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SPECIFIC ALDOSTERONE BINDING IN RAT KIDNEY AND PAROTID

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SUMMARY

The specific intracellular binding of [^{3}H]-aldosterone was studied in tissue slices of kidney and parotid from adrenalectomized rats. Specific aldosterone binding proteins were isolated from (1) cytosol by G-50 Sephadex chromatography, (2) nuclei by an initial osmotic shock procedure (2·2 M sucrose followed by extraction with 0·1 M tris–3 mM $CaCl_2$ and 50% $(NH_4)_2SO_4$ precipitation = "soluble nuclear") and (3) nuclei by a subsequent 0·4 M KCl–3 mM $CaCl_2$ extraction and 50% $(NH_4)_2SO_4$ precipitation ("chromatin bound"). In both tissues, the time course of uptake into the three intracellular compartments was studied by incubation at 25°C for 0–4 h with $5{\cdot}2 \times 10^{-9}$ m [^{3}H]-aldosterone. Both kidney and parotid show the same three-step time sequence of specific intracellular binding—first cytosol, then soluble nuclear, and then chromatin bound. The time course and extent of [^{3}H]-aldosterone binding in kidney slices was unaffected by concentrations of cycloheximide sufficient to lower protein synthesis by 67%. Cytosol binding proteins in kidney and parotid have an identical affinity for aldosterone but their concentration per g wet weight tissue in the kidney is twice that in the parotid. Despite this difference in cytosol donor concentration, and the presumed identity of active sites, levels of intranuclear [^{3}H]-aldosterone–protein complexes are considerably higher in parotid than in kidney (soluble nuclear × 2, chromatin bound × 15).

INTRODUCTION

ON THE basis of physiological, biochemical and histological studies, a model of the molecular pathways in the action of aldosterone has been proposed comprising (1) formation of an intranuclear aldosterone-receptor complex, (2) stimulation of DNA-dependent RNA synthesis and RNA-mediated protein synthesis and (3) enhanced transepithelial Na^+ transport[1]. This paper is concerned with the putative first steps in this model whereby the steroid binds specifically to a cytosol protein and subsequently appears bound in the intranuclear compartment.

Sterospecific binding of aldosterone to cytosol and nuclear proteins has been observed in a variety of mammalian tissues[2–5]. That the formation of these aldosterone-protein complexes initiates the physiological action of the hormone has been inferred in the main from correlations between the relative affinities of a wide range of steroids for the binding sites and their potencies as mineralocorticoid agonists or antagonists. Recently, a rat kidney slice technique has been developed allowing the definition of the time-course of intracellular binding of aldosterone[6].

We have exploited this technique to obtain information on the temporal sequence of intracellular binding of aldosterone–cytosol, then soluble nuclear (released by osmotic shock), then chromatin-bound—over a four hour period. Secondly, the role of continued normal levels of protein synthesis in the pattern

of formation of these complexes has been examined. In addition, we have compared the rates of generation and the quantities of intracellular steroid-protein complexes in two physiological target tissues, kidney and parotid.

Our results are consistent with the postulate of sequential intracellular aldosterone binding, ultimately to a posited effector site on chromatin which initiates the physiological response.

METHODS AND RESULTS

Preparation of tissue slices, incubation and isolation of aldosterone-binding proteins

Male Sprague–Dawley rats were used in all experiments. At the time of use, the rats weighed 140–200 g and had been adrenalectomized for at least five days. Under ether anesthesia, both kidneys were removed, decapsulated, halved, blotted, and placed in incubating solution in ice to await slicing. The parotid glands were removed en bloc; the glandular tissue was separated by fine dissection from the fibrous capsule and interspersed lymph nodes. Slices of 275 μ thickness were made of both organs and rinsed thoroughly in iced incubating solution. The incubating solution consisted of: $Na^+ = 133$, $K^+ = 6$, $Ca^{++} = 1$, $Mg^{++} = 0{\cdot}5$, $Cl^- = 134$, $H_2PO_4^- = 6$, Tris HCl = 5 and glucose = 5 (all in mM); pH = 7·4. The rinsed slices were incubated at 25°C (0·15–0·75 g wet wt. tissue/10 ml of solution), with added [^{3}H]-aldosterone and such other reagents as described, with continuous agitation at 150–200 rpm. At the end of the period of incubation, the tissue slices were drained under suction and homogenized in 2·5 ml of 0·25 M glucose–3 mM $CaCl_2$ in a teflon-glass homogenizer. This procedure, and all subsequent steps, was carried out in ice.

To assay the tissue slices for cytosol aldosterone-binding protein content the homogenates were centrifuged at 600 g for 10 min; the supernatants were decanted from the crude nuclear pellet and recentrifuged at 30,000 g for 30 min; the supernatant of this spin was regarded as cytosol. In a series of parallel experiments on aliquots of the same homogenates, no difference in the recovery of ^{3}H-aldosterone-protein complexes was found between supernatant after centrifugation at 30,000 g for 30 min and that prepared by the conventional "cytosol" centrifugation at 105,000 g for 60 min. Accordingly, we routinely used the shorter procedure. To separate free [^{3}H]-aldosterone from that protein-bound, 1 ml aliquots of cytosol were passed through 3·6 ml of G-50 Sephadex (fine mesh) in 5 ml serological pipettes. The protein-bound steroid was recovered in the external volume of the column. Aliquots were taken for radioassay by liquid scintillation spectrometry[7] and for determination of protein concentration by the method of Warburg and Christian[8].

To assay for nuclear binding of aldosterone, the crude nuclear pellets obtained from the initial spin at 600 × g were resuspended in 30 ml. of 2·2 M sucrose–3 mM $CaCl_2$ and centrifuged at 105,000 G for 60 min. The soluble nuclear aldosterone-protein complexes were isolated as described previously[2]. The purified nuclear pellets were resuspended in 0·1 M tris HCl–3 mM $CaCl_2$ for 15 min and recentrifuged at 19,000 g for 10 min. The tris-extractable complexes were precipitated from the supernatant fractions by addition of an equal volume of saturated $(NH_4)_2SO_4$. The 50% saturation $(NH_4)_2SO_4$ solutions were incubated for 30 min and then centrifuged at 19,000 g for 10 min. The precipitates were resuspended in 0·1 M tris HCl–3 mM $CaCl_2$, allowed to stand for 10 min, and

recentrifuged at 19,000 g for 10 min to remove protein aggregates. Aliquots of these supernatants were assayed for ^3H content and protein concentrations as described above.

To quantify the chromatin-bound aldosterone-binding protein, the nuclear pellets after extraction with tris-$CaCl_2$ were resuspended in 2 ml of 0·1 M tris HCl–3 mM $CaCl_2$, and recentrifuged to ensure that the chromatin-bound species was not significantly contaminated with the tris-soluble species. The washed pellets were resuspended in 3 ml of 0·4 M KCl–3 mM $CaCl_2$ and allowed to stand for 20 min. The supernatants which contained the 0·4 M KCl–extractable protein were separated from the residual nuclei by centrifugation and brought to 50% saturation with $(NH_4)_2SO_4$. The subsequent procedure for isolating and assaying the quantity of aldosterone-protein complex extracted from chromatin was identical to that detailed after $(NH_4)_2SO_4$ precipitation of the tris-extractable fraction. That extraction with high salt concentrations releases the chromatin-bound species has been shown in earlier studies by Swaneck *et al.*[4].

Recovery experiments. To compare the binding protein content of diverse tissues the quantity of protein or DNA extracted should be equivalent in the tissues under comparison. Aliquots of kidney and parotid slices prepared from adrenalectomized rats were incubated at 25°C for 40 min in $5{\cdot}2 \times 10^{-9}$ M ^3H-aldosterone (specific activity = 50 C/m mole). After washing in ice-cold saline, the slices were homogenized, aliquots taken for analysis and purified nuclear fractions prepared by centrifugation through 2·2 M sucrose–3 mM $CaCl_2$. The nuclear fractions were extracted successively in 0·1 M tris HCl–3 mM $CaCl_2$ and 0·4 M KCl–3 mM $CaCl_2$. The concentrations of DNA and protein were determined by the methods of Webb and Levy[9] and Lowry[10], respectively. The results summarized in Table 1 indicate a nuclear recovery (based on DNA content) of 82% for kidney and 64% for parotid. The recoveries of protein in the various renal and parotid fractions are given in Table 2. On a gm wet wt. basis, the recovery of cytosol protein was the same in both tissues but the parotid nuclei yielded 3-fold greater quantities of protein on extraction with tris-$CaCl_2$,

Table 1. DNA content and recovery in kidney and parotid. Results are expressed as the mean of four experiments with each tissue in mg DNA per g wet tissue wt. Recovery denotes the percent of the total homogenate DNA found in the purified nuclear sample

Tissue	Total homogenate	Purified nuclei	Recovery (%)
Kidney	2·00	1·65	82%
Parotid	1·71	1·10	64%

Table 2. Protein extracted in the three subcellular fractions from kidney and parotid. Results are expressed as the mean of four experiments with each tissue in mg protein per g wet wt. of tissue

Tissue	Cytosol	Soluble nuclear	Chromatin bound
Kidney	28·1	0·23	0·07
Parotid	29·7	0·68	0·40

and 6-fold greater quantities on extraction with KCl. The significance of these differences will be discussed below in the context of the yield of [^{3}H]-aldosterone-protein complexes from kidney and parotid nuclei.

Time course of binding of [^{3}H]*-aldosterone in rat kidney and parotid slices*

In the time course studies, the experiments were so arranged that on any one day the incubations were of 5, 10, 20 and 40 min duration or of 40, 80, 160 and 240 min duration. To obviate between day variation, all values were corrected for the deviations in forty minute values obtained on different days.

The rates of formation of the specific [^{3}H]-aldosterone-protein complexes of the cytosol, tris-soluble and chromatin fractions is shown in Fig. 1. The results in Fig. 1 are mean values at each time point expressed as cpm of [^{3}H]-aldosterone bound per g wet wt of tissue. From 7 to 14 observations were made at each time point for each tissue and averaged. Specific activities (cpm/mg protein) have been converted to cpm/g wet wt. of tissue on the basis of the nuclear and protein recoveries detailed in Tables 1 and 2. A comparison (Fig. 2) of the rates of formation of the three complexes in kidney and parotid was made by normalizing the earlier time points to the mean value at 240 min, which was taken as 100%. The significance of differences in rates of formation of the complexes was evaluated

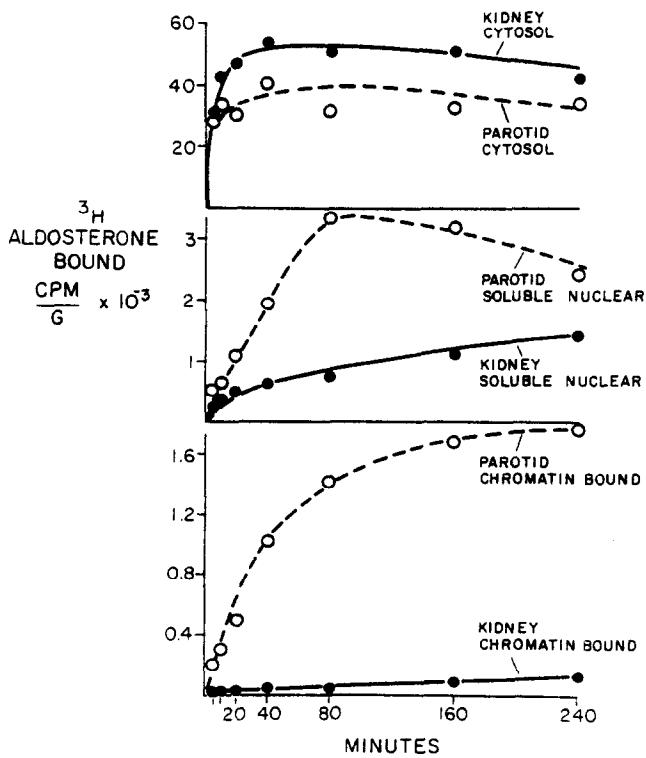

Fig. 1. Specific binding of [^{3}H]-aldosterone in kidney and parotid cytosol (upper panel), soluble nuclear (center panel) and chromatin bound fractions (lower panel). Tissue slices incubated for 5–240 min at 25°C with $5{\cdot}2 \times 10^{-9}$ M [^{3}H]-aldosterone. Results expressed as cpm [^{3}H]-aldosterone bound per g wet wt. of tissue. Each value represents the mean of 7–14 experiments.

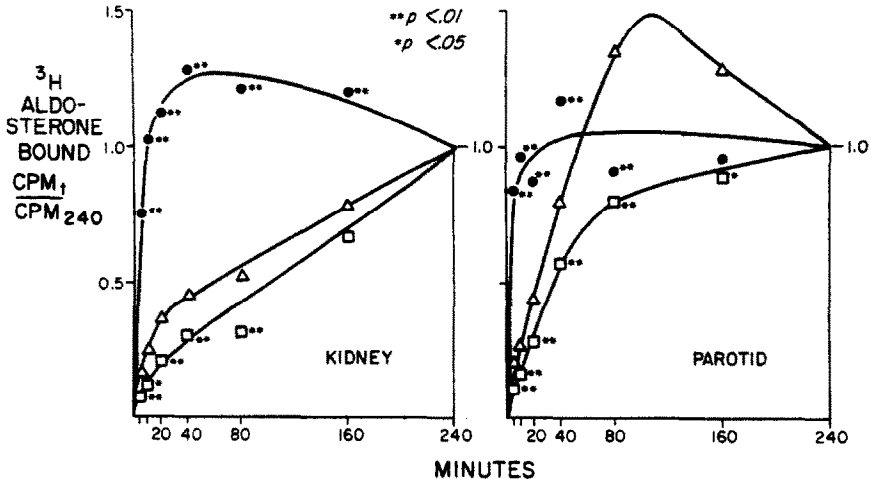

Fig. 2. Specific binding of ^{3}H-aldosterone in kidney and parotid cytosol (●), soluble nuclear (△), and chromatin bound fractions (□). Values expressed as fractions of the appropriate 240 min value. Asterisks denote significant differences between cytosol and soluble nuclear, and between chromatin bound and soluble nuclear, at each time point.

by paired t-tests[11] of the cytosol vs. soluble-nuclear and of soluble-nuclear vs. chromatin-bound species in each tissue at each time point. The following inferences can be drawn from the data shown in Figs. 1 and 2: (1) cytosol binding is rapid, values greater than half-maximal being achieved in both tissues after 5 min incubation, and is maximal after 40 min, (2) binding in the soluble-nuclear fraction is significantly slower than in the cytosol in both tissues, (3) still slower, and significantly different from the rate of binding in the soluble-nuclear fraction, is the rate of binding to chromatin in both tissues.

A comparison of the two panels in Fig. 2 suggests that there are consistent differences in rate of binding between tissues within compartments. Parotid cytosol binds more rapidly than kidney and a similar precocity is apparent in the soluble nuclear and chromatin compartments. The soluble nuclear fraction of the parotid peaks after 80 min incubation; values for the kidney rise throughout the 4 h of observation. The chromatin-bound species approaches a steady state level in parotid after 80 min. whereas in the kidney this species continues to be generated at a linear rate even after 160 min.

Role of protein synthesis in the formation of renal [^{3}H]*-aldosterone-protein complexes*

In kidney slices, the quantity of aldosterone-protein complexes in the two intranuclear compartments continues to increase over the entire period of incubation (Fig. 1). The sequence of formation of the three species, cytosol then soluble-nuclear than chromatin-bound, suggests that the cytosol complex may give rise to the two intranuclear forms. Since maximal cytosol binding is achieved in 40 min and declines slowly thereafter, the continuing rise in nuclear binding raises the possibility that the pool of aldosterone-specific binding protein in cytosol is continuously replenished by ongoing protein synthesis. This possibility was evaluated by comparing post-40 min rates of formation of the renal [^{3}H]-

aldosterone-protein complexes with and without cycloheximide (Actidione R, Nutritional Biochem. Corp.) in the incubation medium. In preliminary experiments, the effect of a range of cycloheximide concentrations (0·1–5·0 μg/ml) and duration of pre-incubation (0, 15, 30 min) was determined on the incorporation into renal proteins of [^{3}H]-leucine (20 min pulse of 4, 5 H-leucine, S.A. = 54 Ci/mmole, Schwarz Bioresearch). The kidney slices were incubated at 25°C with $5{\cdot}2 \times 10^{-9}$ M d-aldosterone. At a concentration of 0·5 mg/ml, and pre-incubation time of 15 min, cycloheximide inhibited [^{3}H]-leucine incorporation by 67%. These conditions with respect to concentration and time of pre-incubation were used in the experiments on the role of protein synthesis in the generation of the ^{3}H-aldosterone-protein complexes. The mean values obtained in 4 experiments are shown in Fig. 3. For incubation times of 40–240 min, the generation of all of the renal complexes was the same whether or not cycloheximide was present. Moreover, the level of non-specific binding of [^{3}H]-aldosterone (measured by addition of 100-fold excess of d-aldosterone to the incubation medium) was unaffected by cycloheximide treatment. These results imply that continued formation of the intranuclear complexes after 40 min involves a pool of pre-formed receptors probably cytoplasmic in origin and does not depend on *de novo* synthesis of these binding proteins.

Number and affinity of cytosol [^{3}H]*-aldosterone binding sites in kidney and parotid*

The total quantity of intra-nuclear complexes formed per unit weight of parotid is considerably greater than in the kidney. Thus at 80–240 min the [^{3}H]-aldosterone bound in the soluble-nuclear component of the parotid is more than twice that of the kidney; the chromatin-bound species of the parotid was 15-fold greater (Fig. 1). These estimates include the appropriate corrections for the

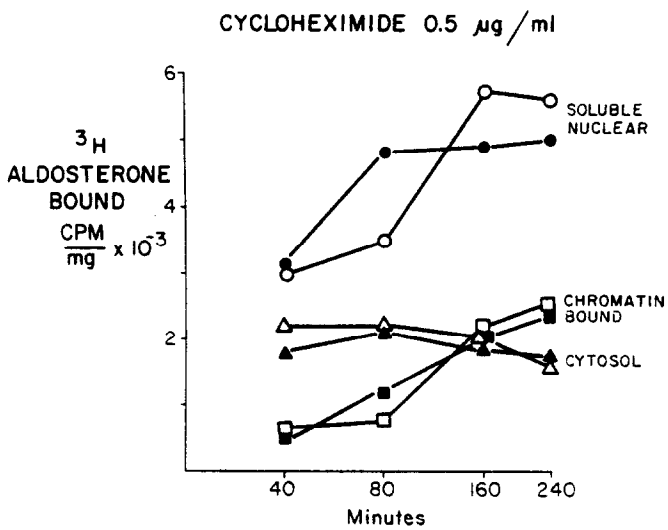

Fig. 3. The effect of cycloheximide (0·5 μg/ml) upon the specific binding of [^{3}H]-aldosterone $5{\cdot}2 \times 10^{-9}$ M in cytosol (△, ▲), soluble nuclear (○, ●), and chromatin bound (□, ■) fractions of kidney slices incubated at 25°C. Open symbols represent controls. Closed symbols, cycloheximide experiments. Results are expressed as specific activities in each fraction – cpm per mg protein – mean of four experiments.

differences in recovery of DNA (Table 1) and protein (Table 2) for the two tissues. Since the cytosol complexes—the presumed precursors of the nuclear species—are present at lower specific activities in the parotid cytosol than in renal cytosol these results imply either (1) than the parotid cytosol has intrinsically greater donor activity or (2) than the parotid nuclei have intrinsically greater acceptor activity. To assess the possibility of differences in the properties of the renal and parotid cytosol binding proteins, estimates were made of the number and affinity for aldosterone of these sites. Tissue slices were prepared as described above and incubated for 40 min with ^{3}H-aldosterone at concentrations of 2×10^{-10} M to 5×10^{-8} M. The results are shown in Figs. 4 and 5 in the form of Scatchard plots[12]. The slope of the calculated line of best fit for the high affinity sites is the same in kidney and parotid; equilibrium constant of dissociation, $K_d = 1{\cdot}8 \times 10^{-9}$ M, at 25°C. The extrapolated intercepts on the X-axis yield more than twice as many [^{3}H]-aldosterone-binding sites in renal cytosol: $8{\cdot}8 \times 10^{-14}$ moles/mg cytosol protein from kidney vs. $3{\cdot}9 \times 10^{-14}$ moles/mg cytosol protein from

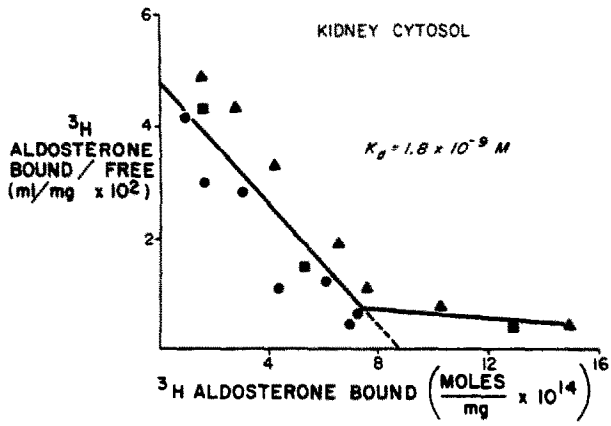

Fig. 4. Scatchard plot of cytosol binding of [^{3}H]-aldosterone to kidney slices after 40 min incubation at 25°C. The symbols represent experiments performed on different days.

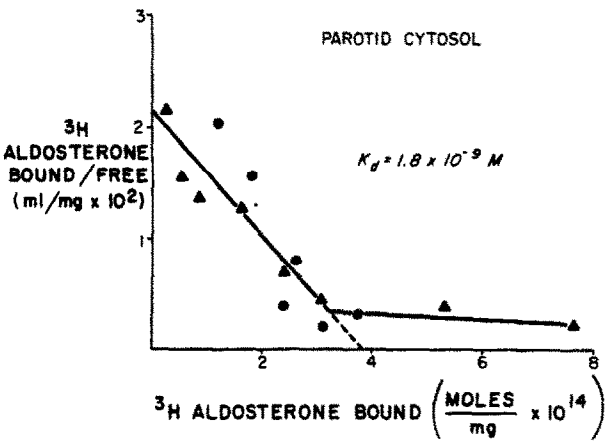

Fig. 5. Scatchard plot of cytosol binding of [^{3}H]-aldosterone to parotid slices after 40 min incubation at 25°C. The symbols represent experiments performed on different days.

parotid. As the recovery of cytosol protein correlated closely with the wet weight of tissue (Table 2), on a weight basis the kidney has ~ twice the high affinity aldosterone binding capacity of the parotid. Despite the higher capacity of renal cytosol and equivalent affinities, parotid nuclei achieved higher concentrations of aldosterone-protein complexes.

DISCUSSION

The mammalian kidney and parotid gland are well-characterized target organs for mineralocorticoids[13, 14]. The present study, which exploited the tissue slice method, revealed the existence of similar three-step aldosterone-binding systems, cytosol, soluble-nuclear and chromatin-bound, in both organs. An unexpected feature of the renal binding system was the finding of a continued increase in the nuclear binding species during the 2nd to 4th h of incubation associated with some decline in the cytosol content of aldosterone-protein complex (Figs. 1 and 2). The failure of cycloheximide (at a concentration that reduced amino acid incorporation into proteins to one-third of the normal levels) to impair the formation of the nuclear complexes (Fig. 3) implies that the cytosol receptors are relatively long-lived, and that the transfer process does not depend on newly synthesized proteins. Alternatively, if the cytosol receptors are short-lived (half life 1–2 h), the results would indicate that their biosynthesis is not impaired by the concentration of cycloheximide used in this experiment.

Although the sequence of appearance of the aldosterone-protein complexes (cytosol, soluble nuclear, chromatin-bound) is the same in parotid and kidney, the rate of formation of these complexes is more rapid and the quantity of nuclear complex generated is greater in the parotid. These differences may be a consequence of greater donor activity in parotid cytosol or greater acceptor activity by parotid nuclei. From the Scatchard plot analysis of the number of cytosol binding sites, there appear to be ~ twice as many per gram tissue in kidney as in parotid after 40 min incubation (Figs. 4 and 5). The identity of the affinity constants calculated from the Scatchard plots for cytosol aldosterone-binding proteins in kidney and parotid, however, implies an identity of active sites. If this is the case, the cytosol donor activity may be the same in both tissues, and the enhanced nuclear binding in parotid would be a reflection of enhanced acceptor activity.

The intranuclear complexes may be generated by transfer of aldosterone from the cytosol receptor to the nuclear receptors or by transfer of at least one protein subunit with the bound steroid from the cytoplasm into the nucleus as suggested by Jensen[15] for the estrogens. If, indeed, the intranuclear and the cytosol binding proteins are in part the same molecule, the finding of plateau levels of cytosol binding protein in the parotid half those in the kidney may reflect differential intracellular transfer or turnover rates, rather than absolute differences in the initial pool of specific cytosol receptors.

The significance of the finding of total nuclear binding in the parotid ~ 4 times that in the kidney, and in the chromatin bound moiety ~ 15 times, is not yet apparent. From the practical standpoint, however, these results suggest the possibility that the parotid may prove to be more suitable than the kidney for future studies on the determinants of cytosol donor and nuclear acceptor activity, and for analysis of the relationship between nuclear binding of aldosterone and regulation of transcription.

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DISCUSSION

Handler: What is the evidence within the model that the donor cytoplasm becomes the activator, that is, not the aldosterone, in the derepressor? I agree that the aldosterone label has the necessary precursor kinetics, but that doesn't indicate whether it is the binding material as well as the aldosterone that is moving into the nucleus.

Funder: Sherman and Atienza (*Fed. Proc.* **30** (1971) 1213A) recently reported an unsuccessful attempt to label estrogen binding protein in cytosol with tritiated amino acids, and by means of selective nuclear uptake to obtain it in a high degree of purity. This attempt actually presumes the question you are raising; that is the identity—at least in part—of the cytosol and nuclear steroid binding proteins. On the other hand, had the experiments succeeded, they would have answered your question directly. There is, however, a good deal of indirect evidence that the aldosterone-protein complex is transferred from cytosol to nucleus rather than protein-free steroid alone. Diana Marver in Dr. Edelman's laboratory has shown that if purified nuclei are heated and then used in a recombination experiment (labelled cytosol plus unlabelled nuclei) they are perfectly competent acceptors for aldosterone. Once the aldosterone is on board the nucleus, heating under identical conditions destroys the specific binding. Secondly, more than half the nuclear aldosterone-protein complexes are extracted into 0·1 M Tris by osmotic shock procedures that I talked about earlier. If nuclear uptake of steroid is a function of a specific non-cytosol intranuclear binding protein, then purified nuclei after the osmotic shock procedure and considerable extraction of protein would be expected to have reduced acceptor activity. In

fact, they are perfectly normal in their behaviour as acceptors. So if we are dealing with an acceptor protein rather than an acceptor process, it is a protein that is heat stable unless the steroid is attached to it, and cannot be extracted from the nucleus with 0·1 M Tris unless the steroid is attached to it. This is why I think it is much more likely that we are seeing a protein-steroid complex transfer process rather than a transmembrane transfer of steroid from the donor protein to a quite different acceptor protein. But this is all indirect evidence.

Rousseau: The fact that parotid nuclei show a greater acceptor activity than the kidney nuclei might be due to the cellular heterogeneity of these tissues. In other words, the proportion of aldosterone-sensitive cells may be higher in the parotid than in the kidney. Since your studies deal with whole organ homogenates, you would then find the total nuclear acceptor activity to be greater in the parotid even if the activity of aldosterone-binding nuclei is the same in this tissue and in the kidney.

Funder: This is an attractive thought. There is evidence from physiological studies that only a relatively small proportion of kidney cells are involved in aldosterone-modulated transepithelial sodium transport, whereas at least under certain circumstances the large bulk of the parotid – both duct and acinar cells – is aldosterone-sensitive. As Dr. Edelman has said, cytosol binding of aldosterone appears almost ubiquitous, with the exception of the anuclear erythrocytes; therefore rough parity in terms of cytosol activity between the two tissues, but enhanced nuclear uptake in the parotid correlating with a higher percentage of aldosterone susceptible cells, is indeed an attractive thought. The only difficulty is the demonstration (Bogoroch and Edelman, unpublished) of a homogeneous distribution of [^{3}H]-aldosterone – both cytoplasmic and intranuclear – throughout the kidney, and not confined to any particular area.

Alberti: I want to make just a brief statement. It concerns work on the toad bladder in contrast to kidney. We were unable to demonstrate mineralocorticoid specific receptors in cytosol of toad bladder. In the cytosol there was a perfectly well demonstrable steroid-binding low-affinity protein and massive capacity which seemed to show no specificity at all. Certainly the procedures you talked about for kidney tissue didn't give us analogous results for toad bladder. And that leads me to my question: what happens if you drop the temperature of your kidney slices even further?

Funder: The reason why we did our studies at 25°C, is that at this temperature the processes of uptake and transfer are slowed down compared to 37°C; and with these processes slowed down, the chances of seeing differences in time course become better. In addition, in tissue slices, 23–27°C seems to be the temperature at which specific binding in all three compartments is quantitatively maximal. In homogenates at 37°C there is virtually no binding due to the extreme thermolability of the binding protein; in whole cell preparations binding at 37°C is slightly more rapid than at 25°C, but both are much faster than at 4°C.

Alberti: You cut out step 3 all together?

Funder: No.

Alberti: So you always take all steps?

Funder: At 0°, 25° and 37°C, if you have noticed