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Relationships between renal cytoplasmic and nuclear aldosterone-receptors

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Relationships between renal cytoplasmic and nuclear aldosterone-receptors. Three ^{3}H-aldosterone receptor complexes have been recovered from rat kidneys: 1) cytosol (high speed supernatants), 2) Tris-soluble nuclear (obtained by an osmotic shock procedure), and 3) chromatin-bound (prepared by extracting post-shock nuclei with 0.4 M KCl). Glycerol density gradient analyses of cytosol labelled *in vivo* or *in vitro* with ^{3}H-aldosterone yielded two specific peaks – 4.5S and 8.5S. These peaks were sensitive to salt concentration; 0.4 M KCl shifted the 8.5S to 4.5S and the addition of Ca^{++} (6 mM) resulted in a further shift to 3.5S. The Tris-soluble nuclear species sedimented at 3S and the chromatin-bound species at 4S. The time-course of generation of the ^{3}H-aldosterone-labelled cytosol and nuclear receptor species was studied *in vivo* and *in vitro* by tissue slice and reconstitution methods. The results obtained are consistent with a three-step mechanism: cytosol (8.5S or 4.5S) → Tris-soluble nuclear (3S) → chromatin-bound (4S). Alternatively, the 3S and 4S complexes may be attached to independent nuclear sites. The formation of the chromatin-bound species was temperature sensitive and failed to form at 0°C. Pre-treatment with DNase but not RNase impaired the generation of both the Tris-soluble nuclear and chromatin-bound species. These results imply a close association between nuclear aldosterone-receptor complexes and intact DNA.

Rapport entre les récepteurs cytoplasmiques et nucléaires d'Aldostérone au niveau du rein. Trois systèmes récepteurs de l'Aldostérone-^{3}H ont été obtenus à partir de reins de rats: 1) cytosol (surnageants d'ultracentrifugation), 2) nucléaires solubles dans le Tris (obtenus par un procédé de choc osmotique) et 3) liés à la chromatine (extraction par KCl 0,4 M après choc des noyaux). L'analyse du cytosol marqué in vivo et in vitro par de l'Aldostérone ^{3}H en gradient de densité sur glycérol a mis en évidence deux pics spécifiques – 4,5 S et 8,5 S. Ces pics dépendent de la concentration en sel: le KCl 0,4 M déplace le pic 8,5 S vers 4,5 S et l'addition de Ca^{++} (6 mM) provoque un nouveau déplacement vers 3,5 S. Le récepteur nucléaire soluble dans le Tris sédimente à 3 S et le récepteur lié à la chromatine à 4 S. La cinétique de formation des récepteurs cytoplasmiques et nucléaires marqués par l'Aldostérone ^{3}H a été étudiée in vivo et in vitro par les méthodes de coupes de tissus et de reconstitution. Les résultats sont compatibles un mécanisme à trois étapes cytosol (8,5 S ou 4,5 S) → avec fraction nucléaire soluble dans le Tris (3 S) → fraction liée à la chromatine (4 S). Une autre hypothèse est que les complexes 3 S et 4 S puissent être fixés à des sites nucléaires indépendants. La formation de le fraction liée à la chromatine est thermodépendante et nulle à 0°. Le traitement préalable par la DNase inhibe la formation de la fraction nucléaire soluble dans le Tris et celle de la fraction liée à la chromatine. La RNase, par contre, est sans action. Ces résultats suggèrent un rapport étroit entre les récepteurs nucléaires de l'Aldostérone et le DNA intact.

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The concept that induction of protein synthesis via DNA-dependent RNA synthesis mediates the action of aldosterone on Na^{+} transport has been supported by a variety of physiological and biochemical studies [1]. The initiating events presumably involve an interaction of the steroid with specific target cell receptors. In this paper, the term "receptor" will be used as synonymous with specific aldosterone-binding protein.

Implication of nuclear-binding sites in mineralocorticoid action was obtained in earlier experiments. Uptake of ^{3}H-aldosterone in the isolated toad bladder system was complete in 15 to 30 min, well within the latent period, and the labelled steroid was preferentially localized in the nuclei of the target mucosal epithelial cells [2–5]. In adrenalectomized rats, the renal nuclear-binding mechanism proved to be specific for mineralocorticoids and saturable at physiological concentrations of aldosterone [6]. Further studies revealed the presence of stereospecific mineralocorticoid binding to macromolecules in the cytoplasmic as well as nuclear fractions of the rat kidney [7]. These binding species are considered to be proteins because of their susceptibility to proteases but not to

nucleases, lipase, phospholipase D or neuraminidase. The intra-nuclear aldosterone-binding system was resolved into two components: 1) a protein that was released by osmotic shock produced by centrifuging the nuclear fraction in 2.2 M sucrose-3 mM $CaCl_2$ and then extraction in 0.1 M Tris-HCl, 3 mM $CaCl_2$, and 2) an acidic protein bound to the chromatin fraction which was obtained by extraction with 0.3 to 0.4 M KCl [7, 8]. These nuclear extracts contained predominately proteins, a small amount of RNA and no detectable DNA.

In this paper we describe the time-course of cytoplasmic and intranuclear binding *in vivo* and in kidney slices *in vitro*. The sedimentation properties of these binding species and transfer studies between cytoplasmic and nuclear fractions are also described. Our results suggest that the intranuclear complexes are derived from the extra-nuclear species.

Methods

In vivo experiments. Male Sprague-Dawley rats (body wt = 125 to 150 g) were adrenalectomized at least three days before use and maintained on 0.9% saline drinking water and standard Purina® chow pellets. Fifteen hours before the experiment, the rats were placed on a low K^+ diet (1.2 μEq of K^+/g dry wt; General Biochem., Inc.) and continued on 0.9% saline drinking water, *ad libitum*. The rats were injected intravenously with 2.6×10^{-10} moles of ^{3}H-aldosterone (30 to 50 Ci/mM) and the kidneys were removed at various times under ether anesthesia. Just before excision, the kidneys were perfused with 20 ml of ice-cold 0.25 M sucrose-3 mM $CaCl_2$ via the abdominal aorta.

Preparation of cytosol and nuclear extracts. The conditions of homogenization and preparation of the renal cytosol (high speed supernatants) were as described previously [7]. All procedures were carried out at 0° to 2° C. To obtain purified nuclear fractions, the crude 600 × G nuclear pellets were suspended in 30 ml of 2.2 M sucrose-3 mM $CaCl_2$ per g of original kidney mass and centrifuged at 65,000 × G for one hr in a Spinco 30 fixed-angle rotor. The 2.2 M sucrose nuclear pellets were osmotically shocked by suspension in 4 to 5 ml of 0.1 M Tris-HCl, 3 mM $CaCl_2$ buffer (pH = 7.2) for 10 min. The nuclei were recollected by centrifugation at 19,000 × G for 10 min. To concentrate the Tris-soluble extracts, the supernatants were diluted with equal volumes of saturated $(NH_4)_2SO_4$ allowed to stand for 30 min and then centrifuged at 19,000 × G for 15 min. The precipitates were collected and resolubilized in 1.5 ml of 0.1 M Tris-HCl, 3 mM $CaCl_2$ and the resuspended extracts were recentrifuged at 19,000 × G for 15 min to remove insoluble material. To obtain the chromatin-bound steroid-protein complexes, the purified nuclear pellets were collected after extraction in 0.1 M Tris-HCl, 3 mM $CaCl_2$ and washed once with the same buffer. The pellets were then resuspended in 1.5 ml 0.3 or 0.4 M KCl for 10 min. The KCl extracts were collected by decantation after centrifugation at 19,000 × G for 10 min as described previously [8]. Earlier studies have shown that 0.3 M KCl extracts the complex that had been tightly bound to nuclear chromatin [8].

Characterization of aldosterone receptors by density gradient centrifugation. Three renal fractions were analyzed: 1) The cytosol fraction (100,000 × G supernatant); 2) the Tris-soluble nuclear fraction precipitated with ammonium sulfate and resolubilized in 0.1 M Tris-HCl, 3 mM $CaCl_2$ buffer; 3) the chromatin-bound complex obtained from the nuclear fraction which had been extracted with Tris buffer and then re-extracted with 0.3 or 0.4 M KCl.

Linear density gradients (total volume = 13 ml) were prepared from spectroquality glycerol in 0.1 M Tris-HCl, 3 mM $CaCl_2$ (pH = 7.2 or 8.0) with a final concentration of 10% glycerol (v/v) at the top and 34% at the bottom of the polyallomer tubes. Linearity was assured by determining the freezing-point depression of the fractions in a Fiske osmometer. Unless specified to the contrary, all gradients contained a background of 1.6×10^{-9} M ^{3}H-aldosterone (30 to 50 Ci/mM) throughout the gradient. The tubes were centrifuged at 283,000 × G (max) for 40 hr at 2° C in a swinging bucket rotor (SB 283) in an International Equipment Co. (IEC) B-60 ultracentrifuge. The density gradients were calibrated with catalase, 11.5S; aldolase, 7.8S; rabbit γ-globulin, 6.8S; alkaline phosphatase, 6.2S; malate dehydrogenase, 4.3S; papain, 2.4S; and cytochrome C, 2.1S [9]. Forty hour centrifuge runs were required to resolve the peaks of lower S value. The background of free ^{3}H-aldosterone minimized the loss of bound material due to dissociation of the complexes during centrifugation. The gradients were separated into 12 to 13 one ml fractions by puncturing the bottom of each tube with a #26 hypodermic needle. Each fraction was cleared of free ^{3}H-aldosterone by passage through 3.6 ± 0.1 ml Sephadex G-50 (fine mesh) in 5 ml serological pipets with glass wool supports. The bound complexes were eluted with 0.1 M Tris-HCl, 3 mM $CaCl_2$ (pH =

7.2) and recovered in the void volume. A 1.0 ml aliquot of the void volume was added to 15 ml Bray's dioxane counting solution and assayed for ^{3}H-activity in a Mark I Nuclear-Chicago Liquid Scintillation Spectrometer with an external standard [6]. The protein content of the void volume was determined from the OD at 260:280 nm in a Zeiss spectrophotometer [10].

In vitro kidney slice experiments. In the experiments on uptake and binding of ^{3}H-aldosterone in kidney slices, the adrenalectomized rats were killed by decapitation. The kidneys were removed immediately, decapsulated and placed in beakers containing standard buffer: NaCl = 135, KH_2PO_4 = 5, Tris base = 5, $MgCl_2$ = 0.5, $CaCl_2$ = 1.0, glucose = 5 (all in mM), pH = 7.4, at 2° C. The kidneys were sliced (275 μ) in an McIlwain tissue slicer (Brinkmann Instrument Co.) and washed in excess ice-cold standard buffer. Slices from one to two kidneys were added to 20 ml of standard buffer solution containing ^{3}H-aldosterone (5×10^{-9} M) with or without unlabelled d-aldosterone (5×10^{-6} M) or spirolactone (5×10^{-5} M). Non-specific adsorption of ^{3}H-aldosterone was estimated by using a "zero-time" control. For the zero-time control, both the slices and steroid-containing buffer were mixed at ice temperature and immediately filtered and washed with ice-cold standard buffer solution. All other flasks were incubated 2 to 40 min at 25° C in a shaking water bath (New Brunswick Scientific Co.). At the end of each incubation period the flasks were removed from the bath, poured into ice-cold stainless steel mesh baskets and washed.

The slices were homogenized in 5 ml of 0.25 M sucrose-3 mM $CaCl_2$, filtered through Nytex (nylon cloth, 132 mesh, Swiss Silk Bolting Cloth Mfg. Co., Ltd.) and centrifuged at 600 × G for 10 min. The supernatants of the 600 × G centrifugation were decanted and respun at 34,800 × G for 10 min. The supernatants of the 34,800 × G centrifugation were used for measurement of cytosol receptor content since this fraction contained the same quantity of bound ^{3}H-aldosterone as that prepared by a conventional preparation of cytosol, i. e., 100,000 × G supernatants. The cytosol fractions were filtered through G-50 Sephadex columns to determine the content of bound ^{3}H-aldosterone. The 600 × G pellets were purified and extracted as described above.

In vitro transfer experiments with reconstituted cytosol nuclear fractions. The 600 × G crude nuclear pellets from adrenalectomized rat kidneys were washed three times with 7 to 10 volumes of 0.25 M sucrose-3 mM $CaCl_2$. The final pellets were resuspended in a minimal volume of the same medium. The 600 × G supernatant was spun at 19,000 × G for 15 min and this supernatant was used to prepare labelled cytosol receptors. In control studies we found that the 19,000 × G supernatants contained about the same quantity of aldosterone receptors as the 100,000 × G supernatants (i. e., "cytosol"). ^{3}H-aldosterone (final concentration = 1.3×10^{-8} M) with or without 9 α-fluorocortisol (final concentration = 1.3×10^{-6} M) was added to the 19,000 × G supernatants and incubated at 0° C for 30 min. In later studies, glycerol (final concentration = 25% v/v) was mixed with these cytosol fractions prior to the addition of the steroids. Washed nuclei from two to three kidneys were transferred to a 25 ml Erlenmeyer flask in a rotary shaking water bath at 25° C and two to four ml of ^{3}H-aldosterone-labelled cytosol was added to each flask. These mixtures were incubated for 0 to 15 min. Following incubation, the samples were centrifuged at 600 × G at 2° C for five min. The supernatants were decanted and one ml fractions put through G-50 Sephadex columns. The nuclei were purified through 2.2 M sucrose-3 mM $CaCl_2$ and extracted with Tris-$CaCl_2$ buffer and 0.4 M KCl as outlined above for the estimation of Tris-soluble and chromatin-bound receptor content.

Preparation of anti-rat plasma antibodies. Female New Zealand white rabbits were injected intradermally at multiple lumbar sites with either 50 mg of rat serum albumin (Mann Research, Fraction V) or 1.0 ml whole rat serum mixed 1:1 (v/v) with Freund's complete adjuvant, type H_3R-Difco. The anti-sera were processed by precipitation of the serum globulins and by dialysis of the solubilized precipitates [11]. Titers were performed by incubating antigen and anti-sera at 4° C overnight, centrifuging at 600 × G for 10 min and washing the precipitate twice with one volume of borate-saline buffer. After draining the tubes for one hr at 4° C, the precipitates were solubilized in one ml of 0.1 N NaOH and protein contents were determined spectrophotometrically from the OD at 280 nm [12].

Materials. d-Aldosterone, 17 β-estradiol and cytochrome C were obtained from Cal-Biochem Co., alkaline phosphatase from Worthington Chem. Co., catalase and papain from Sigma Chem. Co., malate dehydrogenase from Boehringer Co., 9 α-fluorocortisol from the Upjohn Co., 17 α-isoaldosterone from Professor J. F. Tait, G-50 Sephadex and aldolase from Pharmacia Chemicals, Inc., and 1,2-^{3}H-aldosterone (30 to 50 Ci/mM) and 1,2-^{3}H-11-desoxycortisol (34 Ci/mM) from New England Nuclear Corp. Water-soluble spirolac-

tone (SC 14266) was a gift from G. D. Searle &Co. The conventional reagents were all analytical grade from Mallinckrodt Co. The rabbit γ-globulin was a gift from Joel Goodman, University of California at San Francisco.

Results

Density gradient analysis. Previous studies demonstrated the existence of cytosol and nuclear receptors in rat kidney and intestinal epithelia [7, 8, 13]. The isolation of these receptors from cytosol and nuclear fractions requires optimal conditions since the complexes are very labile. Four conditions proved helpful in stabilizing these complexes: 1) low temperature (0° C), 2) pH 7 to 8, 3) addition of glycerol (15 to 30%) to the suspending media, and 4) addition of ^{3}H-aldosterone to the suspending media [14]. Accordingly, these conditions were used in sedimentation analysis of the steroid-protein complexes.

The sedimentation properties of the cytosol and Tris-soluble nuclear receptors were characterized in glycerol density gradients. These gradients were calibrated with protein markers of known sedimentation coefficients (Fig. 1). The S values of the aldosterone receptors were obtained by reference to this standard regression line and are accurate to ±0.5S units.

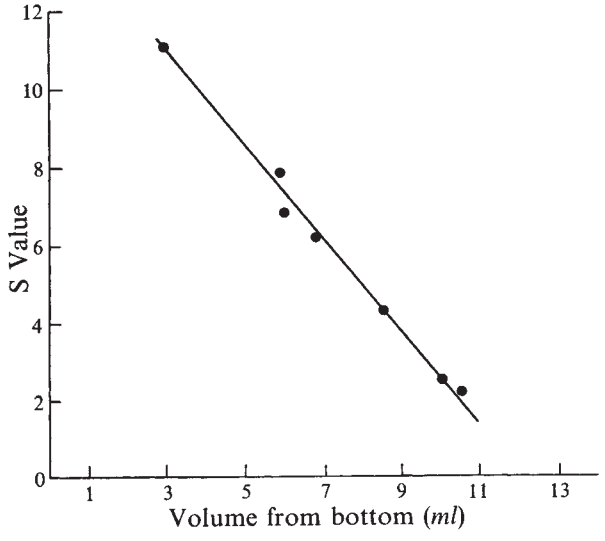

Fig. 1. *Calibration of the glycerol density gradient system.* Linear glycerol density gradients, 10–34% (v/v) were prepared in 0.1 M Tris-HCl, 3 mM $CaCl_2$, pH=7.2. The markers were layered onto separate tubes and centrifuged at 283,000×G for 40 hr in an SB 283 rotor in an IEC B-60 ultracentrifuge. The data points represent the final positions of catalase (11.5S), aldolase (7.8S), rabbit γ-globulin (6.8S), alkaline phosphatase (6.2S), malate dehydrogenase (4.3S), papain (2.4S) and cytochrome C (2.1S).

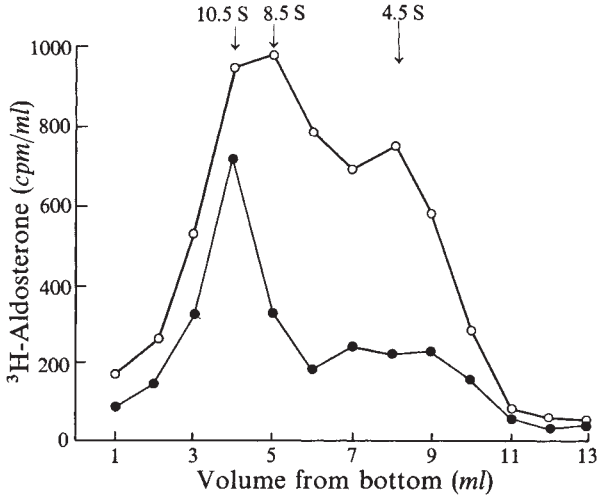

Fig. 2. *Glycerol density gradients of cytosol labelled in vitro with ^{3}H-aldosterone.* Labelled renal cytosol was prepared by incubation with ^{3}H-aldosterone (5×10^{-9} M) with or without added 9α-fluorocortisol (5×10^{-7} M) at 0°C for 30 min. The glycerol density gradients were made up with a background of free ^{3}H-aldosterone (1.6×10^{-9} M) with or without a 100-fold excess of 9α-fluorocortisol. The gradients were centrifuged for 40 hr at 283,000×G. One ml fractions were collected and filtered through G-50 Sephadex to remove free ^{3}H-aldosterone. The gradients prepared without 9α-fluorocortisol (-o-) yielded peaks at 10.5S, 8.5S, and 4.5S. The gradients prepared with 9α-fluorocortisol (-•-) yielded a major peak at 10.5S and a small residual in the 4 to 8S region.

Labelled cytosol was prepared from adrenalectomized rat kidneys after intravenous injection of ^{3}H-aldosterone or after incubation of unlabelled renal cytosol with ^{3}H-aldosterone. Fig. 2 shows the density gradient distribution of cytosol ^{3}H-aldosterone complexes with and without a 100-fold excess of 9 α-fluorocortisol. In addition to two broad specific peaks, one at 4.5S and the other at 8.5S, there is a non-specific peak at 10.5S as evidenced by the residual peak in the presence of excess 9 α-fluorocortisol (lower curve).

Since the high salt, 0.3 to 0.4 M KCl, and millimolar concentrations of $CaCl_2$ have been shown to alter the sedimentation properties of 17 β-estradiol receptors [15], we determined the effect of KCl and $CaCl_2$ on the distribution of ^{3}H-aldosterone-labelled renal cytosol receptors in the density gradients. Renal cytosol fractions (19,000×G supernatants) were labelled *in vitro* with ^{3}H-aldosterone and then layered on 13 to 34% glycerol density gradients made up in 0.1 M Tris-HCl, 1.5 mM Na_4EDTA, pH=7.2. Three simultaneous gradients were run: 1) with no further additions, 2) constant background of 0.4 M KCl, and 3) a constant background of 0.4 M KCl and 6 mM $CaCl_2$. As shown in Fig. 3, in the presence of 0.1 M Tris-1.5 mM EDTA,

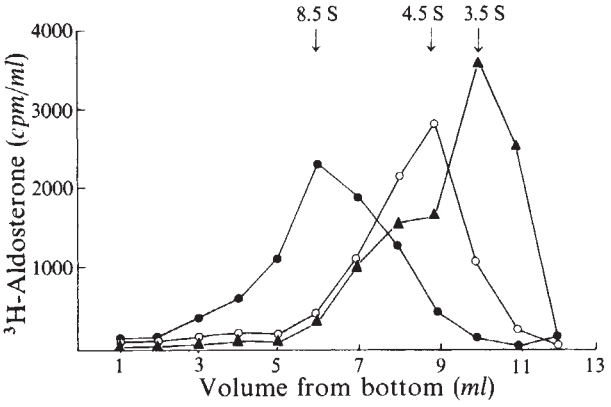

Fig. 3. *The effects of high salt and Ca^{++} on the glycerol density gradient patterns of cytosol labelled in vitro with ^{3}H-aldosterone.* Adrenalectomized rat kidneys were homogenized in 0.1 M Tris-HCl, pH = 7.2. The cytosol fraction was prepared by centrifugation at 19,000 × G for 15 min and labelled by incubation with 1×10^{-8} M ^{3}H-aldosterone for 30 min at 0°C. Aliquots were layered on 13 to 34% glycerol gradients containing 0.1 M Tris-HCl pH = 7.2, and 1.6×10^{-9} M ^{3}H-aldosterone, and: a) 1.5 mM EDTA (-•-); or b) 1.5 mM EDTA and 0.4 M KCl (-○-); or c) 1.5 mM EDTA, 0.4 M KCl, 6 mM $CaCl_2$ (-▲-). One ml fractions were collected and filtered through G-50 Sephadex to remove free ^{3}H-aldosterone.

the bound ^{3}H-aldosterone migrated as a single, broad peak with a maximum at 8.5S. The recovery of a single 8.5S peak (Fig. 3) as compared to the double 8.5S and 4.5S peaks (Fig. 2) may be attributed to the elimination of $CaCl_2$ and the use of 1.5 mM EDTA in the homogenizing solutions and in the density gradients. Addition of 0.4 M KCl shifted the 8.5S peak to 4.5S and with 6 mM $CaCl_2$, there was a further shift, resulting in a peak at 3.5S with a shoulder at 4.5S. These results suggest that the 8.5S and 4.5S cytosol receptors found in low salt gradients (*cf.* Figs. 2 and 3) are aggregate or deaggregate forms of the same species.

The sedimentation characteristics of the Tris-soluble nuclear receptors were also defined in glycerol density gradients. Adrenalectomized rats were injected with 2.6×10^{-10} moles of ^{3}H-aldosterone and either, a) saline diluent, or b) competing cold steroids as indicated in the legend of Fig. 4. The Tris-soluble nuclear extracts were prepared and subjected to density gradient analysis as described above. In the absence of added cold steroids or in the presence of a 10-fold excess of 17 α-isoaldosterone or a 78-fold excess of 17 β-estradiol, the Tris-soluble complex migrated as a single sharp peak with a maximum at 3S. A 10-fold excess of d-aldosterone or a 78-fold excess of 9 α-fluorocortisol eliminated the 3S peak. In contrast to d-aldosterone and 9 α-fluorocortisol, 17 α-isoaldosterone and 17 β-estradiol which are devoid of mineralocorticoid activity at these concentrations did not diminish the 3S peak [16]. The 3S nuclear complex, therefore, is stereospecific and formation of the radioactive complex is inhibited by excess cold mineralocorticoids. Additional experiments were carried out on the effect of the ionic strength of the medium on the sedimentation properties of the Tris-soluble nuclear receptors. After extraction of the nuclei with 0.1 M Tris-HCl, 3 mM $CaCl_2$ the extracts were layered on glycerol density gradients with a uniform background of 0.4 M KCl and centrifuged for 40 hours at 283,000 × G. These complexes sedimented at 3S in the high salt medium and thus proved to be insensitive to this shift in ionic strength.

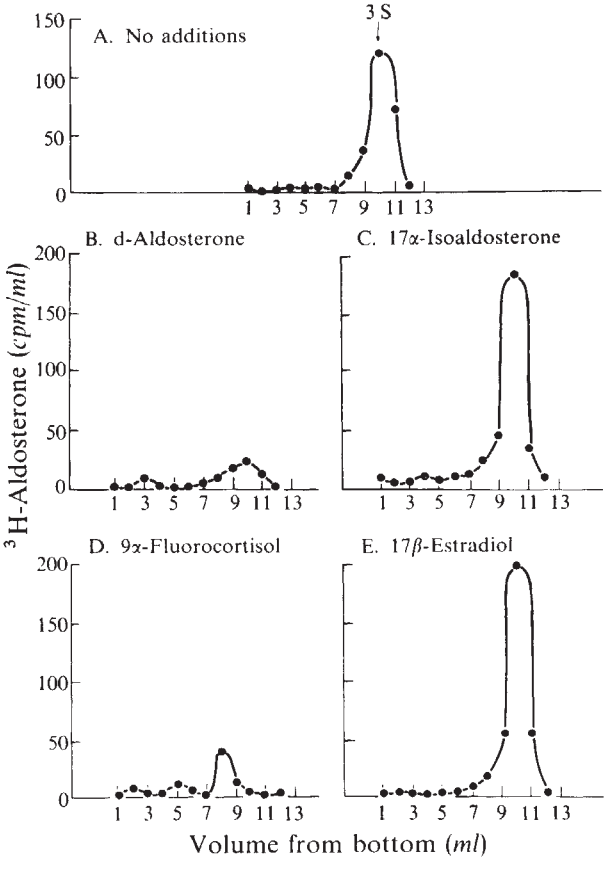

Fig. 4. *Glycerol density gradients of Tris-soluble nuclear extracts labelled in vivo with ^{3}H-aldosterone.* Renal nuclear Tris-soluble extracts were prepared after injection of adrenalectomized rats with 2.6×10^{-10} moles ^{3}H-aldosterone (see Methods) and either: 1) no additional steroid (Panel A), 2) 2.6×10^{-9} moles d-aldosterone (Panel B), or 3) 2.6×10^{-9} moles 17α-isoaldosterone (Panel C), or 4) 2×10^{-8} moles 9α-fluorocortisol (Panel D), or 5) 2×10^{-8} moles 17β-estradiol (Panel E). The glycerol density gradients 10 to 34%) were made up in 0.1 M Tris-HCl, 3 mM $CaCl_2$, pH = 8.0, and 1.6×10^{-9} M ^{3}H-aldosterone with or without a 10- or 78-fold concentration of the appropriate competitor. The gradients were centrifuged at 283,000 × G for 40 hr. One ml fractions were collected and filtered through G-50 Sephadex to remove free ^{3}H-aldosterone.

Swaneck, Chu, and Edelman [8] showed that ^{3}H-aldosterone was specifically bound to chromatin and that this steroid-receptor complex was extracted with 0.3 M KCl. In glycerol density gradients with a constant 0.3 M KCl background, the species extracted from chromatin migrated at 4S; in KCl-free gradients, this complex aggregated and formed a pellet at the bottom of the gradient tube. Thus, three classes of aldosterone receptors have been characterized, a 4.5S (in high salt) cytosol species, a 3S Tris-soluble nuclear species and a 4S (in high salt) chromatin-bound species.

Studies with rat plasma antibodies. Aldosterone binds to serum albumin and other serum proteins that have sedimentation constants in the 4 to 8S range [9]. To evaluate the possibility of contamination of tissue fractions with serum proteins, we used anti-rat serum and anti-rat serum albumin antibodies. Renal cytosol and Tris-soluble nuclear extracts, and whole rat serum and rat serum albumin, labelled *in vivo* with ^{3}H-aldosterone were subjected to glycerol density gradient centrifugation (low salt). These gradients were separated into one ml fractions and 0.7, 0.1, and 0.05 ml of each one ml fraction were added to a constant volume of antiserum. The volume of antiserum was chosen so that at least one of the aliquots would yield optimal precipitation. The mixtures were incubated at 4° C for 16 hr. The precipitates were collected, washed, resolubilized in 0.1 N NaOH and analyzed for protein content. Anti-rat serum and anti-rat serum albumin formed precipitates with fractions from the 3S region of gradients layered with rat serum albumin or rat serum but not in those layered with Tris-soluble renal nuclear extracts. Fractions obtained from cytosol gradients yielded no precipitates in the 8S region but detectable precipitates in the 5S region. These results imply that the specific ^{3}H-aldosterone binding proteins of cytosol and Tris-soluble nuclear extracts are not serum protein contaminants.

Time-course of formation of ^{3}H-aldosterone-receptor complexes in vivo. If the three previously identified aldosterone-receptor complexes are interrelated (e. g., if one is derived from the other), the time-course of formation could indicate precursor-product relationships.

Adrenalectomized rats were injected intravenously with 2.6×10^{-10} moles of ^{3}H-aldosterone with or without a competing dose of 9 α-fluorocortisol (2.6×10^{-8} moles). At various times after injection, blood was collected by cardiac puncture and the kidneys were removed under ether anesthesia. The cytosol, Tris-soluble nuclear and chromatin-bound

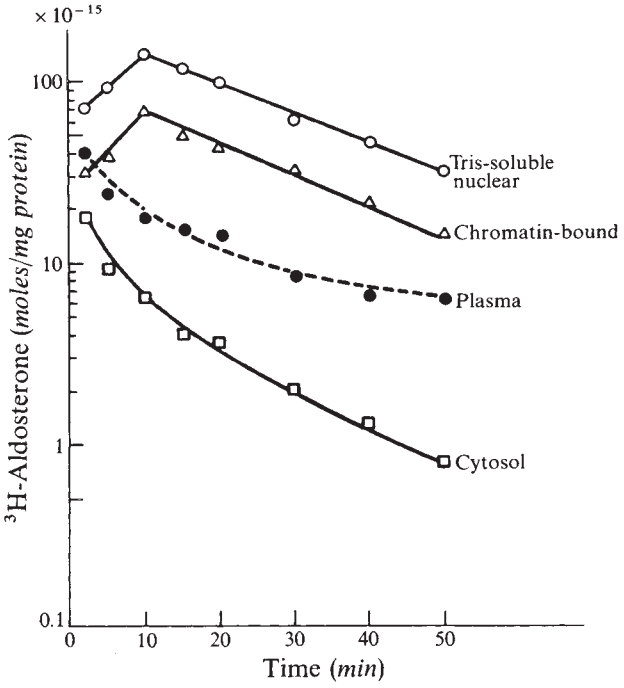

Fig. 5. *Time-course of formation of renal ^{3}H-aldosterone-receptor complexes in vivo.* The labelled cytosol (-▫-), Tris-soluble nuclear (-○-), and chromatin-bound (-△-) fractions were prepared as described under Methods. Adrenalectomized rats were injected with 2.6×10^{-10} moles of ^{3}H-aldosterone with or without 2.6×10^{-8} moles 9α-fluorocortisol and sacrificed 2 to 50 min after injection. The renal fractions were corrected for non-specific labelling. The concentration of ^{3}H-aldosterone in plasma, both free and bound, is shown as a dashed line (--●--). The receptor content in each fraction was corrected for variations in plasma ^{3}H-aldosterone concentration by a factor which was the ratio of the plasma concentration in that sample to the average concentration of all of the plasma samples at that time point. Each point represents an average of five experiments.

receptors were assayed as described above. As shown in Fig. 5, binding of ^{3}H-aldosterone to cytosol receptors attained a maximum level prior to the earliest time point of two min and declined progressively, thereafter. In contrast, both intranuclear receptors continued to increase in specific activity and reach a maximum at 10 min, despite the fall in the content of bound ^{3}H-aldosterone in both plasma and cytosol in the interval between 2 and 10 min. The Tris-soluble nuclear and chromatin-bound species rise and fall in close parallel. To assess this finding in more detail, a separate series of experiments were performed in which adrenalectomized rats were injected with ^{3}H-aldosterone and the kidneys were excised at two min intervals, 6 to 14 min after injection. These results confirmed the findings in Fig. 5, to wit: both intra-nuclear species attained peak specific activities at 10 min and thereafter declined in parallel. The interval in which nuclear binding increases as

cytosol activity decreases suggests that the nuclear receptors may be derived from the cytoplasm by a transfer mechanism. To examine this possibility in greater detail and to try to resolve differences in time-course of formation of the two intra-nuclear species, we exploited a tissue slice technique.

Formation of ^{3}H-aldosterone-receptor complexes in kidney slices in vitro. Slices of adrenalectomized rat kidneys were prepared and incubated with ^{3}H-aldosterone (5.2×10^{-9} M) at 25° C as under Methods. Two types of controls were used to distinguish between specific and non-specific binding: 1) a "zero-time" control was included with each set of incubations in which the slices were kept at 0° C and were processed as soon as the ^{3}H-aldosterone was added to the flask, and 2) steroidal specificity was determined by incubating parallel flasks with d-aldosterone (5.2×10^{-6} M) or spirolactone (5.2×10^{-5} M). Table 1 lists the results obtained with 30 min incubations to indicate the relative magnitudes of specific and non-specific binding. Addition of d-aldosterone (1,000:1) to the media reduced binding by 94% in the cytosol, 90% in the Tris-soluble nuclear and 76% in the chromatin-bound fractions. Spirolactone, at a concentration of 10,000:1, was almost as effective as d-aldosterone as a competitor of ^{3}H-aldosterone. The time-course of specific binding to the three species of renal receptors is shown in Fig. 6. Binding of aldosterone to the cytosol receptors was rapid and attained steady state levels in ~15 min. The Tris-soluble nuclear complex formed at an intermediate rate and accumulated quasi-linearly over the 40 min period of observation. The chromatin-bound species was generated at the slowest rate and appeared in measurable quantities after a lag period of ~5 min. These results are consistent with the possibility of a three-step mechanism in which the cytosol receptors react with the nucleus to give rise to the Tris-soluble complex, which in turn reacts with chromatin to yield the chromatin-bound receptor complex.

Table 1. Binding of ^{3}H-aldosterone to renal receptors *in vitro* (tissue slice method)[a]

Additions	Cytosol	Tris-soluble	Chromatin-bound
	$\times 10^{-15}$ *moles/mg protein*		
None	26.1	25.0	6.7
d-Aldosterone	1.6	2.6	1.6
% of Control	6.1%	10.4%	23.9%
None	37.2	28.2	8.5
Spirolactone	4.1	5.8	2.7
% of Control	11.0%	20.6%	31.8%

[a] Adrenalectomized rat kidney slices were incubated at 25° C for 30 min, in 5.2×10^{-9} M ^{3}H-aldosterone $\pm$ 5.2×10^{-6} M d-aldosterone or $\pm$ 5.2×10^{-5} M spirolactone. Values shown are moles ^{3}H-aldosterone bound/mg protein at 30 min for the cytosol, Tris-soluble nuclear, and chromatin-bound fractions (prepared as under Methods).

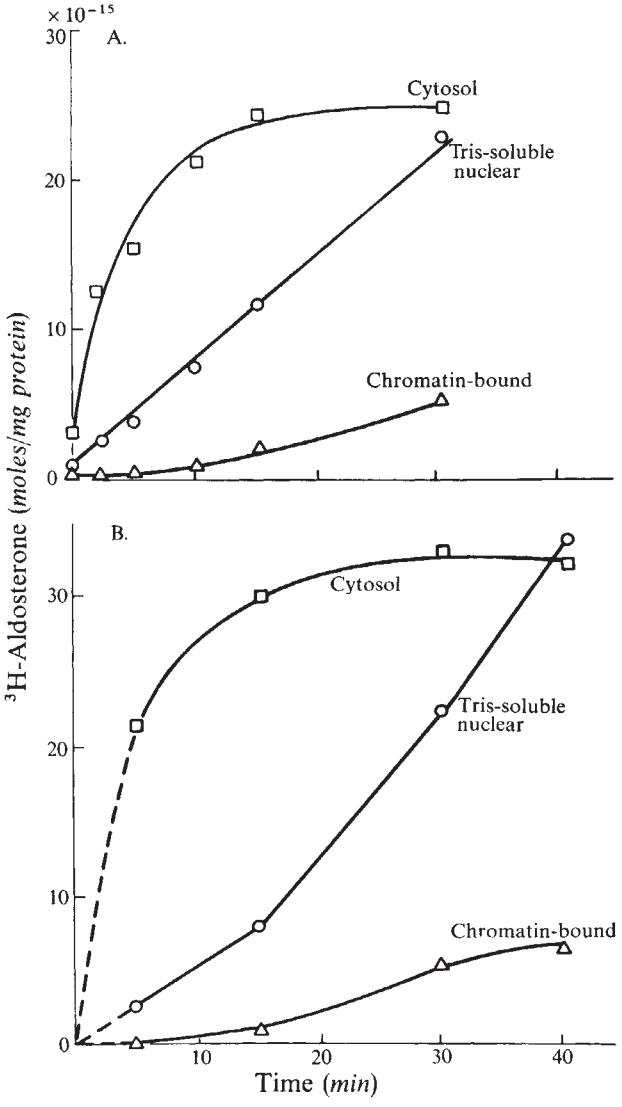

Fig. 6. *Time-course of formation of ^{3}H-aldosterone-receptor complexes in kidney slices, in vitro.* Kidney slices, 275 μ in thickness, were incubated in 5.2×10^{-9} M ^{3}H-aldosterone at 25°C. The labelled cytosol (-□-), Tris-soluble nuclear (-○-) and chromatin-bound (-△-) fractions were prepared as described under Methods. The results represent binding that is sensitive to competition by d-aldosterone and spirolactone. These curves were constructed from experiments in which slices were incubated with or without a 1,000-fold excess of d-aldosterone (Panel A) or 10,000-fold excess of spirolactone (Panel B).

If this three-step mechanism represents the physiological receptor system, a quantitative relationship should exist between the potency of a steroid as a mineralocorticoid, and the concentration required to generate maximum levels of steroid-receptor complexes. To test this prediction, we assayed the concentration dependence of the generation of the steroid-receptor complexes, extra- and intra-nuclear, with ^{3}H-aldosterone and ^{3}H-11-desoxycortisol. 11-Desoxycortisol (compound S) was chosen because it is a weak mineralocorticoid with moderate glucocorticoid activity [17]. In parallel experiments, aliquots of pooled adrenalectomized rat kidney slices were incubated at 25° C for one hr in various concentrations of either steroid. The results were corrected for non-specific labelling with 1,000-fold excess of d-aldosterone in flasks containing ^{3}H-aldosterone and 1,000-fold excess of 11-desoxycortisol in flasks containing ^{3}H-11-desoxycortisol. As shown in Fig. 7, at a concentration of 5×10^{-10} M, all three receptor systems (cytosol, Tris-soluble nuclear and chromatin-bound) were more heavily labelled with ^{3}H-aldosterone and the differences diminished progressively at higher steroid concentrations. Moreover, the pattern of convergence was similar in all three receptor components.

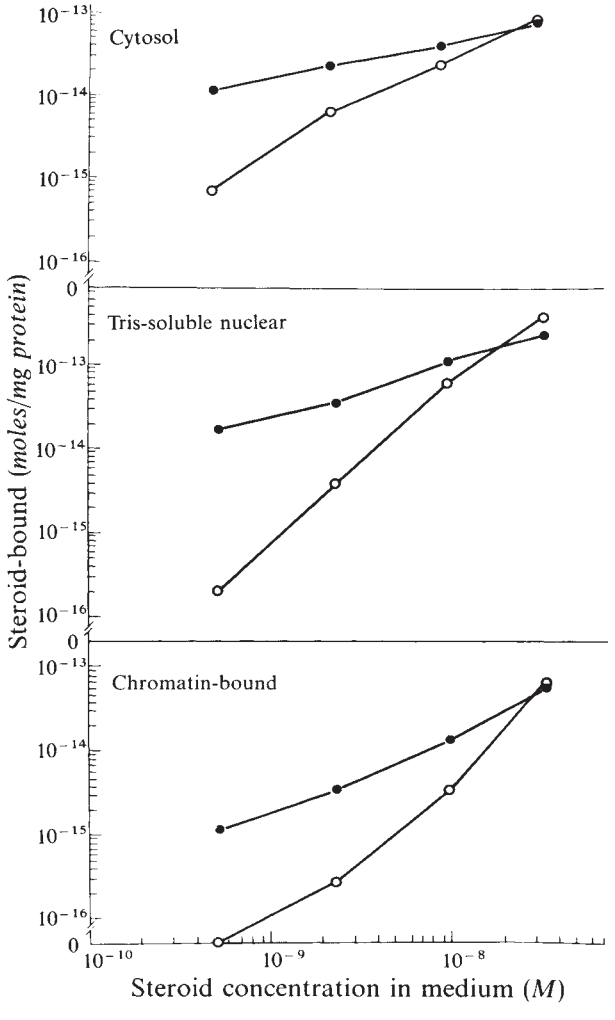

Fig. 7. *A comparison of the formation of ^{3}H-aldosterone vs. ^{3}H-11-Desoxycortisol steroid-receptor complexes in kidney slices.* Kidney slices were incubated at 25° C for one hr in various concentrations of either steroid. The results were corrected for non-specific labelling by parallel incubations with 1,000-fold excess of d-aldosterone or 11-desoxycortisol in flasks containing the same ^{3}H-labelled steroid. The ^{3}H-aldosterone-receptor complexes are denoted by -•- and the ^{3}H-11-desoxycortisol complexes by -o-. Each point represents the average of two experiments.

To test the temperature dependence of the three steps in the generation of labelled receptors, rat kidney slices were incubated for one hr at either 0° C or 25° C in a rotary shaking water bath. ^{3}H-Aldosterone (5.2×10^{-9} M) was added to the flasks with or without d-aldosterone (5.2×10^{-6} M). The results are shown in Table 2. In both cytosol and soluble nuclear compartments binding at 0° C was about 70% of that at 25° C. In the chromatin fraction, however, binding was much more temperature sensitive; at 0° C the chromatin-bound receptor complex was reduced to 16% of that at 25° C. These findings can be interpreted in terms of the three-step model as indicating an energy requirement for the generation of the chromatin-bound species. To explore this model further, we assayed labelled cytosol donor activity and unlabelled nuclei for acceptor activity in reconstitution experiments.

Formation of ^{3}H-aldosterone-receptor complexes in reconstituted mixtures of cytosol and nuclear fractions. Cytosol fractions ($19{,}000 \times$ G supernatants) were pre-

Table 2. Temperature dependence of ^{3}H-aldosterone binding in rat renal cytosol and nuclear fractions[a]

Temperature	Cytosol	Tris-soluble	Chromatin-bound
	$\times 10^{-15}$ moles/mg protein		
0° C	22.5	19.0	2.1
25° C	31.4	28.4	12.9
0° C/25° C	71.7%	66.9%	16.3%

[a] Adrenalectomized rat kidney slices were incubated at either 25° C or 0° C for 1 hr in 5.2×10^{-9} M ^{3}H-aldosterone with or without 5.2×10^{-6} M d-aldosterone. The cytosol, Tris-soluble nuclear, and chromatin-bound fractions were prepared as under Methods. The quantity of ^{3}H-aldosterone bound has been corrected for non-specific labelling. The results are the mean of five experiments.

pared from adrenalectomized rat kidneys and labelled with ^{3}H-aldosterone alone or with added 9 α-fluorocortisol. The pre-labelled cytosol and unlabelled nuclear fractions were mixed and incubated at 25° C for periods up to 16 min. The fractions were separated by centrifugation at 2° C and the receptor complexes were isolated and quantified as described under Methods. Separation of the cytosol and nuclear fractions took six min. Thus, the "zero-time" point actually represents a contact time of six min between cytosol and nuclei; the initial temperature at the time of contact was 25° C and the final temperature at the time of separation was 2° C. The average temperature during this six min of contact was indeterminate. The results are shown in Fig. 8 and are corrected for non-specific binding. There was a progressive decrease in cytosol receptor content, an early rise in the Tris-soluble nuclear and a later appearance of the chromatin-bound species. It is noteworthy that cytosol receptor content was depleted by incubation with nuclei (Fig. 8). Cytosol labelled with ^{3}H-aldosterone was incubated at 25° C in 0.25 M sucrose-3 mM $CaCl_2$ without added nuclei for 16 min. This fraction contained 4×10^{-14} moles of specifically bound ^{3}H-aldosterone/mg of cytosol protein at zero-time and there was no change in the content of bound steroid after incubation for 16 min. Thus, depletion of cytosol receptor content is a result of contact with the nuclear fraction. On a quantitative basis, we found that nuclear uptake in the form of Tris-soluble and chromatin-bound receptors accounted

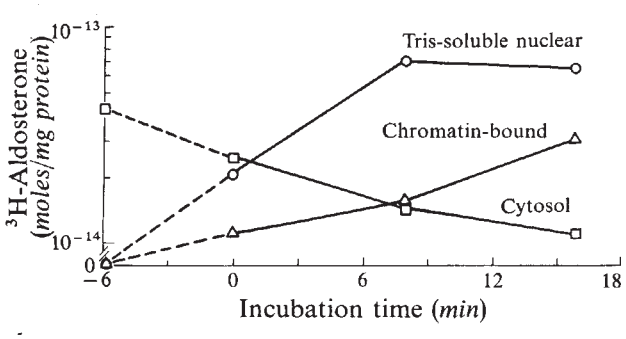

Fig. 8. *Time-course of formation of nuclear ^{3}H-aldosterone-receptor complexes in reconstituted mixtures of cytosol and nuclear fractions.* Cytosol fractions were prelabelled with ^{3}H-aldosterone (1.3×10^{-8} M), with and without 9α-fluorocortisol (1.3×10^{-6} M) for 30 min at 0° C. The labelled cytosol fractions were mixed with washed renal nuclear fractions and incubated for various times at 25° C. The "zero-time" values represent measurements obtained on mixtures of labelled cytosol and unlabelled nuclei that were processed immediately after mixing at 25° C. An extrapolated "true zero" point of −6 min has been included to take into account binding developed during the 6 min required to process the mixture. The results are the average of 5 experiments.

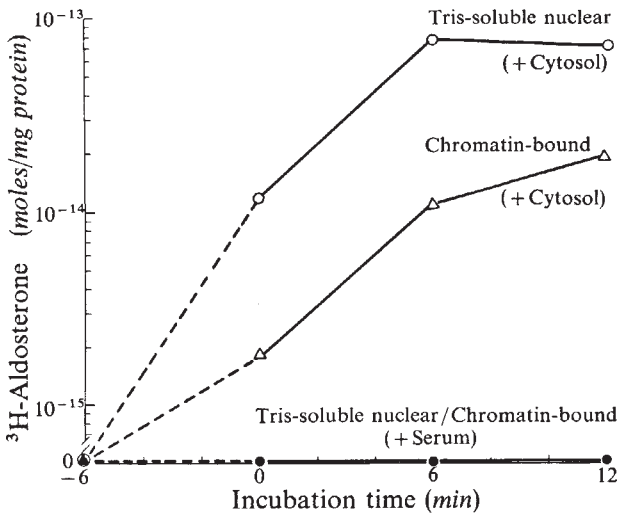

Fig. 9. *A comparison of the formation of nuclear ^{3}H-aldosterone receptors in the presence of labelled serum vs. cytosol.* Rat cytosol and serum were labelled by preincubation with ^{3}H-aldosterone (10^{-8} M) with and without 9α-fluorocortisol (10^{-6} M) at 0° C for 30 min. The labelled serum and cytosol were mixed with washed renal nuclear fractions and incubated in parallel for various times at 25° C. The results are the average of two experiments. The time-scale is defined in the legend of Fig. 8.

for about 60% of the receptor content lost from the cytosol fraction.

Several control studies were performed to ensure that generation of the nuclear receptor species was dependent on the presence of cytosol receptor complexes. Rat serum labelled with ^{3}H-aldosterone was prepared and incubated with unlabelled nuclei according to the same protocol for mixing of labelled cytosol and unlabelled nuclei. The results are shown in Fig. 9. Although the serum and cytosol fractions contained approximately equal amounts of protein and the serum contained 10-fold more bound ^{3}H-aldosterone than the cytosol fractions, only the nuclei incubated with cytosol yielded the Tris-soluble and chromatin-bound labelled receptor fractions. That cytosol donor activity depends on the presence of intact ^{3}H-aldosterone-receptor complexes was tested in three further experiments in which washed renal nuclei were mixed with: 1) ^{3}H-aldosterone in 0.25 M sucrose-3 mM $CaCl_2$ with no added cytosol, 2) dissociated cytosol ^{3}H-aldosterone-receptor complexes prepared by prewarming these complexes at 37° C for 20 min, and 3) labelled cytosol in glycerol; the incubation mixture of cytosol and nuclei contained 25% glycerol (v/v). Control flasks containing nuclei and intact labelled cytosol were incubated under the same conditions in each of these experiments. No specific intra-nuclear receptor complexes were recovered on

incubation in cytosol-free media or in pre-warmed cytosol (Table 3). In separate experiments, we found that pre-warming labelled cytosol to 37° C for 20 min completely and irreversibly dissociated the specific ^{3}H-aldosterone-receptor complexes. These results imply that generation of the intra-nuclear complexes and cytosol donor activity depends on intact cytosol receptor complexes. In addition, glycerol increased the yield of both intra-nuclear complexes. This finding is similar to the previously noted stabilizing effect of glycerol on receptor complexes labelled *in vivo* [14].

Table 3. Formation of labelled nuclear receptors from cytosol receptor complexes[a]

Donor	Tris-soluble	Chromatin-bound
	$\times 10^{-15}$ *moles/mg protein*	
Cytosol	80.0	20.1
Standard Medium	−11.9	−2.3
Cytosol	15.7	5.1
Cytosol preheated to 37 °C	−2.1	−1.0
Cytosol	33.6	12.3
Cytosol + glycerol	87.0	31.1

[a] Renal nuclei were incubated for 12 min at 25° C with cytosol prelabelled with ^{3}H-aldosterone (10^{-8} M) with and without a 100-fold excess of 9α-fluorocortisol. The nuclei were purified and extracted as under Methods and the resultant nuclear binding compared to that obtained when nuclei were incubated with ^{3}H-aldosterone ± 9α-fluorocortisol, and 1) 0.25 M sucrose-3 mM $CaCl_2$ (standard medium), 2) cytosol which had been depleted of labelled receptors by heating to 37° C for 20 min, and 3) cytosol in 25% glycerol (v/v). The results are the mean of three experiments and are corrected for non-specific labelling. The negative values indicate that the fraction contained less bound ^{3}H-aldosterone than the parallel fraction with 100-fold excess of 9α-fluorocortisol.

To define the species formed in the nuclear fractions, and depleted from the cytosol, the complexes isolated after mixing of the pre-labelled cytosol and unlabelled nuclear fractions were subjected to glycerol density gradient analysis. The glycerol density gradient patterns are shown in Fig. 10. Tris-soluble nuclear extracts prepared from nuclei incubated with ^{3}H-aldosterone alone (no cytosol) or with labelled cytosol pre-warmed at 37° C for 20 min (heat dissociated) yielded no radioactive peak. The Tris-soluble extract from nuclei incubated with intact cytosol-receptor complexes, however, contained the characteristic 3S peak (Fig. 10; lower panel). Labelled cytosol incubated in 0.25 M sucrose-3 mM $CaCl_2$ at 25° C for 16 min gave the usual

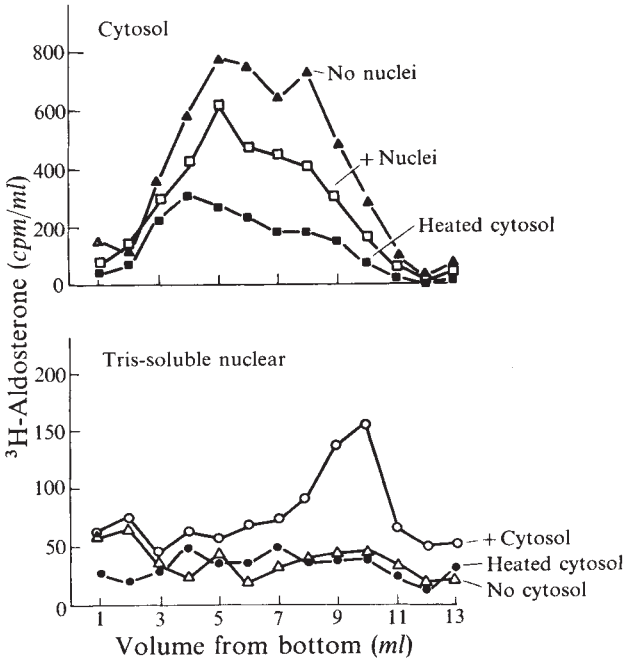

Fig. 10. *Glycerol density gradients of labelled cytosol and Tris-soluble nuclear extracts from reconstituted mixtures of cytosol and nuclear fractions.* The following mixtures (volume ratio of 3.5:1, cytosol:diluent were prepared: 1) cytosol labelled with ^{3}H-aldosterone (1.3×10^{-8} M) and the diluent, 0.25 M sucrose-3 mM $CaCl_2$, 2) cytosol labelled with ^{3}H-aldosterone (1.3×10^{-8} M) and washed renal nuclei in 0.25 M sucrose-3 mM $CaCl_2$, 3) cytosol preheated to 37°C for 20 min and then labelled with ^{3}H-aldosterone (1.3×10^{-8} M) and washed renal nuclei in 0.25 M sucrose-3 mM $CaCl_2$, 4) cytosol-free diluent which consisted of ^{3}H-aldosterone (1.3×10^{-8} M) in 0.25 M sucrose-3 mM $CaCl_2$ and washed renal nuclei in the same diluent. These mixtures were incubated at 25°C for 16 min. Cytosol and Tris-nuclear extracts were prepared as described under Methods and layered on glycerol density gradients made up in 0.1 M Tris HCl-3 mM $CaCl_2$, pH 7.2 and 1.6×10^{-9} M ^{3}H-aldosterone. The gradients were centrifuged at 283,000 × G for 40 hr. One ml fractions were collected and filtered through G-50 Sephadex to remove free ^{3}H-aldosterone.

8.5S and 4.5S peaks (Fig. 10; upper panel). Pre-warming the labelled cytosol at 37° C for 20 min resulted in almost complete loss of both peaks. Incubation of the labelled cytosol with unlabelled nuclei produced significant diminution in both peaks of about equal magnitude. These findings indicate that formation of the intra-nuclear species is associated with disappearance of the specific cytosol ^{3}H-aldosterone-receptor complexes and lend credence to the postulate that the nuclear receptors are derived at least in part (one or more peptide chains) from the cytosol receptors.

Mixing experiments were also used to determine the conditions needed for nuclear acceptor activity. Previous studies have shown that labelled nuclear receptors are thermolabile and dissociate irreversibly when

heated for as little as 15 min at temperatures as low as 30° C [7, 8, 14]. If receptors sensitive to these influences are resident in unlabelled nuclei, pre-heating of the nuclear fractions should result in a loss of acceptor activity. Accordingly, washed nuclear fractions were incubated at 37° C for 20 min prior to mixing with ^{3}H-aldosterone-labelled cytosol. The cytosol-nuclear mixtures were then incubated at 25° C for 12 min and analyzed for formation of labelled nuclear receptors. Pre-heating of the nuclear fractions produced no significant loss in acceptor activity (Table 4). In contrast, heating of the nuclear fraction *after* incubation with labelled cytosol reduced the Tris-soluble receptor content to 40% and the chromatin-bound content to 23% of their respective control values. These results suggest that the heat-sensitive receptor peptides are derived from the cytosol rather than the nuclear fraction. If this were the case, the nuclear receptors released by osmotic shock (centrifugation in 2.2 M sucrose-3 mM $CaCl_2$ followed by extraction in 0.1 M Tris-HCl, 3 mM $CaCl_2$) should not be resident in nuclei prior to exposure to cytosol. This inference was explored by subjecting the nuclear fraction to osmotic shcok before mixing with labelled cytosol. Unlabelled crude nuclear fractions were mixed with 2.2 M sucrose-3 mM $CaCl_2$, centrifuged at 65,000 × G for one hr and the pellets were resuspended in 0.1 M Tris-HCl, 3 mM $CaCl_2$ for 10 min at 0° C. The nuclei were recovered by centrifugation at 600 × G for 10 min. The Tris extracts of these unlabelled nuclei contained the same amount of protein (~0.25 mg/ml) as the extracts of nuclei which had been labelled with ^{3}H-aldosterone either *in vivo* or *in vitro*. The control nuclei were similarly purified by centrifugation through 2.2 M sucrose-3 mM $CaCl_2$ but were spared osmotic shock by stepwise dilution to a final concentration of 0.25 M sucrose-3 mM $CaCl_2$. The pre-shocked and control nuclei were mixed with ^{3}H-aldosterone-labelled cytosol and incubated at 25° C for 12 min. The nuclei were then assayed for the content of Tris-soluble and chromatin-bound receptor complexes. The data in Table 4 indicate that the pre-shocked nuclei exhibited no loss in acceptor activity. This result is also consistent with the inference that nuclear receptor peptides are derived from the cytoplasmic steroid-receptor complex. This conclusion assumes that aldosterone does not uniquely confer susceptibility to heat and osmotic shock. Pre-heating of the cytosol, however, destroys binding activity either in the presence or absence of aldosterone (Marver, D., and Edelman, I. S.: Unpublished observations).

Table 4. Effect of pretreatment of nuclei on nuclear acceptor activity[a]

Nuclear Treatment	Tris-soluble	Chromatin-bound
	$\times 10^{-15}$ *moles/mg protein*	
Control	27.8	9.9
Pre-heated, 37° C	38.8	12.6
Post-heated, 37° C	11.0	2.2
Control	166.6	25.0
Pre-osmotic shock	209.1	37.0
Control	67.0	40.0
+DNase	19.1	3.7
+RNase	74.6	40.4

[a] Unlabelled renal nuclei were pretreated either by 1) heating to 37° C for 20 min, or 2) osmotic shock in 2.2 M sucrose-3 mM $CaCl_2$ followed by dilution in 0.1 M Tris-HCl, 3 mM $CaCl_2$, or 3) incubation with RNase or DNase (250 μg/ml) at 37° C for 20 min. After pretreatment, the nuclei were incubated at 25° C for 12 min with cytosol pre-labelled with ^{3}H-aldosterone (10^{-8} M) ± 100-fold excess of 9α-fluorocortisol. Glycerol (25%) was present only in the osmotic shock experiment. Following incubation at 25° C, the nuclei were centrifuged through 2.2 M sucrose-3 mM $CaCl_2$ and extracted with Tris buffer and 0.4 M KCl as under Methods. The results are the mean of two to three experiments and have been corrected for non-specific binding.

The nuclear receptor complexes fall into two classes, those released by osmotic shock and soluble in 0.1 M Tris-HCl, and those released from chromatin-bound sites by extraction with 0.3 to 0.4 M KCl. In view of the evidence suggesting that the nuclear aldosterone receptors may be involved in the regulation of transcription, nuclear DNA and/or RNA may play an important role in the binding process or in the generation of nuclear receptor complexes from cytosol complexes. In earlier studies the nucleases, DNase and RNase, did not accelerate the release of bound ^{3}H-aldosterone from intact renal nuclei or the dissociation of the ^{3}H-aldosterone-protein complexes extractable from intact nuclei or isolated chromatin fractions [6–8]. In the present study, we tested the effect of pre-treatment of unlabelled renal nuclei with DNase and RNase on nuclear acceptor activity. Crude nuclear pellets, obtained by centrifugation of renal homogenates at 600 × G for 10 min, were suspended in 0.25 M sucrose-3 mM $CaCl_2$, 50 mM Tris-HCl, pH = 7.0, with or without added DNase or RNase (final concentration = 250 μg/ml). These suspensions were incubated at 37° C for 20 min, and the nuclei were recollected by centrifugation at 2° C for 10 min. The nuclear fractions were

washed with 7 to 10 volumes of 0.25 M sucrose-3 mM $CaCl_2$ and purified by centrifugation in 2.2 M sucrose-3 mM $CaCl_2$. The purified nuclei were resuspended in 0.25 M sucrose-3 mM $CaCl_2$, mixed with cytosol fractions pre-labelled with ^{3}H-aldosterone and incubated at 25° C for 12 min. Non-specific nuclear uptake was determined in parallel incubations that contained 100-fold excess of 9 α-fluorocortisol over ^{3}H-aldosterone. These mixtures were analyzed for Tris-soluble and chromatin-bound receptor content by the usual procedures. Aliquots of the nuclear fractions were taken for analysis of DNA and RNA content. Treatment with RNase increased the DNA/RNA ratio by 21% (from 3.2 to 3.9) but had no effect on the generation of either the Tris-soluble nuclear or chromatin-bound receptors (Table 4). In contrast, treatment with DNase which lowered the DNA/RNA ratio by 50% (from 3.2 to 1.6) decreased formation of Tris-soluble receptors to 28% and the chromatin-bound receptors to 9% of the control values. Loss of acceptor activity due to DNase treatment is probably not a consequence of the loss of measurable quantities of protein since the RNA/protein ratio was unchanged. These findings imply that the Tris-soluble nuclear receptors, as well as the chromatin-bound receptors, are associated with the DNA backbone of chromatin.

Nuclear acceptor activity can be conceived as consisting of two processes: 1) transformation of the complex from the cytoplasmic form (8.5/4.5S) to the nuclear form (3S or 4S), and 2) binding of the transformed complex to chromatin. The results obtained with DNase may signify either that the transformation of the cytoplasmic to the nuclear forms is DNA dependent or that binding of the transformed complexes to chromatin is DNA dependent.

Discussion

The initiation of steroidal regulation of Na^+ transport probably involves formation of chromatin-bound receptors and regulation of DNA-dependent RNA synthesis [1]. Moreover, very similar initiation mechanisms, consisting of binding to tissue receptors and regulation of RNA and protein synthesis, have been described for virtually all of the steroid hormones [18, 19]. Stereospecific aldosterone binding proteins have been isolated from cytosol and nuclear fractions of rat kidney after adrenalectomy [7, 8]. These receptors are distinguishable into three classes by their sedimentation properties in low and high salt media. By glycerol density gradient analysis, the cytosol receptor is 8.5S in 0.1 M Tris-buffer and 4.5S in 0.3 M KCl (Fig. 3). The Tris-soluble nuclear receptor released by osmotic shock sediments at 3S (Fig. 4) in either low salt (0.1 M Tris-HCl) or high salt (0.1 M Tris-HCl, 0.4 M KCl). Swaneck et al. [8] found that the chromatin-bound receptor was not released by osmotic shock but was extractable with 0.3 to 0.4 M KCl and sedimented at 4S in the presence of high salt (0.4 M KCl). In low salt, the chromatin-bound receptor aggregated and sedimented to the bottom of the gradient tube. The sedimentation properties in low and high salt media imply that one or more of the subunits of the cytosol receptors differ from that of the nuclear receptors. The nature of the differences are not yet clear and may consist either of 1) addition or subtraction of subunits or adhering peptides, or 2) modification of the subunits by chemical reactions (e. g., phosphorylation or acetylation). An example of the former type of change was provided by the recent work of Harris [20]. She found that in the presence of polyanions, the estradiol-protein complex extracted from uterine chromatin sediments as an 8S peak in low salt media. The polyanion appears to complex basic proteins which contribute to aggregation of the complex in low salt.

We have presented evidence in support of the view that the cytosol-steroid-receptor complex is the precursor of the intranuclear forms and that the process may consist of three steps: aldosterone + cytosol receptor → active cytosol complex (8.5/4.5S) → Tris-soluble nuclear complex (3S) → chromatin-bound complex (4S). In the reconstitution experiments, the aldosterone-receptor content of the cytosol fraction diminished coincident with the appearance of the intra-nuclear species despite the presence of excess free ^{3}H-aldosterone in the medium (Fig. 8). Moreover, the intranuclear aldosterone-receptor complexes are thermolabile but neither pre-heating or pre-osmotic shock of unlabelled nuclei impaired nuclear uptake of the cytosol complex (Table 4). In contrast, pre-heating of cytosol destroys both aldosterone binding and donor activity. These results suggest that the complex is transferred from the cytoplasm into the nucleus and is transformed to the 3S and 4S forms in the process. In support of this inference, mass balance analysis (i. e., total protein × cpm/mg protein) revealed that the pool of aldosterone-receptor complexes was greater in the cytosol than in the soluble nuclear fraction and in turn the pool was greater in the soluble nuclear than in the chromatin fraction. It is of interest that in the slice experiments the content of cytosol receptor complexes attained steady state levels with no reduction during the continued rise in the content of nuclear

receptor complexes (Fig. 6). The failure to detect cytosol depletion coincident with nuclear uptake in the slice experiments may reflect the existence of a slowly reacting pool of cytosol receptors or simply that the quantity of extra-nuclear receptors is large compared to the amount taken up by the nucleus. From our results and the protein content of each fraction, we have calculated that the latter explanation is tenable.

If the three-step mechanism is valid, the interactions of the receptor complexes with chromatin should initiate the induction phase of the physiological response. All of the physiologically active steroids share in common the property of binding to specific receptors that attach to chromatin and of inducing protein synthesis *de novo* [21]. The participation of intact DNA in nuclear binding of the receptor complex is indicated by the finding that pre-treatment with DNase, but not RNase, impaired nuclear acceptor activity (Table 6). Harris [20] found that uterine nuclear estradiol-receptor complexes are released by the action of DNase in Mg^{++}-free solutions. Moreover, Mainwaring and Mangan [22] showed that cytosol receptor-dihydrotestosterone complexes bind specifically to deproteinized prostatic DNA. These results support the inference that regions of DNA provide a surface for the binding of steroid-receptor complexes to chromatin. The participation of chromosomal proteins in the binding of steroid-receptor complexes to chromatin has been inferred from chromatin reconstitution experiments. O'Malley et al. [23] found that the elimination of the nonhistone proteins from reconstituted oviduct chromatin resulted in a considerable decrease in the extent of binding of progesterone-receptor complexes prepared from oviduct cytosol.

The precise relationship, if any, between the binding process and induction of DNA-dependent RNA synthesis by aldosterone remains to be elucidated. There also remains the question of the relationship between the Tris-soluble nuclear receptors (3S) and the chromatin-bound species (4S). The time-course of formation of these species is consistent with a precursor-product relationship. The dependence of both forms on intact DNA suggests that both are bound to chromatin in the intact nucleus. Further studies are needed to distinguish between the possibility that the 3S and 4S attach to independent sites on chromatin as opposed to a precursor-product relationship.

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