Stereospecific Binding of Aldosterone to Renal Chromatin*

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SUMMARY

In an earlier study, 51% of total renal nuclear content of 3H-aldosterone was recovered bound to nuclear proteins readily extractable with Tris-3 mM CaCl2. Modifications in the methods used to prepare renal nuclear fractions resulted in the isolation of 3H-aldosterone bound to chromatin to the extent of 55% of the total nuclear content of the steroid. The chromatin binding system was stereospecific for aldosterone and related mineralocorticoids. In competition studies, the order of affinities for the 3H-aldosterone binding sites was d-aldosterone > 9α-fluorocortisol > cortisol > 17α-estradiol ≅ progesterone = 17α-isoaldosterone. Actinomycin D did not compete for the 3H-aldosterone binding sites in renal chromatin. The binding affinities were in accord with the relative potencies of these steroids as mineralocorticoids. In addition, spirolactone blocked the binding of 3H-aldosterone to chromatin at the molar ratio needed to block the action of the steroid on sodium transport. Based on differential susceptibility to specific hydrolases (i.e. DNase, RNase, trypsin, and chymotrypsin), CsCl density centrifugation, chemical analysis of 0.3 M KCl extracts of labeled chromatin, and glyceral density centrifugation, the primary binding unit appears to be a 4 S, nonhistone chromosomal protein. The aldosterone-binding protein is heat-stable and tends to be stabilized by 20% glycerol. The possibility that the formation of the aldosterone-chromosomal binding system initiates steroidal action on active sodium transport is discussed.

The available evidence suggests that aldosterone and related mineralocorticoids regulate active sodium transport in anuran and mammalian target tissues by inducing RNA and protein synthesis (1–3). It is probable that these events are initiated by the formation of specific steroid-receptor complexes. Recent studies indicate that an early step in the mechanism of action of aldosterone involves noncovalent binding of the native steroid to nuclei of target cells, e.g. rat kidney and urinary bladder of the toad (1, 4–7). The molecular events in the initiation process, however, remain to be elucidated.

Stereospecific aldosterone-binding proteins with many of the expected properties of the physiological receptors have been isolated from nuclear and cytosol fractions of rat kidneys and other tissues (8, 9). For obvious reasons, our attention was directed to the possibility of direct interactions between the steroid-receptor complexes and the chromatin system. We were encouraged to study the binding of aldosterone to renal chromatin by earlier studies on the binding of androgens and estrogens to chromatin of their respective target tissues (10, 11).

In this paper, we report some of the characteristics of stereospecific binding of aldosterone to rat kidney chromatin and the isolation of a steroid-protein complex from the chromatin fraction.

EXPERIMENTAL PROCEDURE

Materials—d-Aldosterone-1,2-3H (25 Ci per mmole) in 90% benzene, 10% ethanol was obtained from New England Nuclear. The solvents were evaporated under N2 and the steroid was dissolved in 0.9% NaCl. Radiochemical purity was 96% in a conventional chromatographic system.1 Progesterone, cortisol, 17β-estradiol, and d-aldosterone were obtained from Calbiochem, 9α-fluorocortisol was from Upjohn, 17α-isoaldosterone was from Dr. J. F. Tait (Worcester Foundation for Experimental Biology, Worcester, Massachusetts), spirolactone (SC-14266) was from G. D. Searle, actinomycin D was from Calbiochem, bovine pancreatic DNase I, RNase, trypsin, chymotrypsin, and lysozyme, as well as other enzymes, were from Worthington, and Sephadex G-50 was from Pharmacia. Dr. H. H. Fudenberg supplied the sample of human IgG myeloma protein. We prepared rat hemoglobin by distilled H2O hemolysis of rat erythrocytes and separation of the ghosts by centrifugation. All of the conventional reagents used were of analytical grade quality.

Procedures—Male adrenalectomized Sprague-Dawley rats (150 to 200 g of body weight) were maintained on 0.9% NaCl drinking water and Purina laboratory chow ad libitum. Sixteen hours before the experiment, the rats were placed on diets of low potassium pellets (1.2 μeq of K+ per g, dry weight, supplied by General Biochemicals). The rats were injected subcutaneously with $2.6 \times 10^{-10}$ mole of 3H-aldosterone in ~1 ml of NaCl solution per 100 g of body weight. Competition studies were carried out by simultaneous subcutaneous injection with d-aldosterone

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1 E. Biglieri, personal communication.
or 17α-isoaldosterone at dosages of 2.6 × 10^{-3} mole/100 g of body weight or with 9α-fluorocortisol, cortisol, progesterone, or 17β-estradiol at dosages of 2.6 × 10^{-3} mole/100 g of body weight. In additional experiments, spirolactone (2.6 × 10^{-6} mole/100 g of body weight) was injected subcutaneously 30 min before "H-aldosterone.

Thirty minutes after injection of "H-aldosterone, the rats were anesthetized with ether and exsanguinated by cardiac puncture. The kidneys were perfused via the renal arteries with 20 ml of ice-cold solution hs, decapsulated, and put into ice-cold solution hs. All subsequent procedures were carried out at 4° and all centrifugations at ~2°. The nuclear fractions were prepared by a modification of the method of Sadowski and Steiner (12). The kidneys were excised longitudinally, blotted with gauze, passed through a stainless steel Harvard tissue press, and homogenized in 6 volumes of solution hs by eight strokes of a Potter-Elvehjem Teflon-glass homogenizer, with a pestle clearance of 0.018 to 0.023 cm, at a speed of 900 rpm. The homogenate was diluted 1:20 (w/v), filtered through silk mesh (Swiss Silk Bolting Cloth, Zurich), and centrifuged at 1000 × g for 8 min in a Sorvall HB-4 swinging bucket rotor. The supernatants were discarded and the pellets were resuspended in 10 ml of solution hs. This suspension was layered over 0.88 M sucrose, 3 mM MgCl₂ (pH 8.0), and centrifuged at 2500 × g for 8 min. The pellets were resuspended in 2.5 ml of 0.88 M sucrose, 3 mM MgCl₂ (pH 8.0) and then well with 30 ml of 2.2 M sucrose, 3 mM MgCl₂ (pH 8.0), and centrifuged for 1 hour in an SW 27 rotor at 25,000 rpm in an L-2 Beckman ultracentrifuge. The supernatants were then discarded and the white pellets were carefully suspended in 0.1 M Tris, 3 mM CaCl₂ (pH 8.0) for extraction of Tris-soluble proteins or put directly into 0.01 M Tris, 8 mM Na₂ EDTA (pH 8.0) for isolation of chromatin. Aliquots were taken for chemical analysis of protein and nucleic acids in 10% trichloroacetic acid and for radioactive assay. The pellets were stained with 0.5% methylene blue and examined in a Zeiss photomicroscope with phase contrast optics.

Tris-soluble nuclear proteins were extracted by suspending the purified pellets in 5 ml of 0.1 M Tris, 3 mM CaCl₂ (pH 8.0) at 0° for 10 min (S). These nuclei were then treated by centrifugation at 20,000 × g for 10 min. The Tris-soluble "H-aldosterone-protein complexes were isolated either by passage through Sephadex G-50 or by precipitation in (NH₄)₂SO₄ at 50% saturation. In a preliminary study, we found that the maximum yield of "H-aldosterone-macromolecular complexes from chromatin was obtained by extraction with 0.3 M KCl. Aliquots of suspensions of purified chromatin were incubated in 0.3 M KCl, 0.01 M Tris (pH 8.0) for 15 min at 0°. The suspensions were intermittently mixed with a Teflon-glass homogenizer at low speed. The residual chromatin was sedimented by centrifugation at 110,000 × g for 60 min. The quantity of bound "H-aldosterone released by extraction with KCl was determined either by passage through Sephadex G-50 columns or by precipitation in 50% saturated (NH₄)₂SO₄. Glycerol density gradient analysis was used to characterize the complexes extracted in 0.3 M KCl. One milliliter aliquots of the extracts were layered on top of a 10 to 35% glycerol gradient (made up in 0.3 M KCl, 0.01 M Tris, pH 8.0) and centrifuged at 280,000 × g for 4 hours at 2° in the SW 40 rotor (Beckman model L2-65B ultracentrifuge). Thirteen-drop (1.0 ml) fractions were collected with a Buchler dripping device. Standards with known sedimentation coefficients, including malic dehydrogenase, alkaline phosphatase, rat hemoglobin, human IgG myeloma protein, or lysozyme were run in parallel tubes with each centrifugation.

Analytical Methods—Protein concentrations were determined by the method of Lowry et al. (15) with bovine serum albumin as the standard, DNA by the method of Burton (16) with calf thymus DNA as the standard, and RNA by the orcinol method of Ceriotti (17) with yeast RNA as the standard.

"H-Aldosterone was recovered from various fractions by radio-assay by extraction with 5 volumes of CHCl₃. The organic phase was transferred to plastic counting vials and air-dried.
TABLE I

Composition of rat kidney nuclei and chromatin

The crude homogenates and purified nuclear and chromatin fractions were prepared as described in the text. Results are given as means plus or minus standard error.

<table>
<thead>
<tr>
<th>Source</th>
<th>No.</th>
<th>RNA-DNA ratio (mg/mg)</th>
<th>DNA-protein ratio (mg/mg)</th>
<th>RNA-protein ratio (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>24</td>
<td>1.08 ± 0.10</td>
<td>0.014 ± 0.001</td>
<td>0.029 ± 0.001</td>
</tr>
<tr>
<td>Purified nuclei</td>
<td>24</td>
<td>1.05 ± 0.088</td>
<td>0.307 ± 0.018</td>
<td>0.067 ± 0.003</td>
</tr>
<tr>
<td>Chromatin</td>
<td>30</td>
<td>0.153 ± 0.01</td>
<td>0.522 ± 0.01</td>
<td>0.081 ± 0.001</td>
</tr>
</tbody>
</table>

a Number of experiments.

TABLE II

Intracellular distribution of \(^3\)H-aldosterone

Adrenalectomized rats were injected with \(^3\)H-aldosterone (2.6 × 10^{-10} mole/100 g of body weight) 30 min before death. The purified nuclear fractions were extracted with 0.1 M Tris-3 mM CaCl\(_2\) (pH 8.0) as described previously (8). Chromatin was prepared from the Tris-CaCl\(_2\)-extracted nuclei as described in the text. "Residual" denotes the quantity of \(^3\)H-aldosterone in the supernatant of the 1.7 M sucrose solutions after sedimentation of the chromatin. Results are given as means plus or minus standard error.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total</th>
<th>Bound(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-CaCl(_2) extract</td>
<td>27.6 ± 0.9</td>
<td>63(^a)</td>
</tr>
<tr>
<td>Chromatin</td>
<td>55.1 ± 2.1</td>
<td>76(^a)</td>
</tr>
<tr>
<td>Residual</td>
<td>17.3 ± 0.3</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Bound (\%) refers to the proportion of the total \(^3\)H-aldosterone in the particular fraction that was bound to a macromolecule.

\(^b\) Determined by precipitation with 50\% saturated (NH\(_4\))\(_2\)SO\(_4\) and Sephadex G-50 gel filtration.

\(^c\) The chromatin was sheared and the resultant soluble nucleohistone was passed through Sephadex G-50 columns.

Fifteen milliliters of counting solution\(^3\) were added to each vial and the samples were assayed in a Mark I liquid scintillation spectrometer (Nuclear-Chicago) at an efficiency of 30\%. Total tritium content was determined by direct addition of aliquots (less than 1 ml) of various fractions to 15 ml of scintillation solution. Correction for quenching in all samples was made with an external \(^24\)Ba standard.

RESULTS

Nuclear Preparation. In earlier studies \(^3\)H-aldosterone-protein complexes were isolated from cytosol as well as nuclear fractions of rat kidney (8). Accordingly, we used both histological and chemical criteria to ensure that the nuclear preparations were minimally contaminated with cytoplasmic elements. In the light microscope, stained smears revealed no discernible contamination. The nuclei were well preserved and round or oval in shape and the nucleoli stained intensely. Based on recovery of DNA, the purified nuclear fraction contained 69.4\% of the nuclei of the crude homogenate. The DNA, RNA, protein ratios are listed in Table I. The purified nuclear fraction consisted of 27\% DNA, 4.4\% RNA, and 65.6\% protein. Minimal extranuclear enzyme activity (i.e. cytochrome c oxidase, glucose 6-phosphatase, and glucose 6-phosphate dehydrogenase) was detected in the nuclear fraction. The quantity of RNA in the crude renal homogenates was 35\% less than that found in rat liver homogenates and the renal nuclear fraction contained proportionately more of the cellular RNA (12). The DNA-protein ratio of the renal chromatin preparations meets the accepted criteria of purity (18). Moreover, the ultraviolet absorption spectrum of the renal chromatin preparation was identical with that of rat liver (18). These results indicate that the nuclear and chromatin fractions conformed to present standards of purity.

Intracellular Distribution of \(^3\)H-Aldosterone—Herman, Finog- nari, and Edelman (8) reported that 81\% of the \(^3\)H-aldosterone content of renal nuclei was extracted by 0.1 M Tris-3 mM CaCl\(_2\) solutions and 63\% of this steroid was recovered in a complex bound to protein. In the present study, preparation of the

TABLE III

Stereospecificity of binding of aldosterone to renal chromatin

Adrenalectomized rats were injected with \(^3\)H-aldosterone (2.6 × 10^{-10} mole/100 g of body weight) alone or simultaneously with a competing steroid (2.6 × 10^{-8} mole/100 g of body weight). The kidneys were removed 30 min after the injections and the quantity of \(^3\)H-aldosterone bound to chromatin was measured as described in the text in units of moles per mg of protein. Results are given as means plus or minus standard error.

<table>
<thead>
<tr>
<th>No.</th>
<th>Competing steroids</th>
<th>Relative specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>(^\alpha)-Isodiol-alosterone</td>
<td>106 ± 10</td>
</tr>
<tr>
<td>12</td>
<td>(^\delta)-Aldosterone</td>
<td>20 ± 3</td>
</tr>
</tbody>
</table>

\(^a\) Number of experiments.

\(^b\) Molar ratio of competing steroid to \(^3\)H-aldosterone is given in parentheses.
nuclear fractions by homogenization of the kidney in a Mg\textsuperscript{2+}-containing medium and serial passage through 0.88 M and 2.2 M sucrose substantially reduced the fraction of \textsuperscript{3}H-aldosterone that was extractable in Tris-CaCl\textsubscript{2} (Table II). Of the total nuclear content of \textsuperscript{3}H-aldosterone, 55% was recovered in association with chromatin and 76% of this subfraction was recovered bound to soluble nucleohistone after passage through Sephadex G-50. The chromatin fractions were also extracted with 5 volumes of CH\textsubscript{3}CO\textsubscript{2}H, and 96% of this \textsuperscript{3}H-aldosterone corresponded to the native steroid as judged by conventional paper chromatography. The nature of the \textsuperscript{3}H-aldosterone in the residual fraction was not investigated further.

Specificity of Binding of \textsuperscript{3}H-Aldosterone to Chromatin—If physiological significance is to be attributed to binding of aldosterone to chromatin, the binding properties of competing steroids should correspond in some way to their potencies as agonists or antagonists with respect to active sodium transport. Accordingly, four classes of steroids were chosen for study as competitors: d-aldosterone and \textsuperscript{9}α-fluorocortisol, potent agonists; cortisol, an agonist of intermediate potency; \textsuperscript{17}α-isoaldosterone, progesterone, and \textsuperscript{17β}-estradiol, inactive as mineralocorticoids; and spirolaactone, an antagonist, when the dose is more than 1000-fold greater than that of d-aldosterone. The results in Table III show that at dose ratios of 10:1 the inactive stereoisomer, \textsuperscript{17α}-isoaldosterone, had no effect on the binding of \textsuperscript{3}H-aldosterone to chromatin, whereas d-aldosterone reduced the binding by 80%. That the aldosterone binding sites in chromatin are mineralocorticoid specific as well as stereospecific is indicated by the data given in Table IV. The order of inhibition of \textsuperscript{3}H-aldosterone binding was \textsuperscript{9}α-fluorocortisol > cortisol > \textsuperscript{17β}-estradiol = progesterone, which is in accord with their relative potencies as mineralocorticoids. The antagonist spirolaactone, at a molar ratio of 10\textsuperscript{4}:1, displaced \textsuperscript{3}H-aldosterone from 79% of the chromatin binding sites. These results, therefore, are compatible with the possibility that the binding of aldosterone to chromatin initiates the sequence of events in the action on sodium transport.

Actinomycin D is a specific inhibitor of the mineralocorticoid action of aldosterone in the toad bladder \textit{in vivo} and in the rat kidney \textit{in vivo} (1, 19). This effect has been attributed to the affinity of actinomycin D for the guanosine residues in DNA and its consequent inhibition of RNA synthesis (19). An alternative possibility is that this antibiotic impairs the binding of aldosterone to physiological receptors in chromatin. To test this possibility, actinomycin D (40 μg/100 g of body weight) was injected subcutaneously into adrenalectomized rats 30 min before injection of \textsuperscript{3}H-aldosterone (2.6 × 10\textsuperscript{-10} mole of \textsuperscript{3}H-aldosterone 30 min before death). The chromat was centrifuged in 2.09 M CsCl and fractionated as described under "Methods."

CsCl Density Gradient Centrifugation—High speed centrifugation of chromatin in concentrated CsCl dissociates and separates most of the chromosomal proteins from the high molecular weight polynucleotides. Maurer and Chalkley (10) exploited this method to characterize the macromolecular species involved in the binding of \textsuperscript{17β}-estradiol to chromatin of calf endometrium.
Table V

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Bound 3H-aldosterone</th>
<th>20% Glycerol-Tris buffer</th>
<th>% of control</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>8</td>
<td>100†</td>
<td>100†</td>
</tr>
<tr>
<td>Trypsin²</td>
<td></td>
<td>6</td>
<td>90.3 ± 3.2</td>
<td>105 ± 4.5</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td></td>
<td>6</td>
<td>11.3 ± 1.1</td>
<td>52.8 ± 8.8</td>
</tr>
<tr>
<td>DNase²</td>
<td></td>
<td>4</td>
<td>98.0 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>RNase²</td>
<td></td>
<td>4</td>
<td>113 ± 10.0</td>
<td></td>
</tr>
</tbody>
</table>

* Number of experiments.

†, ‡ In the absence of enzymes (controls), the per cent of radioactivity isolated as bound 3H-aldosterone (bound/total) after incubation was 41% in Tris buffer and 67% in glycerol-Tris buffer. The extent of proteolysis with trypsin was 45% in Tris buffer and 36% in 20% glycerol-Tris buffer.

The extent of proteolysis with chymotrypsin was 56% in Tris buffer and 41% in 20% glycerol-Tris buffer.

The extent of hydrolysis was 80% with DNase and 20% with RNase.

Table VI

Specific activities of 3H-aldosterone-protein complexes

Thirty adrenalectomized rats were injected with 3H-aldosterone (2.6 × 10⁻⁴ mole/100 g) 30 min before death. The fractions were obtained from rat kidneys as described in the text. Results are given as means plus or minus standard errors.

| Fraction       | Specific activity
|----------------|------------------|
| Homogenate     | 1.8 ± 0.1
| Nuclear        | 6.0 ± 0.4
| Chromatin      | 10.8 ± 0.8
| 0.3 M KCl extract | 49.9 ± 5.8
| 0.3 M KCl extract-(NH₄)₂SO₄ precipitate | 80.9 ± 10.7

* The precipitate formed in 50% saturated (NH₄)₂SO₄ contained 57% of the 3H-aldosterone in the 0.3 M KCl extract.

We applied the CsCl density gradient centrifugation technique to the problem of identifying the aldosterone-binding species in renal chromatin. The results are shown in Fig. 1. The nucleic acids sedimented to the bottom of the tube and only a negligible fraction of the 3H-aldosterone was associated with this pellet. A reproducible 3H-aldosterone peak was obtained in Fraction 5 (δ = 1.30) that corresponded to the high density side of the protein peak (centered at δ = 1.28). There was, in addition, a background of 3H-aldosterone distributed throughout the gradient, presumably as a result of partial dissociation of 3H-aldosterone from the binding sites during the prolonged centrifugation. The possibility that the 3H-aldosterone peak was a result of nonspecific adherence of the steroid to aggregated nucleic acid was tested by incubating the 3H-aldosterone chromatin complexes at 37°C for 30 min prior to centrifugation in CsCl. Under these conditions, the 3H-aldosterone was completely dissociated from the chromatin binding sites as judged by passage through Sephadex G-50 at 4°C. As shown in Fig. 2, the protein peak of the heat-treated chromatin was unchanged in its position in the CsCl gradient by 3H-aldosterone was uniformly distributed throughout the gradient. Thus, the 3H-aldosterone-protein complex that banded in the CsCl gradient represented a bound complex and did not represent nonspecific adsorption of the steroid to protein aggregates. These results suggest that native chromosomal proteins are responsible for the binding of aldosterone to chromatin.

Susceptibility of 3H-Aldosterone-Chromatin Complex to Enzymatic Hydrolysis—As a further test of the inference that chromosomal proteins provide primary aldosterone binding sites, we studied the effects of specific hydrolytic enzymes on the rate of dissociation of the steroid-chromatin complex. Extensive cleavage of the specific binding species should result in dissociation of 3H-aldosterone from the binding sites. Incubation of 3H-aldosterone-chromatin complexes with either pancreatic DNase I or RNase A had no significant effect on the spontaneous rate of dissociation of the complex (Table V). In contrast, trypsin reduced the recovery of bound 3H-aldosterone to 60% and chymotrypsin to 11% of the control level. We suspected that trypsin reduced the recovery of bound 3H-aldosterone by an indirect effect on the stability of the primary steroid-macro-molecular complex. Herman et al. (8) found that soluble nuclear aldosterone-binding proteins were rapidly denatured at 20°C and that 25% glycerol stabilized the complex significantly. Accordingly, the effects of trypsin and chymotrypsin were re-tested in 20% glycerol-Tris buffer. Under these conditions, the spontaneous rate of dissociation was reduced to 43% per 30 min of incubation at 20°C, and trypsin had no effect on this rate of dissociation. Chymotrypsin, however, almost halved the recovery of bound 3H-aldosterone. Moreover, the extent of proteolysis achieved with trypsin and chymotrypsin in glycerol-Tris buffer was only modestly reduced compared with that in Tris buffer. In view of the high degree of susceptibility of histones to trypsin digestion (21), these results suggest that aldosterone binds to nonhistone chromosomal proteins.

KCI Extraction of 3H-Aldosterone-Protein Complex from Chromatin—Salt extraction of chromosomal proteins has been widely used, including isolation of a 5 S bound 3H-estradiol complex from a uterine nuclear-myofibrillar fraction (22). We found that the optimum conditions for extraction of soluble, bound 3H-aldosterone from chromatin were to incubate in 0.3 M KCl, pH 8.0, at 0°C for 15 min. Under these conditions, 82.7% of the 3H-aldosterone and 15% of the chromosomal protein remained in the supernatant after sedimenting the extracted chromatin at 110,000 × g for 20 min. Sixty-seven per cent of the extracted 3H-aldosterone was recovered bound in a macromolecular complex on filtration through Sephadex G-50. The extent of purification...
FIG. 3. Stability of $^3$H-aldosterone complexes extracted from chromatin. Chromatin was prepared from kidneys of adrenalectomized rats injected with $2.6 \times 10^{-10}$ mole of $^3$H-aldosterone 30 min before removal. KCl extracts, 0.3 M, were prepared as described under "Methods." Aliquots of the extracts were incubated in 0.3 M KCl, 0.01 M Tris (pH 8.0) (O-O), and in the same solution made up to 20% glycerol (O-O) at 0°C for the time periods as indicated. The percent bound was determined from the total $^3$H content of each sample and by filtration through Sephadex G-50 columns. Each point is an average of four separate samples.

achieved by these procedures is given in Table VI. The specific activity of the chromatin fraction was twice that of the nuclear fraction and 6-fold that of the crude homogenate. Extraction in KCl and precipitation of the complex in 50% (NH$_4$)$_2$SO$_4$ yielded an 8-fold increase in specific activity compared to chromatin and 45-fold that of the crude homogenates. To characterize the macromolecular composition of the 0.3 M KCl extracts, conventional analyses of DNA, RNA, and protein content were undertaken. The protein concentration was 56.1 ± 2.4 μg per ml of extract (n = 24); the DNA concentration was below the limit of detectability (i.e. < 0.25 μg per ml), and the RNA concentration was 0.78 ± 0.10 μg per ml, or less than 2% that of the protein concentration by weight. In view of the limited susceptibility of the RNA in chromatin to hydrolysis by RNase (see Table V) and the presence of a small quantity of RNA in the 0.3 M KCl extracts, the possibility that RNA participates in chromosomal binding of aldosterone deserves further study.

The stability of the $^3$H-aldosterone-protein complex obtained by extraction of chromatin with 0.3 M KCl was studied in standard Tris buffer and in 20% glycerol-Tris buffer at 0°C. The results in Fig. 3 show that the $^3$H-aldosterone-protein complex dissociated logarithmically in Tris buffer; only 23% remained bound at the end of 48 hours. In glycerol-Tris buffer, however, 75% of the complex was intact after 48 hours. Lower concentrations of glycerol were much less effective in preventing dissociation of the complex.

The thermal stability of $^3$H-aldosterone-protein complex extracted in 0.3 M KCl was tested by incubation for 30-min intervals at various temperatures. The results in Fig. 4 indicate a high degree of thermal lability at temperatures above 20°C.

FIG. 4. Thermal stability of $^3$H-aldosterone complexes extracted from chromatin. $^3$H-aldosterone-protein complexes were prepared as described in the legend of Fig. 3. Aliquots of each sample were incubated for 30 min at various temperatures in 0.3 M KCl, 0.01 M Tris (pH 8.0) (measured at 22-23°C) (O-O) and in the same solution made up to 20% glycerol (O-O). (See Footnote 4 in text.) The quantity of bound $^3$H-aldosterone was determined by filtration through Sephadex G-50 columns.

FIG. 5. Glycerol density gradient centrifugation of 0.3 M KCl extract of chromatin. $^3$H-aldosterone-protein complexes were prepared as described in the legend of Fig. 3. A 1-ml extract was layered over a 10-55% glycerol gradient in 0.3 M KCl, 0.01 M Tris (pH 8.0). Centrifugation and fractionation of the gradients are described under "Methods."
Thus the pH shift between 0° and 40° was found to be stable in the pH range 6.5 to 9.2 (23). The effect of temperature shifts on the stability of the complexes extracted from chromatin is not ascribable to the pH shift, as nuclear binding of 3H-aldosterone remained at the top of the tube corresponding in position to free 3H-aldosterone (Fig. 6), which negates the possibility of nonspecific adsorption.

The possible relevance of the 4 S chromosomal, 3H-aldosterone-protein complex to physiological action was assessed by competition studies with mineralocorticoids. Adrenalectomized rats were injected with 3H-aldosterone (2.6 × 10^-14 mole per rat) and either d-aldosterone (2.6 × 10^-14 mole per rat) or 9α-fluorocortisol (2.6 × 10^-14 mole per rat). The 0.3 M KCl extracts of renal chromatin were prepared as described above and analyzed in identical 10 to 35% glycerol (0.3 M KCl, 0.01 M Tris, pH 8.0) density gradients. The 4 S protein peak under these conditions contained no detectable 3H-aldosterone. These results indicate that 3H-aldosterone binds to mineralocorticoid-specific sites in a 4 S chromosomal, heat-labile protein.

**DISCUSSION**

Renal chromatin has been shown to possess a stereospecific and mineralocorticoid-specific binding system. Based on CsCl density gradient centrifugation, susceptibility to loss of binding activity by specific hydrolases (i.e. trypsin, chymotrypsin, DNase, and RNase), and chemical analysis of 0.3 M KCl extracts of chromatin, the primary binding substance appears to be a 4 S nonhistone chromosomal protein. The possibility that chromosomal RNA is involved in the aldosterone-binding system deserves further study. The thermal lability of the 3H-aldosterone-protein complex indicates that the native state of the protein is a requirement for binding of the steroid. It is probable that aldosterone is bound as the native, unmetabolized steroid since CHCl₃ readily extracted 90% of the labeled steroid from renal chromatin, which was identified as native aldosterone chromatographically. Moreover, in the urinary bladder of the toad, the active form of aldosterone has been identified as the unmetabolized steroid (24). In contrast, the active form of the androgens in prostate is dihydrotestosterone, a metabolite of testosterone (11), and that of the D vitamins in intestinal epithelia is a polar metabolite of vitamin D (25).

No direct evidence is available on the role of binding of aldosterone to chromatin in the mechanism of action on active sodium transport. The specificity of the binding system is, however, in accord with the possibility of direct involvement in the initiation of the physiological response. The agonists, d-aldosterone and 9α-fluorocortisol, and the competitive antagonist, spironolactone, displaced 3H-aldosterone from the chromatin binding system but the inactive analogues, 17α-isoadosterone, progesterone, and 17β-estradiol, were without effect on the binding process. Cortisol, a relatively weak mineralocorticoid, partially blocked the binding of 3H-aldosterone.

There is a marked similarity in the relative affinities of diverse steroids for the chromosomal aldosterone-binding proteins and the previously isolated soluble nuclear aldosterone-binding proteins and cytoplasmic aldosterone-binding proteins (8). In addition to the similarities in their affinities for steroids, all three binding systems are equally susceptible to digestion by chymotrypsin and resistant to trypsin, DNase, and RNase. It is possible, therefore, that the binding proteins are located in only one intracellular site, in vivo, and are a single binding system that isartifactually distributed between the cytoplasm.
and nucleus during preparation of the cell fractions. There is some evidence, however, to suggest that the three binding systems may exist in vivo and perhaps form a coordinated mechanism for the initiation of the action of aldosterone.

It is now evident that stringently specific chromosomal binding systems are present in the target organs for most, if not all, steroids. The characteristics of chromatin binding have been described for 17β-estradiol in the immature uterus (10), dihydrotestosterone in the prostate (11), and vitamin D metabolite in the intestinal mucosa (25). Radiographs obtained with 3H-aldosterone show selective and mineralocorticoid-specific accumulation in the nuclei of intact target epithelial cells of toad bladders (7). Evidence has been presented by Gorski et al. (26) and Jensen et al. (27) for a two-step binding mechanism for 17β-estradiol involving an 8 S cytoplasmic and a 6 S nuclear binding protein.

In time course studies on binding in vitro of 3H-aldosterone in kidney slices, Goodman and Edelman4 recently found that the cytoplasmic aldosterone-binding protein complex is formed first, followed by formation of the soluble nuclear aldosterone-binding protein complex and finally by formation of the chromatin aldosterone-binding protein complex. Additional studies are needed to elucidate the relationships between the cytoplasmic and nuclear steroid-binding systems.

The evidence implicating activation of DNA-dependent RNA synthesis in the regulation of active sodium transport by aldosterone has been summarized recently (3). The fact that aldosterone binds rapidly and specifically to chromosomal and nuclear steroid-binding systems.

An approximate estimate of the minimum number of chromosomal aldosterone binding sites per nucleus can be made from the data presented. Based on the specific activity of the purified nuclear fraction of 6 × 10⁻¹⁴ mole per mg of protein (Table VI), a DNA-protein ratio of 0.40 (Table I) and the estimate of 6.6 × 10⁻¹² g of DNA per nucleus (28), we calculated that ~600 molecules of aldosterone were bound per renal cell nucleus at the dosages and sampling time of this study. This estimate is similar in magnitude to the number of vitamin D metabolite binding sites of intestinal mucosa (25) and of 17β-estradiol binding sites of calf and rat uterus (10, 29), which ranged from ~500 to ~2000 sites per nucleus.

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Stereospecific Binding of Aldosterone to Renal Chromatin


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