine pool or to a decrease in RNA degradation but apparently reflect an increased synthesis.

Wilce, Rossier, and Edelman also studied (32) the effect of 70 μM aldosterone on the uptake of H^3-uridine into phenol-chloroform extracted, poly-A containing cytoplasmic RNA (mRNA) in toad bladder epithelial cells. Before chromatography on oligo(dT)-cellulose, they observed the aldosterone-induced peak of radioactivity at 9-12S as noted above. Sucrose gradient analysis of the poly(A)-containing fraction of RNA showed three peaks (at approximately 25S, 18S, 12S) following incubation in aldosterone for 30 minutes but only two peaks (approximately 9S and 4S) when the 30 minute incubation was followed by a 150 minute "chase". Labeling of RNA was greater after the first 30 minutes than after 150 minutes of exposure to the steroid. It was not determined whether the peaks of similar RNA's found after the longer labeling period were degradation products of the larger RNA's or if they were a different class of RNA synthesized at a later time. Yu and Feigelson (33) also found that cortisone causes an initial burst of synthesis of hepatic (A + U)-rich-RNA during the first hour of treatment which waned during the next 5 hours.

Edelman's group (34,35) further studied the actions of aldosterone on rRNA and Na^+ transport in mucosal cells of the toad bladder. They

reported that 7 X 10^{-8}M aldosterone promotes methylation of 18S, 28S and 40S nuclear rRNA and pre-rRNA within 90 minutes. After 240 minutes, aldosterone increases the methylation of 28S cytoplasmic rRNA, but no effect was detected in any fraction of total cytoplasmic RNA on a sucrose gradient profile after incubation for 30 or 90 minutes. Very little incorporation of H^3-methyl groups was seen in these cytoplasmic fractions, however, until 240 minutes. Aldosterone had little effect on the size of the acid-soluble H^3-methyl methionyl or C^{14}-uridyl fractions at any time tested. Examination of the published gradient profiles suggests that aldosterone increased the incorporation of H^3-methyl groups into both nuclear and cytoplasmic 4S RNA at 90 and 240 minutes. C^{14}-uridine incorporation into 4S, 18S, 28S cytoplasmic RNA is greater in tissues incubated in aldosterone for 4 hours. The translational activity of the ribosomes was tested in a cell-free protein synthesis system, to which equal amounts of ribosomes from aldosterone-treated (240 min.) or control toad bladder epithelial cells were added. With and without inhibitors of re-initiation, the aldosterone-treated ribosomes translated more protein than those of the control tissue. This study implies that aldosterone increases the pool of actively translating ribosomes.

Recently, Rossier, Wilce, and Edelman (37) reported that spironolactone (SC-9420) at 7 X 10^{-6}M had no effect on SCC during 180 minutes of incubation, nor did that dose interfere with H^3-uridine incorporation into cytoplasmic RNA except at the 4S (and maybe at the 18S) peaks. Higher concentrations (2.5 X 10^{-5}M) depressed the SCC by 28% compared to control values. This high concentration slightly depressed H^3-uridine incorporation (30 minutes pulse - 140 minutes chase) into

cytoplasmic RNA in all fractions compared to control bladders. The combination of SC-9420 plus aldosterone caused much less incorporation into all fractions of cytoplasmic RNA than did aldosterone alone. No control group was presented for this data. The fractional change in SCC after 180 minutes was correlated with the fractional change of the specific activity of the cytoplasmic 12S RNA (used here as an arbitrary mean RNA fraction) in paired hemibladders. A straight line was obtained SC-9420 (7×10^{-6}m) depressed by 20% the incorporation of H^3-uridine into cytoplasmic RNA compared to control or aldosterone-treated tissue. SC-9420 depressed slightly incorporation of H^3-uridine into all fractions of poly(A)-rich, cytoplasmic RNA compared to either control or aldosterone-treated mRNA sedimenting at 12S. These data are in accord with the observation of Sakauye and Feldman (51) that although some spirolactones are partial agonists, SC-9420 is an antagonist to mineral ocorticoids with no agonist activity on the transport properties of toad bladder.

Benjamin and Singer (38) reported that 10^{-6}M aldosterone (a dose high enough to give glucocorticoid effects) increased the incorporation of S^{35}-methionine incorporation into a 12,000 dalton protein isolated from epithelial cells of the toad's urinary bladder. Dexamethosone (10^{-6}M) did not induce the increased S^{35}-methionine incorporation into this protein; this observation is inconsistent with the well-established properties of the steroid as a potent mineralocorticoid in this tissue (53). Insulin (1 mU/ml) increased both the SCC and the incorporation of S^{35}-methionine into the "aldosterone-induced protein."

Sapirstein and Scott (39) examined the membrane fraction of MR or G cells for the presence of aldosterone-induced protein by using a

double-label technique. Aliquots of separated MR and G cells were incubated with ^{3}H- or with ^{35}S-methionine, with aldosterone added to one set. After the incubation, the sets of MR cells were mixed, sonicated and centrifuged. The pellets were extracted with chloroform-methanol (1:1) and washed with 2M KCl. The washed extract containing proteolipids was chromatographed on a column of Sephadex LH-20 equilibrated with chloroform-methanol-HCl (1:1:0.01). The sets of G cells were analyzed by the same procedures. Three distinct peaks of labeled proteolipid were eluted from the aldosterone-treated MR cells whereas the control tissue yielded only two peaks. The peak of aldosterone-induced proteolipid was not observed in G cell extracts nor was it induced in either MR or G cells by corticosterone.

Kirstein, Kirsten, Leaf, and Sharp (40) studied the effect of aldosterone on the activities of certain enzymes of the citric acid cycle in the toad bladder. Two hours after aldosterone (10^{-7}M), the activities of "condensing enzyme" (citrate synthetase), isocitrate dehydrogenase, glutamate-oxaloacetate transaminase and malate dehydrogenase increased. The increases in enzyme activities following aldosterone had approximately the same time course as the increases in the SCC. Citrate synthetase activity was particularly enhanced; giving a significant positive correlation with the stimulation of the SCC. The stimulation of the enzyme activity was independent of the presence of Na^{+} in the mucosal bath, and, therefore, independent of Na^{+} transport. Actinomycin D and puromycin inhibited both the increased SCC and the increased activity of these enzymes following aldosterone, although direct evidence that the increased enzyme activity was due to increased synthesis of enzymes was not obtained.

Fimognari, Porter, and Edelman (55) reported that in substrate-depleted bladders, aldosterone's effect on SCC was evident only after either glucose, pyruvate, β-hydroxy butyrate, acetoacetate or oxaloacetate was added to the bathing medium. Leached bladders which had been exposed to aldosterone for a period of time longer than the latent period, responded to the addition of substrate with an immediate increase in SCC which paralled the response usually caused by aldosterone, but without the latent period. Acetate, alpha-ketoglutarate, succinate, aspartate, and propionate were ineffective substrates. The authors concluded that the effects of aldosterone on Na^+ transport were mediated by an increase in the activity of the rate-limiting steps of the Krebs cycle between condensing enzyme and alpha-ketoglutarate dehydrogenase.

Kirchberger, Martin, Leaf, and Sharp (42) reported that aldosterone, in a dose-dependent fashion, decreased the activity of the pentose phosphate pathway (PPP) in the toad bladder, with a time course similar to that of aldosterone's effect on the SCC. The presence of sodium in the mucosal medium was not required for the effect of aldosterone as with the stimulation of the Krebs cycle. Actinomycin D blocked aldosterone's inhibition of the PPP. The effect was termed "mineralocorticoid-specific" since de-oxycorticosterone (DOC) and dexamethasone inhibited the PPP while progesterone had no effect. The response of the system to glucorocticoids was not reported.

Kirchberger, Chen and Sharp (42) examined whether the effects of aldosterone on the PPP (a decrease in activity) and on the Krebs cycle (an increase in activity) were either related or coincidental effects, and whether these were "glucocorticoid" effects. Spirolactone, a specific inhibitor of mineralocorticoid activity, had no effect on the

PPP, but inhibited the aldosterone-induced decrease in the PPP. Cortisone and cortexolone (10^{-7}M), both glucocorticoids with weak mineralocorticoid activity in the toad bladder, had no effect on either the PPP or Krebs cycle in the toad bladder. Cortisol, which has no mineralocorticoid effect on the toad bladder, also had no effect on glucose metabolism in this tissue.

Because cAMP had been reported to inhibit the PPP in adipose tissue as well as to increase the SCC in the toad bladder, Kirchberger, Witcum and Sharp (44) examined the effects of the nucleotide upon the PPP and Krebs cycles. They found that cAMP decreased the PPP and decreased the activity Krebs cycle in the toad bladder and that vasopressin, which stimulates the adenylate cyclase, had similar effects. There were no significant increases in the intracellular cAMP following aldosterone.

Snart (45) noted that an increased oxygen consumption was seen with concentrations of aldosterone lower than the minimally effective mineralocorticoid dose. He confirmed Sharp, Komack, and Leaf's observation (4) that there are binding sites in the toad bladder epithelial cell nucleus, one of which is saturated below the minimum effective dose of aldosterone. Snart speculated that this binding site is involved in aldosterone's effect of increasing oxygen consumption. The Na^+, K^+-ATPase specific activity of the toad bladder epithelial cells was not increased by aldosterone. Hill, Cortas and Walser (46,47) also failed to demonstrate a stimulation by aldosterone of Na^+ -K^+ATPase in the toad bladder. Aldosterone (10^{-6}M) stimulated the SCC, but neither the activity, the number of sites, nor the Kdiss of the Na^+, K^+-ATPase.

Stoff, Handler and Orloff (48) studied the permissive effect of aldosterone (2×10^{-7}M) on the stimulation by ADH and theophylline of the SCC. They, as Kirchberger, Witcum, and Sharp (44), observed no significant increase in cAMP following aldosterone. However, steroid-depleted bladders responded with minimal increases in the SCC response to exogenous cAMP, ADH, or theophylline compared to large and immediate increases in the SCC in aldosterone pre-treated bladders. Aldosterone exhibited its maximal permissive effect after 18-20 hr. Dexamethasone was as effective as aldosterone in permitting the maximal response to cAMP or ADH. Incubation in cycloheximide for 2.5 hrs. depressed the response of the tissue to ADH and the permissive effect of aldosterone for ADH. Although they were unable to directly measure an increase in the tissue levels of cAMP in response to aldosterone, the authors speculated that aldosterone inhibits phosphodiesterase.

Stoff, Handler, Preston and Orloff (49) tested that hypothesis. Toad bladders treated with aldosterone plus ADH had higher levels of cAMP than tissues treated with ADH alone. Phosphodiesterase activity was lower in the aldosterone-treated bladders than in steroid depleted bladders.

Voûte, Hanne, and Ammann (50) observed aldosterone-induced morphological changes in the mitochondria-rich epithelial cells in the frog skin and toad bladder. In the toad bladder, these cells comprise about 10% of the cell population and believed by some to be the site of aldosterone's action on the SCC.

In summary then, direct evidence for aldosterone's stimulation of mRNA and protein synthesis has been obtained. The exact role of the newly synthesized protein is unknown however. The cellular site of the mRNA has not been established.

VI. Insulin

The disorders of protein and amino acid metabolism are so prominent in diabetes mellitus that this disease has often been called "muscle wasting disease." Cahill, Awki and Marliss[51] report that insulin's effects on skeletal muscle include increased active transport of amino acids across the sarcolemma (52), increased protein anabolism, and decreased protein catabolism. The increased active transport of amino acids has been shown to be independent of the presence of glucose in the medium (53), and independent of protein and RNA synthesis (54).

The insulin-induced increases in sugar and amino acid transport in skeletal muscle are associated with an increase in resting potential (hyperpolarization) and an increase in K^+ uptake; the latter cannot account for the changes in potential (55-57). Insulin and lactate have been shown (58) to increase K^+ uptake and O_2 consumption in frog skeletal muscle. Kernan(59,60) showed that insulin increased Na^+ extrusion from muscle under conditions which indicated that insulin increased the amount of energy available to the "Na^+ pump". The pump in skeletal muscle may be electrogenic, and its stimulation by insulin would explain the increased resting membrane potential. Creese (61) demonstrated that insulin (20 mU/ml) increased the rate constant for Na^+ efflux from rat diaphragm by 50%, indicating increased Na^+ active transport.

Wool's laboratory has reported (62) that insulin restores muscle protein synthesis to normal in diabetic rats after five minutes, pre- presumably by a direct action on the ribosome. Identical numbers of skeletal muscle ribosoms from normal, from insulin-treated diabetic and from non-treated diabetic animals were tested for relative rates of protein synthesis. The rate of C^{14}-amino acid incorporation into protein using

muscle ribosomes from diabetic animals was only 1/3 of the rate observed with ribosomes from normal and from insulin-treated diabetic rats. The rates of synthesis of all muscle proteins were reduced to the same extent. Ribosomes from diabetic animals were less effective at all Mg^{++} concentrations tested in translating exogenous mRNA. Experiments in which 40S and 60S ribosome submits from diabetic and normal animals were dissociated, separated, and recombined revealed that the 60S particle was responsible for the insulin effect and that the peptidyl transferase of the 60S particle was not involved. Diabetic ribosomes were found to bind less peptidyl - tRNA and could not use amino-acyltransferase I as effectively. There were no differences apparent in the electrophoretic analysis of the ribosomal proteins.

Manchester (63) isolated ribosomes from muscle cells incubated in vitro with and without insulin. Insulin reduced the extent of polysome disaggregation during incubation and enhanced the initiation of nascent pepticle chains. The fact that incubation of the tissue without insulin did not lessen the response of ribosomes to poly-(U) suggests that lack of insulin does not directly affect the functional capacity of the ribosomes and that the initiation defect is due to some other factor. Incubation of muscle cells with amino acids at 5 times normal plasma concentration enhanced the synthetic capacity of the isolated ribosomes and diminished the rate of disaggregation of polysomes. Denervation uniquely causes a transient hypertrophy of diaphragm skeletal muscle along with a stimulation of the excretion of Na^{+} at the sarcolemma (61). The hypertrophy is characterized by increased uptake of amino acids, increased protein synthesis and an increase number (by 80%) of ribosomes. Denervated diaphragm muscle has a diminished responsiveness of insulin.

Baseman, Paolini and Amos (68) reported that insulin increased H^3-uridine incorporation into 45S and 32S pre-ribosomal RNA, into 28S, 18S, 4S ribosomal RNA and into 5S tRNA in cultured chick fibroblasts. Insulin's effect was evident within two hours. Insulin also increased the incorporation of labelled amino acids into protein after two hours and labelled thymidine into DNA after twnety hours in this tissue.

The effects of insulin in the isolated mucosa of the small intestine were studied by Fromm, Field and Sanders (69). Absorption of Na^+, Cl^- and 3-0 -methyl glucose were increased while the SCC decreased 20 minutes after the addition of insulin. The decrease in SCC was greater than could be accounted for by Na^+ or Cl^- movements, implicating at least one other ion in insulin's effect. The transcellular transport of alanine was not apparently affected in this short time period although intracellular alamine concentrations and protein synthesis rates were measured. The effects on SCC in this tissue may be associated with increases in cGMP. Brasitus, Field and Kimberg (70) found that stimulation of α-adrenergic and muscarinic cholinergic receptors in rabbit ileal mucosa in vitro produced 5 to 15-fold increases in cGMP which were maximal in 2 minutes and dissipated after 30 minutes, and that cholecystokinin octapeptide and insulin produced similar increases in cGMP. All of these agents have been shown to decrease SCC, electrical resistance, and HCO_3^- secretion,

while increasing active absorption of NA^+ and Cl^-. These effects are opposite to the effects of addition of cAMP. The intestine is the only tissue in which cGMP has been directly implicated as a second messinger to insulin. In the isolated perfused liver, insulin has been shown to increase by greater than 2-fold the cellular uptake of non-metabolizable amino acids after a two hour incubation (71, 72).

The effect in liver was much greater than the 50-60% increases in uptake which insulin induced in heart muscle and diaphragm (71). The effect of insulin in liver seemed to be of a different mechanism than the increase in AIB uptake seen in liver treated with hydrocortisone since phenoxybenzamine, an α-adrenergic blocker, inhibited the hydrocortisone response but not the insulin response. Actinomycin D, however, partially inhibited the insulin but not the hydrocortisone effect on AIB uptake. Further, the effects of the two hormones were additive.

The insulin-induced increase in the uptake of glucose and inhibition of release of free fatty acids in adipose tissue is well known. Beigelman and Hollander (73-75) have found that isolated fat cells also increase their resting membrane potentials on stimulation by insulin. Latarte and Renold (76) have reported that if Na^+ is replaced by choline, the insulin stimulated entry of glucose into isolated fat cells is decreased, thereby linking the ionic events with glucose transport.

Herrera (77), in 1965, was the first to report that insulin increased the short-circuit current and Na^{22} transport in the toad urinary bladder. He also showed that insulin had no apparent effect on sodium entry into the mucosal cells and did not affect water permeability. The time course of insulin's effect was intermediate between that of ADH and that of aldosterone. These data indicate a different mode of action for insulin and either aldosterone or ADH.

Crabbé presented more evidence for insulin and aldosterone having separate mechanisms of action upon sodium transport (78) Toad skin, which responds to aldosterone and insulin similarly to the bladder, was bathed in solutions of lowered sodium content while the SCC was measured. Insulin-stimulated skin was more sensitive than the control

to variations in sodium content; the aldosterone-treated tissue was less sensitive than the control to reductions in the external sodium concentration. Amiloride, which blocks Na^+ entry into mucosal cells through the apical membrane, had a greater effect upon the insulin-stimulated skin than the aldosterone-stimulated skin. Further, in aldosterone-treated, substrate-depleted bladders and skin, glucose addition results in a large stimulation of SCC, but glucose does not affect SCC in insulin-treated substrate depleted bladders. Aldosterone did not change the relationship of the sodium pool size to SCC, while insulin decreased the sodium pool relative to the increase in the SCC. These observations were interpreted as evidence that insulin works close to the sodium pump site whereas there is much evidence that aldosterone's major site of action is on the sodium permeability of the apical membrane (79).

Crabbé (80) subsequently observed that pre-incubation with either actinomycin D or puromycin did not inhibit the subsequent stimulation of SCC by insulin. Edelman had previously shown that these agents completely blocked aldosterone's effects upon sodium transport in the toad bladder (24). Crabbé also reported that insulin decreased the excretion of sodium, potassium and water in the isolated perfused dog kidney without altering plasma flow, GFR, plasma sodium or potassium concentration. The change in water excretion was attributed to an effect on the distal nephron.

Crabbé, et al (81), reported that the insulin stimulation of SCC in toad bladder and skin was much more sensitive to inhibition by ouabain than were ADH-or aldosterone-treated tissues. They also noted that the effect of aldosterone plus glucose was greater than that of aldosterone alone, but that the extra stimulation of glucose over aldosterone was abolished by ouabain. Previous studies had determined that the cellular

concentrationof ATP was unaltered by insulin or by aldosterone plus glucose.

Siegel and Civan (82) measured the driving force (E_{Na}) of the Na^+ pump using a technique developed in their laboratory. They report that aldosterone did not increase the driving force of the sodium pump, but that insulin significantly increased this value. These observations were interpreted as further evidence that aldosterone affects the passive mucosal entry of sodium into toad bladder epithelial cells, while insulin affects the pump directly.

In a recent series of studies, Weismann, Sinha and Klahr (83-85) studied the effects of insulin, ADH and cAMP on the toad bladder. Toads were starved three weeks prior to use to decrease endogenous levels of insulin. They found, in contrast to Herrera, that insulin was only effective from the serosal side and implied that the effect of mucosal insulin observed by Herrera was due to leakage of the ptptide through "edge damanged" in areas of the tissue in the Ussing chambers. They also observed that the time course of insulin's action was dose dependent : no effect was seen from 50mU/ml insulin; 100mU/ml (7×10^{-7}M) caused an increase in SCC which began in 5 minutes, peaked at 30 minute and fell back to baseline at 60 minutes; 250mU/ml caused a 30% increase in SCC in 60 minutes for at least 3 hours; 500mU/ml caused a peak increase in SCC of 55% greater than control at 60 minutes, and also remained above baseline SCC and greater than the 250mU/ml response for longer than 3 hours. Although these levels of insulin are high, most observers have reported that, for a given magnitude of effect, a greater concentration of hormone is required <u>in vitro</u> than <u>in vivo</u>. These authors also report a bladder exposed to 250 or 500 mU/ml of insulin is refractory to another

dose of insulin but is still sensitive to ADH. Substrate-depleted bladders respond with a 20% increase in SCC following insulin compared to 40% rise in fresh bladders. Pyruvate, a substance whose intra-cellular concentration is independent of insulin, restores the insulin response in substrate-depleted bladders to normal while glucose does not. This suggests that insulin's effect is not due to the secondary effect on glucose entry into the epithelial cells. Insulin and ADH add together cause much greater increase in SCC than the sum of either alone. Insulin plus cAMP were more effective together than the sum of either alone. Insulin added 25 minutes after ADH had no synergistic effect. Insulin did not change the intracellular levels of cAMP with, or without ADH when compared to the appropriate control. Finally, actinomycin D did not block the onset or the peak response of insulin on the total bladder, but it did abolish the sustained increase in SCC of the larger doses of insulin. The response to insulin appears to have two phases: 1) a membrane effect different from and synergistic to the effect of ADH; and 2) an effect requiring RNA synthesis and probably protein synthesis which sustains the elevated sodium transport.

Benjamin and Singer (37) demonstrated that insulin induced the synthesis of a protein in mucosal cells of the toads urinary bladder. The protein, which had an apparent molecular weight of 12,000 daltons, was also induced by 10^{-6}M aldosterone. Insulin was observed, however, to increase overall incorporation of S^{35}-methionine into total protein which which may mean that the induction of synthesis of the aldosterone-induced protein was part of a non-specific growth effect typical of the effects of insulin in other epithelial tissues.

Cox and Singer (86) reported that insulin was less effective in the

mucosal medium of the *in vitro* toad bladder than in the serosal medium and attributed any effect of mucosal insulin to a leakage across the membrane (edge damage). They obtained responses from doses of insulin as low as 100 μU/ml (7×10^{-10}M), and showed that the response was independent of the presence of glucose in the bathing medium. Both the aldosterone and insulin responses were dependent on serosal (but not mucosal) K^+ concentration over the range 2-7 mg K^+/L. Aldosterone stimulated SCC after a maximal insulin effect and *vice versa* indicating separate mechanisms of action. Cycloheximide, which inhibited 80-95% of protein synthesis, did not affect the SCC response to insulin for the period of time (120 minutes) that measurements were reported.

Finally, it has recently been shown by Goldfine, et al. (87) that I^{125}-insulin was bound to nuclei of human cultured lymphocytes. The binding was specific, temperature-sensitive and accounted for 15-20% of the I^{125}-insulin uptake of the entire cell. Purified nuclei from lymphocytes specifically bound 7% as much I^{125}-insulin as the whole cell. The binding was freely dissociable and the rate of dissociation was higher if the incubation was carried out at 5°C than at 37°C. Half-maximal inhibition of nuclear binding occurred at a hormone concentration 250 μU/ml (2×10^{-9}M). Autoradiography revealed an increase in nuclear and a decrease in cytosol grain counts with time (5 min. compared to 120 min.). Finally, specific total cellular uptake of labelled insulin was characterized as almost instantaneous, while specific nuclear binding was half maximal at 5 minutes and maximum at 90 minutes. The paper confirmed the results of Lee and Williams reported 20 years ago and of the more recent work of Argulla, who also noted I^{125}-insulin uptake into nuclear fractions.

Goldfine, et al. (88), working with rat liver cells, found immunological evidence that the specific binding sites for insulin in the plasma membrane are immunologically different from the specific binding sites in the nucleus and smooth endoplasmic reticulum. The antiserum used in these studies contained antibodies directed against many membrane proteins, so the "specificity" of their binding is in question.

In summation, insulin would seem to have two kinds of effects. The first type of effect is on the cell membrane, increasing active transport of both ions and nutrients. The second effect is similar to that of steroid hormones and thyroxine, which are specifically bound in the nucleus and cause translational and transcriptional events in the course of their action upon target cells.

Methods

I Labeling of Ribonucleic Acids Using Intact Bladders

Female toads (Bufo marinus) from the Dominican Republic were kept in 0.6% saline for 2-4 days before use. After double pithing, paired hemi-bladders were removed and placed in Amphibian Ringers (90mM NaCl, 3mM KCl, 25 mM $NaHCO_3$, 0.5mM $MgSO_4$, 0.5mM KH_2PO_4, 1mM $Cacl_2$, and 6mM glucose, pH 7.4). The paired hemi-bladders were mounted on Luer-locks, filled with 2ml Ringers solution and placed in one of three beakers containing 50 ml Ringers. After one hour for equilibration, 0.07 mCi/ml of H^3(5,6)-uridine was added to each beaker. Twenty minutes later, aldosterone ($7x10^{-8}$M), spironolactone ($1.4x10^{-5}$M) plus aldosterone ($7x10^{-8}$M), or diluent were added to the bladders. After 1 hour of incubation, the bath was changed to EDTA-Ringers (2mM EDTA replacing the $CaCL_2$) at 0-4° containing 2', 3'-CMP (2mM) as an inhibitor of intracellular RNases (172). 45 minutes later, epithelial cells were removed and the bladders rinsed in EGTA Ringers (100 µM EGTA replacing EDTA) containing 2', 3'-CMP (2mM). The cells were then layered over discontinuous Ficoll gradients (1.017, 1.035, and 1.067 g/cm^3) in EGTA Ringers and centrifuged at 25K for 45 minutes at 0-4°C in a Beckman SW 27 rotor. A fraction enriched in MR cells was recovered from the 1.017-1.035 interface and a fraction enriched in G cells was recovered from the 1.035-1.067 interface. The MR and G cells from the aldosterone and control treated bladders were each placed in separate tubes an and pelleted at 7000g for 10 minutes. The cells were rinsed in BOMB solution (0.25M NaCl, 0.01M Tris HCl, pH 7.4, 10^{-4}M uridine, 0.002M Mg Cl_2) and re-pelleted at 1000 g for 10 minutes.

Disruption of the cells was carried out in 2ml BOMB solution in a Parr Bomb by nitrogen cavitation after 15 minutes at 1350 psi. One ml of Add I (20 ml Bomb solution, 300 µl Triton X-100, 6mM 2', 3'-CMP) was added to each broken cell suspension and centrifuged at 12,000 g for 10 minutes. The pellet was dissolved in 2ml Beckman Tissue Solubilizer and an aliquot was taken for tritium counts. One ml of Add II (1mg/ml RNase-free protease, 40 mM EDTA, 200 mg% heparin, 1.25 M Na Cl, 0.01 Tris, pH 7.4) was added to the supernate which was incubated at 37° for 30 minutes. The supernatant fraction was then chromatographed on an affinity column (5x25) of oligo-dT-cellulose. The column was rinsed with 8 ml of High Salt buffer(0.5 M NaCl , 0.01 Tris, pH 7.4, 0.01 M EDTA, 50 mg% heparin) and collected together with the chromatographed 4 ml of supernatant. The poly (A)-rich cytoplasmic RNA (mRNA) was recovered by developing the column with 3 ml of Low Salt buffer (0.01 Tris, pH 7.4, 0.01 M EDTA, 50 mg of heparin). Aliquots were taken from both High and Low salt fractions for liquid scintillation counting. The tRNA and rRNA was precipitated from the High Salt fraction with 2 volumes of ethanol overnight at -17°C. The precipitate was recovered by centrifugation at 12,000g for 10 minutes in a Sorvall centrifuge. The RNA pellet was re-dissolved in 4ml buffer and aliquots were taken for H^3-RNA dtermination.

The mRNA fraction, immediately after chromatography, was heated to 60° for 20 minutes, and layered over 15 ml linear sucrose gradients (0-27%) and centrifuged for 16 hours on an SW 27 rotor at 27,000. The samples were fractionated and the sucrose concentrations was determined on a Bausch and Lomb Abbé refractometer. Aliquots were mixed with 0.5 ml of Beckman Tissue Solibilizer and counted in a Beckman LS-9000 liquid scintillation counter using a toluene-base cocktail.

II. Labeling Experiments Using Separated Mucosal Cells

Hemibladders from 12-30 female Bufo marinus toads of Dominican origin were incubated in EDTA-Ringers (34 mM NaCl, 17.5mM $NaHCO_3$, 4mM KCl, 0.8mM $MgCl_2$, 0.8mM KH_2PO_4, 2mM EDTA, 0.011M glucose) for 45 minutes. The mucosal cells were removed by gentle agitation and the bladders were rinsed with Ringers with 100mM EGTA replacing the EDTA. The cells were pelleted by centrifugation at 2,000g for 10 minutes, resuspended in EGTA-Ringers, and layered over discontinuous Ficoll gradients (1.017, 1.035 and 1.067 g/cm^3) in EGTA Ringers. The preparations were centrifuged at 27,000 rpm for 35 minutes in a Beckman SW27 rotor. The bands of material enriched in MR cells (density = $1.035 g/cm^3$) and G cells (density = $1.067 g/cm^3$) were collected and diluted with Ca^{++}-Ringers (0.002M $CaCl_2$ replacing the EGTA) and centrifuged at 7,000xg for about 10 minutes.

The MR cells and the G cells were each resuspended in approximately 2 ml of Ca^{++} Ringer containing 0.12 mCi ($9x10^{-7}M$) (3H-5,6)-uridine and divided into three aliquots. Each G cell preparation contained approximately 10^{10}cells and each MR preparation, 10^9. After twenty minutes of incubation at room temperature, aldosterone ($7x10^{-8}$), aldosterone ($7x10^{-8}M$) plus spironolactone ($1.4 \times 10^{-5}M$), or diluent was added to each each of the three sets of cells. After incubation for varying periods of time (20 to 240 minutes), the cells were pelleted at 2,000g for 3 minutes, and washed 3 times in BOMB solution (1.25M NaCl, 0.01M Tris HCl, pH7.4, $10^{-4}M$ Uridine, 0.002M Mg Cl_2) at 4^oC. The cells were suspended in 2ml of Bomb solution, equilibrated with nitrogen (1500 psi for 15 minutes) in a Parr Bomb (Parr Co., Moline,Ill.) at 4^oC, and disrupted by cavitation. On ml of ADD I (20 ml Bomb solution, 300 µl Triton X-100, 6mM 2', 3'-CMP) was added to each broken cell suspension

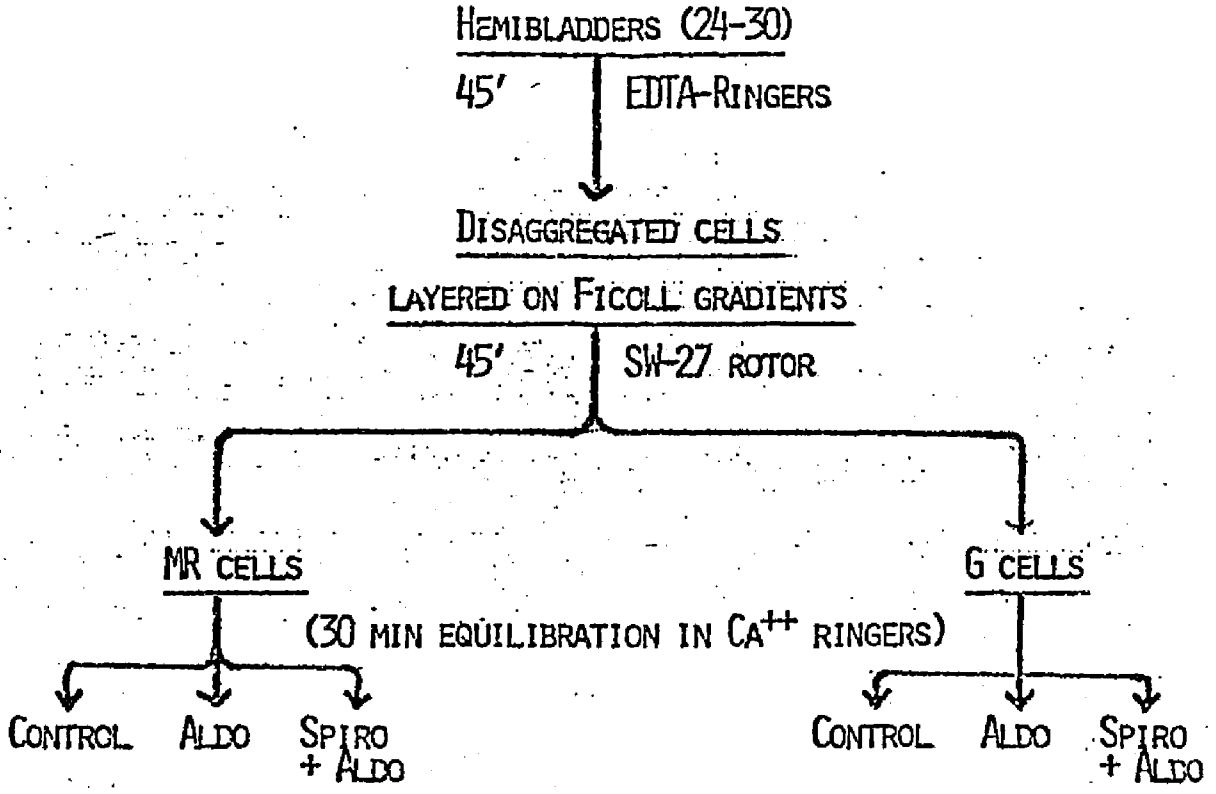
and centrifuged at 12,000g for 10 minutes. One ml of ADD II (1mg/ml RNase-free protease, 40mM EDTA, 200mg% Heparin, 1.25M N aCl, 0.01M Tris, pH 7.4) was then added to the supernate which was then incubated at 37°C for 30 minutes. The supernatant fraction was chromatographoed on a column (5x25mm) of either poly-(U)-Sepharose 4B or oligo-dT-cellulose and the column was rinsed with 8mL of High Salt buffer (0.5M NaCl, 0.01M Tris, pH 7.4, 0.01M EDTA, 50mg% heparin). Poly (A)-rich cytoplasmic RNA (mRNA) was recovered by developing poly-(U)-Sepharose 4B columns with 8ml of Low Salt buffer (0.01 M Tris, pH 9.0, 0.01 EDTA, 50 mg% heparin, containing 75% formamide) or oligo-dT-cellulose columns with 3 ml of the same buffer at pH 7.4 without formamide. The presence of formamide necessitated a buffer change which was accomplished by pervaporation of the sample for 5 hours against powdered Ficoll using 1/4" dialysis tubing. The concentrated mRNA fraction was dissolved in 3ml of Gradient Buffer (0.25M NaCl) 0.01M EDTA, 50% Heparin, 0.01M Tris, pH 7.4). The sample containing mRNA obtained from either oligo-dT-cellulose column or the poly-(U)-Sepharose column was layered over a linear 0-30% sucrose gradient and centrifuged at 25,000 rpm for 16-24 hours in a Beckman SW27.1 rotor. The samples were fractioned and the concentration of sucrose measured in each sample using a Bausch and Lomb Abbé refractometer. Samples taken from these fractions and from column eluates were mixed with 0.5 ml Beckman Tissue Solubilizer and counted in a Beckman LS-9000 liquid scintillation counter using a toluene-base fluid.

III. Double-label Experiments Using Separated Mucosal Cells

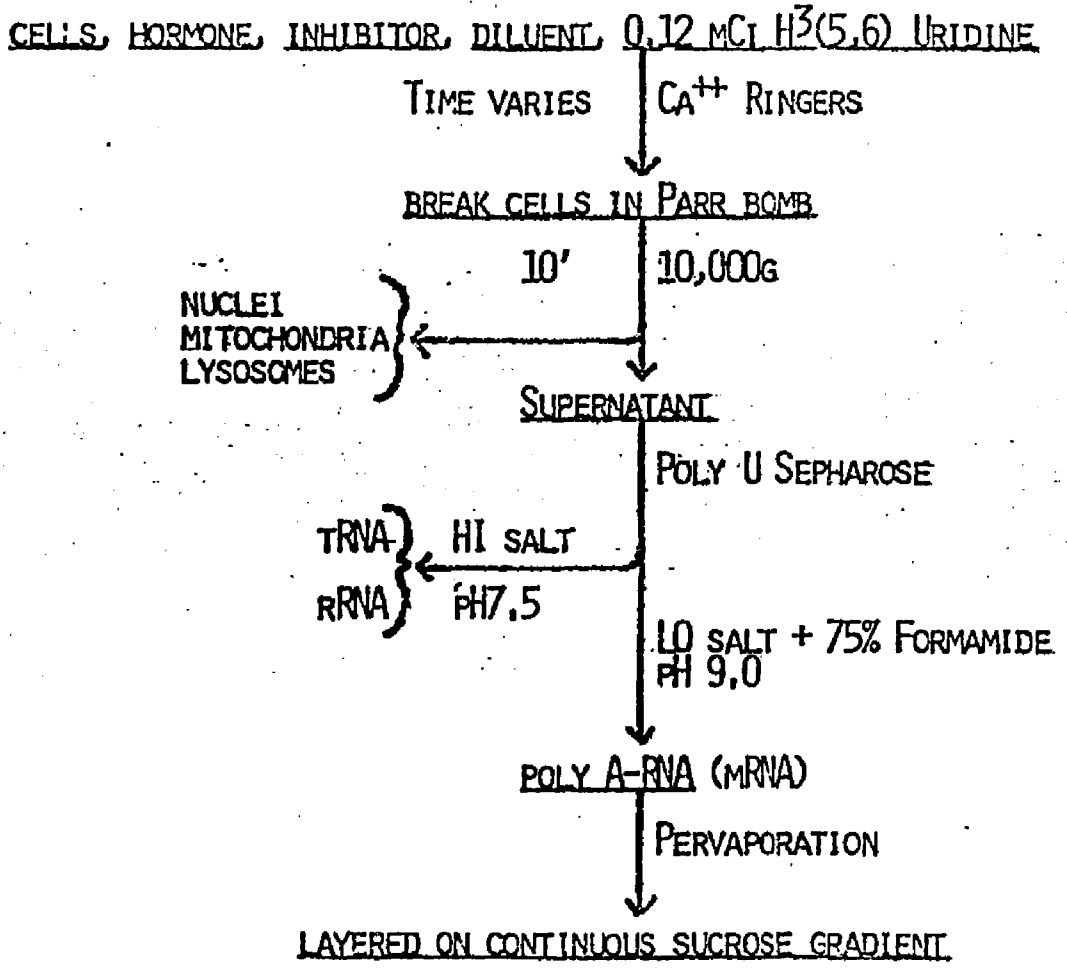
Cells were removed from hemibladders and separated using Ficoll gradients as described above. Twenty minutes before the addition of hormones, 0.06 mCi/ml of H^3(5,6)-uridine and 0.025 mCi/ml of a mixture of 14^C-labeled amino acids (NEC-445, New England Nuclear) neutralized with Tris base were added to each aliquat of epithelial cells. At time zero, hormone was added and the cells were incubated for varying lengths of time at 22°C. At the end of the incubation the cells were cooled to 0°C, pelleted, washed and disrupted by nitrogen cavitation as before. In these experiments, however, no protease was included in ADD II. The nuclei and mitochondria were pelleted at 12,000g and the supernatant was chromatographed on an oligo-dT-cellulose column. The initial eluate and 8ml of High Salt wash were combined. An aliquot was taken for $H^{-3}$and C^{14}-dpm determination in 14 ml 5/8" x 3" nitrocellulose tubes and then the sample was made 30mM Mg Cl_2 (20mM greater than the 10mM EGTA concentration). Ribosomes were pelleted at 38,000 rpm in a Beckman 40 rotor for 90 minutes. The supernatant fluid, containing tRNA, cytosol proteins, membranes and vesicles, was poured into a 15 ml Corex centrifuge tube. The tRNA and proteins were precipitated with 1/9 volume of 10% (W/V) TCA at 0-4°C for 4 hours. The supernate was discarded. This pellet and the ribosomal pellet were dissolved in 2ml Beckman Tissue Solubilizer (BTS) and aliquots were taken for H^3-rRNA and C^{14} protein determination.

Isolation of mRNA from Toad Bladder Mucosal Cells

I. Preparation of Cells

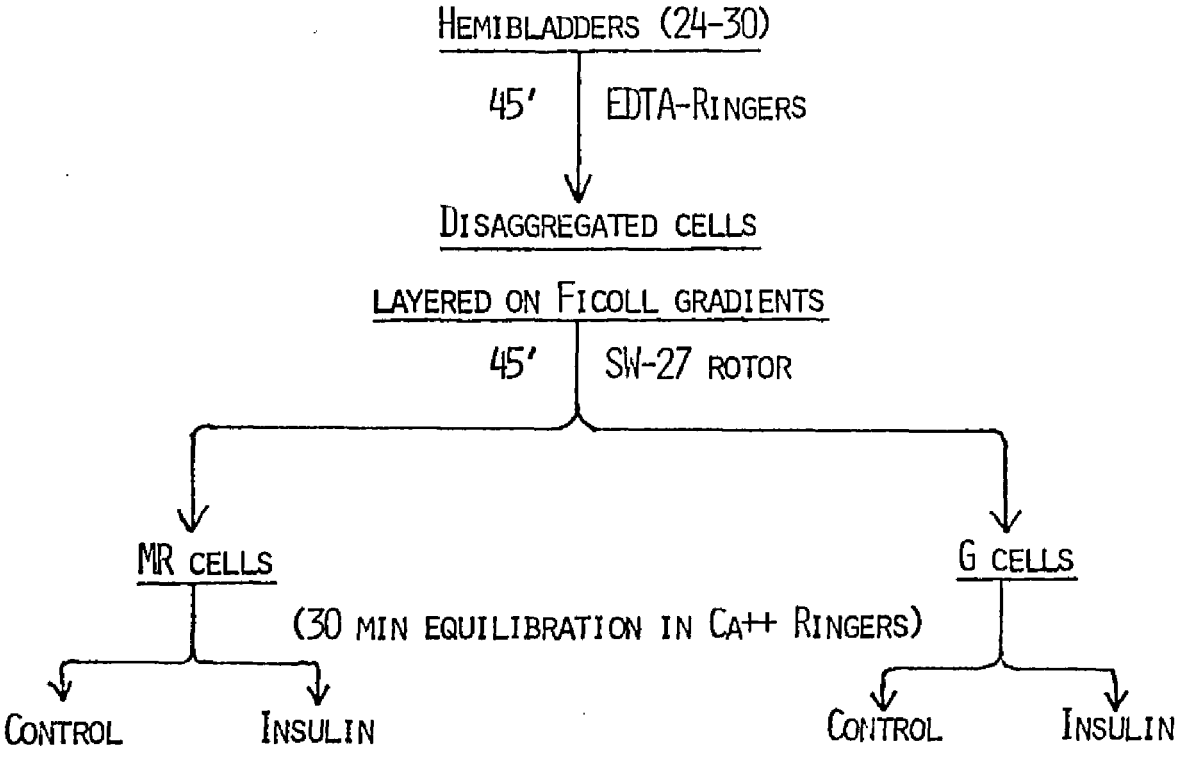


II Fractionation of RNA (procedure same for all 6 cell groups)

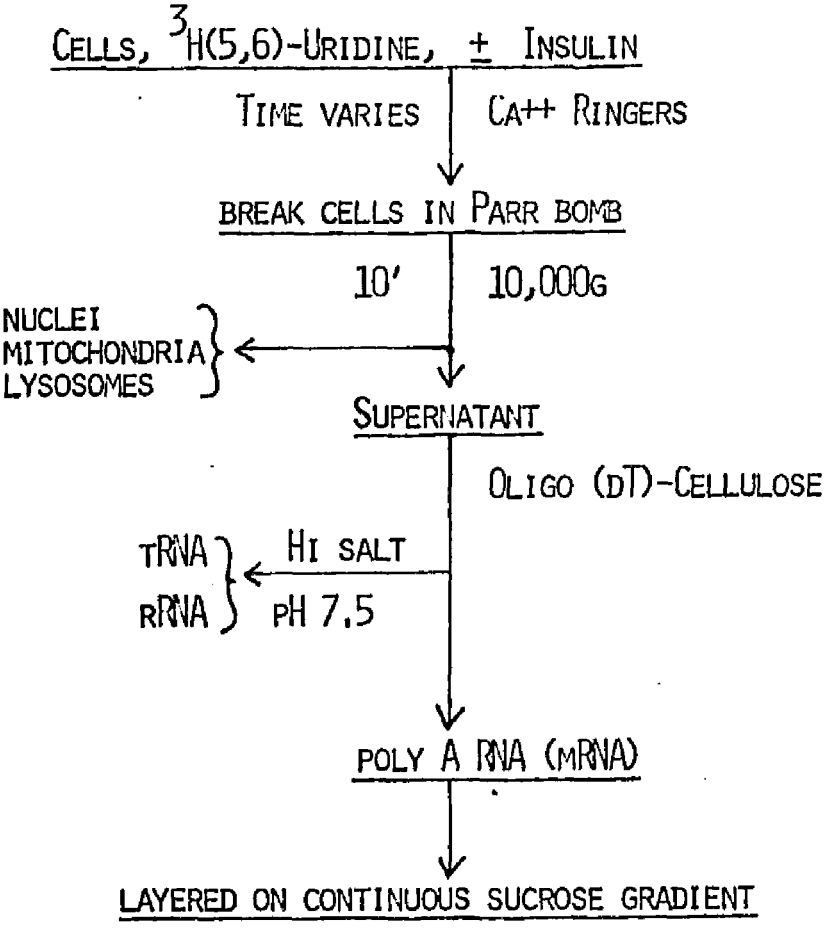


ISOLATION OF mRNA FROM TOAD BLADDER MUCOSAL CELLS

1. PREPARATION OF CELLS



2. FRACTIONATION OF RNA (PROCEDURE SAME FOR ALL CELL GROUPS)



IV. Short circuit current measurements

Paired hemibladders were prepared from toads that had been immersed in 0.6% NaCl for longer than two days. The tissues were mounted on glass tubing and suspended in (17-20 ml) Ca^{++} Ringers. The potential difference was measured using a Keithey 610BR electrometer with Beckman calomel electrodes connected by 1% agar-saturated KCl salt bridges in polyethylene tubing. Short-circuiting current was applied from a Heathkit high voltage power supply (model IP-17) through saturated KCl-agar bridges to the bladder. Readings were obtained intermittantly from up to nine pairs of hemibladders. The method is essentially that described by Bentley (128).

V. The Effects of Insulin Upon the Uptake of H^3-Uridine and C^{14}-Amino Acids Separated Mucosal Cells

24 ml (MR cells) or 48 ml (G cells) of Ca^{++}-Ringers containing cytidine, guanosine, adenosine, H^3-(5,6)-uridine (0.06 mCi/ml) and Cl4-amino acid mixture (0.01 mCi/ml) were added and the incubation continued for another 20 minutes at which each set of cells was divided into two equal fractions. Insulin (250 mU/ml or 100 mU/ml) was added to one of each pair. Aliquots (6 ml) of the cell suspension were removed at 30, 60, and 120 minutes after which 6 ml of fresh Ca Ringers plus nucleosides was added and the remaining cells were collected at 180 minutes.

After incubation at 22°C the cells were pelleted and washed twice in 5 ml Ca^{++}-Ringers and once in 5ml of Bomb solution. The cells were then suspended in 3 ml Bomb solution and disrupted at 1350 psi in the Parr bomb. Add II (1.5 ml) was added and the solution was vortexed. A 500 λ sample was taken for total protein determination (in triplicate) after precipitation and washing in 10% TCA, and redissolving the pellet in 1% NaOH overnight. The rest of the disrupted cell solution was centrifuged at 12,000g for 10 minutes and the supernate poured into a separate 15 ml Corex centrifuge tube. The cytosol fractions were made 10% in TCA. Fifty µl of the TCA supernate was taken in duplicate to get a rough measure of the size of the cytosol pools of H^3-uridine and C^{14} amino acids. The TCA pellets from the cytosol fractions and the nuclear pellets were each washed in 5 ml of 10% TCA and 5 ml of 100% ethanol and dissolved in 2 ml of Beckman Tissue Solubilizer. H^3 and C^{14} were each determined in a 500 µl aliquot using 10 ml of a toluene-base counting solution, with PPO and POPOP as fluors.

Results

I. Incorporation of Uridine into Ribonucleic Acids of Intact Bladders

In four separate experiments, the effects of aldosterone (7×10^{-8} M) upon the incorporation of H^3-(5,6)-uridine into the RNA of mucosal cells using intact hemibladders was determined. After one hour incorporation into cytoplasmic poly-adenylated RNA (mRNA) of MR cells was increased an average of 68% (Table 1). This effect of aldosterone was blocked by the simultaneous addition of SC-9420, a specific mineralocorticoid inhibitor. Aldosterone had no apparent effect on the labelling of this fraction of cytoplasmic RNA prepared from G cells.

The High Salt fraction includes ribosomal and transfer RNA and in addition, many H^3-uridyl compounds. The amount of labeled uridine this fraction prepared from MR cells was increased by aldosterone in 3 of the 4 experiments to an average 33% greater than the control tissues (Table 2). SC-9420 inhibited aldosterone's action in MR cells in each of these experiments. Aldosterone caused no change in the amount of labelled uridine in this High Salt fraction prepared from G cells.

The RNA (tRNA plus rRNA) was precipitated from the High Salt fraction and the radioactivity measured. Aldosterone caused an average 51% increase (Table 3) in incorporation of H^3(5,6)-uridine into this fraction in MR cells. The effect was blocked by SC-9420. Aldosterone did increase the incorporation of uridine into the tRNA plus rRNA of G cells.

The 12,000g pellet of these cells includes nuclei and mitochrondria, both of which contain RNA. Aldosterone caused an increased uptake of H^3(5,6)-uridine into all MR cells 12,000g pellets (average = +63%) and into 3 of 4 G cell pellets (average = + 69%, Table 4) SC-9420 seemed to inhibit H^3(5,6)-uridine incorporation in both MR and G cell nuclei.

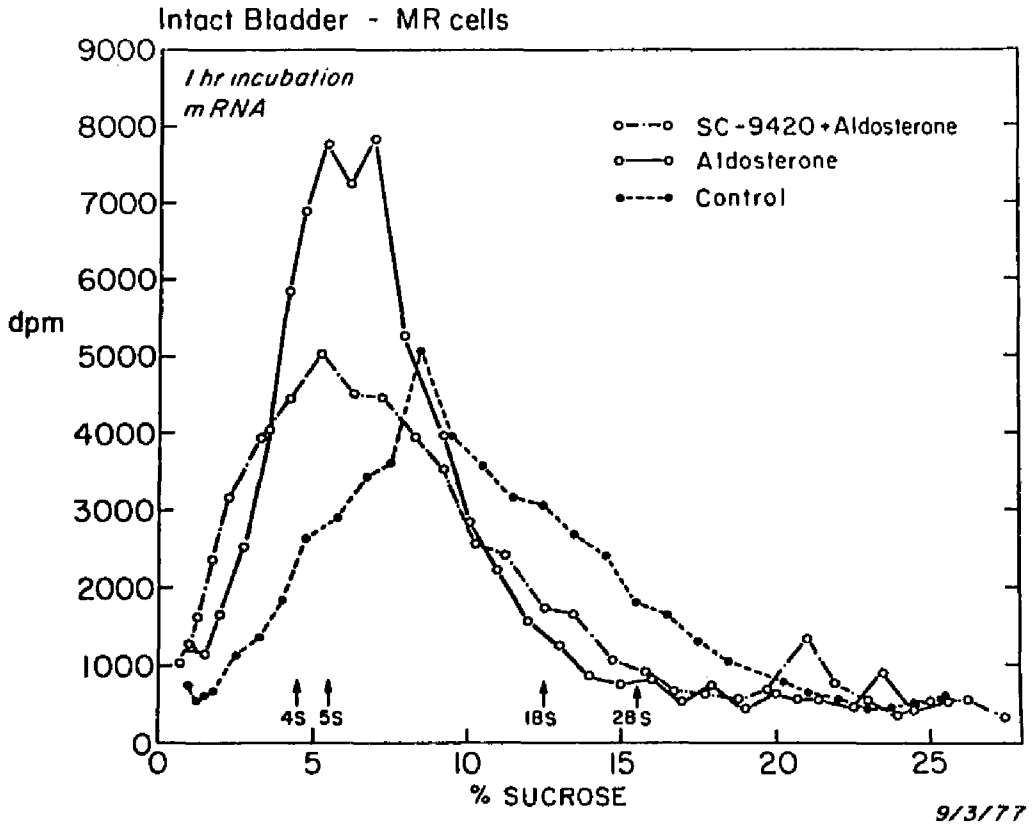


Fig. 1 Sucrose gradient profile of MR cell cytoplasmic poly-A containing RNA. In this experiment, SC-9420 (1.4×10^{-5}M) was added simultaneously with aldosterone (7×10^{-8}M) to one set of hemibladders, while aldosterone (7×10^{-8}M) or diluent were added to two other sets of equal numbers of bladders. The cytoplasmic, poly-A RNA was obtained the same way as in Figure 1.

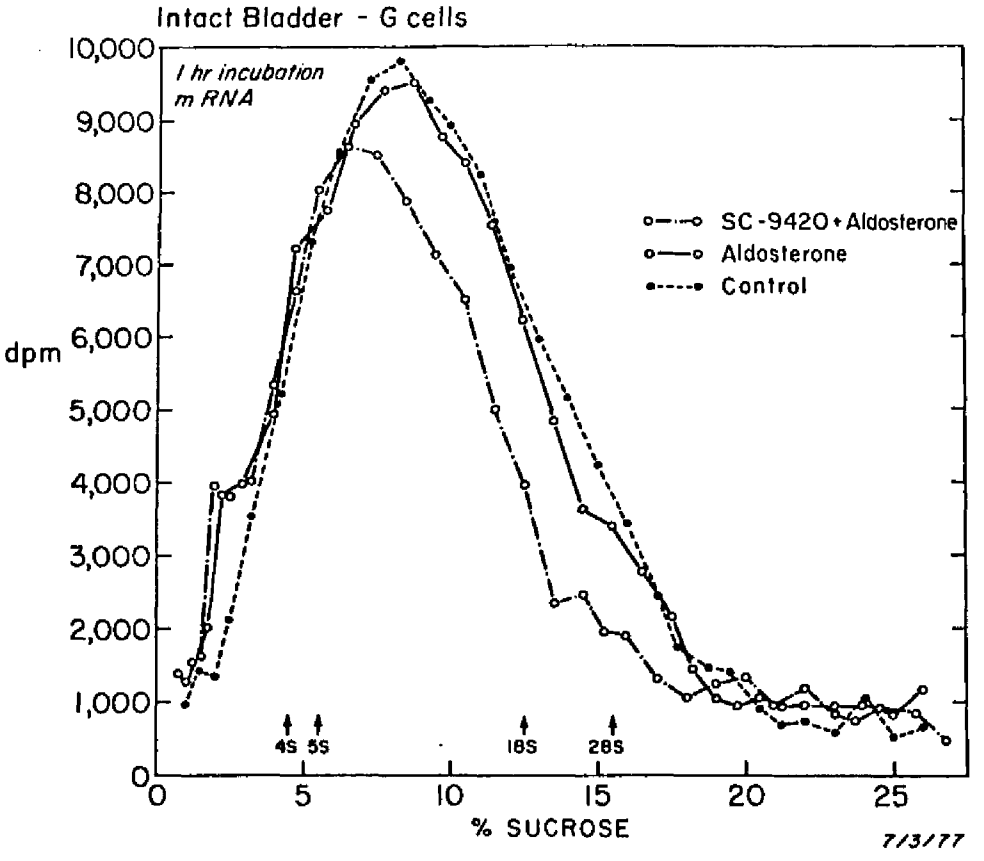


Fig. 2 Sucrose gradient profile of cytoplasmic poly A-containing RNA from G cells obtained the same way as Figure 2 and obtained the same day as the MR cell poly-A RNA in Figure 1.

Table 1 H^3 -poly A-RNA

	Control	Aldosterone		SC-9420 + Aldosterone	
	(dpm)	(dpm)	(%change)	(dpm)	(%change)
MR cells	6,525	9,375	+ 44%	--------	--------
	1,440	3,440	+139%	2,080	+ 42%
	25,335	35,442	+ 40%	7,099	- 72%
	42,320	54,240	+ 28%	44,160	+ 4%
		Mean =	+ 63%	Mean =	- 8%
G cells	42,755	41,850	- 2%	-------	-------
	4,000	4,240	+ 6%	2,320	- 42%
	137,524	124,320	- 10%	124,818	- 9%
	102,480	95,680	- 7%	72,320	- 29%
		Mean=	- 3%	Mean=	- 27%

Table 1 Cytoplasmic H^3 -poly A-RNA. After incubations of intact hemibladders with H^3-uridine and either $7x10^{-8}$M aldosterone, aldosterone plus spiromolactone (1:200), or diluent, cells were removed, separated, disrupted and cytosol fractions obtained. These cytosol fractions were chromatographed on oligo-(dt)-cellulose columns and the fraction containing cytoplasma poly A-rich RNA (in RNA) was obtained. An aliquot was taken for H^3dpm before layering over sucrose gradients. The results obtained from those aliquots in four different experiments are represented above.

Table 2 High Salt Wash

MR cells	1,255,800	1,227,240	- 2%	-----	-----
	552,480	610,560	+ 11%	587,520	+ 6%
	1,450,800	2,631,840	+ 81%	1,355,520	- 7%
	2,424,720	3,384,720	+ 40%	2,157,120	- 11%
			Mean=+33%		Mean= - 4%
G cells	4,215,680	2,631,440	-37%	-----	-----
	672,240	612,720	- 9%	586,320	- 13%
	4,095,120	3,982,080	- 3%	3,998,400	- 2%
	4,339,200	5,303,280	+22%	3,720,480	- 14%
			Mean= - 7%		Mean= - 10%

Table 2 High Salt Wash. After incubations of intact bladders with H^3-uridine and either 7 x 10^{-8}M aldosterone, aldosterone plus spironolactone (1:200), or diluent, cells were removed, separated, disrupted and cytosol fractions obtained. These cytosol fractions were chromatographed on oligo-(dt)-cellulose columns and the initial wash plus 3 rinses of high salt buffer were pooled. This fraction contains H^3-uridine and many H^3-uridyl compounds including H^3-rRNA and H^3-tRNA. An aliquot was taken and H^3-dpm determined. The results are expressed above.

Table 3 tRNA + rRNA

	Control	Aldosterone		SC-920 + Aldosterone	
	(dpm)	(dpm)	(%change)	(dpm)	(%change)
MR cells	106,200	141,040	+ 33%	-----	-----
	-----	-----	-----	-----	-----
	151,760	316,240	+108%	142,600	- 6%
	655,080	730,320	+ 11%	499,680	- 24%
			Mean= + 51%		Mean= - 15%
G cells	470,400	329,760	- 30%	-----	-----
	-----	-----	-----	-----	-----
	486,200	619,600	+ 27%	451,440	- 7%
	925,000	916,480	- 1%	649,520	- 30%
			Mean= - 1%		Mean= -18%

Table 3 tRNA and rRNA. After incubations of intact hemibladders with H^3-uridine and either 7 x 10^{-8}M aldosterone, aldosterone plus spironolactone (1:200), or diluent, cells were removed, separated, disrupted, and cytosol fractions obtained. These cytosol fractions were chromatographed on oligo-(dt)-cellulose columns and the high salt fraction obtained. After aliquots were taken for total H^3-tRNA and H^3-rRNA were precipitated with 2 volumns ethanol overnight at -70°C. After centrifugation, the RNA pellet was re-dissolved in Ringers and H^3-dpm's were determined. The results are represented above.

Table 4 12,000g pellet

MR cells	296,110	372,850	+26%	-----	-----
	109,180	214,550	+97%	102,730	- 6%
	642,590	1,197,670	+86%	420,590	- 35%
	1,049,020	1,515,820	+44%	970,020	- 8%
			Mean=+63%		Mean= -16%
G cells	942,420	781,500	-17%	-----	-----
	167,150	282,310	+69%	108,250	- 35%
	1,420,790	2,579,140	+81%	1,447,910	+ 2%
	1,052,300	2,578,750	+145%	1,997,970	+ 90%
			Mean= +69%		Mean= +19%

Table 4 12,000g pellet. After incubations of intact bladders with H^3-uridine and either 7 x 10^{-8}M aldosterone, aldosterone plus spironolactone (1:200), or diluent, cells were removed, separated, disrupted, and a 12,000g pellet was obtained after 10 minutes centrifugation. This pellet contains nuclei, mitochondria, and lysozomes. It was washed in 100% ethanol, dissolved in 2 ml Beckman tissue solubilizer and H^3-dpm determined. The results are expressed above.

II. Incorporation of Uridine into Ribonucleic Acids of Isolated Mucosal Cells

A. Effects of Aldosterone in Mitochondria-rich cells

Mitochondria-rich (MR) and granular (G) mucosal cells recovered from Ficoll gradients were incubated in calcium-Ringers for period of 30 to 180 minutes. 3H(5,6)-uridine was added to the baths and, after 20 minutes, diluent, aldosterone, or aldosterone plus spironolactone (1:200) were added to the cell suspensions. After various periods of incubation (20, 40, 60, 70, 90, 120, 150, 180, 210, 240 minutes), the cells were pelleted, washed and disrupted in the cold (0 - 4^{o}C). The nuclei, mitochondria and lysozomes were pelleted and the salt concentration adjusted to the 0.5M NaCl and 0.01M EDTA. The supernatant fractions were analyzed by affinity chromatography followed by sucrose density centrifugation.

Aliquots of the low salt wash of the affinity columns (Fig. 4)show that aldosterone (7 X 10^{-8}M) stimulates the incorporation of H^3 uridine into poly-A-containing cytoplasmic RNA (mRNA) with the earliest significant increase appearing after 40 minutes. The maximum effect is obtained after 90 minutes, and then falls steadily to insignificant levels at 4 hours. The effects of aldosterone on this mRNA is mineralocorticoid specific since spironolactone given simultaneously with aldosterone blocks the effect.

Aliquots from the high-salt wash (Fig. 5), which contains many H^3-uridyl compounds including tRNA, rRNA, the smaller H^3RNA degradation products, H^3-uridine, H^3-UMP, H^3-UDP, H^3-UTP, and others, show variable effects after aldosterone. The tendency was an increase in the radioactivity in this fraction, but the variability in results is large. The simultaneous addition of SC-9420 and aldosterone (200:1) always reduced the labeling of this fraction compared to aldosterone alone.

Ethanol precipitates of the High Salt wash, which contain t-RNA, rRNA and possibly some partially degraded RNA molecules, were dissolved and the radioactivity measured. The results from MR cells, summarized in Fig. 6, indicate that aldosterone stimulates the incorporation of H^3-uridine into these cytoplasmic RNA molecules after 40 minutes. The radioactivity in this fraction increased further at 60 and 90 minutes, but the effect was apparently somewhat diminished after 120 and 150 minutes. However, single observations after 3 and 4 hours showed enormous increases in this value. SC-9420 blocked completely the effects of aldosterone except at the 180 minute and the 240 minute time periods. These data indicate that some of the control ethanol precipitate may have been lost. Even so, taking the SC-9420 +aldo values as control values, the increase in tRNA and rRNA is +53% for the 180 and +148% for the 240 minute time period, large effects indeed.

Counts of the 12,000 g pellet from MR cells (Fig.3) reveals much the same time course of aldosterone's effect as does the poly A-RNA fractions.

II. B. Effects of Aldosterone in Granular Cells

Granular cell-enriched fractions were prepared from the same tissues and incubated under conditions identical to those described above for the mitochondria-rich cells. The G cells were disrupted, chromatographed, and the samples analyzed as above. Aliquots from the ply A-RNA fractions (Fig. 4), the High Salt washes (Fig. 5), precipitates from High Salt washes (Fig. 6), and from the 12,000g pellets (Fig. 3) showed no increase in labeling with H^3-uridine following exposure to aldosterone.

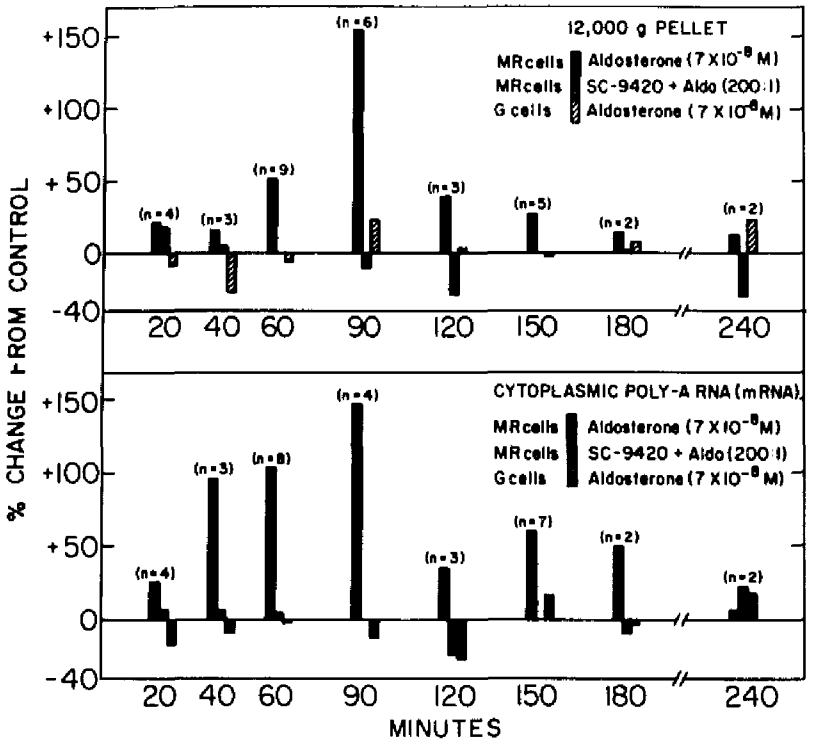


Fig. 3 (top) H^3-dpm from the 12,000g pellet obtained just after cell disruption. Results from (n) experiments are expressed as per cent change from control values at each incubation time.

Fig. 4 (bottom) H^3-dpm from the low salt fraction of affinity chromatography containing poly A-rich, cytoplasmic RNA. Results from (n) experiments are expressed as per cent change from control values at each time period.

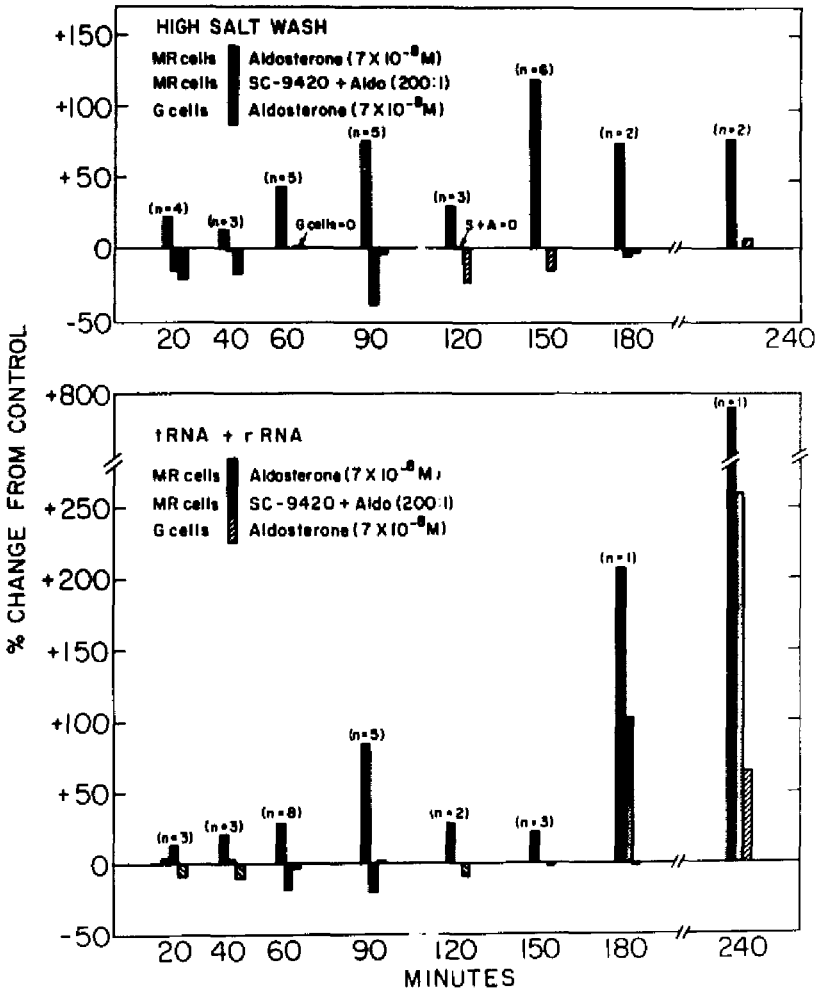


Fig. 5 (top) H^3-dpm from the high salt fraction of affinity chromatography. Results from (n) experiments are expressed as per cent change from control values at each time period.

Fig. 6 (bottom) H^3-dpm from the ethanol precipitate of the high salt wash which was re-dissolved and counted. Results from (n) experiments are expressed as per cent change from control values at each time period.

C. Analysis of MR Cell mRNA by Sucrose Density Gradient Centrifugation

The effects of aldosterone upon the labeling of mRNA or MR cells incubated for varying periods of time, are illustrated in figures 7-16. The labeled cytoplasmic ply A-rich-RNA was sedimented in linear gradients of sucrose and the samples were fractionated and the radioactivity measured.

Commercially obtained yeast cell RNA was re-extracted with chloroformphen 1, precipitated at -70°C overnight with 2 volumes of ethanol. The RNA pellet obtained after centrifugation was dissolved in buffer and layered over sucrose gradients and centrifuged as were the mRNA fractions. The gradients were fractionated and read at 260 nm to obtain 5S, 18S, and 28S values. After 20 minutes, no consistent mRNA peaks are apparent. After 40 minutes incubation (Fig. 7), there is more incorporation of H^3-uridine into the aldosterone-treated MR cell 7S to 18S mRNA as compared to control. While the relative heights of the peaks at 40 minutes were not consistent from experiment to experiment, a peak at about 7S was always seen, and a 12S peak and some increase in labeling between 18S and 28S were seen in all but one experiment. Also, the smaller mRNA species are generally dominant at these two times. The inconsistency in labelling profiles may be due to the fact that the relatively short incubation periods are followed by some 35 minutes of centrifugation and cell disruption before the synthesis and degradation of RNA can be stopped completely with the addition of heparin and pronase. The cells are kept at 4°C during these operations but the length of time these operations took varied slightly from experiment to experiment and

this may have influenced the results of these shorter incubations. Figure 7 also shows the uptake of H^3-uridine into mRNA after 90 min utes incubation in the same batch of MR cells as the 40 minute data. These profiles clearly show that only the smaller mRNA species appear in the cytoplasm at 40 minutes, that it takes a full 90 minutes for the full profile to appear, and that aldosterone affects both the smaller mRNA peaks (7S and 12S within 40 minutes) and the larger peaks (18S and 25S).

Figures 8 and 9 illustrate mRNA profiles from MR cells incubated for 60 minutes. The dominant aldosterone-induced peak is usually seen at 25S.

Figure 9 depicts one of two experiments in which sedimentation profiles were prepared from MR cells exposed to five different concentrations of aldosterone for 60 minutes. The results, along with the data in Table 5, indicate that the amount of labelled uridine incorporated into the poly-A-RNA in the cytoplasm after 60 minutes exposure to aldosterone is dependent on the dose level of the mineralocorticoid used.

Figures 7, 10-12 illustrate profiles of cytoplasmic poly-A-RNA or MR cells incubated for 90 minutes in hormone.

Figure 12 illustrates a 90 minute incubation, but in this experiment, low salt wash of the affinity column was layered over a 0-8% continuous sucrose gradient in 70% foramamide - 30% sucrose buffer. Only two peaks of aldosterone-induced mRNA are seen, but it was felt that the SW27 rotor was not capable of sufficiently separating the various mRNA peaks in 16 hours, and that th major aldosterone-induced peak represents several in RNA species.

After 90 minutes incubation with aldosterone, the peak of aldosternoe-induced poly-A-RNA found in the cytoplasm of MR cells shifts progressively to the left, indicating that progressive degradation of these mRNA's is taking place. At 120 minutes (Fig. 13) the peak at 25S is gone, leaving a broad peak between 5S and 18S. At 3 hours (Figs. 23 and 24) the major peak is at approximately 5S with remnants of the 25S peak still extant. After 4 hours incubation, no distinct peaks could be discerned (Fig. 25).

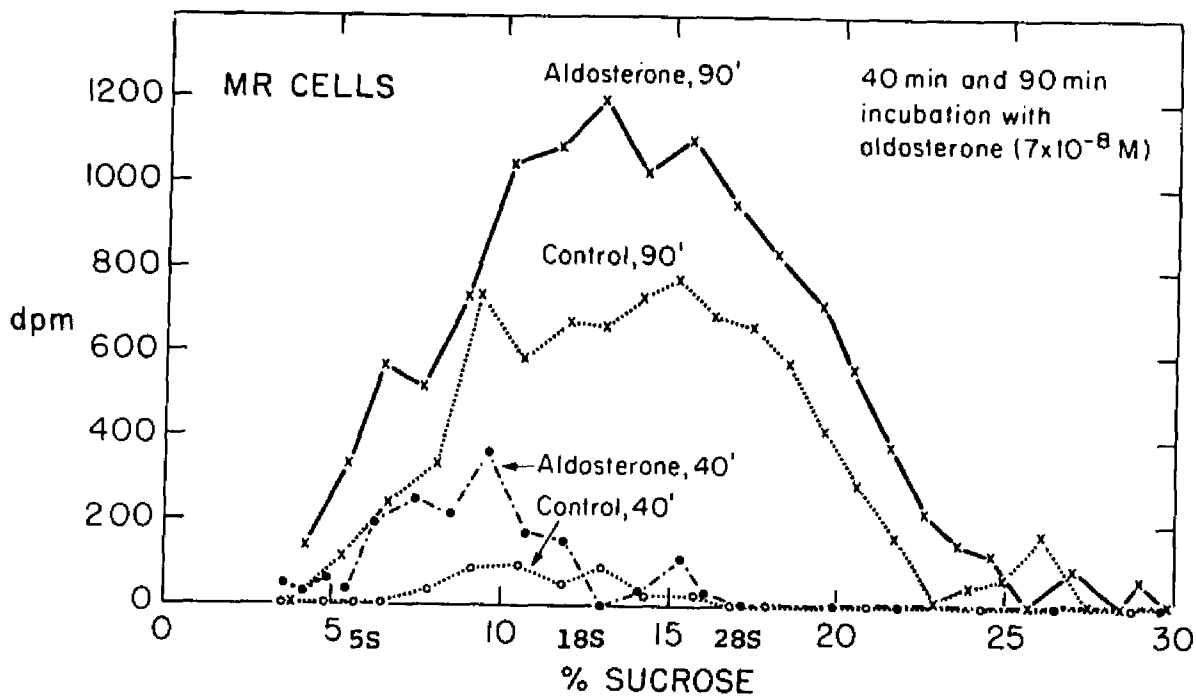


Fig. 7 Sucrose gradient profiles of cytoplasmic poly-A containing RNA from separated MR cells. The cells were incubated in H^3(5,6) uridine and either aldosterone or diluent for 40 or 90 minutes. Isolation and chromatography of the RNA was carried out as described in Methods, sec. II.

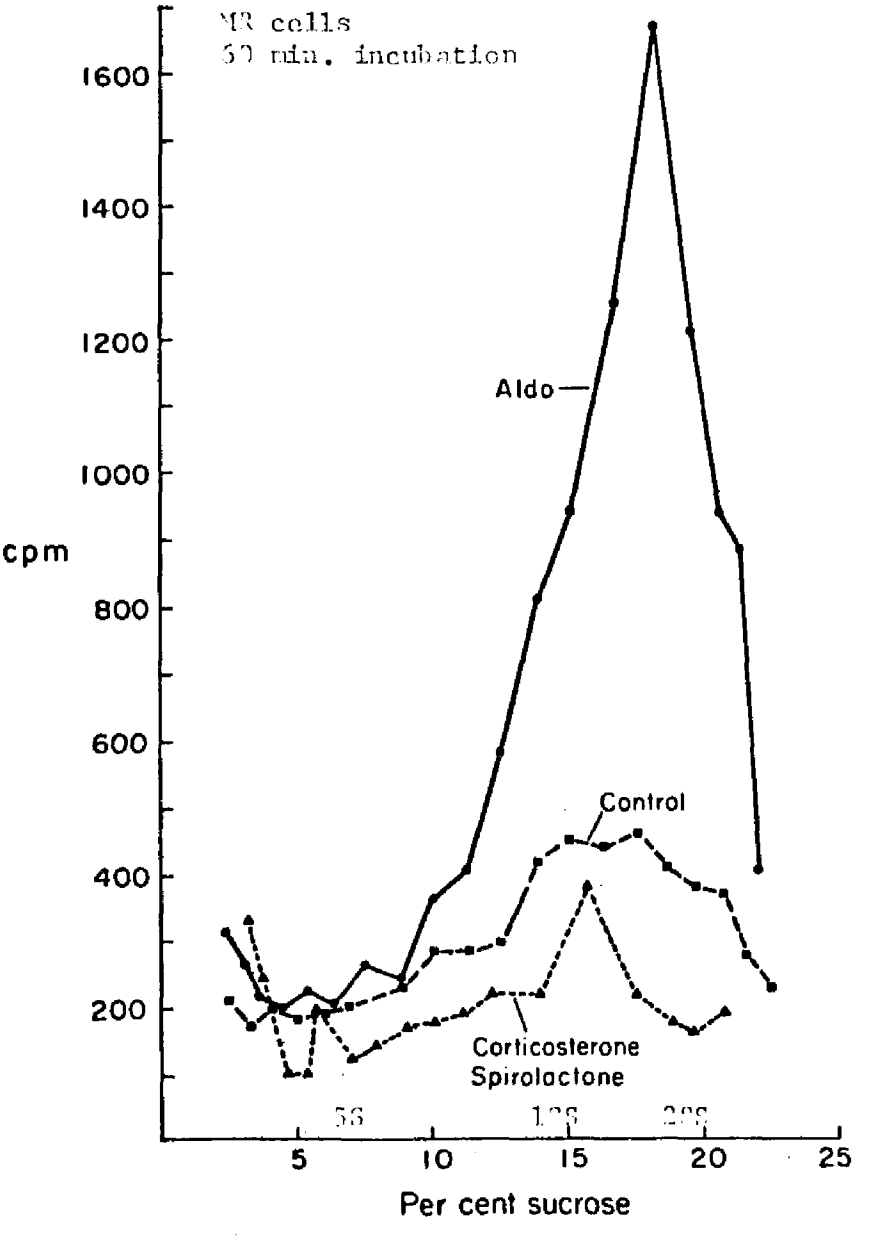


Fig. 8 Sucrose gradient profile of cytoplasmic poly A-containing RNA from separated MR cells. The cells were incubated in H^3(5,6) uridine and either 7×10^{-9}M aldosterone, 7×10^{-8}M corticosterone plus 1.4×10^{-5}M SC-9420, or diluent for 60 minutes. Isolation and affinity chromatography was carried out as described in Methods, Sec. II.

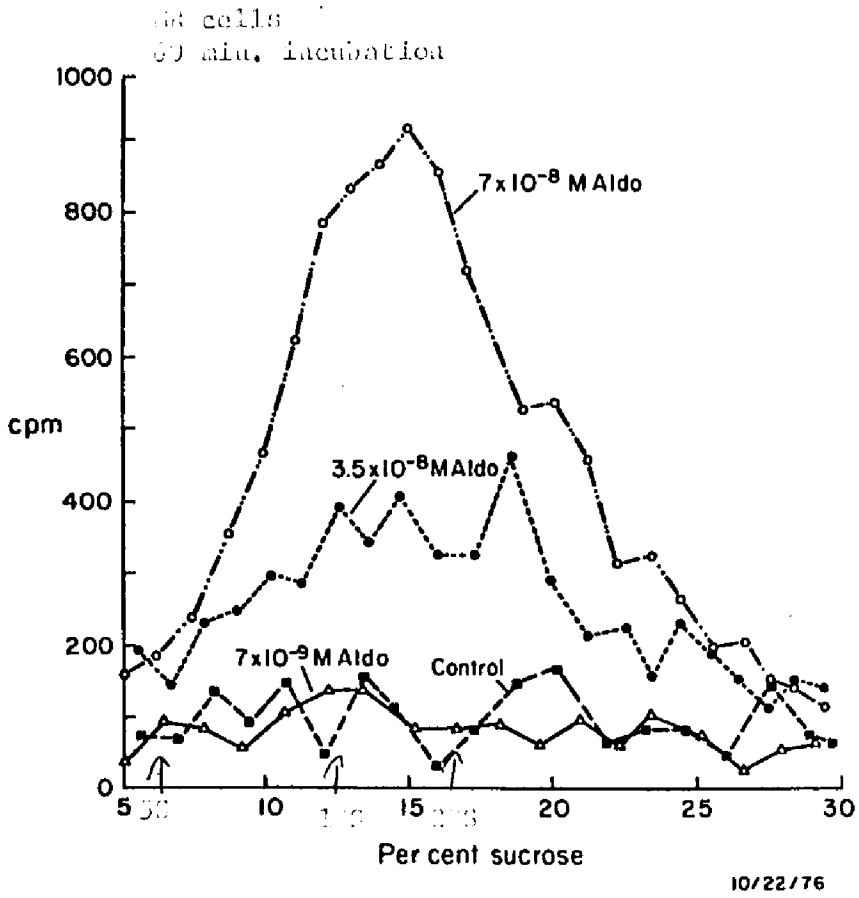


Fig. 9 Sucrose gradient profile of cytoplasmic, poly A-containing, [illegible] from separated [illegible] cells. The cells were incubated in [illegible]-(5,6) uridine and either aldosterone at 7 x [illegible], 3.5 x [illegible] or 7 x [illegible] or diluent for 60 minutes. Isolation and affinity chromatography of the [illegible] was carried out as described in Methods, sec. II.

Table 5 [illegible]

Dose	12,000g pellet dpm	% change	mRNA dpm	% change	High Salt dpm	% change	tRNA + rRNA dpm	% change
Experiment 1								
7×10^{-3} rad/s	235,590	+53%	21,040	+166%	1,001,440	+42%	120,160	+59%
3.5×10^{-3} rad/s	212,240	+38%	19,220	+152%	900,000	+20%	95,240	+17%
7×10^{-4} rad/s	143,250	- 7%	11,630	+ 47%	713,760	- 6%	81,047	+ 8%
Control	154,320	-	7,920	-	750,120	-	75,120	-
Experiment 2								
3.5×10^{-2} rad/s	358,740	+122%	33,071	+237%	-	-	-	-
7×10^{-3} rad/s	253,250	+53%	19,760	+165%	-	-	-	-
1.4×10^{-3} rad/s	213,400	+30%	14,300	+53%	-	-	-	-
Control	161,110	-	9,310	-	-	-	-	-
3.5×10^{-2} rad/s		+122%		+237%		-		
7×10^{-3} rad/s		+53%		+116%		+42%		+59%
3.5×10^{-3} rad/s		+38%		+152%		+20%		+17%
1.4×10^{-3} rad/s		+30%		+ 53%		-		-
7×10^{-4} rad/s		- 7%		+ 47%		- 6%		+ 8%

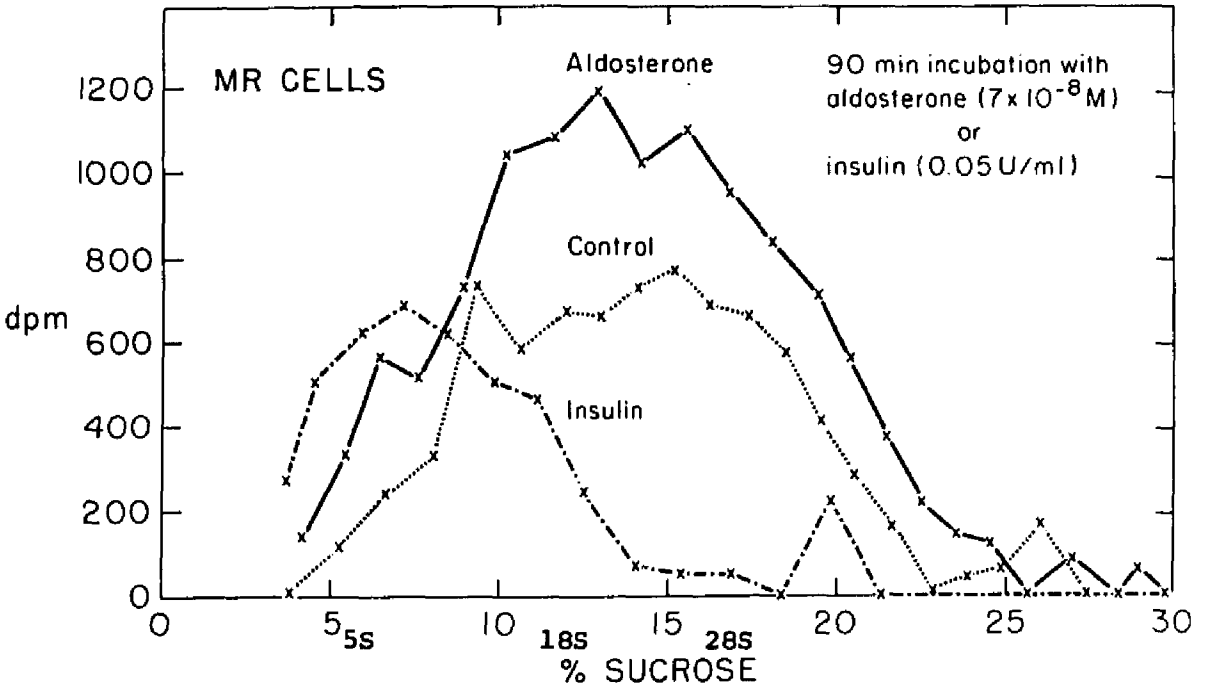


Fig. 10 Sucrose gradient profiles of cytoplasmic poly A-containing RNA from separated MR cells. The cells were incubated in H^3(5,6) uridine and either aldosterone (7×10^{-8}M), insulin (50 mU/ml) or diluent for 90 minutes. Isolation and affinity chromatography were carried out as described in Methods, sec. II.

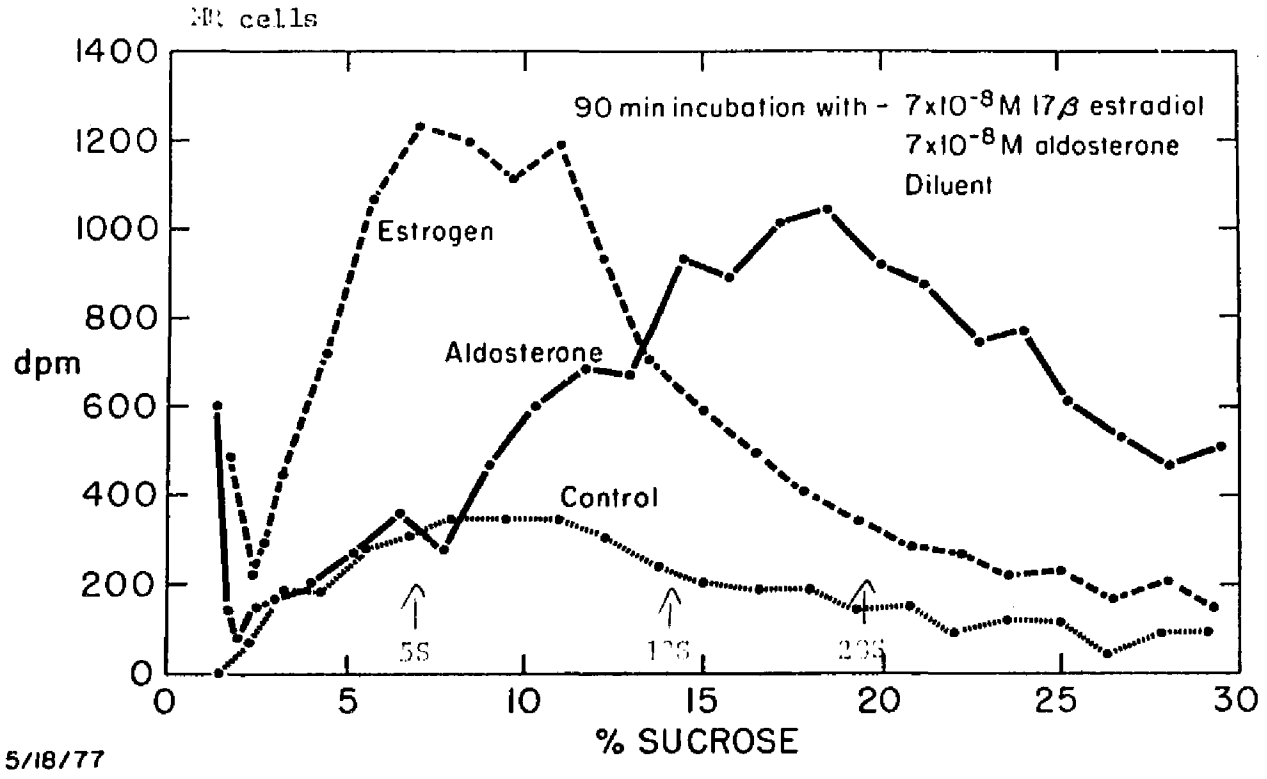


Fig. 11 Sucrose gradient profiles of cytoplasmic poly A-containing RNA from separated MR cells. The cells were incubated in Ca^{++} Ringers with H^3(5,6) uridine and either 7×10^{-8} M aldosterone, 7×10^{-8} M aldosterone, 7×10^{-8} M 17 -estradiol or Diluent for 90 minutes. The RNA was isolated and chromatographed as described in Methods, sec. II.

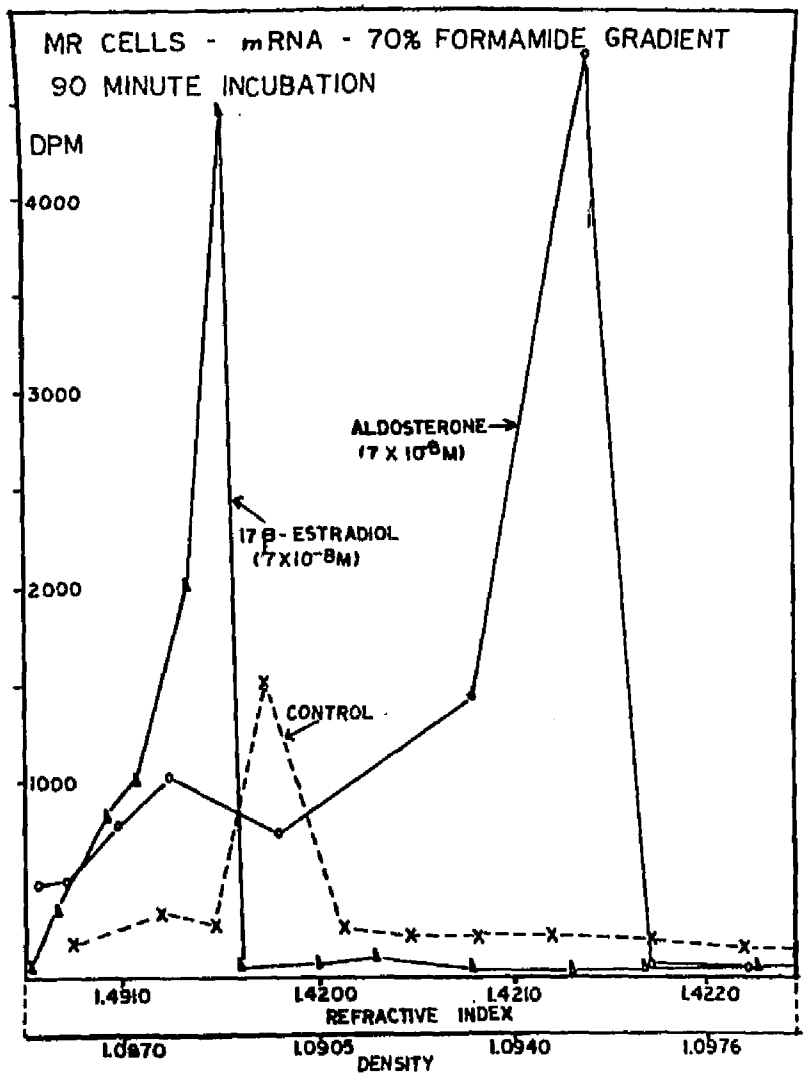


Fig. 12 70% formamide sucrose gradient (0-10% sucrose) profiles of cytoplasmic, poly A-containing RNA from separated MR cells. The cells were incubated for 90 minutes in Ca^{++} Ringers containing $H^3(5,6)$ uridine and either 7×10^{-8}M aldosterone, 7×10^{-8}M 17β-estradiol, or diluent. The RNA was isolated and chromatographed as outlined in Methods, sec. II.

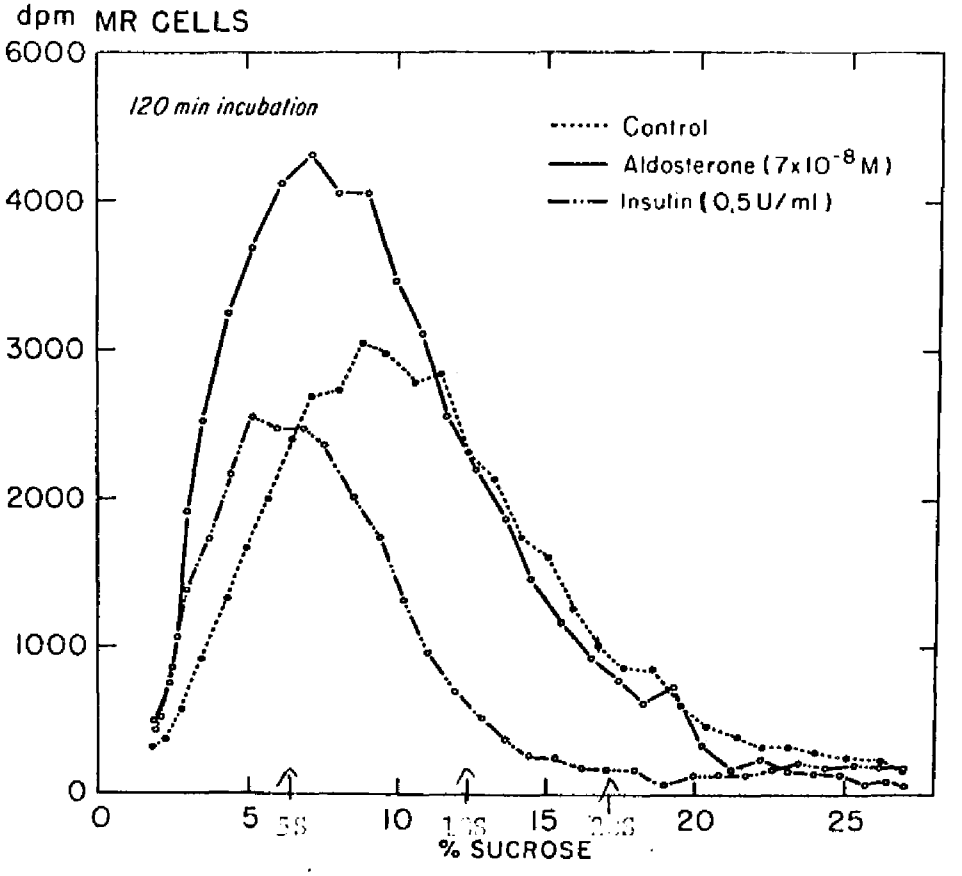


Fig. 13 Sucrose gradient profiles of cytoplasmic poly A-containing RNA from separated MR cells. The cells were incubated in Ca^{++} Ringers containing H^3(5,6) uridine and either 7×10^{-8}M aldosterone, 0.3 U/ml insulin, or diluent for 2 hours. The RNA was isolated and chromatographed as described in Methods, sec. II.

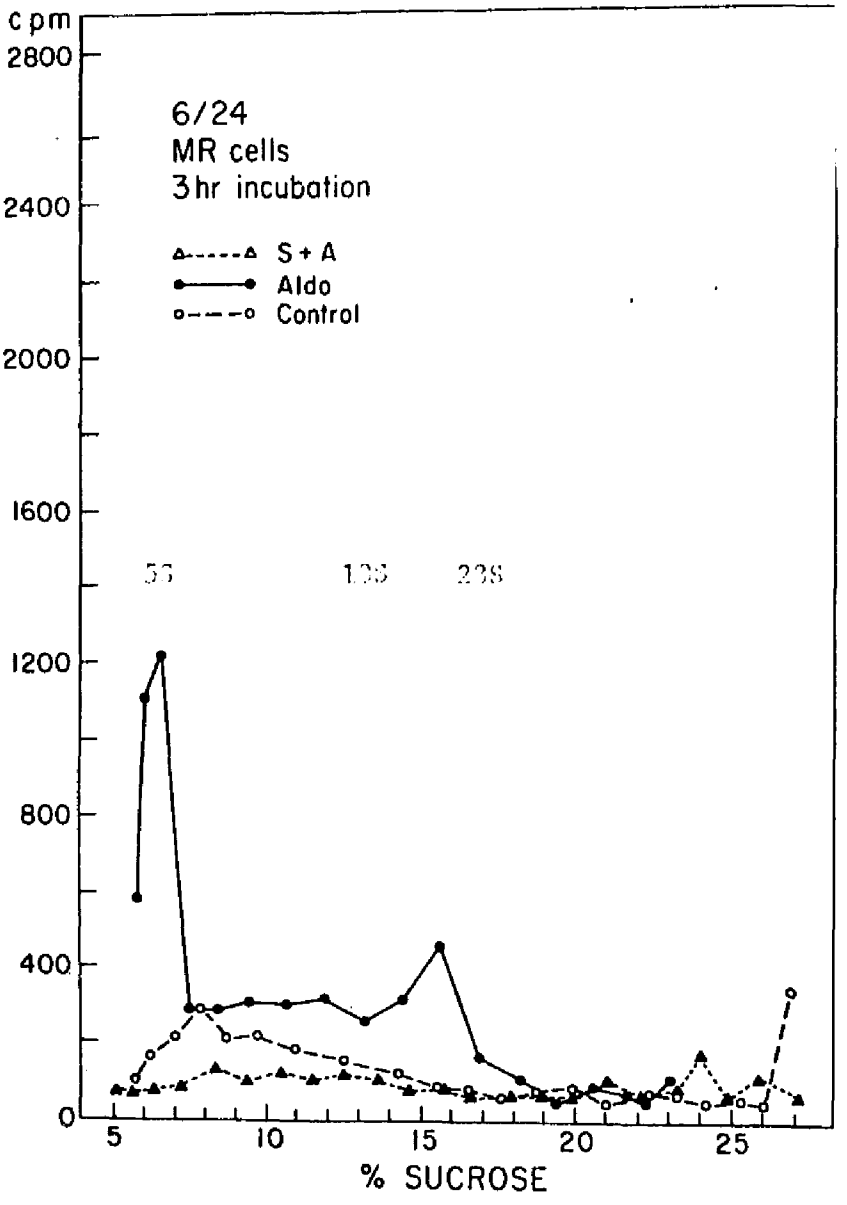


Fig. 14. Sucrose gradient profiles of cytoplasmic poly A-containing RNA from separated MR cells. The cells were incubated in Ca^{++} Ringers containing H^3(5,6) uridine and either 7×10^{-9} M aldosterone, 7×10 aldosterone plus 1.4×10^{-5} M SC-9420, or diluent. The RNA was isolated and chromatographed as described in Methods, sec. II.

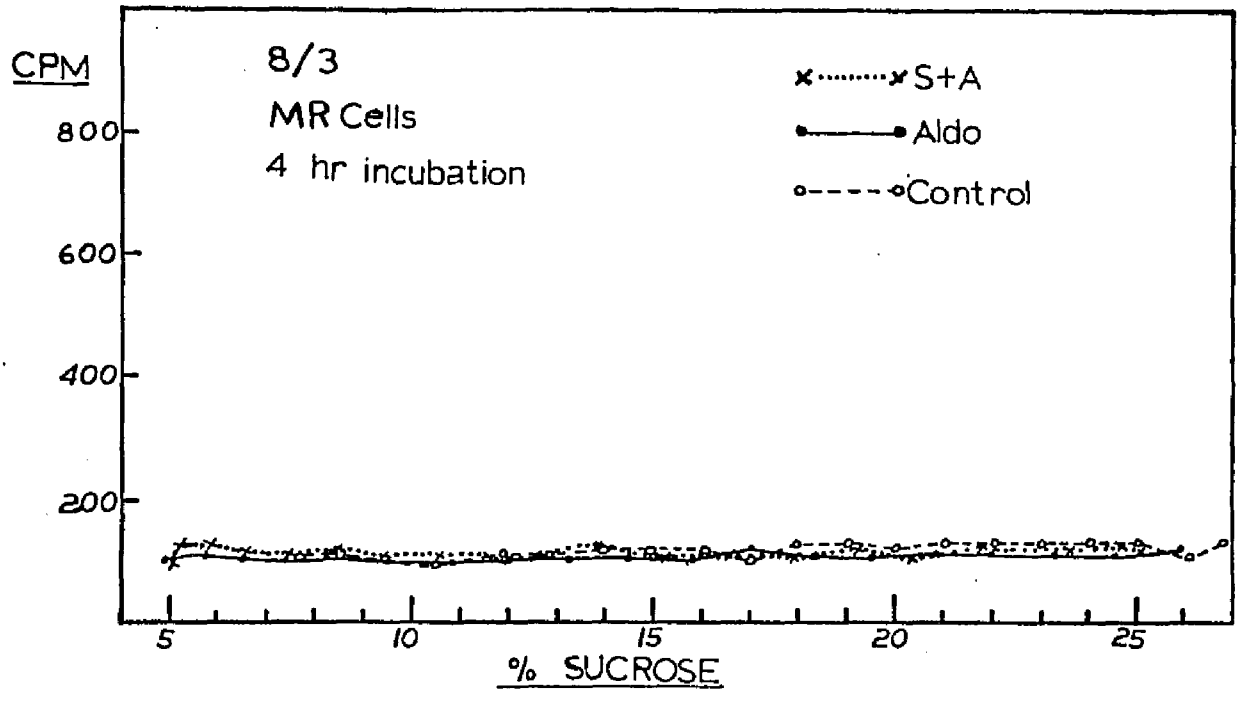


Fig. 15 Sucrose gradient profiles of cytoplasmic, poly A-containing RNA from separated MR cells. The cells were incubated for 4 hours in Ca^{++} Ringers which included H^3(5,6) uridine and either 7×10^{-8} M aldosterone, 7×10^{-8} M aldosterone plus 1.4×10^{-5} M SC-9420, or diluent. The RNA was isolated and chromatographed as described in Methods, Sec. II.

II. D. Sucrose Gradient Profile - Mixed Cells - mRNA

Mixed MR and G cells were incubated for 90 minutes in 4 ml Ca^{++}Ringers containing 10^{-4}M each of adenosine, guanosine, cytosine, and H^3-uridine for 20 minutes before the addition of $7x10^{-8}$M aldosterone. The sucrose gradient profile of the mRNA (Fig.16) reveals several peaks of aldosterone-induced mRNA. The position of these mRNA peaks corresponds to the position of the mRNA peaks reported by Rossier, Wilce, and Edelman(31,32), who obtained their cytoplasmic, poly-A-RNA from mixed mucosal cells scraped from the toad bladder instead of being obtained by removing the calcium as wasdone here. A further difference in isolation technique, their use of a chloroform-phenol extraction proceedure before the cytosol fraction was applied to the oligo(dT)-cellulose column, did not seem to affect the comparability of their and my results.

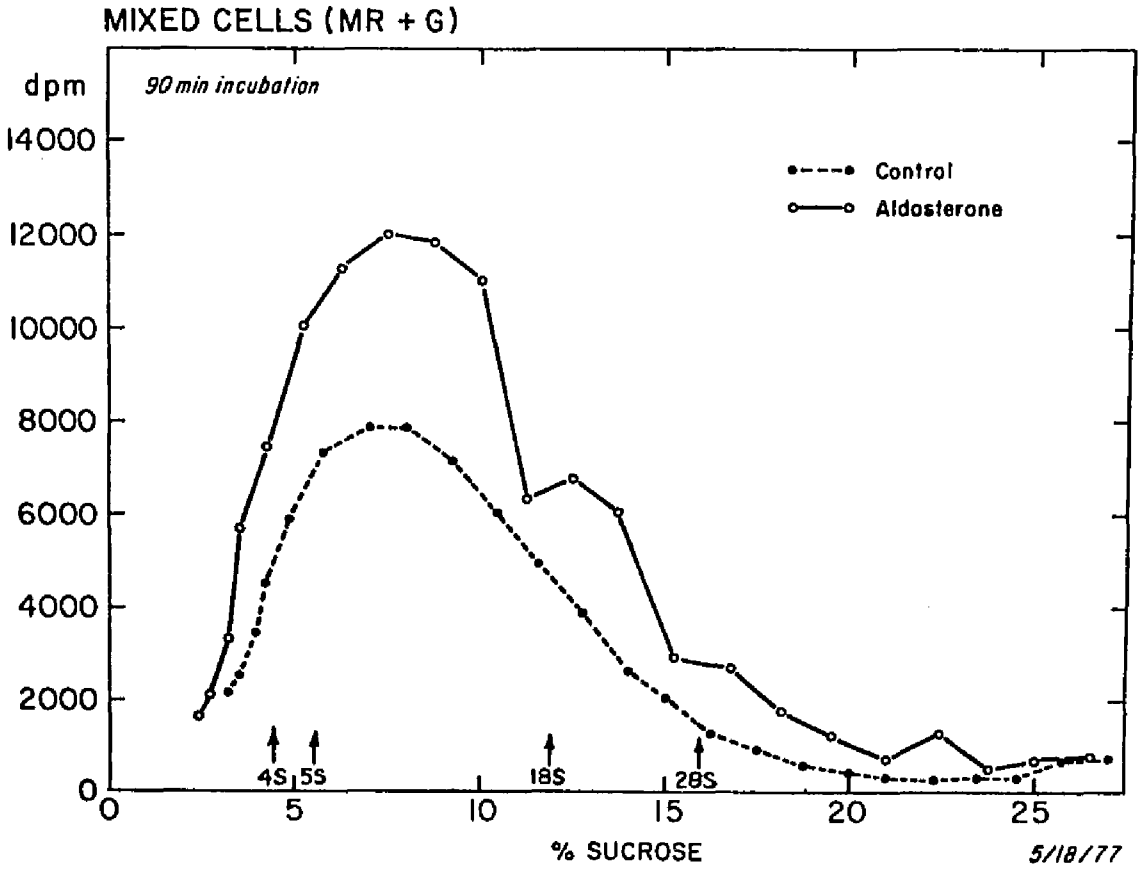


Fig. 16 Sucrose gradient profiles of cytoplasmic poly A-containing RNA from a mixed population of toad bladder epithelial cells which had been removed from the bladder wall and incubated in Ca^{++} Ringers containing H^3(5,6) uridine and either 7×10^{-8}M aldosterone or diluent. The isolation and chromatography of the RNA is described in Methods, sec. II.

II. E. Sucrose Gradient Profiles - G Cells - mRNA

Sucrose gradient profiles of G cells reveal that aldosterone does not have a measurable effect on cytoplasmic poly-A-RNA metabolism in this population of cells. No evidence for an aldosterone-induced mRNA was seen after 20,60,120, or 240 minutes incubation in media containing physiologic levels of aldosterone (Figs. 17-20).

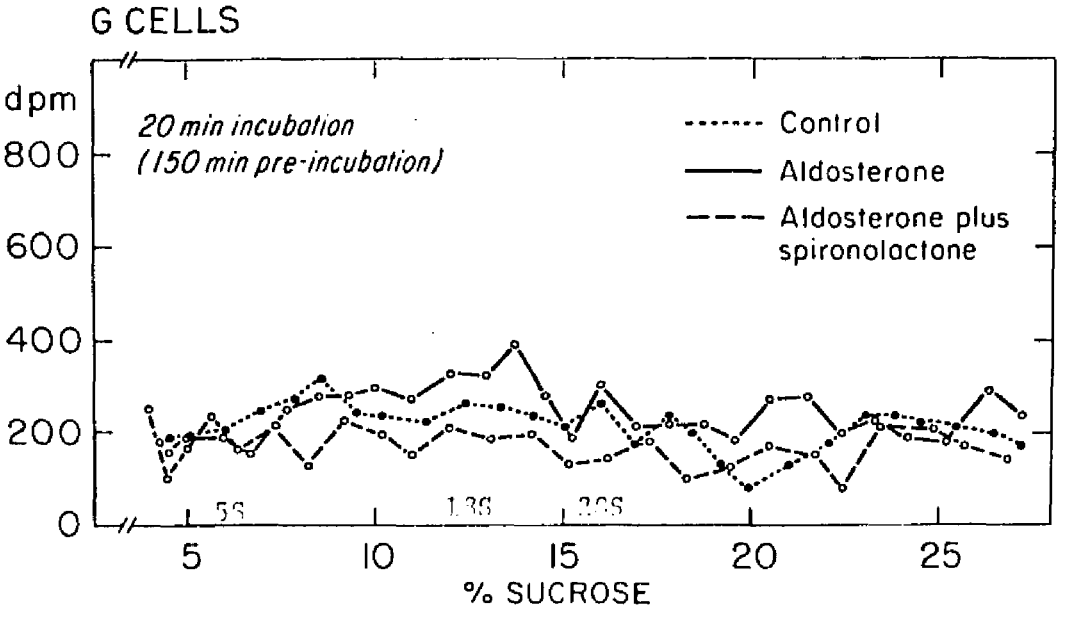


Fig. 17 Sucrose gradient profiles of cytoplasmic, poly A-containing RNA from separated G cells. The cells were pre-incubated for 2½ hours in Ca^{++} Ringers. During the last 20 minutes of the pre-incubation, H^3(5,6) uridine was added. After the addition of either 7×10^{-8} M aldosterone, 7×10^{-8} M aldosterone plus 1.4×10^{-5} M SC-9420, or diluent, the cells were incubated for 20 minutes more. The RNA was isolated and chromatographed as outlined in Methods, sec. II.

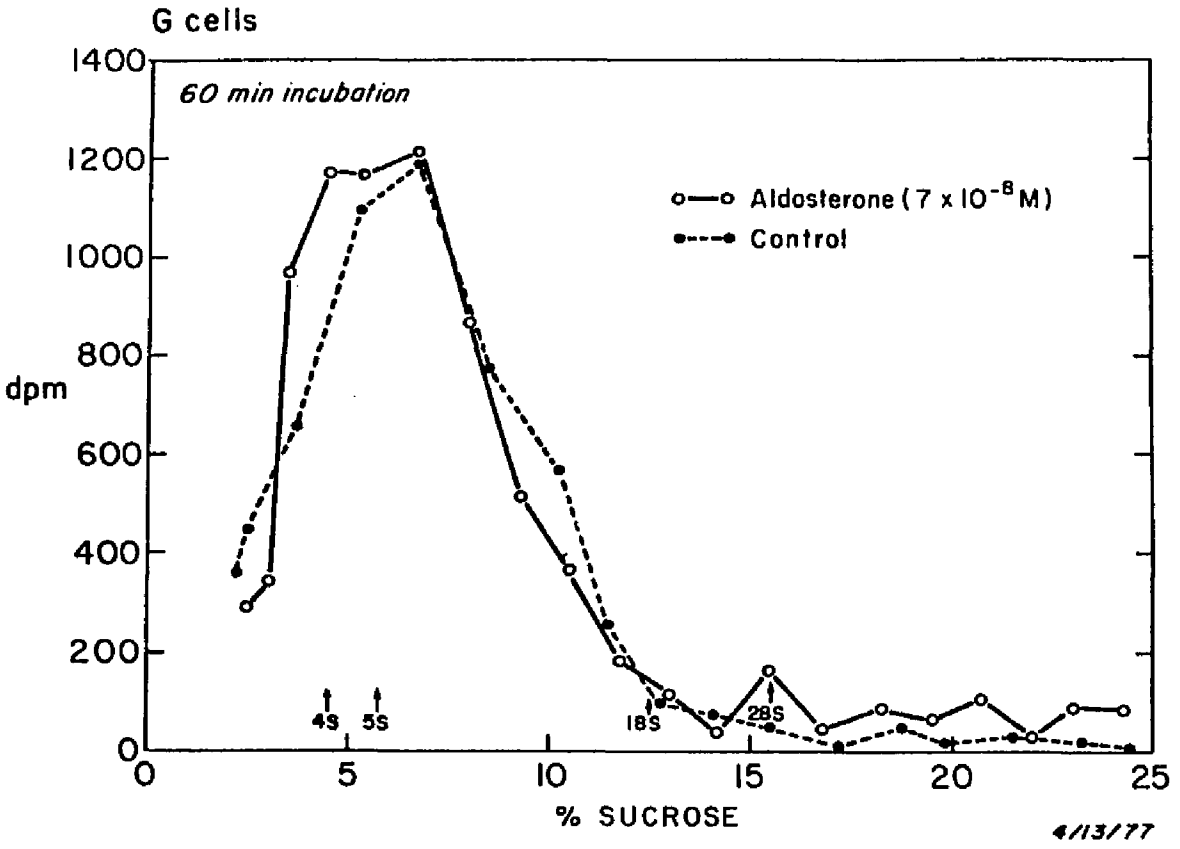


Fig. 18 Sucrose density profiles of cytoplasmic, poly A-containing RNA from separated G cells. The cells were incubated for 1 hour in Ca^{++} Ringers containing H^3(5,6) uridine and either 7 x 10^{-8}M aldosterone or diluent. The RNA was isolated and chromatographed as outlined in Methods, sec. II.

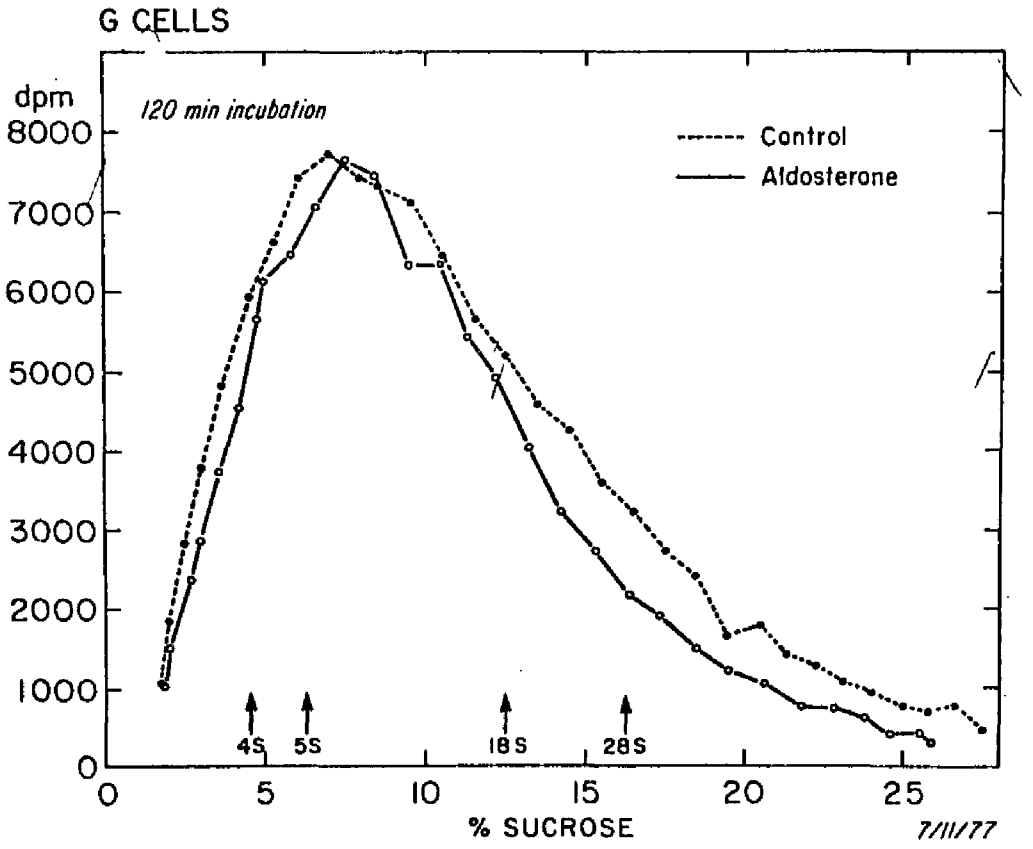


Fig. 19 Sucrose gradient profiles of cytoplasmic, poly A-containing RNA from separated G cells. The G cells were incubated in Ca^{++} fingers containing ^{3}H(5,6) uridine and either 7×10^{-8} M aldosterone or diluent for 2 hours. The RNA was isolated and chromatographed as described in Methods, sec. II.

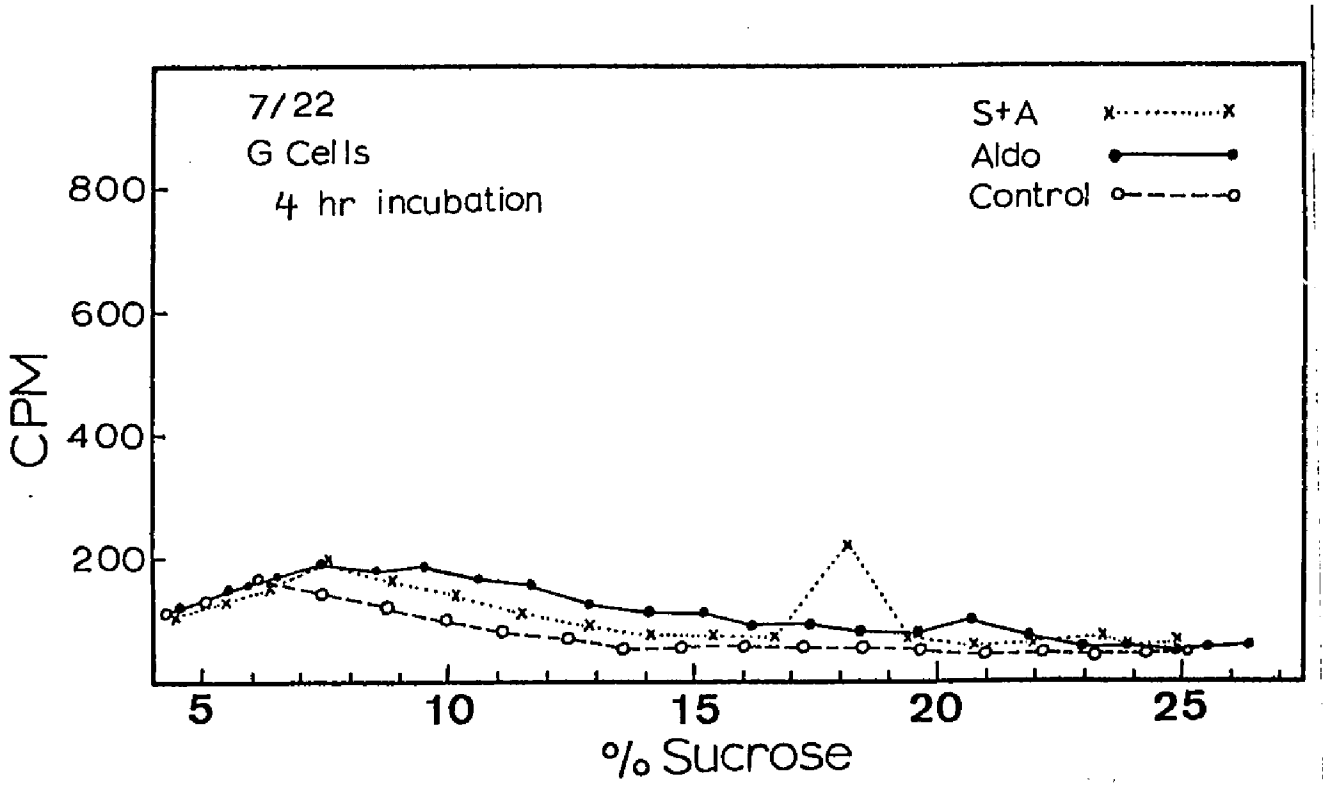


Fig. 20 Sucrose gradient profiles of cytoplasmic, poly A-containing RNA from separated G_3 cells. The cells were incubated in Ca^{++} Ringers containing H^3 (5,6) uridine and either 7×10^{-8} M aldosterone, 7×10^{-8} M aldosterone plus 1.4×10^{-8} M SC-9420, or diluent for 4 hours. The RNA was isolated and chromatographed as described in Methods, sec. II.

II. F. Sucrose Gradient Profile - MR Cells - tRNA and rRNA

The ethanol precipitate of the High Salt wash from MR cells incubated 120 minutes in aldosterone or diluent were re-dissolved in sucrose buffer and layered over contiuous sucrose gradients. The samples were centrifuged at 25,000 rpm for 16 hours and fractionated. The profiles (Figure 21) indicate that aldosterone had its major effect on 5S tRNA with no effect on 18S and 28S ribosonal RNA at these incubation times.

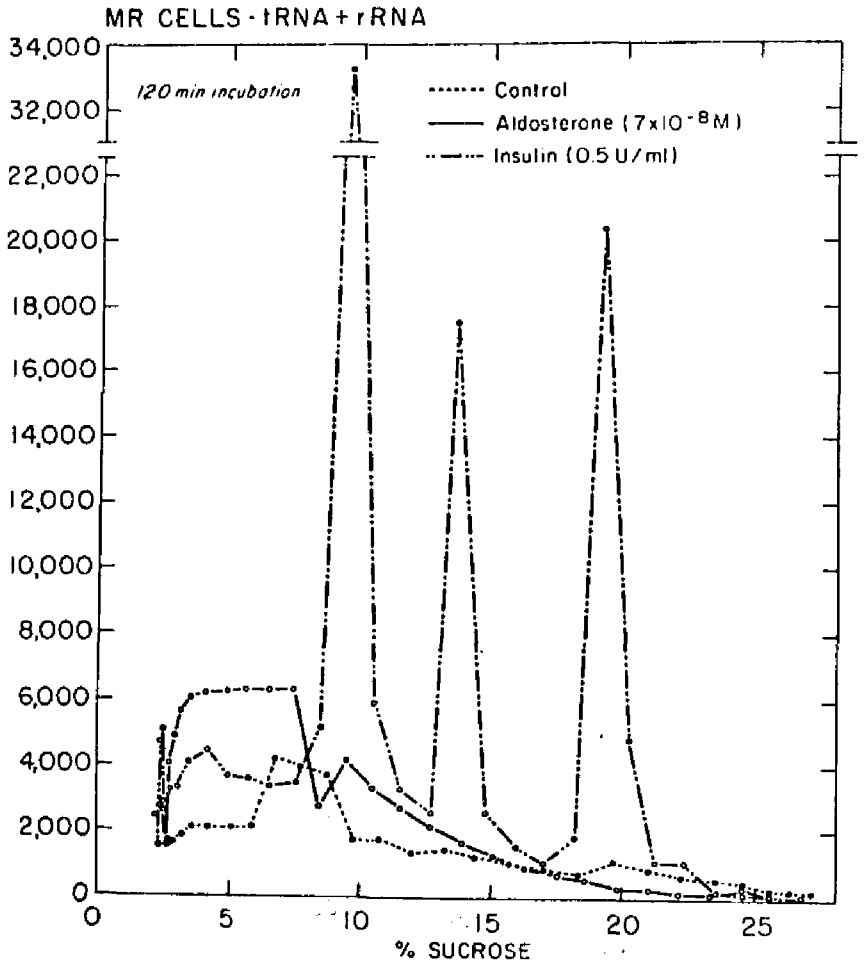


Fig. 21 Sucrose gradient profiles of cytoplasmic tRNA and rRNA from separated MR cells. The cells were incubated in Ca^{++} Ringers containing H^3(5,6) uridine and either 7×10^{-8}M aldosterone, 0.5 U/ml insulin or diluent for 2 hours. The RNA was obtained in the same fashion as that of Figure 42.

II. G. Corticosterone Plus Spironolactone

In an effort to dissect out the glucocorticoid from the mineeralocorticoid effects of the other natural corticosteroid of *Bufo marinus*, corticosterone was given to separated epithelial bladder cells for 63 minutes at the low dose of 7×10^{-8}M along with 1.4×10^{-5}M SC-9420, the specific mineralocorticoid blocking agent.

Table 6 lists the effects of corticosterone plus spironolactone (200:1) on the various cell fractions. No significant effect is seen on any fraction, including the sucrose profi les MR cells (Fig. 8).

Table 6 Corticosterone (7×10^{-8}M) plus Spironolactone (1.4×10^{-5}M)
60 Minute Incubation

	Control (dpm)	S + SC-9420 (dpm)	(% change)	Mean % Change
Poly A-RNA				
MR cells	15,760	19,920	+ 26%	
	21,760	36,000	+ 65%	
	7,500	6,550	- 13%	+ 26%
G cells	6,295	4,100	- 35%	
	38,720	60,000	+ 55%	
	22,720	21,600	- 5%	+ 5%
High Salt Wash				
MR cells	488,000	342,000	- 30%	
	1,029,600	1,115,760	+ 8%	
	459,600	474,480	+ 3%	- 6%
G cells	578,000	784,000	+ 36%	
	1,656,240	1,308,800	- 21%	
	1,034,160	1,022,400	- 6%	+ 3%
tRNA + rRNA				
MR cells	116,000	117,000	+ 1	
	158,760	149,960	- 6%	
	106,320	124,240	+ 17%	+ 4%
G cells	116,000	118,000	+ 2%	
	180,920	142,040	- 21%	
	72,480	78,440	- 1%	- 7%
12,000 G pellet				
MR cells	778,130	332,880	- 57%	
	820,680	850,590	+ 4%	
	313,450	352,320	+ 12%	- 14%
G cells	622,000	654,210	+ 9%	
	677,460	537,770	- 21%	
	573,040	542,400	- 6%	- 6

II. H. Progesterone

The general lack of effect of a 90 minute incubation of progesterone ($7x10^{-8}$M) on separated epithelial cells of the toad bladder are seen summarized in Table 7. Sucrose gradient profiles of MR and G cells are seen in figures 24 and 28, respectively. No effect of progesterone at 7×10^{-8}M is apparent in either MR or G cells.

Table 7 Progesterone (7×10^{-5} M) 30 Minutes Incubation

	Control (dpm)	Progesterone (dpm)	(% change)
Poly A RNA			
HR cells	12,071	13,393	+ 3%
C cells	103,738	114,158	+ 11%
High Salt			
HR cells	7,414,830	6,457,[illegible]	- 12%
C cells	21,409,160	19,566,340	- 9%
t-RNA + rRNA			
HR cells	1,034,739	844,[illegible]	- 18%
C cells	3,839,540	3,[illegible]	- 9%
11,000 g pellet - C^{14} uridyl compounds			
HR cells	7,315,710	8,464,[illegible]	+ 16%
C cells	37,[illegible]	37,[illegible]	+ 9%
11,000 g pellet - C^{14} amino acyl compounds			
HR cells	9,721,[illegible]	11,[illegible]	+ 14%
C cells	11,767,[illegible]	13,[illegible]	+ 16%

II. I. Estrogen

MR cells, but not G cells, incubated in $7X10^{-8}$M 17β-estradiol respond with a quick increase in the 12,000 g pellet and poly-adenylated cytoplasmic RNA. The effect on mRNA peaks at 90 minutes and is gone in $2^1/2$ hours (Table 8). This hormone seems to have no effect on the other fractions of RNA in either cell type.

Sucrose profiles (Figure 11 and 12) reveal that the increase in mRNA is localized in a peak at about 7S.

No effect was seen from 17β-estradiol or SCC of Bufo marinus paired hemibladders in incubations of up to 15 hours (Table 9).

Table 8 The Effects of 17β Estradiol (7×10^{-8}M) upon Labeling of RNA

Time of Incubation (min)	Control (dpm)	17β Estradiol (dpm)	(% change)	
Poly(A)-Rich RNA				
MR cells				
60'	2,320	3,400	+ 47%	
90'	4,470	11,490	+157%	
150'	4,400	4,000	- 9%	
150'	11,200	20,560	+ 84%	+ 38%
G cells				
60'	6,000	6,880	+ 15%	
150'	3,920	3,680	- 6%	
150'	11,600	9,760	- 16%	- 11%
High Salt Wash				
MR cells				
60'	147,000	152,000	+ 3%	
90'	2,537,280	1,856,880	- 27%	
150'	1,060,800	1,075,200	+ 1%	
150'	567,120	495,600	- 13%	- 6%
G cells				
60'	610,000	506,000	- 17%	
150'	855,120	746,160	- 12%	
tRNA + rRNA				
MR cells				
60'	19,680	18,400	- 7%	
90'	395,150	810,200	+105%	
150'	318,640	315,200	- 1%	
G cells				
60'	44,680	38,080	- 14%	
150'	283,000	227,720	- 20%	
12,000 g pellet				
MR cells				
20'	330,200	373,260	+ 13%	
60'	74,650	88,700	+ 19%	
90'	696,310	886,970	+ 27%	
150'	49,100	66,000	+ 34%	
	134,240	154,400	+ 15%	+ 25%
G cells				
20'	222,920	190,520	- 15%	
60'	225,920	225,270	0%	
90'	4,578,130	5,693,760	+ 24%	
150'	31,200	20,400	- 35%	
	77,200	67,130	- 13%	- 24%

Table 9 The effect of 17B estradiol on the Short Circuit Current of paired hemibladders from toads immersed in 0.6% NaCl for 3 days or longer.

Time	Treatment	Relative Change in SCC Value Relative to Initial	N	Paired t Test significance
		Mean ± S.E.M.		
90'	Estradiol	- 8% ± 6.1%	6	N.S.
	Control	- 5% ± 5.7%	6	
150'	E_2	- 17% ± 6.4%	13	0.10 P 0.05
	C	- 1% ± 5.2%	13	
180'	E_2	- 17% ± 6.5%	13	N.S.
	C	- 6% ± 8.6%	13	
855'	E_2	- 30% ± 9.1%	7	0.20 P 0.10
	C	- 54% ± 11%	&	

III. Insulin's Effects on the Toad Bladder

A. RNA Metabolism and Insulin

1. Mitochondria-Rich Mucosal Cells

Insulin was added, at doses of 50, 250 and 500 mU/ml, to separated cell fractions and incubated for periods of 45 minutes to three hours. Although both MR and G cells responded with increases in incorporation of H^3-uridine into all fractions of RNA examined, the extent and time course of insulin's effects on MR cells were qualitatively different from its effects on G cells.

After 90 minutes of 50 mU/ml insulin (Table 10), incorporation of H^3-uridine into poly A-containing, cytoplasmic RNA was depressed in MR cells but the sucrose gradient profile of the mRNA (Fig. 10) reveals a peak of 4S to 12S mRNA induced by insulin. Incorporation of H^3-uridine into the 12,000g pellet is increased by 23% at this time period (Table 11). While little change is induced by insulin in the high salt wash, incorporation of labeled uridine into the ethanol precipitate of the high salt wash (Table 12), which includes tRNA and rRNA, is increased by this dose of insulin.

As the levels of insulin were increased, there were progressively greater increases in H^3-uridine incorporation into mRNA in MR cells (Table 10; Figs. 22,24, and 25). At 250 mU/ml, the position of the peak in figures 22 and 25 indicates a smaller S value than the experiment illustrated in figure 24 and figure 10. In the latter studies, pronase was added to the post-mitochondrial supernatant fraction to digest RNases, but in the former experiments pronase was not used because the incorporation of C^{14}-amino acids into proteins was also examined. The apparent smaller size of the control and insulin-

induced mRNA peaks in figures 22 and 25 indicate their partial degradation by endogenous RNases. The incorporation of C^{14}-amino acids in these experiments indicates that the poly-A-containing RNA has a small amount of labeled protein associated with it. The low levels of C^{14}-amino acids in this fraction indicates also that the C^{14}-acyl-tRNA or C^{14}-ribosomes contamination of the mRNA fraction is negligible. Insulin-induced increases labelling, parallel in both time course and magnitude, are found at this dose in the incorporation of both labels, H^3-uridine and C^{14}-amino acids, into the 12,000g pellet (Table 11), the high salt wash (Table 12), the ribosomes pelleted from the high salt wash (Table 14), and the tRNA and proteins of the high salt wash (Table 15).

Increasing the insulin levels to 500 mU/ml results in an increase in the incorporation of H^3-uridine into mRNA at 45 minutes (Table 10), followed by an inhibition at later time periods.The sucrose gradient profile of a 45 minute incubation shows peaks of insulin-induced mRNA (Fig. 23) which sediments at approximately 5S and 7-12S. The sucrose gradient profile of an incubation performed for 90 minutes shows the insulin-stimulated band of mRNA to be quite broad (Fig. 24) as compared to other studies. This may be due to the formation of mRNA aggregates in this sample.

Insulin, at 500 mU/ml, increases the uptake of H^3-uridine and C^{14}-amino acids into all MR cell fractions tested at all incubation times with the following exceptions: total H^3-dpm in mRNA at 120 minutes (Table 10); H^3-uridine into 12,000g pellet at 45 minutes (Table 11); and amino acid uptake into ribosomes at 45 minutes. The other MR cell fractions incubated with insulin showed much greater uptake than control

tissues, but the steady increase in uptake of label with time was not found at this dose level.

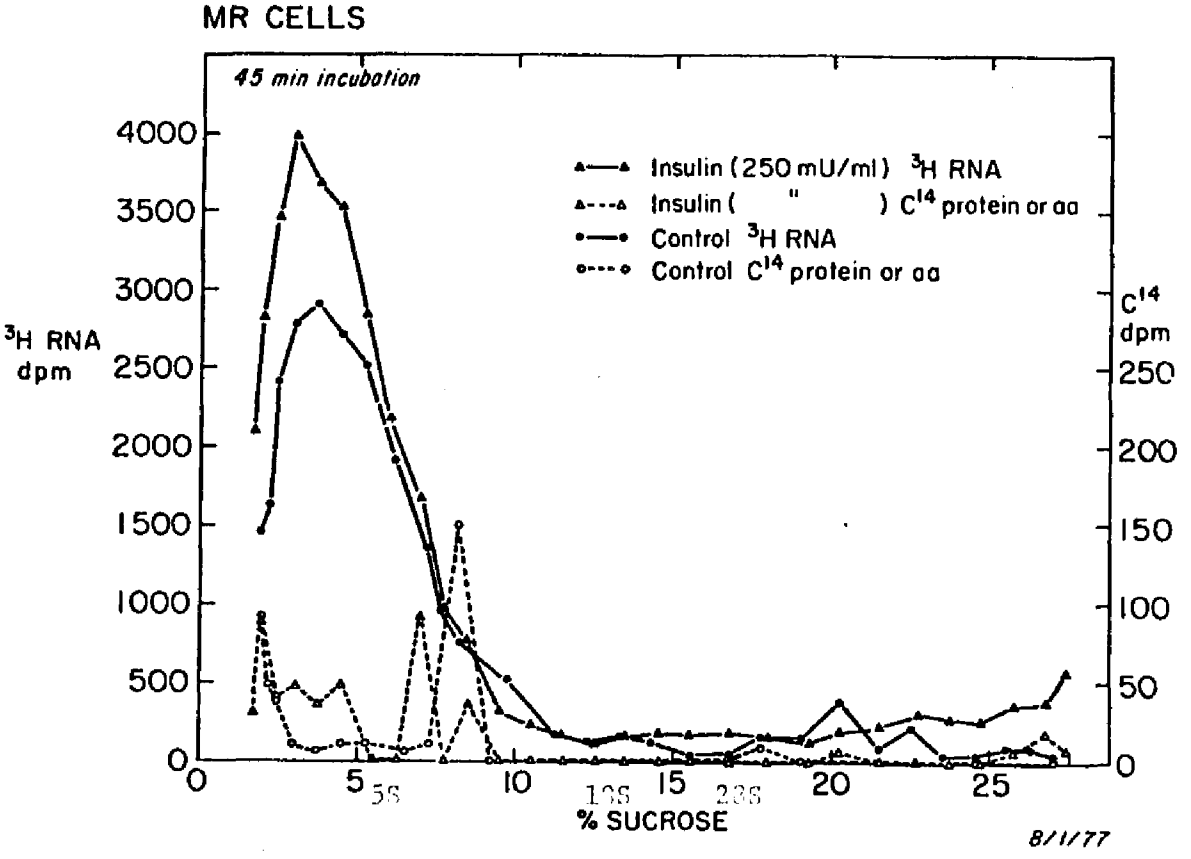
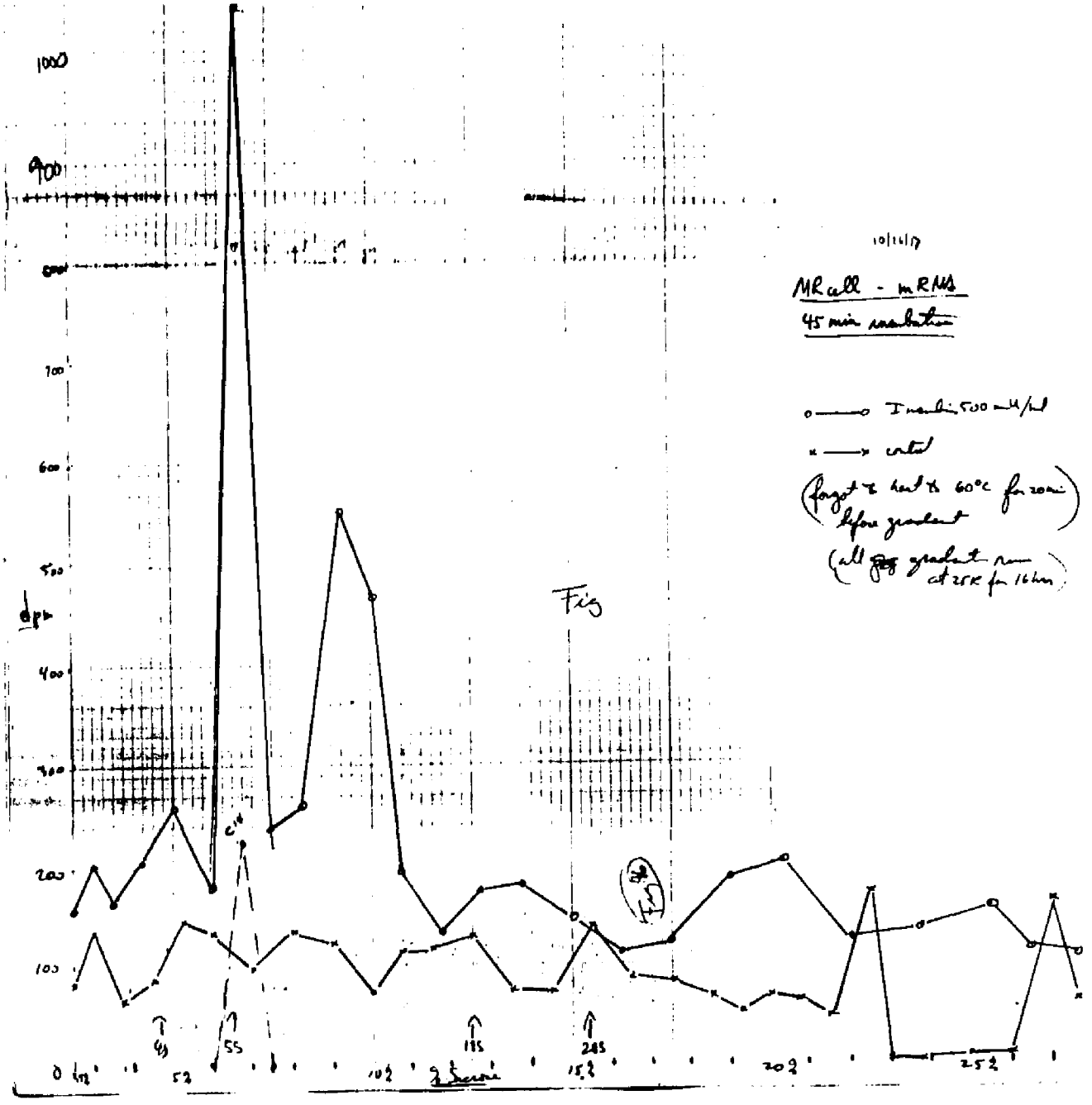


Fig. 22 Sucrose gradient profiles of cytoplasmic, poly-A containing RNA from separated MR cells. The cells were incubated in Ca^{++} Ringers containing $H^3(5,6)$ uridine, C^{14} amino acid mixture and either 250 mU/ml insulin or diluent. The RNA was isolated and chromatographed as described in Methods, sec. III.

Fig. 23 Sucrose gradient profiles of cytoplasmic, poly-A containing RNA from seperated MR cells. The cells were incubated in Ca-Ringers containing H^3(5,6) uridine, C^{14} amino acid mixture and either 500 mU/ml insulin or diluent. The RNA was isolated and chromatographed as described in Methods, sec. III.



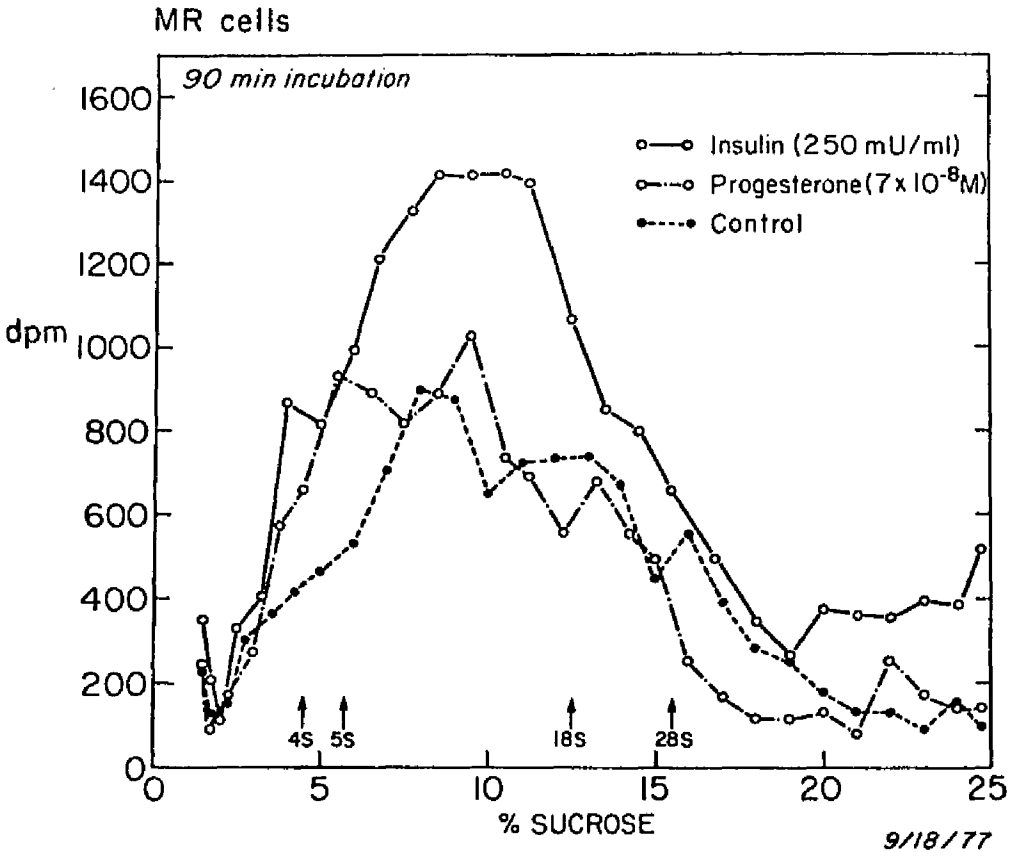


Fig. 24 Sucrose density profiles of cytoplasmic, poly A-containing RNA from separated MR cells. The cells were incubated for 90 minutes in Ca^{++} Ringers containing H^3(5,6) uridine and either 7 x 10^{-8}M progesterone, 250 mU/ml insulin, or diluent. The RNA was isolated and chromatographed as outlined in Methods, sec. II.

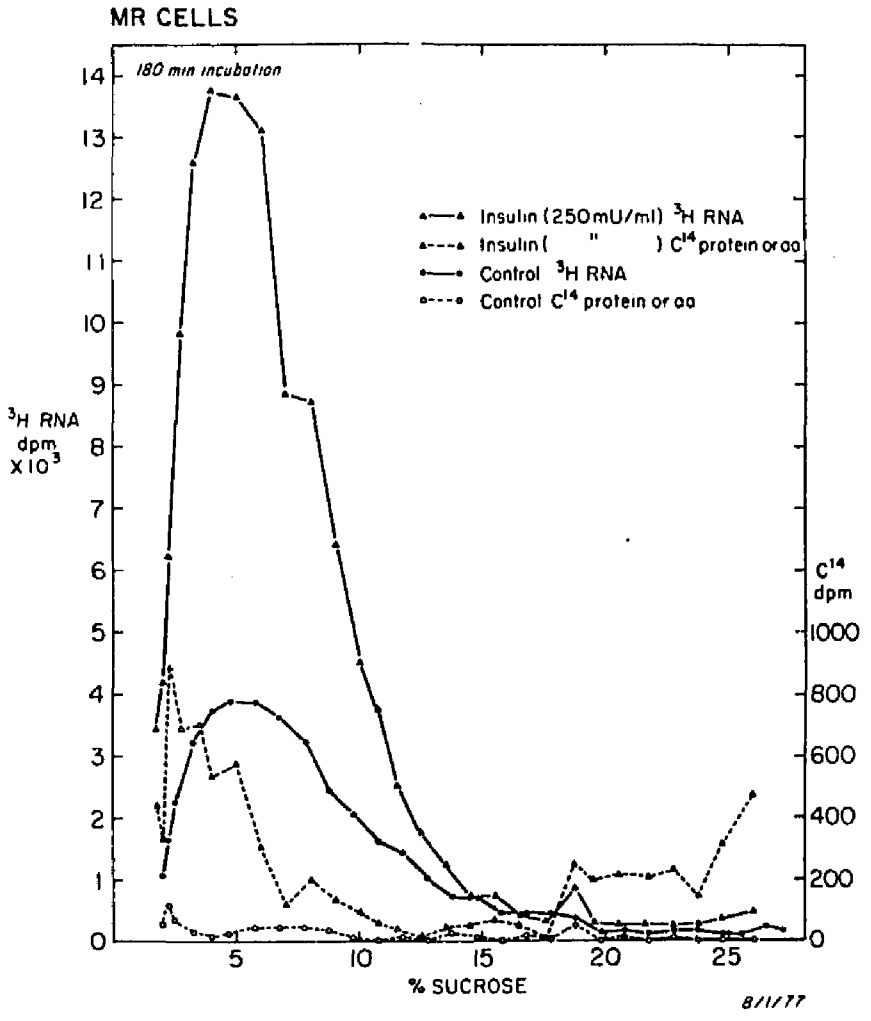


Fig. 25 Sucrose gradient profiles of cytoplasmic, poly-A containing RNA from separated MR cells. The cells were incubated in Ca^{++} Ringers containing H^3(5,6) uridine, C^{14}-amino acid mixture and either 250mU/ml insulin or diluent for 180 minutes. The RNA was isolated and chromatographed as described in Methods, sec. III.

2. RNA Metabolism and Insulin in G Cells

Insulin's effects on the metabolism of G cell RNA were rapid in onset and declined to baseline levels within 2 hours. The sucrose gradient profiles of G cell mRNA prepared after incubation of the mucosal cells for 45 minutes is presented in figure 26. The preparations show a large increase from control values. Figures 27 and 28 illustrate mRNA profiles from three separate experiments in which G cells were incubated in baths containing various levels of insulin. Insulin at 250 mU/ml (Figs. 27 and 28) resulted in a moderate increase of the labeling of the mRNA after 90 minutes but less than the same dose at 45 minutes (Fig. 26). The 500 mU/ml insulin dose had an effect similar to the 250 mU/ml dose on the mRNA profile at 90 minutes. H^3-uridine incorporation into the G cell total mRNA fraction (Table 10) reflects the results illustrated in the sucrose gradient profiles. At 4S and 90 minutes, insulin induces a large increase in H^3-uridine uptake into mRNA. Thereafter, the uptake of H^3-uridine into mRNA in insulin-treated G cells drops to or below control levels. The uptake of C^{14}-amino acids into the various protein fractions studied behaves similarly (Tables 11-15).

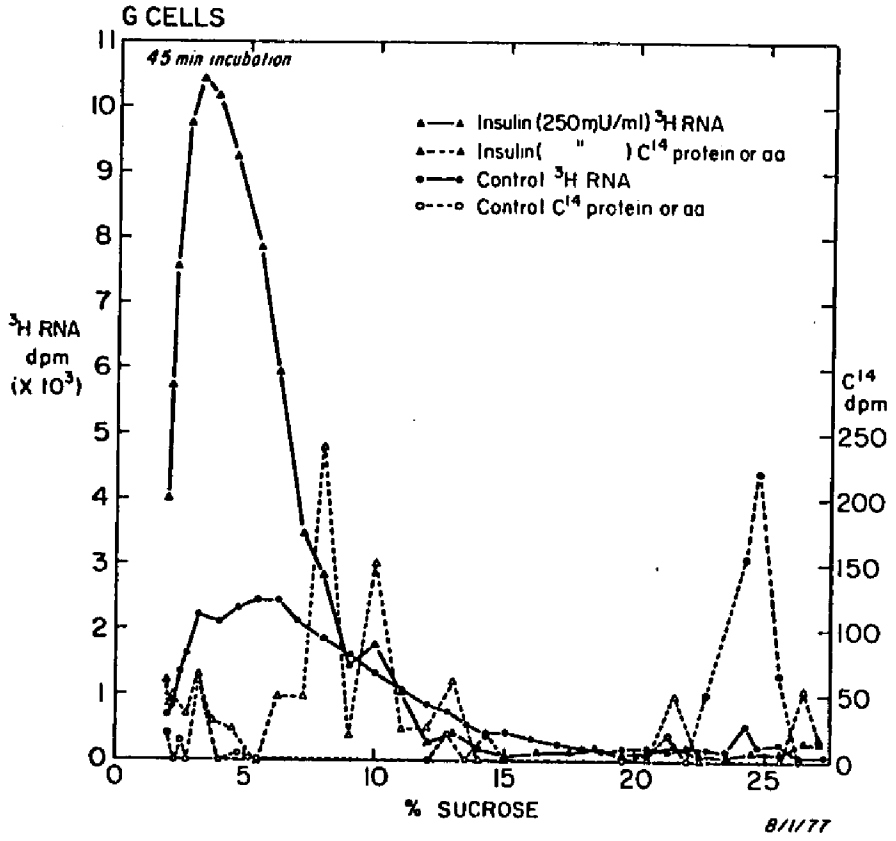


Fig. 26 Sucrose gradient profiles of cytoplasmic, poly-A containing RNA from separated G cells. The cells were incubated in Ca^{++} Ringers containing H^3(5,6) uridine, C^{14} amino acid mixture, and either 250mU/ml insulin, or diluent for 45 minutes. The RNA was isolated and chromatographed as described in Methods, sec. III.

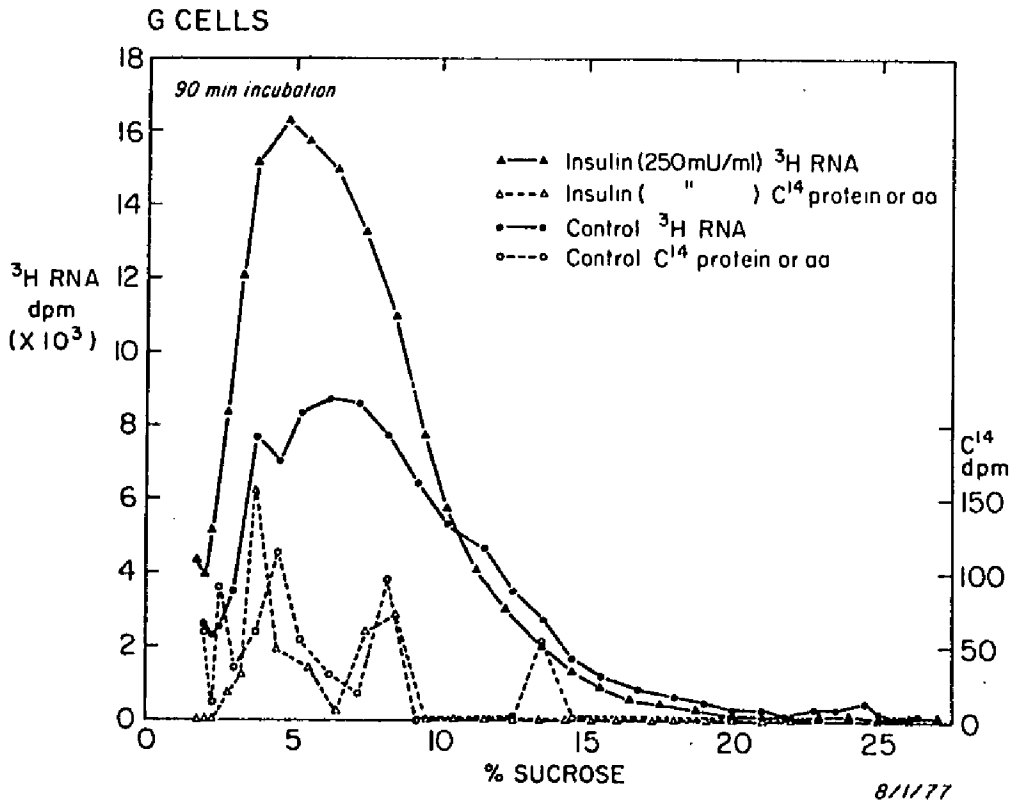


Fig. 27 Sucrose gradient profiles of cytoplasmic, poly-A containing RNA from separated G cells. The cells were incubated in Ca^{++} Ringers containing H^3(5,6) uridine, C^{14} amino acid mixture, and either 250mU/ml insulin or diluent for 90 minutes. The RNA was isolated and chromatographed as described in Methods, sec. III.

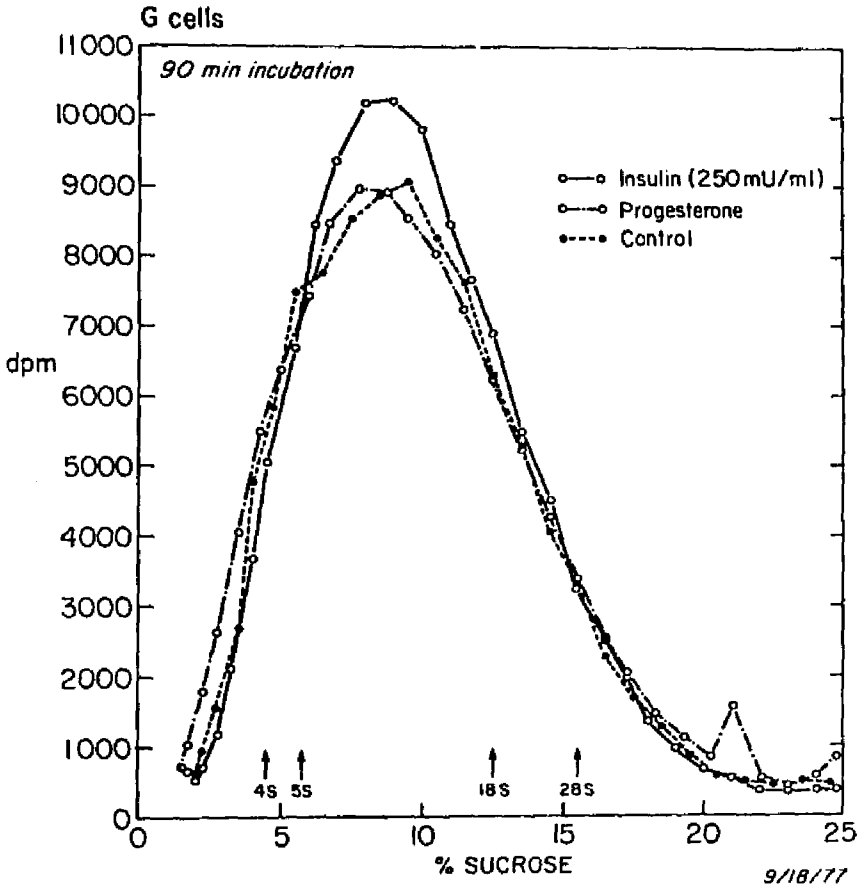


Fig. 28 Sucrose density profiles of cytoplasmic, poly A-containing RNA from separated G cells. The cells were incubated for 90 minutes in Ca^{++} Ringers containing H^3(5,6) uridine and either 7×10^{-8}M progesterone, 250 mU/ml insulin, or diluent. The RNA was isolated and chromatographed as outlined in Methods, sec. II.

TABLE 10 mRNA - Insulin Effects

	Dose (mU/ml)	Control (dpm)	Insulin (dpm)	% Change
MR Cells				
45 min.	250	24,885	37,056	+ 49%
	500	3,086	6,910	+124%
90 min	50	3,086	6,910	- 45%
	250	12,071	21,967	+ 82%
	500	21,274	33,540	+ 56%
120 min	500	43,240	30,030	- 31%
180 min	250	48,060	141,375	+194%
G Cells				
45 min	250	31,890	86,145	+170%
	500	25,314	38,595	+ 52%
90 min	50	52,688	58,279	+ 11%
	250	98,940	155,595	+ 57%
	250	103,788	117,005	+ 13%
	500	56,132	38,908	- 31%
	500	135,570	149,880	+ 11%
180 min	250	2,932	6,181	+111%
	250	12,480	19,194	+ 54%

TABLE 11 12,000g Pellet - Insulin Effects

	Dose (mU/ml)	H^3-RNA (dpm)	% Change from Control	C^{14}Protein (dpm)	% Change from Control
MR Cells					
45 min	250	3,241,150	+ 64%	1,236,770	+ 60%
	500	1,062,100	- 14%	409,685	+ 88%
90 min					
	50	1,127,490	+ 23%	-	-
	250	11,044,460	+ 51%	14,171,110	+ 46%
	500	8,933,220	+100%	1,759,105	+ 69%
120 min	500	4,092,790	+ 79%	-	-
180 min	250	5,216,130	+181%	6,531,570	+208%
G Cells					
45 min	250	4,072,200	+155%	600,050	+107%
	500	7,689,165	+ 82%	1,694,365	+ 21%
90 min	50	11,451,710	+ 12%	-	-
	250	31,450,340	+ 24%	14,956,500	+ 27%
	250	7,261,090	+ 86%	724,470	+ 57%
	500	7,300,070	+ 20%	-	-
	500	22,726,580	+ 26%	3,920,830	- 19%
180 min	250	116,589	+18%	-	-
	250	1,236,833	+52%	-	-

TABLE 12 High Salt Wash

Insulin Effects

	Dose (mU/ml)	H^3-dpm	% Change from Control	C^{14}-dpm	% Change from Control
MR Cells					
45 min	250	5,447,640	+ 71%	1,313,160	+ 46%
	500	3,528,480	+ 58%	291,120	+ 47%
90 min	50	489,960	+ 1%	-	-
	250	8,667,110	+ 17%	-	-
	500	11,791,680	+ 59%	888,240	+ 33%
120 min	500	4,930,800	+ 15%	-	-
180 min	250	20,165,880	+224%	7,367,760	+225%
G Cells					
45 min	250	13,168,560	+ 78%	1,725,720	+ 56%
	500	16,785,600	+ 40%	913,680	+ 21%
90 min	50	3,411,360	+ 12%	-	-
	250	22,492,770	+ 5%	-	-
	250	17,294,400	+ 34%	2,090,880	+ 22%
	500	10,848,000	+ 41%	-	-
	500	27,305,040	+ 14%	2,451,120	+ 29%
180 min	250	5,164,600	+ 5%	-	-
	250	10,809,120	+74%	-	-

TABLE 13 Insulin - tRNA + rRNA

Dose (mU/ml)	Time	Control (dpm)	Insulin (dpm)	(% Change)
MR Cells				
500	90'	417,500	1,025,640	+146%
50	90'	188,880	221,240	+ 17%
50	120'	78,850	111,850	+ 42%
G Cells				
500	90'	1,486,880	2,673,120	+ 80%
50	90'	1,934,200	2,899,640	+ 50%
50	120'	763,515	812,560	+ 6%

TABLE 14 Ribosomes - Pelleted from High Salt Wash in 40 Rotor

Time	Insulin Dose (mU/ml)	H^3-rRNA (dpm)	% Change	C^{14} Protein (dpm)	% Change
MR Cells					
45'	250	1,522,000	+5%	101,000	-3%
	none	1,447,000	-	104,000	-
	500	40,696	+63%	3,012	+3%
	none	24,957	-	2,913	-
90'	500	281,482	+54%	16,121	+48%
	none	183,288	-	10,866	-
180'	250	16,848,850	+281%	1,766,940	+272%
	none	4,426,085	-	479,330	-
G Cells					
45'	250	2,399,000	+141%	190,400	+141%
	none	994,750	-	78,790	-
	500	170,983	+96%	11,243	+240%
	none	87,438	-	3,299	-
90'	250	3,856,100	+51%	343,150	+49%
	none	2,539,285	-	168,145	-

TABLE 15 TCA ppt of High Salt (After Ribosomes Pelleted)

	Time	Insulin Dose (mU/ml)	H^3 tRNA (dpm)	% Change	C^{14} Protein (dpm)	% Change
MR Cells						
	45'	500	30,365	+ 111%	102,625	+ 13%
		none	14,415	-	90,920	-
	90'	500	130,085	+ 59%	443,335	+ 51%
		none	81,770	-	292,570	-
	180'	250	2,973,150	498%	4,736,780	+269%
		none	497,100	-	1,282,060	-
G Cells						
	45'	250	115,830	+ 68%	351,820	+ 66%
		none	68,750		211,720	-
		500	92,280	+ 7%	710,510	+ 72%
		none	86,130		411,960	
	90'	500	320,085	+ 20%	1,714,910	+ 60%
		none	266,175		1,069,775	-

3. Effects of Insulin on the Incorporation of H^3-Uridine into RNA and C^{14}-Amino Acids into Protein

Preparations of MR cells and G cells were incubated with H^3-uridine and C^{14}-amino acids. One set of cells was used as a control and a paired set was treated with insulin (250 mU/ml or 100 mU/ml). Aliquots of cells were removed after 30,60,120, and 240 minutes and chilled. The cells were washed and disrupted in the Parr bomb. An aliquot was taken for total protein (Lowry) determination, and a 12,000g pellet and post-mitochondrial supernate (cytosol) were prepared. Protein and RNA were precipitated from each with TCA (10% w/v final concentration). The supernatant fraction of the TCA-treated post-mitochondrial supernate was used to obtain an estimate of the H^3-uridine and C^{14}-amino acid pools. The aliquot for total protein was precipitated with TCA and the pellet dissolved in 1% NaOH and Lowry protein determinations were performed in triplicate and all results were normalized to µg protein. Each G cell fraction contained four to five times as many cells as MR cell fractions as reflected in the values for total protein in a typical experiment: MR cells, 90.3 $\pm$ 9.8 µg protein; G cells, 449,1 $\pm$ 27.9 µg protein.

Uptake of C^{14}-amino acids into the cytosol fraction (Figs. 29, 35,36) plateaued after 60 minutes regardless of treatment. Insulin increased the uptake of C^{14}-amino acids in both cell types in a dose-related manner, 250 mU/ml (Fig. 29) having a greater effect than 100 mU/ml (Figs. 35,36). The uptake of H^3-uridine into the cytosol fraction (Figs. 30,37,38) was linear over 3 hours in MR and G cells given 250 mU/ml insulin, but tended to plateau in cells given 100 mU/ml insulin and in control cells. Insulin stimulated the uptake of H^3-uridine

2-3 fold at 250 mU/ml and 25-50% at 10 mU/ml in both cell types. Incorporation of H^3-uridine into every fraction of RNA measured is increased by insulin in a dose-related fashion (Figs. 32,34,40,42). Incorporation of C^{14}amino acids into all fractions of protein studied was also increased by insulin in a dose related way (Figs. 41,43,49, 51).

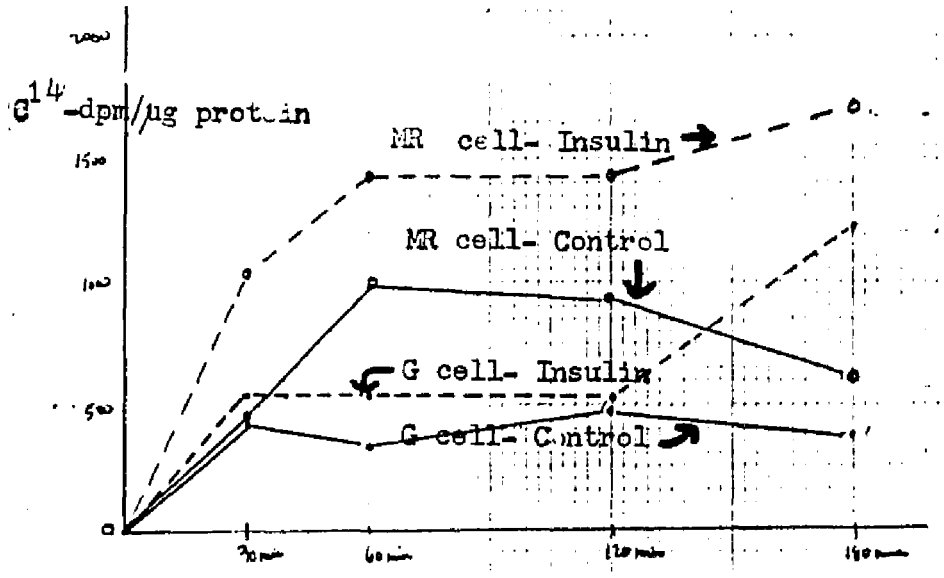


Fig. 29 Seperated cell uptake experiment #1 - C^{14}-amino acid pool of post-mitochondrial supernate (from supernatant of TCA precipitate).

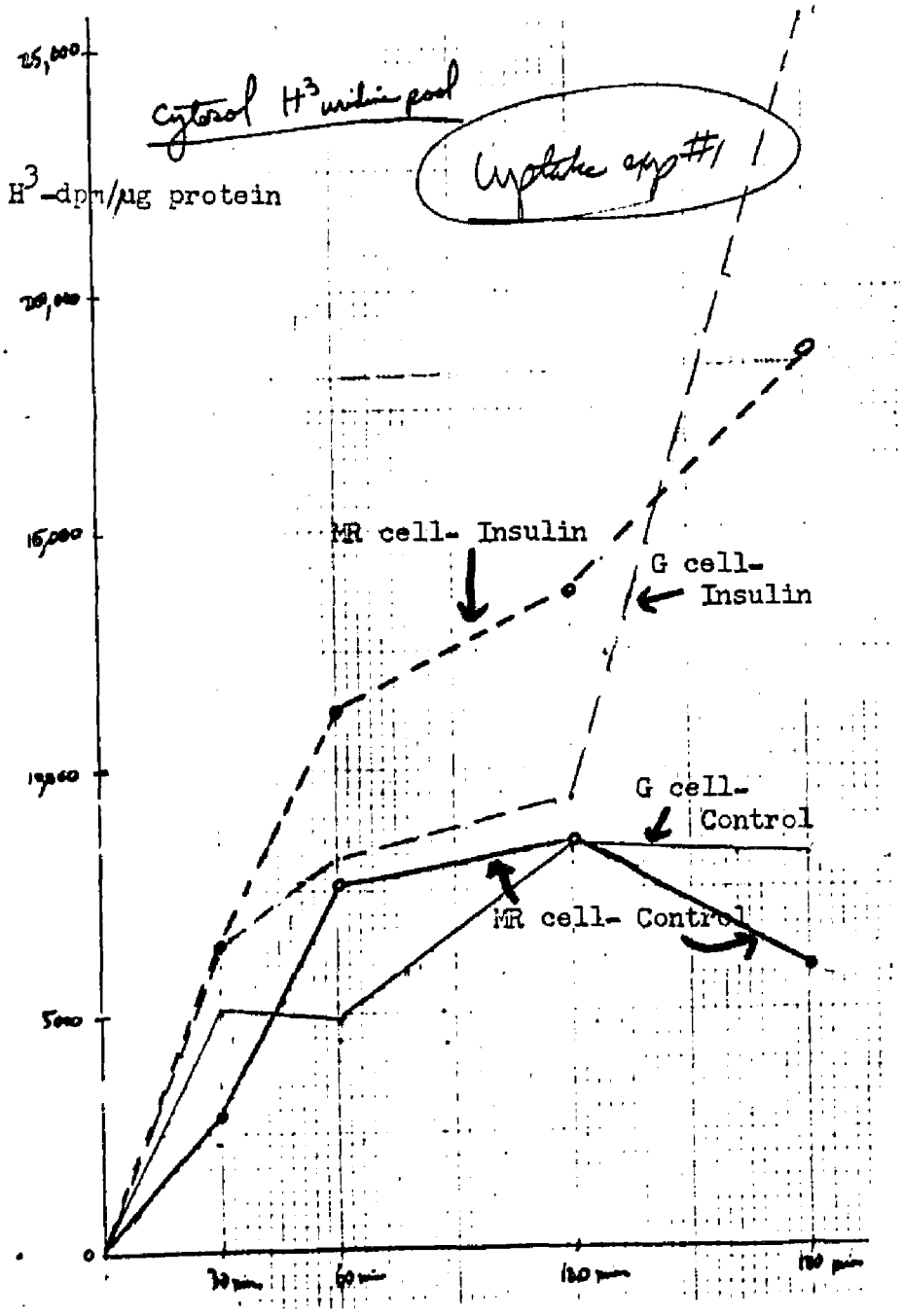


Fig. 30 Seperated cell uptake experiment #1 - H^3-uridine pool of post-mitochondrial supernate (from supernatant of TCA precipitate).

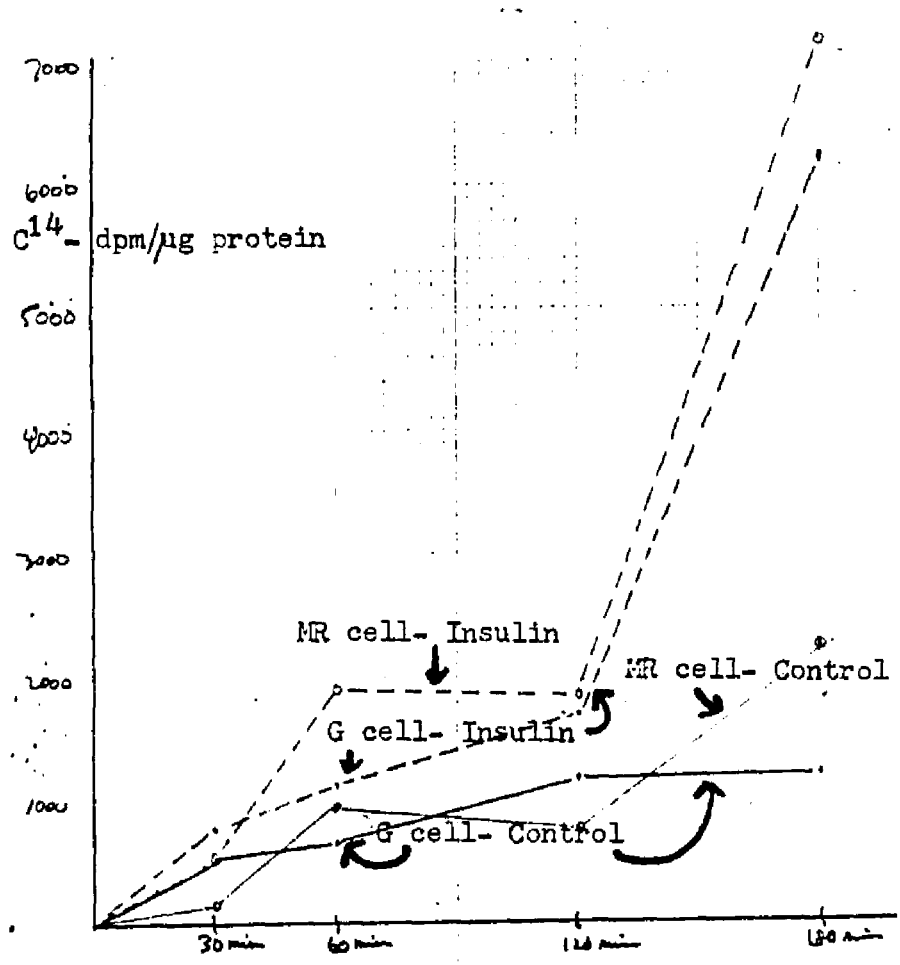


Fig. 31 Seperated cell experiment #1- Incorporation of C^{14}-amino acid into protein in post mitochondrial supernatant.

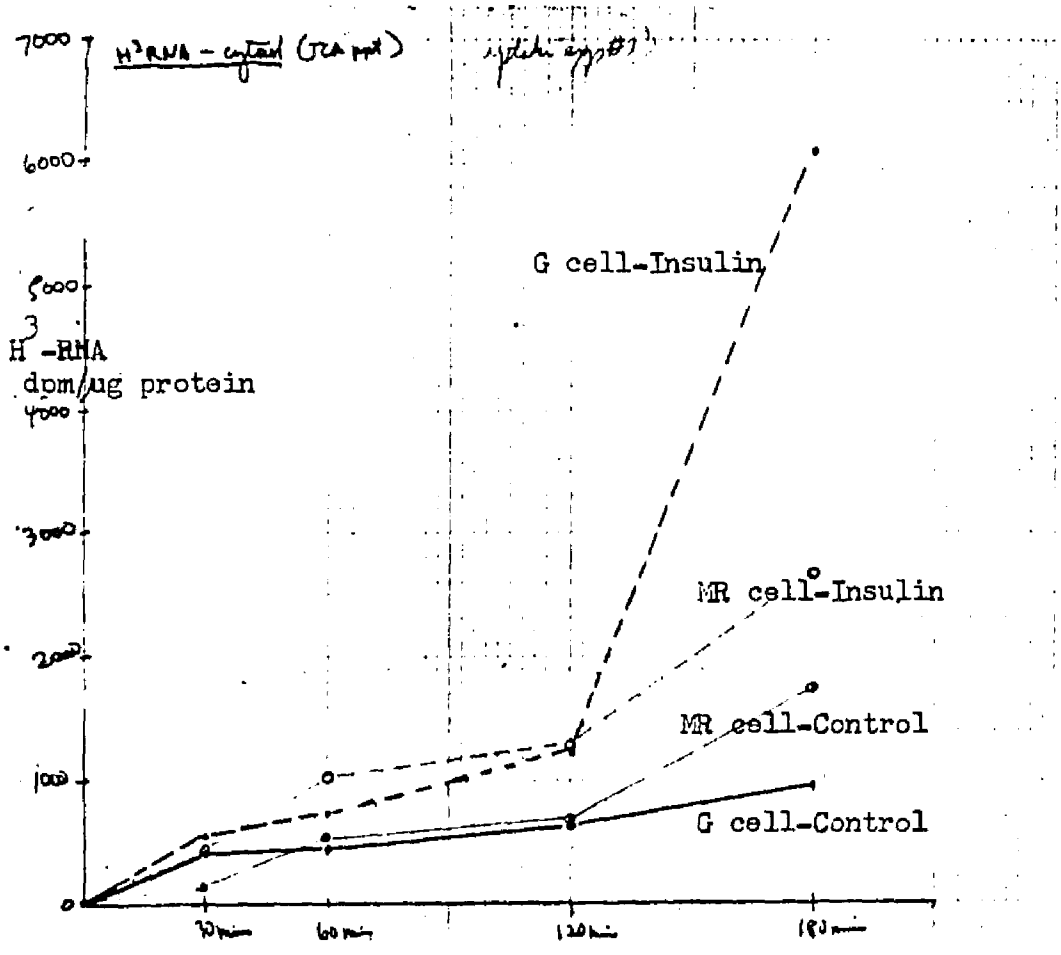


Fig. 32 Seperated cell experiment #1 - Incorporation of H^3-uridine into RNA of post-mitochondrial supernate.

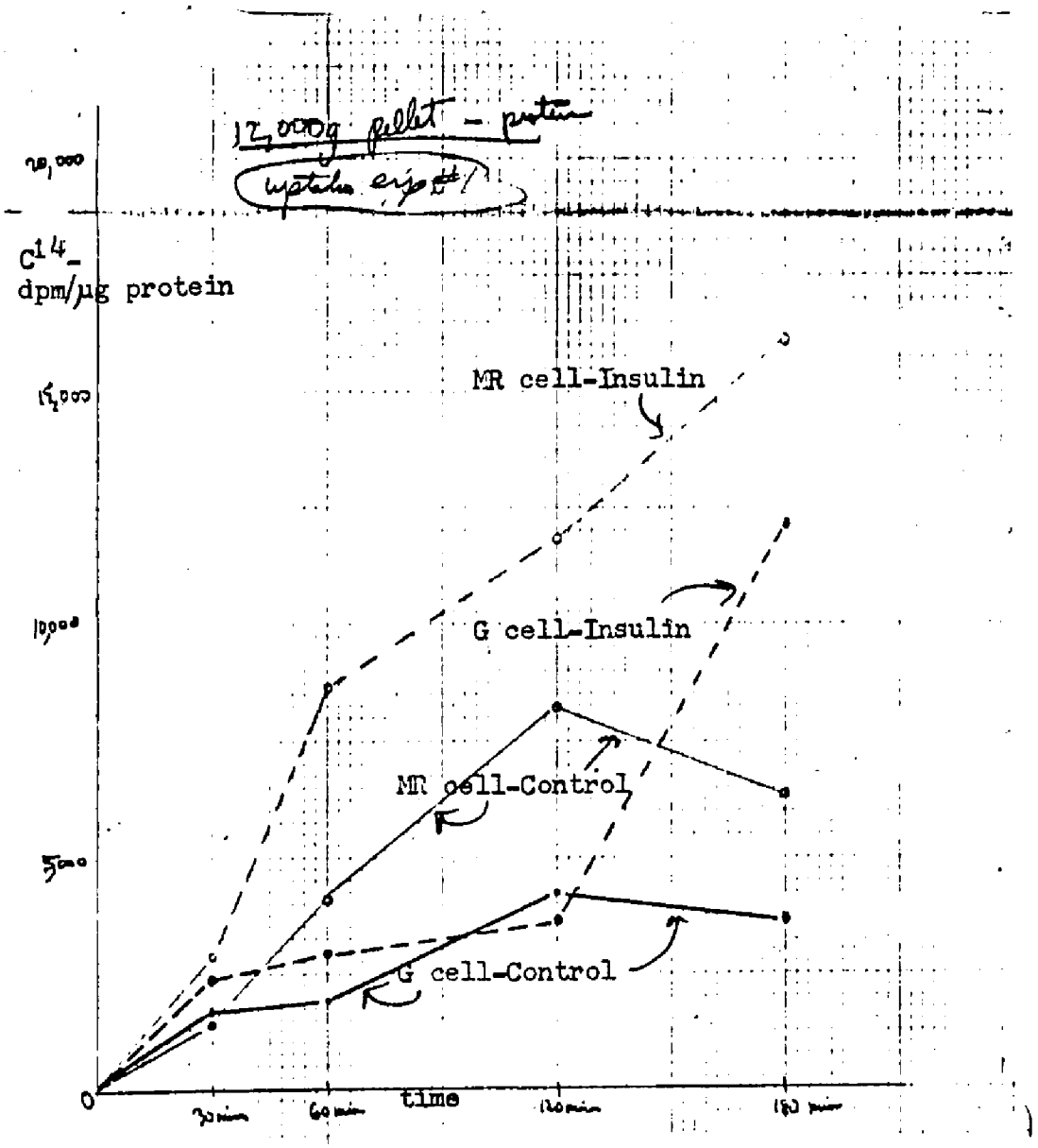


Fig. 33 Seperated cell experiment #1 - Incorporation of C^{14}-amino acids into the 12,000g pellet.

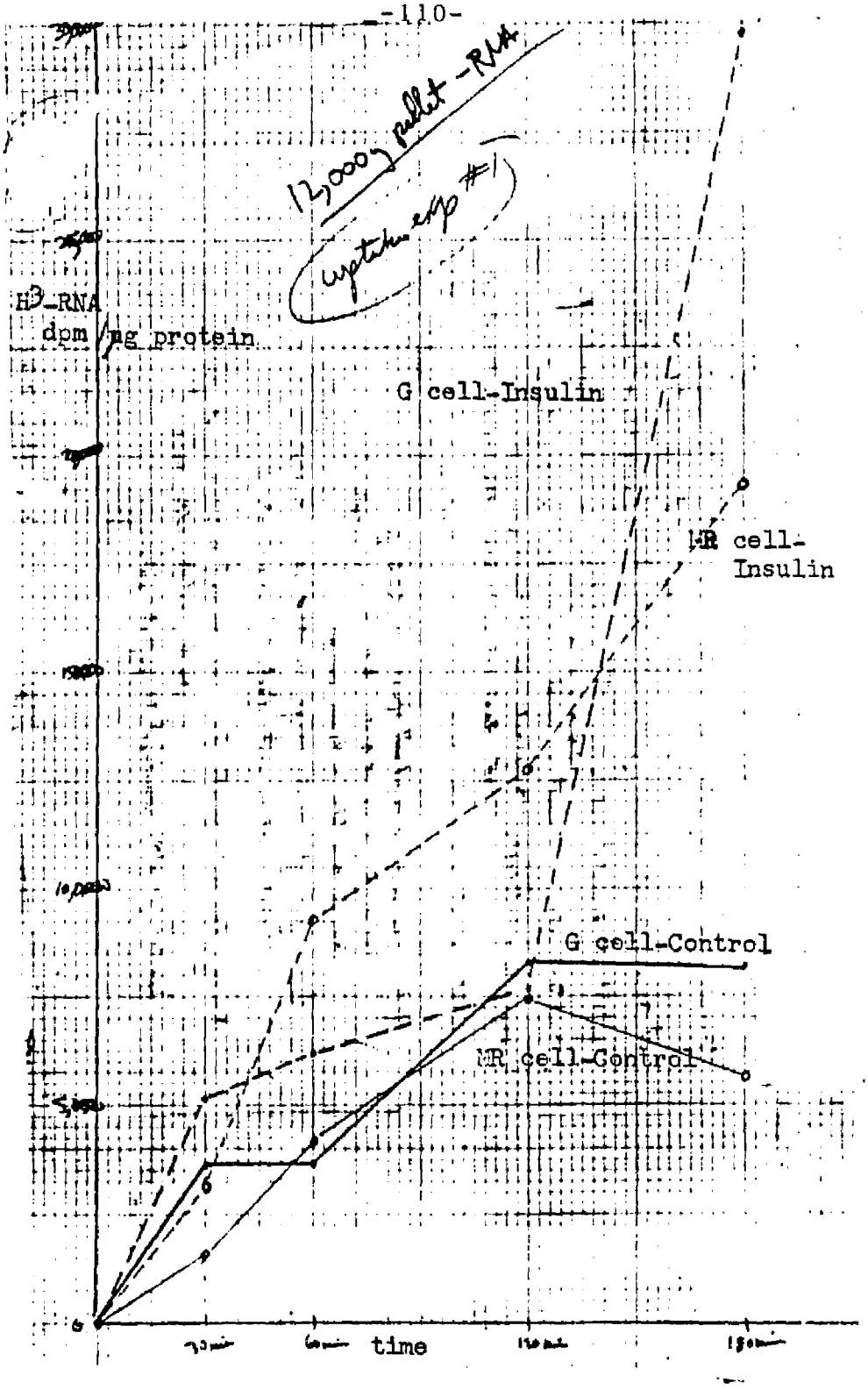


Fig. 34 Separated cell experiment #1 - Incorporation of H^3-uridine into RNA in the 12,000g pellet.(washed with 10%TCA and 100% EtOH).

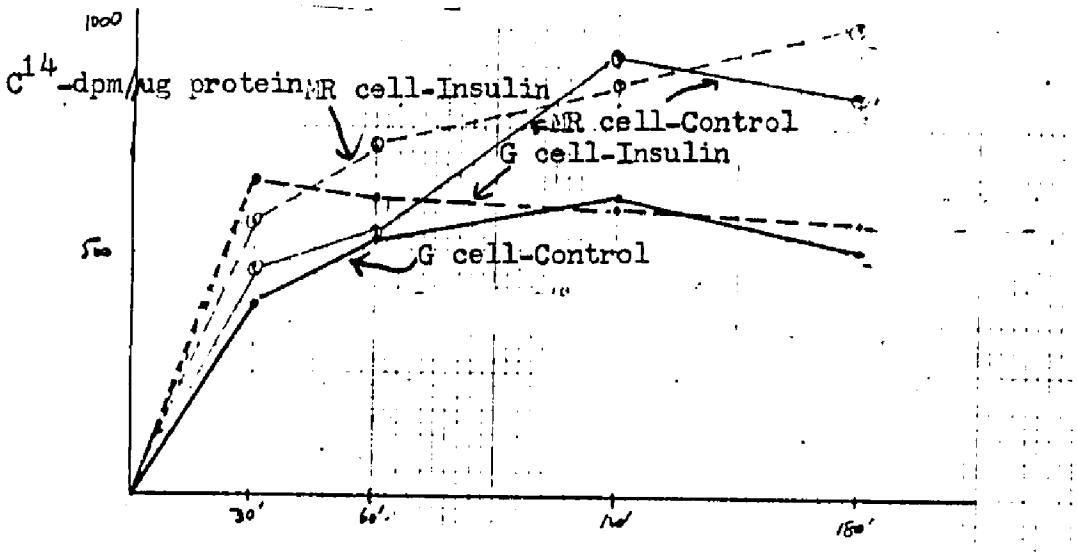


Fig. 35 Separated cell uptake experiment #2 - Uptake of C^{14}-amino acids into post-mitochondrial supernatant (from supernatant of TCA precipitate)

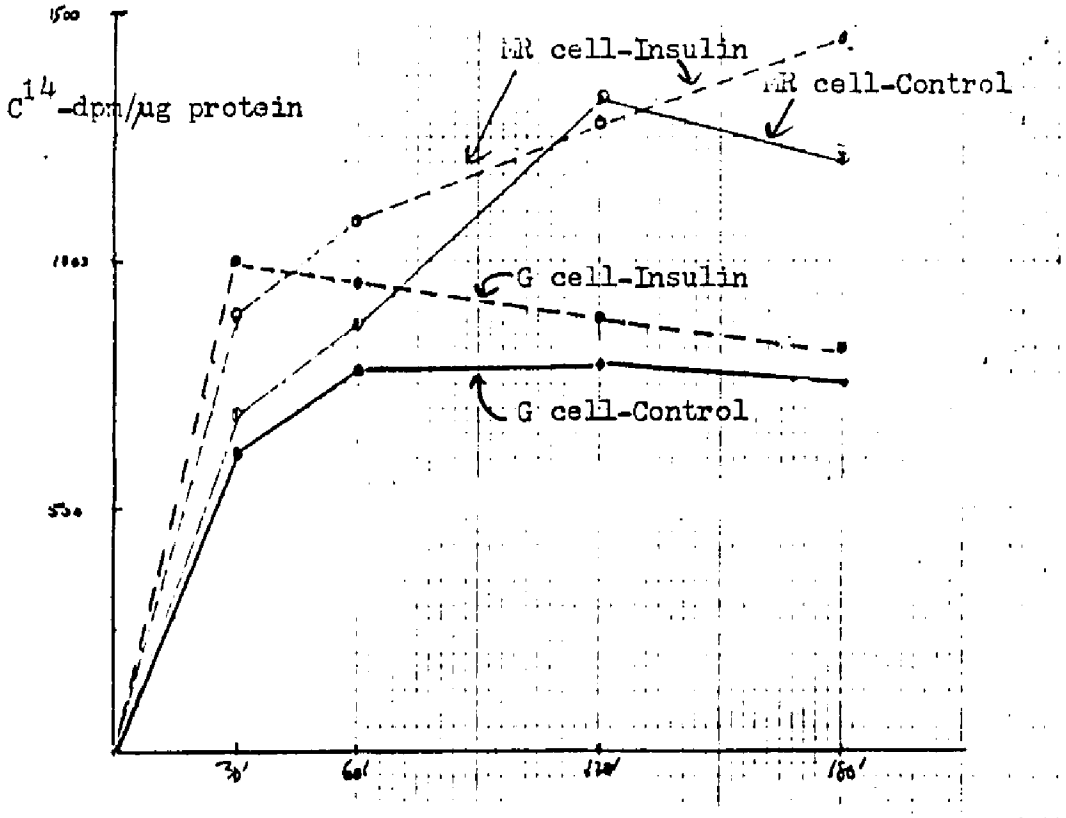


Fig. 36 Seperated cell uptake experiment #2 - Uptake of C^{14}-amino acids into whole cell (from Lowry aliquots after TCA precipitation)

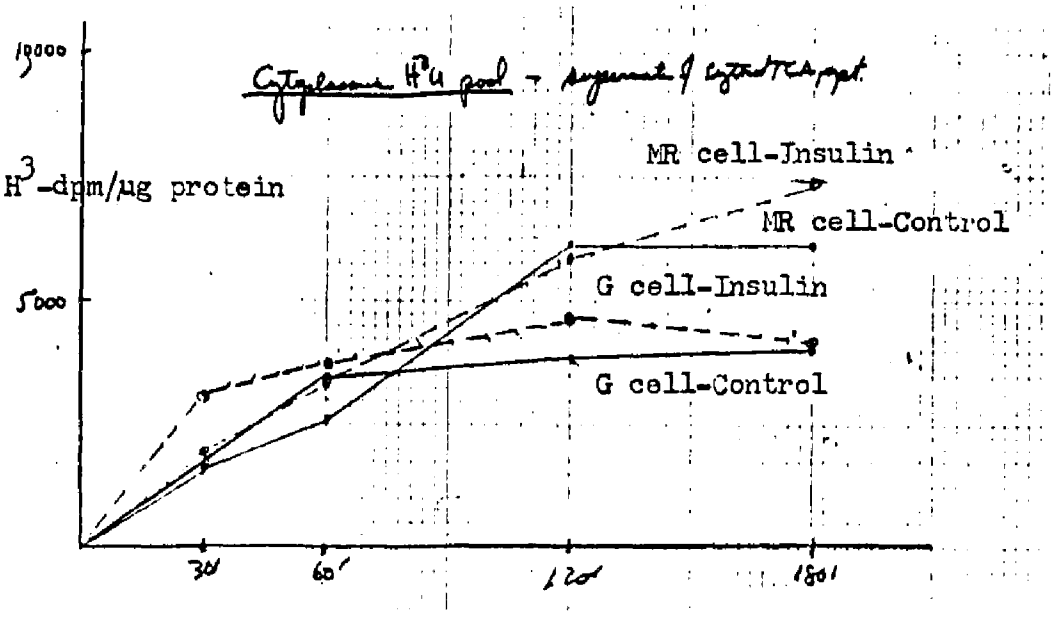


Fig. 37 Separated cell experiment #2- Incorporation of H^3-uridine into post-mitochondrial supernatant (from supernatant of TCA precipitate) - an approximation of cytoplasmic H^3-uridine pool size.

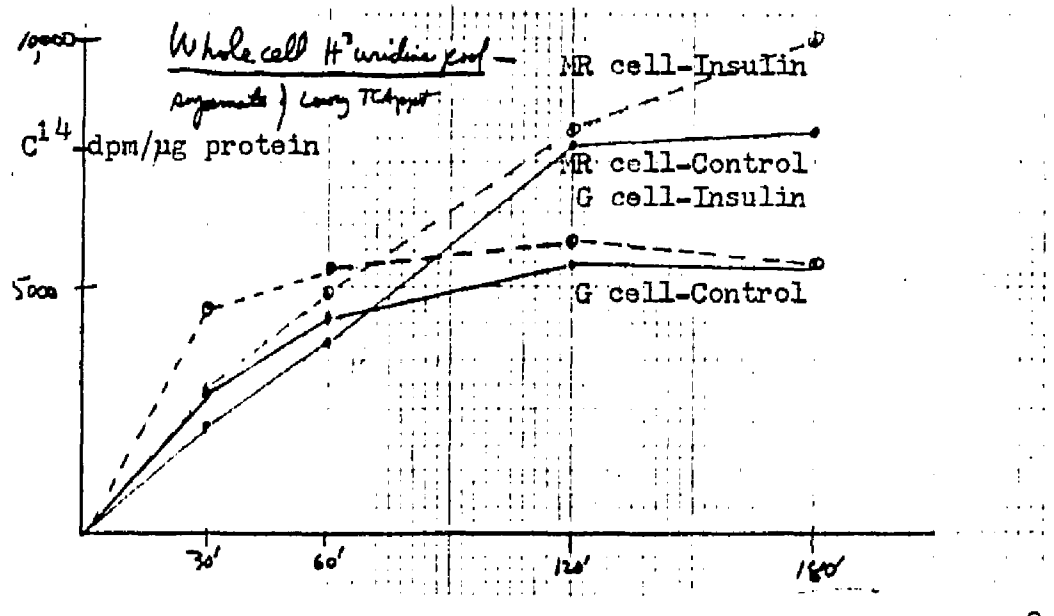


Fig. 38 Separated cell experiment #2 - Incorporation of H^3-uridine into whole cell (from Lowry aliquot) - an approximation of whole cell H^3-uridine pool size.

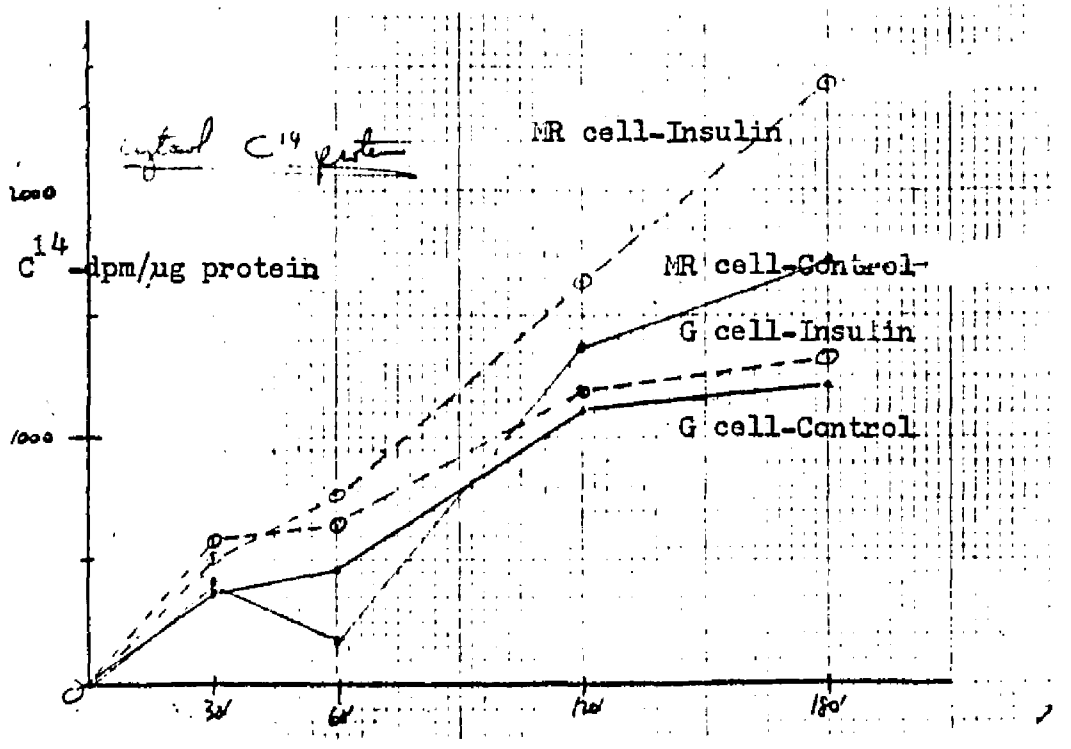


Fig. 39 Separated cell experiment #2 - Incorporation of C^{14}-amino acids into proteins of post-mitochondrial supernatant (from pellet of TCA precipitate).

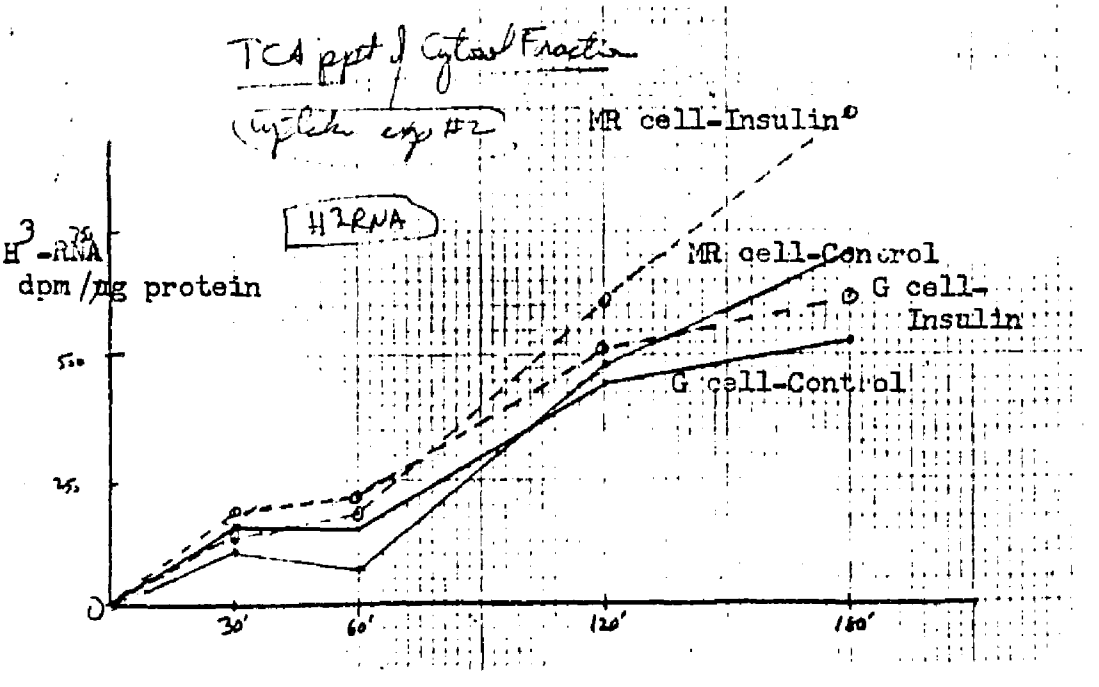


Fig. 40 Separated cell experiment #2 - Incorporation of H^3-uridine into RNA in post mitochondrial supernatant (from pellet of TCA precipitate).

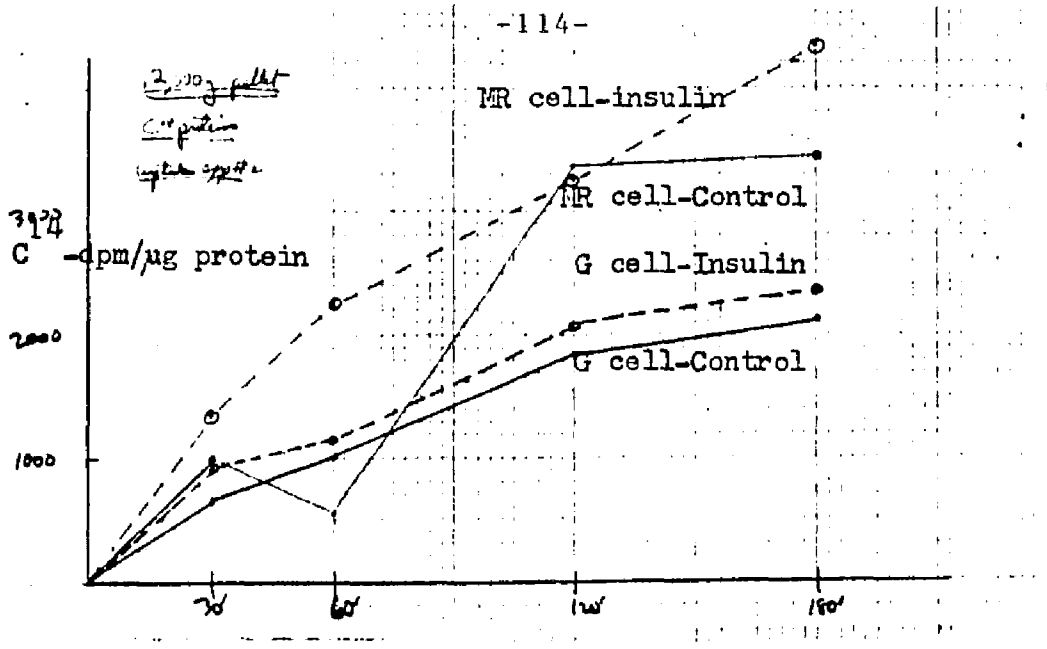


Fig. 41 Separated cell experiment #2 - Incorporation of C^{14}-amino acids into the 12,000g pellet (washed with 10% TCA and 100% EtOH).

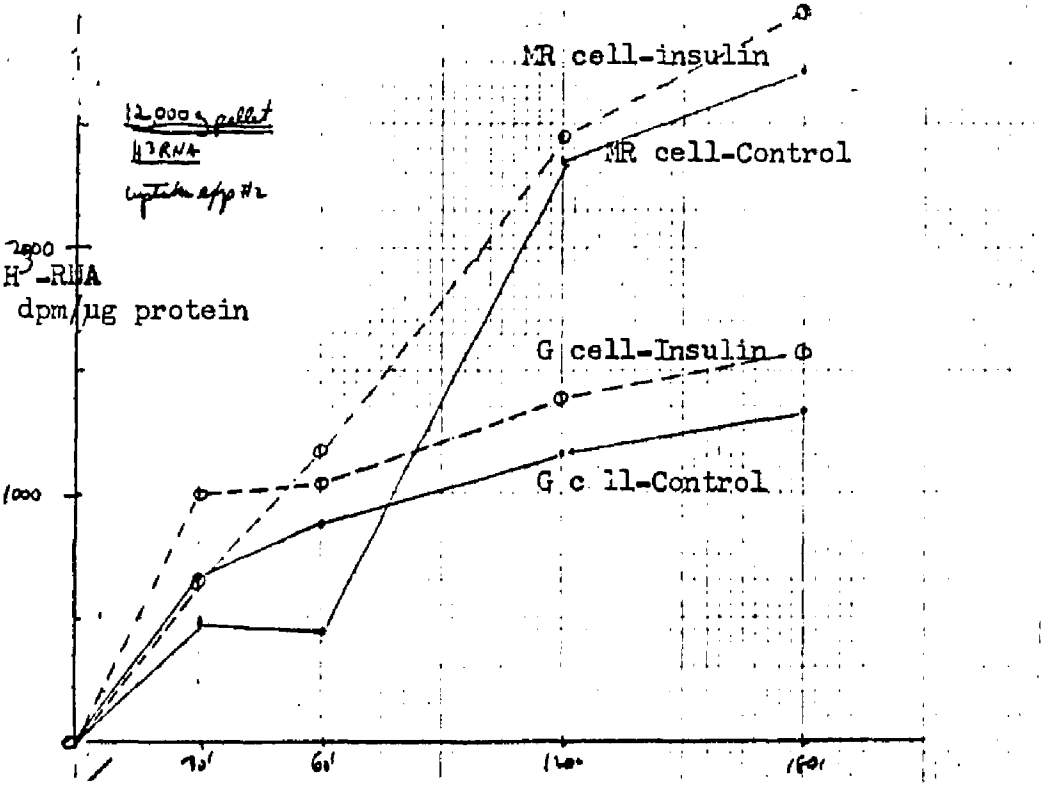


Fig. 42 Separated cell experiment #2 - incorporation of H^3-uridine into RNA of the 12,000g pellet (washed in 10% TCA and 100% EtOH).

III.

B. Insulin and Short Circuit Current (SCC).

The effects of insulin upon the potential difference (P.D.) and short circuit current (SCC) were studied using the "bag" technique described by Bentley (128). Pairs of hemibladders were used, with one of each pair of tissues serving as a control. The addition of insulin caused a stimulation of both the SCC and PD. Within 30 minutes the values were approximately 40% greater than control levels and steadily rose to 73% greater than control levels after 17 hours (Table 24). The duration of insulin's effect on SCC has not previously been reported. The minimum effective dose of insulin seen in these experiments was between 10^{-8}(1mU/ml), which stimulated 1 of 8 bladders, and 2×10^{-7}M (20mU/ml) which stimulated 5 of 8 bladders. The stimulation of SCC was not sustained for the long period observed with higher doses of insulin (Tables 24, 25, 26, 28). A progressive increase in the concentrations of insulin from 20mU/ml (Table 27) to 250mU/ml (Table 24) was associated with an increase in both the peak of SCC and PD obtained as well as the rate at which these parameters rose (Tables 24-28).

The effects of several inhibitors of protein and RNA synthesis upon insulin's stimulation of transport was also examined. Actinomycin D (20 ug/ml) blocked the initial effects of insulin (Table 27) and caused a progressive decay of baseline SCC. This dose of actinomycin has previously been reported to cause decay in baseline SCC by Fimognari and Edelman (14). Puromycin (30 ug/ml) temporarily blocked insulin's effect on SCC. The puromycin inhibitor lasted about 3 hours after which the insulin-induced increased SCC begins to appear and is present full-blown at 21 hours (Table 28). The dose of puromycin was moderate but effective in blocking toad bladder protein syntheis (28). Chloramphenicol (50 ug/ml), a moderate dose(91,146)had no effect on baseline SCC, nor did it

block the insulin-induced increases in SCG (Table 26). If anything, it helped protect the bladder from bacterial infections at long incubation times. 3'-deoxycytidine, at 30 ug/ml (Table 25), did not affect the early insulin-stimulated SCC (2 hours), but knocked out SCC readings at 10-50 minutes(89,90). Lastly, preliminary results show that the neutral amino acids, alamine and glycine, when added to the serosal fluid of substrate-deplted bladders in a glucose-free Ringers cause increases in SCC (Table 29) with a time course and magnitude of effect similar to insulin. Insulin added after the amino acids or amino acid added after insulin had no further effect, implying that their increased SCC's are related in mechanism of action.

Lastly, preliminary results show that insulin (250 mU/ml) does not stimulate SCC in substrated-depleted bladders (i.e., - bladders dissected out of the toads, left in aerated Ringers overnight) which are mounted in glucose-free buffer ($n=9$; mean SCC ±SEM at time when 250 mU/ml insulin added = 110± 11 uA; mean SCC ±SEM 45 minutes later = 94 ± 11uA; 4 remained same; 5 decreased). Further, glucose and uridine added to 5mM final serosal concentration, each caused a transient increase in SCC which peaked in 10 minutes (glucose caused a 93% increase in SCC; uridine caused increases of 11% and 28% at its peak) and fell back to baseline in 30 minutes. Most interesting of all, however, was that glycine and alanine additions (to final serosal concentrations = 5mM) both increased toad bladder SCC but only if 5mM glucose had been given first. The time course and magnitude of the effect of the serosal addition of neutral amino acids was very similar to that of insulin's effect on SCC in the toad bladder. The serosal addition of these amino acids caused 37%, 21%, 16%, and 22% increases in SCC after 30-45 minutes.

Table 17. Short Circuit Current - 3'-deoxycytidine + Insulin

	-60 min	0	60 min	120 min	1050 min	Δ_{1050}
1	3'-deoxycytidine	Insulin	139%	130%	30%	
	-	"	118%	124%	106%	76%
2	3'-deoxycytidine	"	130%	130%	8%	
	-	"	127%	127%	102%	94%
3	3'-deoxycytidine	"	160%	184%	87%	
	-	"	182%	209%	215%	128%
4	3'-deoxycytidine	"	186%	163%	27%	
	-	"	237%	237%	115%	88%
5	3'-deoxycytidine	"	136%	139%	26%	
	-	"	125%	146%	91%	65%
6	3'-deoxycytidine	"	131%	111%	13%	
	-	"	128%	124%	76%	63%
7	3'-deoxycytidine	"	175%	163%	150%	
	-	"	171%	174%	214%	64%
3 Deoxycytidine + Insulin		$\bar{X}$ = 151% ± 8.6% (SEM)	146% ± 9.5%		49% ± 20%	$\bar{X}$ A=83% ±8.9%
Insulin alone		$\bar{X}$ = 155% ± 16%	163% ± 17.1%		131% ± 22% (n=7)	

3'-deoxycytidine = 30 µg/ml

insulin = 250 mU/ml

Table 18 Short Circuit Current - Chloramphenicol + Insulin

Toad	-60 min	0 †	60 min	150 min	1050 min
1	CAP(50µg/ml)	Insulin(250 mU/ml)	145%	153%	363%
	-	"	158%	170%	290%
2	CAP	"	118%	104%	219%
	-	"	138%	153%	233%
3	CAP	"	135%	119%	144%
	-	"	111%	108%	199%
4	CAP	"	138%	105%	151%
	-	"	158%	165%	306%
5	CAP	"	160%	181%	277%
	-	"	196%	217%	29%
6	CAP	"	88%	105%	142%
	-	"	99%	134%	118%
7	CAP	"	92%	66%	220%
	-	"	97%	61%	21%
8	CAP	"	96%	70%	211%
	-	"	108%	89%	179%

Mean Values (+ SEM)

	0 min	60 min	150 min	1050 min
CAP + Ins.		121.5% ± 9.6%	112.9% ± 13 7%	215.9% ± 26.8%
Insulin		133.1% ± 12.5%	137.1% ± 17.7%	172% ± 38%
CAP (50 mg/ml)		91% ± 7.7%	117% ± 6.4%	118% ± 8.3%

† The SCC values in µA at time = 0 are normalized to be 100%

Table 19 Short Circuit Current - Actinomycin D + Insulin

Toad #	-60 min	0 min	90 min	91 min	180 min	930 min
1	Actinomycin D (20 μg/ml)	Insulin(10^{-8}M)	82%	Insulin(2×10^{-7}M)	35%	14 %
		"	106%	"	99%	37%
2	Actin. D	"	101%	"	76%	12%
	-	"	126%	"	146%	110%
3	Actin. D	"	114%	"	66%	15%
	-	"	111%	"	122%	12%
4	Actin. D	"	59%	"	40%	33%
	-	"	71%	"	58%	16%
5	Actin. D	"	96%	"	70%	8%
	-	"	89%	"	111%	77%
6	Actin. D	"	73%	"	65%	18%
	-	"	89%	"	127%	44%
7	Actin. D	"	92%	"	81%	12%
	-	"	93%	"	138%	47%
8	Actin. D	"	85%	"	75%	15%
	-	"	106%	"	145%	71%
Mean % change Actin. D + Insulin ± SEM			88% ± 6%		64% ± 6%	16% ± 2.7%
Mean % change Insulin alone ± SEM			99% ± 6%		118% ± 10% (136% ± 5% for those who responded)	52% ± 11.6%

TABLE 20 Puromycin and Insulin - Effects on SCC

Pair #	-90 min	0	30 min	60 min	90 min	180 min	270 min	1260 min
1	-	Insulin	137%	151%	150%	116%	116%	249%
	Puromycin	"	107%	91%	78%	134%	261%	318%
	Difference		30%	60%	72%	-18%	-145%	-69%
2	-	"	124%	153%	142%	109%	126%	178%
	Puromycin	"	114%	74%	68%	107%	243%	210%
	Difference		10%	79%	74%	2%	-117%	-32%
3	-	"	120%	128%	135%	119%	119%	138%
	Puromycin	"	115%	95%	84%	107%	-133%	30%
	Difference		5%	33%	51%	12%	-14%	108%
4	-	"	117%	117%	141%	102%	124%	127%
	Puromycin	"	91%	76%	69%	58%	73%	459%
	Difference		26%	41%	72%	44%	51%	-332
5	-	"	135%	142%	159%	149%	134%	196
	Puromycin	"	108%	95%	98%	81%	89%	205%
	Difference		27%	47%	61%	68%	45%	- 9%
6	-	"	156%	192%	229%	179%	184	75%
	Puromycin		113%	79%	57%	64%	103%	103%
	Difference		43%	113%	172%	115%	81%	-28%
7	-	"	112%	121%	128%	128%	118%	85%
	Puromycin	"	121%	113%	110%	88%	162%	352%
	Difference		- 9%	8%	18%	40%	-44%	-267%
8	-	"	105%	118%	105%	112%	127%	204%
	Puromycin	"	108%	83%	94%	123%	198%	162%
	Difference		- 3%	35%	11%	-11%	-71%	42%
	Insulin dose ($\bar{X}$)		126%	140%	149%	127%	131%	157%
	(100mU/ml) (± SEM)		± 6	± 9%	±13%	± 9%	± 8%	±21%
Puromycin + Insulin	($\bar{X}$)		110%	88%	82%	95%	158%	230%
(30μg/ml (100mU/ml)	(±SEM)		± 3%	± 6%	±10%	±25%	±49%	
	Difference ($\bar{X}$)		16%	52%	66%	30%	-27%	-73%
	(± SEM)		± 6%	±11%	±17%	±17%	±29%	±53%

DISCUSSION

I. Distribution of Function Between MR and G Cells

Saladino, Bentley, and Trump (145), studied the effects of amphotericin B on the ultrastructure and function of the mucosal cells of the toad bladder. They reported that amphotericin caused electrophysiologic changes (a decreased resistance and an increased SCC) within two minutes. There were pronounced morphological changes in MR cells after eight minutes but there were no morphological changes in the G cells until after 120 minutes. The alterations in MR cells at eight minutes included increased cell volume, expansion of the apical surface area associated with disarray of microvilli architecture, and displacement of mitochondria toward the apical area. The alterations in the morphology of the MR cell mitochondria was also striking. The matrix compartment, which normally is a finely granular matrix compartment, appeared contracted, condensed, and convoluted. Accompanying this condensation of the matrix was a relative expansion of the outer compartment so there was little change in total mitocondrial volume. Further, the rough endoplasmic reticulum and portions of the Golgi apparatus became distended and the nuclei were enlarged. Degenerative changes in the structure of the apical plasma membrane occurred in the MR cell before they occurred in the G cell. When granular cells became swollen after two hours, there was a slight decrease in SCC, not an increase as when similar morphologic changes occurred in MR cells. The authors speculated that, since heterogeneous pathways for sodium transport had been previously demonstrated (146, 147), and, since a large portion of active sodium transport is highly dependent upon respiration, that one cell type, the MR cell, may be responsible for the bulk of the sodium transport across the toad bladder. This would agree with their observation that amphotericin B-induced changes in SCC are much more closely associated in time with the morphologic changes observed in MR cells than with alterations in

in the G cells.

Mullen, et al, (92) studied the structure and function of the urinary bladder of the aquatic urodele, Amphiuma. Only 5% of the mucosal cells were MR cells, compared to 15-20% in the toad bladder, and the potential difference and the short-circuit current were approximately 45mV (mucosa negative) and 20 μAmperes/cm^2, respectively. These values, compared to those usually found in the toad's urinary bladder (e.g., 60-90 mV and 80-200 μA/cm^2) suggest that the MR cells are quantitively related to the electrophysiological properties of the amphibian bladder, particularly to the active transport of sodium.

Voute, Hanni and Amman (50) noted that the percentage of MR cells in frog skin dropped from 35% to 23% as the animals were kept in water, 0.6% saline, or 0.6% saline plus exogenous aldosterone. They described two types of MR cells in toad bladders and frog skin; one was designated "unstimulated MR cells" because of their relative predominance (70% of all MR cells) from toads or frogs kept in 0.6% saline, and the other "stimulated MR cells" which predominated in tissues of toads or frogs kept in water or 0.6 NaCl plus aldosterone. The "stimulated MR cell" had more abundant mitochondria, electron-dense bodies in the cytoplasm, and abundant ribosomes.

The acidification of urine by the mucosal cells of the toad urinary bladder generates a PD and SCC opposite in direction and smaller in magnitude than those associated with sodium transport. The "reversed" PD and SCC of acidification is unmasked by treatment of the bladder with amiloride, which blocks Na^+ transport (93). Acetazolamide, the well known inhibitor of carbonic

anhydrase, completely inhibits the "reversed" PD and SCC associated with acidification. This acidification is stimulated significantly by aldosterone. Dexamethosome, a potent mineralocorticoid in toad bladder epithelium (94) was also effective at 10^{-7}M in stimulating the reversed PD and SCC associated with acidification, but ADH (100mU/ml) and 17β-estradiol (10^{-7}M) had no effect. Thus, the acidification processes of the bladder are apparently regulated by mineralocorticoids.

Scott, Sapirstein, and Yoder (94) developed a method for the preparation of MR enriched and G cell enriched fractions from toad urinary bladders on discontinuous Ficoll gradients. They found a three-fold enrichment of carbonic anhydrase in the MR cell fraction compared to the G cell fraction and further, they found that only the MR cells responded to oxytocin with an increase in cAMP. There is considerable evidence that the cyclic nucleotide acts as a "second messenger" of oxytocin's effect on sodium transport (95). Handler and Preston (96) confirmed the cellular distribution of carbonic anhydrase found by Scott, Sapirstein and Yoder, but found that G cells as well as MR cells responded to ADH with increased in cAMP. However, these effects were "judged small and inconsistent" in both cell types. Both cell types contained cAMP phosphodiesterase. Murther (97) and Voûte, Thummel and Brenner (98), have reported that only MR cells show any histochemical evidence of carbonic anhydrase. The most likely site of the acetazolamide-inhibitable acidification is, then, in the MR cell.

Sapirstein and Scott (99) showed that only MR cells showed displaceable nuclear H^3-aldosterone binding and that aldosterone induces both soluble proteins

and proteolipids only in MR cell fractions (100).

Scott, et al, (148) using a plasma/membrane preparation developed by Reich, et al (in press, 1978), in this laboratory has reported that aldosterone induces increased labelled amino acid incorporation into proteins in that membrane fraction. Analysed on SDS-acrylamide gels, the molecular weights of the aldosterone-induced levels were 170,000, 85,000 and 23,000 daltons.

Slatin reports that cells isolated after mucosal incubation in I^{125}-lactoperoxidase mixture, incorporated I^{125} into the 100,000 and 23,000 dalton plasma membrane proteins (personal communication). This indicates that at least two of these aldosterone induced plasma membrane proteins are exposed to the urine on the apical border of the MR cell.

Because of all the following data: aldosterone binding and aldosterone-induced protein being localized to the MR cell: the high titers of carbonic anhydrase in the MR cell: the acetazolamide-inhibitable, aldosterone-stimulated acidification of the urine, and evidence presented in this thesis that only MR cells respond to aldosterone with increased incorporation of H^3-uridine into RNA: it is proposed that the MR cell is the site of aldosterone-sensitive systems involving Na^+ and H^+ pumps.

The function of the G cell and the MR cell - G cell interrelationships are not understood. The following are a list of papers which report effects of various agents, mainly neurohypophyseal peptides, on G cells. Since these effects are mainly associated with water fluxes, the G cell could be the site of the increased water fluxes induced by ADH-like hormones while the MR cells may be the site of their short-circuit effects.

A report by Spinelli, Grosso and deSousa (101) reported that the G cells swell and their surface becomes modified after stimulation by oxytocin, while the MR cells remain unchanged. DiBona, Civan and Leaf (102) report that when the mucosal solution is made hypotonic and ADH is added, only the G cells swell.

Dissociation of the neurohypophyseal hormonal effects on Na^+ transport from the effects on water flux have been observed by several investigators. Bourguet and Morel (103) noted that, in the urinary bladder of the frog, there is a discrepancy in the time of onset of oxytocin and argenine vasotocin when the two agents are given in equivalent doses. In addition, when an individual preparation is treated alternately with two peptides in doses selected to have equal effects on water transport, there is a marked difference in the response of the SCC. Further, Peterson and Edelman (104) reported that dissociation of vasopressin's effects on sodium and water transport can be demonstrated by elevating the calcium concentration in the serosal bathing medium. Under these conditions, the net flow of water in response to ADH is reduced while the stimulation of sodium transport is unimpaired. The higher calcium concentrations do not affect the hydroosmotic response to cAMP. Pietras, Seeler and Szego (105) reported that 3.3×10^{-6}M cortisol inhibited the hydroosmotic and membrane conductance but not the SCC effects of ADH. Cortisol inhibited the ADH stimualted rise in cathepsin B1 and acid phosphatase activities which were released from cells previously isolated from the bladder. The lysosomal enzymes were released only from G cells and not MR cells.

Davis, et al (106), observing the surface characteristics of the mucosal aspect of the toad bladder epithelium with the scanning electron microscope, noted changes only in the surface morphology G cells after the addition of ADH.

Bourguet, Chevalier and Huzon (107) used freeze-etching to study intramembrane particle distribution in the apical membrane of the bladder of the frog, Rana esulenta. They had previously noted differences between the apical and latero-basal membranes in that tissue and no differences in the tight junctions on ADH stimulation. Oxytocin or cAMP caused intramembranous particles in the apical membrane to aggregate. The magnitude of this effect was correlated with the peak in hydro-osmotic flux of water after neurohypophyseal hormones. The clumping of particle was present even in the absence of an osmotic gradient indicating that they were not formed secondary to water flux. The cell membranes were thought to belong to G cells.

Taylor, et al (108,109) observed microtubules and microfilaments in all epithelial cell types in the bladders. The apical sub-plasmalemmal region of the G cells contains a network of microfilaments and microtubules which are associated with the granules. Agents which disrupt microfilaments (cytochalasin B) or microtubules (vinblastine, colchicine) inhibit the ADH-induced increase in water flux but not the increase in SCC.

Wade, Discala and Karnovsky (110) examined the particle composition of freeze-fractured membranes of granular cells in the toad bladder. The particle size distribution was different in the apical and basolateral mem-

branes of G cells. They also noted clumping or clustering of particles in the apical membrane on addition of ADH.

The data suggests that the physiologic transport processes of the tissue are distributed between the MR and G cells as follows. G cells, which swell after the addition of ADH, are responsible for the hydro-osmotic response. Particles in the fluid apical membrane clump, perhaps forming water channels between the particles or the areas containing clumped particles may be new membrane added by the process of exocytosis of the G cell particles (111). The G cell may have its own neurohypophyseal receptor and respond to ADH with endogenous production of cAMP as indicated by the experiments of Handler and Preston (96) in separated cells, or only the MR cell may respond to ADH to produce cAMP which reaches the G cell through intracellular channels in the junction complex as may follow from Scott, Sapirstein and Yoder's results (95). Since ADH's effects on SCC and hydro-osmotic responses can be separated by a manipulation like raising the (Ca^{++}) which could affect the gap junction (104, 112) such a hypothesis is not so far from being credible.

The MR cell apparently is associated with Na^{+} transport. The presence of many mitochondria indicates that the cell would be a more reasonable choice for the site active transport of sodium which requires much ATP, the primary product of mitochondria. At least the increases in active sodium transport associated with aldosterone much be localized in the MR cell, since the G cell neither specifically binds aldosterone, nor responds to it with the common steroidal biochemical response of making new RNA and protein molecules.

II. Aldosterone

The G cell which comprises 75-85% of the mucosal cell population does not, apparently, respond by alterations in RNA metabolism. This result was obtained whether the RNA was isolated from hemibladders incubated with labeled uridine and hormone, or whether the mucosal cells were isolated first and then incubated in labeled uridine and aldosterone. These conclusions are consistent with previous studies in which the G cells were found to contain no specific mineralocorticoid binding sites (99), nor any evidence of aldosterone-induced protein synthesis (100,134-138). The G cells, then, do not appear to function directly in the sequence of events associated with the physiologic response to mineralocorticoid hormones. Goodman, et al (139), using an immunofluorescent technique for the localization of tissue cyclic AMP, suggested that cyclic AMP synthesized in the MR cell in response to ADH was transmitted to the adjacent G cells. A similar transfer of material from the MR cell could involve the G cells in the mineralocorticoid response, but the presence of a process of this kind is, at the moment, highly speculative.

The MR cells have previously been shown to contain specific mineralocorticoid receptors (99) and to respond to physiologic doses of aldosterone with the incorporation of labelled amino acids into several fractions of MR cell protein (100, 134-138). In the present study, only MR cells showed evidence of aldosterone-induced mRNA synthesis. This completes the identification in the MR cell of the primary sequence of events - hormone binding, mRNA synthesis, protein synthesis - that are evidently necessary for the stimulation of sodium transport and thus identify the MR cells as the primary mineralocorticoid responsive cell of the toad urinary bladder. It is not known whether this division of labor amongst the two cell types has an analogy in the "light" and "dark" cells of the collecting duct of the mammalian nephron.

The time course of aldosterone's effect on the nuclear RNA and the cytoplasmic, poly-A containing RNA (mRNA) (Fig. 4) is especially interesting in view of the 90 minute latent period in the course of aldosterone's effect on sodium transport. Aldosterone has little or no effect on H^3 (5, 6) uridine incorporation into mRNA until 40 minutes, and the peak effect is found at 90 minutes after hormone at the time when the short-circuit current may not yet have even begun to rise. The aldosterone-induced mRNA rapidly decays after 4 hours, at a time when the hormone has its peak effect on the SCC. The data support the well-accepted theory that the action of steroid hormones is dependent on transcription of steroid sensitive genes.

The amount of ^{3}H-uridine incorporated into the mRNA (Table 5, Figure 9), and into 12,000g pellet (containing the nuclei) (Figure 3) of the MR cell is directly related to the concentration of hormone to which the tissue was exposed. This implies that the magnitude of the response to different levels of aldosterone is regulated by the number of mRNA molecules transcribed which, in turn, could be regulated by the number of hormone-receptor complexes which are bound to the nucleus.

The shape of the sucrose gradient profiles of the aldosterone-induced mRNA prepared using isolated MR cells indicate that several species of mRNA are being transcribed in response to this hormone. The peaks are very broad, so it is impossible to determine by these sedimentation procedures the number of species of mRNA molecules being produced in response to this stimulus. However, it appears that the first aldosterone-induced mRNA peaks to appear in the cytoplasm (Figure 7) are small, approximately 7S and 12S, and remain as minor peaks at the peak aldosterone effect at 90 minutes. The aldosterone-induced mRNA peak comprising the labeled molecules largest in size (25S) which becomes apparent after 60 minutes, contains the largest

amount of incorporated uridine. It remains the dominant peak until 3-3 1/2 hours as it diminishes in height to become overshadowed by a 3-5S peak which probably represents partially degraded mRNA molecules. The 25S peak is also accompanied by a smaller 18S peak at 60-90 minutes.

The sucrose gradient profiles of mRNA isolated from MR cells prepared from intact hemibladders incubated in labeled uridine and aldosterone vary from experiment to experiment and the major aldosterone-induced peak has a smaller S value than in the experiments using separated cell. The mRNA sedimentation profiles of mRNA fron control MR cells vary similarly to that of treated MR cells. These discrepancies are presumably due to endogenous RNases partially degrading the labeled mRNA during the 3-4 hours required for the isolation and separation of the mucosal cells.

Spironolactone (SC- 9420), a specific anti-mineralocorticoid which binds to mineralocorticoid receptors, (35, 113, 114) to inhibit the binding and subsequent induction of transcription of the genes coding for the aldo-induced protein, is an effective antagonist of aldosterone effects upon transport. Spironolactone (10^{-5}M) does not bind to glucocorticoid receptor (131). Several investigators (35,36) have found that SC-9420 has no mineralocorticoid agonist properties, and that it is the most potent mineralocorticoid antagonist currently available. Porter (114) reported that the inhibition constant (K_I) which represents the concentration of SC-9420 at which the physiologic effects of $7x10^{-8}$M aldosterone are reduced by half, is $5x10^{-6}$M. The concentration which was used in these experiments, 1.4×10^{-5}M was about three-fold greater than the K_I, and should be at or near the 90% inhibition level. Therefore, the inhibition of aldosterone's effects on RNA metabolism in MR cells upon the prior addition of a 200:1 excess of SC-9420 is consistant with the mineralocorticoid antagonistic properties

of the compound. Generally, the effects of aldosterone on RNA metabolism described in these studies are in agreement with those reported by Rossier, Wilce and Edelman (31, 32, 34, 35) using mixed mucosal cells, scraped from bladders. After incubations in H^3-uridine and C^{14}-methyl methionine, they noted that tissues with 7 x 10^{-8}M aldosterone exhibited increased H^3-uridine incorporation into 9-18S non-methylated RNA. Oligo-(dT) - cellulose chromatography of cytosol RNA revealed increased labeling of mRNA with peaks at 7S, 12S and 18S after 30 minutes of incubation with aldosterone. Careful examination of their published data also reveals small increases in labelling of cytoplasmic poly-A RNA sedimenting at about 25S. After a 30 minute labelling pulse followed by a 150 minute chase in labeled mRNA peaks are seen at 4S and 12S, indicating that the aldosterone-induced mRNA's are being degraded by endogenous RNases. SC-9420 blocked the labeling of these induced mRNA peaks. And the fractional increases in specific activity of 9-12S peaks showed significant correlation with the fractional increase in SCC in paired hemibladders. The acid-soluble H^3-uridine content of epithelial cells was increased slightly but to a lesser extent than incorporation of H^3-uridine into 9-12S RNA, and RNase activity was not measurably altered by aldosterone treatment. Therefore, the increases in content of labeled uridine probably results from an increase in the synthesis of mRNA, rather than a reduction in its degradation. Edelman's group also reported that aldosterone increases total RNA synthesis by about 20%, but little of this increase was seen in cytoplasmic 18S and 28S rRNA within the latent period. There was a moderate aldosterone-induced increase in incorporation of H^3-uridine into 5S tRNA, 18S and 28S rRNA after 240 minutes. Our own data

indicate that all of the above results, which Rossier, Wilce and Edelman obtained from mixed mucosal cells were localized in the MR mucosal cells.

The molecular weights of the aldosterone-induced mRNA molecules, and the maximum size of the proteins for which they code can be estimated by using the following approximations. Brawerman (140) reported that poly(A) segments of mRNA molecules are from 20-250 adenylate residues in length, most being between 100 and 160 residues when they first appear in the cytoplasm. Poly(A) segments of 125 and 75 adenylate residues migrate as 3.3S and 2.6S, respectively. The poly(A) section of ovalbumin mRNA was, on the average, 62 residues in length (142). A conservative estimate, then, for the length of a poly(A) "tail" is 150 adenylate residues which at 347 daltons per residue, amounts to about 50,000 daltons. The rRNA of the pea migrates as a 25.1S particle in sucrose gradients and has a molecular weight of 1.34×10^6 daltons, and the rRNA of the rabbit rRNA measures 24.5S and 1.27×10^6 daltons (141). The 25S aldosterone-induced MR cell mRNA should, then, be approximately 1.25×10^6 daltons without the poly(A) segment. The largest size mRNA yet reported (140) is in the 45S-65S range (5×10^6 daltons) and codes for the silk protein, fibroin (1.7×10^5 daltons). One well-studied 18S mRNA, that coding for ovalbumin (142), has a molecular weight reported to be 650,000 daltons. This molecule contains 1900 nucleotides, or approximately 625 codons if adenylate residues are accounted for in the poly(A) "tail". This mRNA which codes for a protein of 387 amino acids (45,000 daltons), would appear to contain an excess of 200 codons. Because no "repetitive" sequences have been found - all of the sequences are "unique" - either some of the codons are not translated, or ovalbumin is first synthesized as a "pre-pro-ovalbumin" and this molecule is hydrolyzed

to produce the mature protein for secretion into the blood. The molecular weight of the 12S aldosterone-induced mRNA can be induced from the data of Swan, Aviv and Leder (143) who report that the 13S mRNA coding for the MOPC-41 light chain peptide (23,760 daltons) contains 850 bases (280 codons), 200 bases (70 codons) more than the minimum number necessary to code for this protein. The molecular weight of 9.5S hemoglobin mRNA is about 210,000 (210 codons). The α-chain of hemoglobin is 141 amino acids and the β-chain is 146 amino acids (M.W. = 17,000 daltons). Using these data, we have estimated the maximum size of proteins coded for on the aldosterone-induced mRNA molecules (Table 29).

Table 22 Estimate of the Molecular Weights of Aldosterone-Induced mRNAs and Proteins

For these calculations, it was assumed that 1) each mRNA contained a poly-A segment of 150 residues (50,000 daltons), 2) the average nucleotide molecular weight is 333 daltons (1000 per codon), and 3) that the mean molecular weight of the amino acids residues is 130g/mole.

Svedberg mRNA	Estimated Mol. Wt.	Maximum# codon	Protein Mol. Wt.
25S	1.30×10^6	1250	162,500
18S	700,000	650	84,500
12S	250,000	200	26,000
7S	100,000	50	6,500
3-4S	60,000	10	1,300

These estimates for the maximum molecular weights of the proteins encoded by the labeled aldo-induced mRNAs are crude, especially for the smaller forms because the allowance for the poly(A)-tail is high. However, they do provide limits within which one may examine proteins as putative aldo-induced molecules.

Some progress has been made in the identification and characterization of aldosterone-induced proteins. Benjamin and Singer described an "AIP" in the toad bladder of about 12,000 daltons as determined on acrylamide gels. Scott and Sapirstein (100, 134-138) reported that aldosterone induced increased incorporation of labelled methionine into MR cell cytosol proteins having molecular weights ranging from 17,000 to 38,000 daltons. Reich, et al, developed a preparation enriched in cell plasma membranes from separated mucosal cells (148). Using this preparation, it was reported (138) that three proteins of the MR, but not of the G cell membrane, show increased incorporation of labelled methionine after incubation in 7×10^{-8}M aldosterone. The estimated molecular weights of these proteins are 170,000, 85,000 and 12,000 daltons. Dr. Chia-Ping Yang, also working in this laboratory has obtained labelled translation products of aldosterone-treated MR cell mRNA. The molecular weight of the major translation product was 168,000 daltons, similar to the estimate for the translation product of the major 25S aldosterone-induced mRNA peak.

III. Steroids Other Than Aldosterone

A. Glucocorticoids

Many glucocorticoids also have mineralocorticoid effects at high concentrations. Corticosterone, the natural glucocorticoid of amphibians (118), is one of these compounds. In an effort to separate its glucocorticoid from its mineralocorticoid effects, SC-9420 at a 200:1 dose excess was added concurrently with $7x10^{-8}M$ corticosterone. Spirolactone competitively blocks binding to mineralocorticoid receptors, but does not itself either compete with glucocorticoids for their receptor or have any intrinsic glucocorticoid activity (131). Corticosterone is restricted by the addition of 200:1 excess of SC-9420 to binding by glucocorticoid receptors. Our results (Figure 8; Table 6) indicate that corticosterone ($7x10^{-8}M$) plus SC-9420 ($1.4x10^{-5}M$) had no effects on RNA metabolism in either major cell for the type. These results are not consistent with previous reports from our laboratory that corticosterone was specifically bound by G cells (99), and that corticosterone appeared to increase the incorporation of labeled methionine into protein in G cells (131).

B. Estrogens and Progesterone

Although 17β-estradiol had no effect on short circuit current up to 17 hours (Table 9), it did increase incorporation of H^3-uridine into poly A-containing cytoplasmic RNA (Figure 10) and into the 12,000g pellet in MR cells (Table 8) after 90 minutes. Other fractions of RNA were not affected in MR cells and no effect on RNA metabolism was seen in G cells.

Estrogens induce increased production of mRNA molecules within an hour in the rat uterus, while total RNA synthesis is not increased until after 6 hours (129). This response in RNA metabolism in MR cells thus is

similar to the response to estrogens in other target tissues. Although 17β-estradiol does not bind to mineralocorticoid receptors (119, 120), this steroid has been reported to cause Na^+ retention in normal (121), "DOCA-escaped" (all mineralocorticoid receptors saturated) and adrenal-extomized dogs (122). DeVries, Ludens and Fanestil have reported (123) specific 17β-estradiol receptors in rat kidney cytosol fractions. They also found that the sodium concentration in the urine was decreased by 35% in adrenalectomized male rats treated for 2 1/2 days with 20 mg 17β-estradiol every 12 hours. Neither potassium excretion nor GFR was affected by this prolonged treatment with estrogen. It is interesting to note that a single injection of 17β-estradiol had no effect on sodium excretion after 4 hours. Neither aldosterone, testosterone, nor corticosterone competed with H^3-estradiol for this binding.

The estradiol-induced mRNA peak and the increased incorporation of H^3-uridine into the 12,000g pellet may then represent the first step in a series of changes in the metabolism of sodium that are related the elevated estradiol levels found during certain phases of the estrus cycle of vertebrates. The time course of the effect on sodium retention, being days, was too long to allow the demonstration of an in vitro effect on SCC in the toad bladder.

Thus, there is some evidence that estrogens interact in with mineral-ocorticoid systems to some extent. Progesterone has little or no effect on RNA metabolism in the cells of the toad bladder. Further, progesterone has no effect on electrolyte balance in normal dogs (121) nor is it specifically bound by receptors in the toad bladder (3, 4) or kidney (3).

IV. Insulin

Insulin, at levels of 50 to 500 mU/ml, causes profound changes in RNA and protein metabolism in isolated mucosal cells of the toad bladder. The insulin-induced mRNA sucrose gradient profile is strikingly different from that of mRNA isolated from either control or aldosterone-treated mucosal cells. The labeling of smaller species of mRNA is stimulated by insulin, while the labelling of the larger mRNA peaks seen in both control aldosterone-stimulated tissues, is inhibited by insulin. As long as care is taken to avoid substrate depletion, both the MR and G cells respond to these levels of insulin with increases in incorporation of labeled precursors into RNA and protein in all cell fractions tested. These results are in accord with those of Benjamin and Singer (37.) who reported that insulin increased the incorporation of S^{35}-methionine into total cell protein in toad bladder mucosal cells. These investigators also described a small (12,000 daltons) protein that was also induced by insulin. Interestingly, the major insulin-induced RNA which we observed is relatively small (7S) and sediments concurrently with a minor aldosterone-induced mRNA peak.

Insulin is a growth promoting substance in many tissues (51, 62, 125) and the large and sustained (up to 3 hours) increases in anabolism of macromolecules in these epithelial cells suggests that the cells may be entering a growth phase preparatory to cell division (G2 phase of cell growth. Insulin's effects on the incorporation of H^3-thymidine into DNA in mucosal cells would reveal whether insulin's effects on these cells included cell division in one or both cell types. These studies should be done <u>in vivo</u>, since present techniques only allow keeping intact

hemibladders up to 30 hours and separated cells up to 6 hours.

Through several lines of evidence, it appears that insulin mediates its effects on the short circuit current of toad bladders through two different mechanisms. In actinomycin D-inhibited toad bladders (83-85), insulin transiently increases the SCC, the effect dissipating in about 45 minutes. Puromycin-treated bladders (Table 20 and Ref. 110) exhibit a similar transient response to insulin. These data imply that insulin has a direct effect on SCC which is of relatively short duration and a second effect of slow onset which is dependent upon protein synthesis and which slowly, but steadily, rises over a period of at least 20 hours. Dose-response data indicate that the lower concentrations of insulin used in this paper (1-20 mU/ml, Table 19) may induce the initial, short-term rise in SCC without initiating the long-term response. Similar results were reported by Weismann, Sinha and Klahr (83-85) who noted that the effect of the lowest concentration of insulin (100 mU/ml) they found effective was transient and that only levels of 250 mU/ml and greater would produce sustained rises in SCC. Goldine, et al, have recently presented evidence that insulin is bound to nuclear receptors in human cultural lymphocytes (87) and that these nuclear inculin receptors are immunologically distinct from the insulin receptors in the plasma membrane (88). They further observed that the time course of binding to the two receptors was different; half-maximal binding of I^{125}-insulin to plasma membrane receptors required 20 seconds and nuclear binding about 3 minutes. The binding remained at maximal levels in both receptors for at least 90 minutes. At identical levels of insulin, maximal nuclear I^{125}-insulin binding was an order of magnitude less than that of the plasma membrane. However,

the level of nuclear binding was twice that observed for triiodothyronine, a hormone known to affect transcription and translation in these cells.

Plasma membrane and nuclear receptors may each be involved in a different phase of insulin's action, and may account for the bimodal stimulus of transport in the toad bladder. The receptor on the external face of the plasma membrane may be involved in the changes in transport of both sodium and substrates (glucose, amino acids, nucleosides) that are observed within minutes after the addition of low doses of insulin. This effect would be analogous to the effects of rapidly acting, membrane-bound hormones such as ADH and epinephrine. The nuclear receptor would then bw involved in initiating transcriptional and translational effects similar to other hormones which are bound in the nucleus, e.g., steroids and thyroxine. The effects of the latter hormones are of slow onest and longer duration. Further, the intracellular location of these nuclear receptors could make the minimum effective dose of insulin greater than that of the membrane receptors.

SUMMARY

Physiological doses of aldosterone affect RNA metabolism in the mitochondria-rich (MR) mucosal cells of the toad's urinary bladder in a manner similar to the effects of other steroid hormones in their respective target tissues. Aldosterone causes the apparent synthesis of several short-lived, cytoplasmic RNA species which contain poly-A segments (mRNA). These appear in the cytoplasm forty to sixty minutes before any effect of aldosterone on SCC is observed, and sediment on sucrose gradient centrifugation as 25S, 18S, 12S and 7S peaks, the first of these representing the major peak. The disappearance of these mRNA peaks over a 4 hour period is associated with the appearance, at 3 hours, and gradual increase in a 3-4S mRNA peak. The latter, presumably represents degradation products of the larger aldosterone-induced mRNAs which appear earlier. Also, their disappearance coincides with the start of the decline of aldosterone's effect on SCC. Incorporation of H^3-uridine into the 12,000g pellet (which includes nuclei) had a time course identical to that of incorporation into MR cell mRNA. Of the other cytoplasmic RNA species, incorporation of H^3-uridine into MR cell tRNA is affected more than into rRNA, with two peaks apparent, a small one at 90 minutes and a more prominent species after 3-4 hours of incubation. These effects on RNA metabolism are mineralocorticoid-specific since they are blocked by prior addition of SC-9420, a specific mineralocoid inhibitor. Of the other steroids tested, corticosterone, progesterone and 17 β-estradiol, only the latter affected mucosal cell RNA metabolism. Estrogen also increased the synthesis of a species of mRNA only in MR cells. No effects

of estrogen were seen on SCC in incubations up to 17 hours.

Insulin increases in SCC are apparent within 5 minutes after addition of hormone. The rate of rise of SCC declines after 45-60 minutes but the SCC continues to increase slowly over the next 20 hours of incubation. The latter slow rise in SCC is blocked by compounds which interfere with synthesis of RNA and protein without affecting the initial rapid rise, indicating separate mechanisms of action. Insulin also increases the uptake of C^{14}-amino acids and H^3-uridine into the cytoplasm of both MR and G cells. The uptake of C^{14}-amino acids plateaus in 30-45 minutes in both insulin-treated and control cells, while the uptake of H^3-uridine in insulin-treated cells continues to rise for 3 hours. Insulin stimulates, in a linear fashion, the incorporation of these precursors into H^3-RNA and C^{14}-protein in all fractions of both MR and G cells. The apparent insulin-induced synthesis of both new ribosomes and a 7S mRNA species in both cell types was an especially interesting finding.

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