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Renal Aldosterone Receptors: Studies with [^{3}H]Aldosterone and the Anti-Mineralocorticoid [^{3}H]Spirolactone (SC-26304)

(kidney/aldosterone receptors/tritiated spirolactone)

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ABSTRACT ***In vivo*, a spirolactone (SC-26304) inhibited the effects of aldosterone on urinary K^+:Na^+ ratios and the binding of [^{3}H]aldosterone to renal cytoplasmic and nuclear receptors. Cytoplasmic binding of [^{3}H]aldosterone and [^{3}H]spirolactone (SC-26304) was similar in magnitude and involved the same set of sites. Under three sets of conditions—(*i*) in the intact rat, (*ii*) in kidney slices, and (*iii*) in reconstitution studies (mixing prelabeled cytoplasm with either purified renal nuclei or chromatin), [^{3}H]spirolactone (SC-26304) did not yield specific nuclear complexes in contrast to the reproducible generation of these complexes with [^{3}H]aldosterone. In glycerol density gradients, cytoplasmic [^{3}H]aldosterone receptor complexes sedimented at 8.5 S and 4 S in low concentrations of salt and at 4.5 S in high concentrations of salt. Cytoplasmic [^{3}H]spirolactone (SC-26304) receptor complexes sedimented at 3 S in low concentrations of salt and 4 S in high concentrations of salt. These results are discussed in terms of an allosteric model of the receptor system.**

Previous studies suggest that the initial events in the action of aldosterone on active Na^+ transport involve binding of the steroid to stereospecific cytoplasmic receptors, temperature-sensitive activation of this complex, transfer of the complex to chromatin acceptor sites, and induction of RNA and protein synthesis (1–4). Feldman *et al.* (5) recently outlined an allosteric model of the aldosterone cytoplasmic receptor system consisting of an active and inactive form in equilibrium. The inactive complex was assumed to be incapable either of entering the nucleus or attaching to chromatin acceptor sites, or initiating the transcriptional event if attachment took place.

The spirolactones are competitive inhibitors of the Na^+-retaining action of mineralocorticoids *in vivo* and in isolated epithelia (6, 7). At concentration ratios of 10^4:1, spirolactone SC-14266 reduced renal cytoplasmic binding of aldosterone by 70% and in physiological studies produced maximal inhibition of the antinatriuresis (1, 6). In the present studies we made use of another spirolactone of known structure (SC-26304), with a relatively high apparent affinity for the aldosterone binding site, to explore the characteristics of the aldosterone receptor system and the mechanism of the inhibitory effect (8).

MATERIALS AND METHODS

Materials. 1,2-*d*-[^{3}H]Aldosterone (54 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass. [^{3}H]SC-26304 (16 Ci/mmol) and unlabeled SC-26304 were generous gifts from the G. D. Searle Co., Chicago, Ill. Low K^+ food pellets (1.2 μeq. of K^+/g) were obtained from General Biochemical, Chagrin Falls, Ohio.

Physiological Studies In Vivo. Sprague–Dawley, male, adrenalectomized rats were maintained on saline-drinking water *ad libitum*. The evening before study the rats were placed on the low K^+ diet. On the morning of study, a urine sample was collected (zero hour collection) and the rats were injected intraperitoneally with SC-26304 (0–600 μg in 0.5 ml of 0.9% saline per 100 g of body weight) with or without *d*-aldosterone (0.3 μg). This dose of aldosterone produces a submaximal effect on urinary Na^+ and K^+ excretion. Pooled 0–5 hr urine samples were analyzed for Na^+ and K^+ on an IL model 143 Flame Photometer (Instrumentation Laboratory, Inc., Boston, Mass.) and for creatinine using a modification of the Taussky method (9). In the studies on binding of [^{3}H]-aldosterone *in vivo*, the rats were injected with 10^{-10} mol (0.036 μg) of [^{3}H]aldosterone per 100 g of body weight and a range of doses of SC-26304. Nonspecific binding of [^{3}H]aldosterone was determined by simultaneous injection with 1000 × *d*-aldosterone; 20 min after injection the rats were anesthetized with ether, exsanguinated, and the kidneys perfused with 15 ml of ice-cold 0.25 M sucrose-3 mM $CaCl_2$ via the abdominal aorta. The kidneys were homogenized in 0.25 M sucrose-3 mM $CaCl_2$, and cytoplasmic and nuclear fractions were analyzed for [^{3}H]aldosterone binding (see below).

In Vivo Labeling with [^{3}H]SC-26304. Adrenalectomized rats were anesthetized with chloral hydrate (0.036 g/100 g of body weight) and at time (t) = −30 sec, the hepatic artery and portal vein were tied off. At t = 0, a 1.5-ml tail vein injection was given of either: 10.4×10^{-9} mol of [^{3}H]SC-26304 (4 Ci/mmol + 10× dexamethasone ± 100 × *d*-aldosterone, or 0.7×10^{-9} mol of [^{3}H]aldosterone (13.5 Ci/mmol) + 10× dexamethasone ± 100× *d*-aldosterone. Excess dexamethasone was used to minimize binding of the ^{3}H-labeled steroids to glucocorticoid receptors. At t = 2 or 10 min, a cardiac blood sample was drawn and the kidneys were removed and assayed for receptor content (see below). To assess whether the bound

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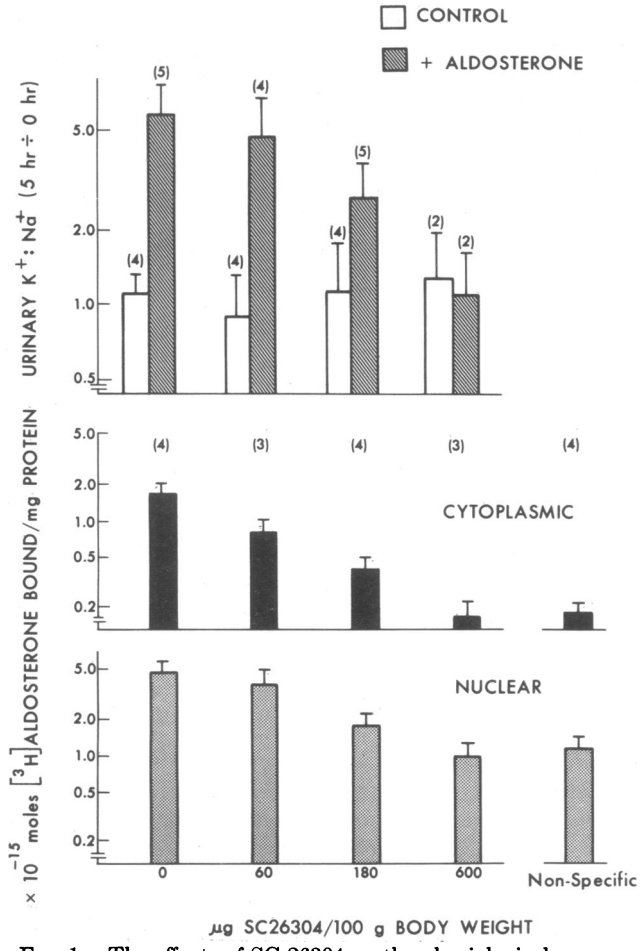

FIG. 1. The effects of SC-26304 on the physiological response to aldosterone and on cytoplasmic and nuclear binding of [^{3}H]-aldosterone. The *upper panel* shows the urinary K^+:Na^+ ratios of pooled 5-hr urine samples after intraperitoneal injection of either saline (control) or 8.3×10^{-10} mol (0.3 μg) of aldosterone and 0 to 600 μg of SC-26304 (/100 g of body weight). The two *lower panels* present the cytoplasmic and nuclear [^{3}H]aldosterone receptor content of kidneys from adrenalectomized rats injected intraperitoneally with 10^{-10} mol (0.036 μg) of [^{3}H]aldosterone and 0–600 μg SC-26304 (/100 g of body weight). The kidneys were removed 20 min after injection and the fractions prepared as described in *Materials and Methods*. The nuclear fraction refers to the sum of the Tris-soluble and 0.4 M KCl-extractable complexes. Nonspecific binding was determined by giving paired rats 1000× concentration of *d*-aldosterone. Results are mean + 1 SEM. The number of rats in each group is indicated in the parentheses.

steroids were native compounds, each fraction was extracted with dichloromethane. Aldosterone metabolites are known to be excluded from the organic phase. Thin layer radiochromatographic analyses of serum and cytoplasmic fractions labeled with [^{3}H]SC-26304 (*in vivo*) showed that 94% or more of the dichloromethane extractable counts were unmetabolized SC-26304 (A. Karim, personal communication).

Five milliliters of spectroquality dichloromethane was added to 1-ml aliquots of labeled serum, cytoplasm and nuclear extracts (0.5–6 mg of protein per ml of water) and the solution was mixed several times at room temperature. After centrifuging at 900 × *g* for 5 min (2°), 2 ml of the organic phase was transferred to a counting vial, evaporated to dryness, and counted as described below.

TABLE 1. *Cytoplasmic and nuclear binding of [^{3}H]aldosterone and [^{3}H]SC-26304, in vivo*

	[^{3}H] Aldosterone		[^{3}H]SC-26304	
	2 min	10 min	2 min	10 min
Cytoplasm	3.1	1.0	6.4	1.2
Tris-soluble	11.3	19.7	−2.2	−0.8
KCl-extract	5.9	9.6	−1.4	0
Serum	10.6	5.9	54.6	23.4

After ligation of the portal vein and hepatic artery, adrenalectomized rats were injected intravenously with either 0.7×10^{-9} mol of [^{3}H]aldosterone + 10× dexamethasone or 10.4×10^{-9} mol of [^{3}H]SC-26304 + 10× dexamethasone, and the kidneys were removed at 2 or 10 min after injection. All fractions were extracted with dichloromethane and corrected for nonspecific labeling based on injections in paired rats with 100× concentration of *d*-aldosterone. The results should be multiplied by 10^{-14} and are expressed in units of mol/mg of protein. The negative values indicate that the nonspecific quantities exceeded the total bound in the absence of excess cold steroid. The serum concentrations (× 10^{-9} M) of the dichloromethane extractable ^{3}H-labeled steroid are given below the line. Mean of two experiments.

Kidney Slice Experiments. Kidney slices (275-μm thick) from adrenalectomized rats were prepared and incubated at 25° for 5–30 min in a rotary shaking water bath as described previously (2). Slices from one or two kidneys were added to 10 ml incubating media containing [^{3}H]SC-26304 or [^{3}H]aldosterone and 10× concentration of dexamethasone ± 1000× *d*-aldosterone. The 4-fold difference in spirolactone:aldosterone concentrations takes into account the differences in affinities of these steroids for the receptor (8). After incubation, the slices were collected on nylon cloth (132 mesh) and transferred to 0.25 M sucrose–3 mM $CaCl_2$ for homogenization and preparation of the cytoplasmic and nuclear fractions (see below).

Preparation of Cytoplasmic and Nuclear Fractions. These procedures were carried out at 0–4°. Homogenization and purification of nuclei were as described previously (2). The resultant purified nuclear pellet was suspended in one of three solutions, depending on the purpose of the nuclear isolation steps: (*i*) 0.25 M sucrose–3 mM $CaCl_2$ if the nuclei were to be used in reconstitution studies; (*ii*) 0.25 M sucrose–0.2% Triton X-100 if they were to be used for preparation of chromatin; or (*iii*) 0.1 M Tris·HCl–3 mM $CaCl_2$ (pH 7.2) and then a high concentration of salt (0.4 M KCl) if the nuclei had been mixed with ^{3}H-labeled steroid and were to be assayed for receptor content (2). Cytoplasmic fractions were prepared by centrifuging the 600 × *g* supernatant at 24,000 × *g* for 15 min. Chromatin was isolated from purified nuclei by a modification of the Spelsberg method (10).

Transfer of Cytoplasmic Complexes to Chromatin in Reconstitution Studies. Unlabeled nuclear or chromatin fractions (from one or two kidneys) were mixed with 1 ml of cytoplasm-glycerol (20%, v/v) containing [^{3}H]SC-26304 or [^{3}H]aldosterone + 10× dexamethasone ± 100× *d*-aldosterone and incubated in a rotary shaking water bath at 25°. The nuclei were recovered by centrifugation at 600 × *g* for 6 min and the chromatin by centrifugation at 10,000 × *g* for 6 min. The pellets were washed three times with 3 ml of ice-cold 0.25 M sucrose–3 mM $CaCl_2$. The nuclear pellets were then suspended

in 2.2 M sucrose, centrifuged, and extracted with 0.1 M Tris buffer and 0.4 M KCl as described previously (2). Chromatin pellets were extracted once with 3 ml of 0.1 M Tris·HCl–3 mM $CaCl_2$ buffer and then with 1.5 ml of a solution containing 0.4 M KCl, 0.05 M Tris·HCl, and 3 mM $CaCl_2$ (pH 7.2).

Density Gradient Analyses. Cytoplasmic complexes were prepared from kidneys perfused with 15–20 ml of iced 0.25 M sucrose via the abdominal aorta. The kidneys were homogenized in 0.02 M Tris·HCl–1.5 mM EDTA (pH 7.5) containing 2.1×10^{-8} M [^{3}H]SC-26304 or 5.2×10^{-9} M [^{3}H]-aldosterone (+ 10× dexamethasone to each). The resultant cytoplasmic fractions were layered on 4 ml of glycerol density gradients. After centrifugation, 0.3-ml aliquots were collected from the bottom of the tube for quantitation of radioactivity and protein.

Assays for Labeled Receptor Content. To remove unbound steroid from labeled cytoplasmic preparations, 1-ml aliquots were processed by gel filtration (2). Protein was estimated from the ratio of A at 260 and 280 nm (11). To measure ^{3}H content, 1-ml aliquots of the various fractions were added to Aquasol (New England Nuclear Corp.) and counted in a Mark I liquid scintillation counter (Nuclear Chicago, Chicago, Ill.) with an efficiency of 28%.

RESULTS

Physiological Studies In Vivo. Administration of SC-26304 alone in doses of 3–600 μg/100 g of body weight had no effect on urinary Na^+:creatinine or K^+:creatinine ratios. In Fig. 1, these results are expressed as urinary K^+:Na^+ ratios. Aldosterone (0.3 μg/100 g of body weight) increased the K^+:Na^+ ratio 5-fold. This increase was significantly inhibited by 180 μg/100 g of body weight of SC-26304 and completely inhibited by 600 μg/100 g of body weight. To correlate inhibitory action and receptor occupancy, the same doses of SC-26304 were given to rats injected with 0.036 μg of [^{3}H]aldosterone. A dose of 180 μg of body weight reduced specific binding of aldosterone in cytoplasmic and nuclear fractions to less than half of the control levels and 600 μg/100 g of body weight eliminated specific binding. The dose of aldosterone used in the physiological studies was about eight times that used in the binding studies, but both doses were well below saturating amounts. Thus, these findings are consistent with the inference that SC-26304 inhibits by competing with aldosterone for the receptor site.

Cytoplasmic and Nuclear Labeling In Vivo. In preliminary studies, adrenalectomized rats were injected intravenously with 10^{-9} mol of [^{3}H]SC-26304/100 g of body weight, and the kidneys were removed 2 min later. Dichloromethane extracts of serum and renal cytoplasmic fractions indicated that more than half of the labeled compound had been converted to water-soluble metabolites and that specific cytoplasmic labeling was low. Rapid metabolism of SC-26304 *in vivo* explains why higher doses of SC-26304 were necessary to inhibit the mineralocorticoid effect and [^{3}H]aldosterone binding *in vivo* than *in vitro*, in the kidney slice experiments described below. To provide sufficient unmetabolized spirolactone for receptor labeling, the hepatic artery and portal vein were ligated just before injection of the ^{3}H-labeled steroids and the dose of spirolactone was increased to 10^{-8} mol/100 g of body weight. The results in Table 1 indicate that the serum concentrations of [^{3}H]SC-26304 were about 4–5 × higher than that of [^{3}H]aldosterone at 2 and 10 min after injection. As reported previously (2), cytoplasmic [^{3}H]-aldosterone-receptor content was higher at 2 than at 10 min and, conversely, the nuclear complexes were more abundant at 10 than at 2 min. Cytoplasmic [^{3}H]SC-26304-receptor content was about twice that of the [^{3}H]aldosterone complex at 2 min and equal at 10 min. However, there were no detectable quantities of intranuclear [^{3}H]SC-26304-labeled receptors at either time. To confirm that binding to the cytoplasmic receptor occurred *in vivo* rather than during homogenization, one of each pair of kidneys, removed 10 min after injection of ^{3}H-labeled steroid, was homogenized in excess *d*-aldosterone (2.6×10^{-6} M) which reduced cytoplasmic receptor labeling by only 18% and 23% for [^{3}H]aldosterone and [^{3}H]SC-26304, respectively.

Binding of [^{3}H]SC-26304 to Renal Receptors In Vitro. Kidney slices were incubated in [^{3}H]aldosterone or in [^{3}H]SC-26304 at 4-fold higher concentrations. As shown in Fig. 2, specific binding of [^{3}H]aldosterone to cytoplasmic receptors attained steady state levels in about 15 min and preceded the formation of the Tris-soluble nuclear and KCl-extractable (chromatin-bound) fractions, as described previously (2). Under the same conditions, [^{3}H]SC-26304 occupied equivalent numbers of cytoplasmic receptor sites but specific nuclear species were not demonstrable. Further studies on the specificity of cytoplasmic binding of [^{3}H]SC-26304 are summarized in Table 2. Nonspecific levels of binding were defined by the addition of 100 × *d*-aldosterone or spirolactone. Equimolar concentrations of corticosterone—the physiological glucocorticoid in rats—decreased specific cytoplasmic binding of [^{3}H]SC-26304 by 9% as compared to a 33% decrease with equimolar concentrations of *d*-aldosterone and a 29% decrease with the same concentration of unlabeled SC-26304. With equimolar concentrations of the competitive steroids the corresponding reductions in cytoplasmic binding of [^{3}H]aldosterone were 8% with corticosterone, 31% with *d*-aldosterone and 27% with SC-26304. Moreover, at 100× concentrations, aldosterone and SC-26304 reduced binding of the ^{3}H-labeled steroids to the same levels. It appears, therefore, that [^{3}H]SC-26304 and [^{3}H]aldosterone occupy the same cytoplasmic sites.

Reconstitution Studies. Renal cytoplasmic fractions were prelabeled with [^{3}H]aldosterone or [^{3}H]SC-26304, mixed with either purified nuclear or chromatin fractions, and incubated at 25°. As shown in Fig. 3, cytoplasmic steroid receptor complex formation was slightly greater with [^{3}H]SC-26304 than with [^{3}H]aldosterone. Uptake of the [^{3}H]aldosterone-receptor complex by nuclei (KCl-extractable) was as reported previously (2). In contrast, the [^{3}H]SC-26304-receptor complex failed to generate any detectable KCl-extractable component. The failure of [^{3}H]SC-26304 receptor complex to transfer to nuclear binding sites was not attributable to hindrance by the nuclear membrane since this complex did not bind to isolated chromatin, whereas the [^{3}H]aldosterone-receptor complex attained high levels of binding (compare upper and lower panels of Fig. 3). The apparent binding of [^{3}H]SC-26304 to chromatin at zero time represents adsorption in the cold (i.e., 3°) which disappears on warming.

Density Gradient Analyses. The sedimentation characteristics of the [^{3}H]SC-26304-labeled and [^{3}H]aldosterone-labeled cytoplasmic complexes are given in Table 3. The [^{3}H]-aldosterone complexes migrated at 8.5 S and 4 S in gradients of low salt concentration and at 4.5 S in gradients of high salt concentration, as reported previously (2). In contrast, the

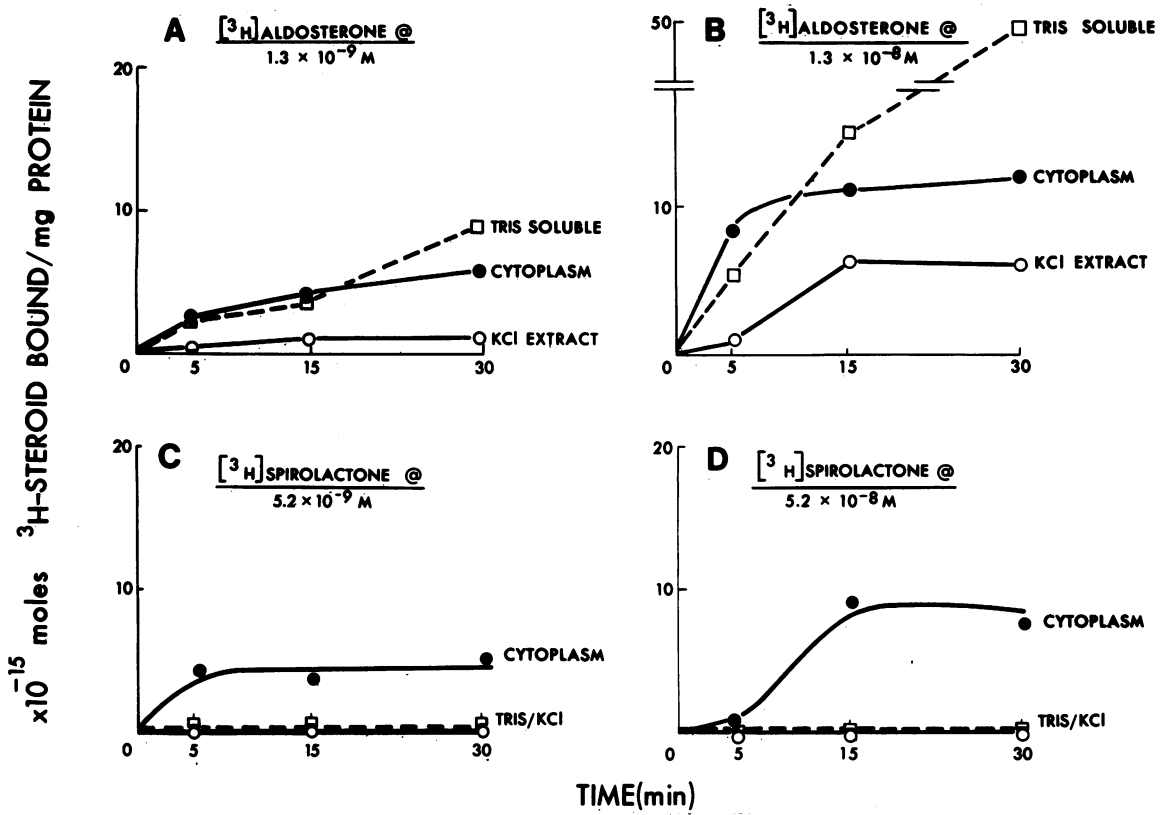

FIG. 2. Uptake of [^{3}H]aldosterone and [^{3}H]SC-26304 in kidney slices. Adrenalectomized rat kidney slices were incubated at 25° in either: (*A*) 1.3 × 10^{-9} M [^{3}H]aldosterone, (*B*) 1.3 × 10^{-8} M [^{3}H]aldosterone, (*C*) 5.2 × 10^{-9} M [^{3}H]SC-26304, or (*D*) 5.2 × 10^{-8} M [^{3}H]SC-26304. All of the media contained a 10× concentration of dexamethasone. The cytoplasmic fractions, and the Tris-soluble and KCl-extractable nuclear fractions were prepared as described in *Materials and Methods*. The quantities bound were corrected for nonspecific binding by parallel incubations in the presence of 1000× concentration of *d*-aldosterone. Each point is the mean of two experiments.

[^{3}H]SC-26304 complex migrated at 3 S in gradients of low salt concentration and 4 S in gradients of high salt concentration. The transitions in a high concentration of salt (i.e., 8.5 S/4 S → 4.5 S for [^{3}H]aldosterone and 3 S → 4 S for [^{3}H]SC-26304) were reversible in that the ionic strength of the incubating solutions had no effect on the density gradient

TABLE 2. *Cytoplasmic binding of [^{3}H]SC-26304 and [^{3}H]aldosterone in kidney slices*

Competing steroid	[^{3}H]SC-26304-bound	[^{3}H]Aldosterone-bound
None	7.8	9.9
1× B	7.2	9.2
1× A	5.6	7.1
1× SC	5.9	7.5
100× A	0.8	1.0
100× SC	1.2	1.5

Adrenalectomized rat kidney slices were incubated at 25° for 45 min in 1.3 × 10^{-9} M [^{3}H]aldosterone or 5.2 × 10^{-9} M [^{3}H]SC-26304 [with 10× concentration of unlabeled dexamethasone (*D*) in all tubes] and equimolar concentrations of corticosterone (1× B), equimolar concentrations of *d*-aldosterone (1× A), or equimolar concentrations of spirolactone (1× SC). The quantities bound in the presence of 10× concentration of dexamethasone and 100× concentration of *d*-aldosterone (100× A) or SC-26304 (100× SC) are considered "nonspecific" binding. Thus "specific" binding would be the difference between the data obtained with 10× D and that with 10× D + 100× A or 100× SC. The results should be multiplied by 10^{-15} and are expressed in units of mol/mg of protein. Mean of two experiments.

TABLE 3. *Density gradient analyses of [^{3}H]aldosterone-labeled and [^{3}H]SC-26304-labeled cytoplasm*

Incubation conditions	[^{3}H] Aldosterone complexes		[^{3}H]SC-26304 complexes	
	Gradient conditions		Gradient conditions	
	−KCl	+KCl	−KCl	+KCl
Low concentration of salt	8.5S/4S	4.5S	3S	4S
High concentration of salt	8.5S/4S	4.5S	3S	4S

Perfused rat kidneys were homogenized in 0.02 M Tris·HCl–1.5 mM EDTA (pH 7.5) containing 2.1 × 10^{-8} M [^{3}H]SC-26304 or 5.2 × 10^{-9} M [^{3}H]aldosterone (+ 10× dexamethasone in all tubes) and incubated for 30 min at 0°. The cytoplasmic fractions to be tested for reversibility of the effects of ionic strength on sedimentation were adjusted to 0.4 M KCl (i.e., incubation conditions of high concentration of salt). Glycerol density gradients (10–34%) contained 2 mM Tris·HCl–1.5 mM EDTA (pH 7.5) (low concentration of salt) or +0.4 M KCl (high concentration of salt). Centrifugations were for 16 hr at 405,000 × *g*. S values given were estimated from previous calibration data using catalase, aldolase, rabbit gamma globulin, alkaline phosphatase, malate dehydrogenase, papain, and cytochrome *c* as markers (2). Each datum is the mean of a minimum of 10 experiments.

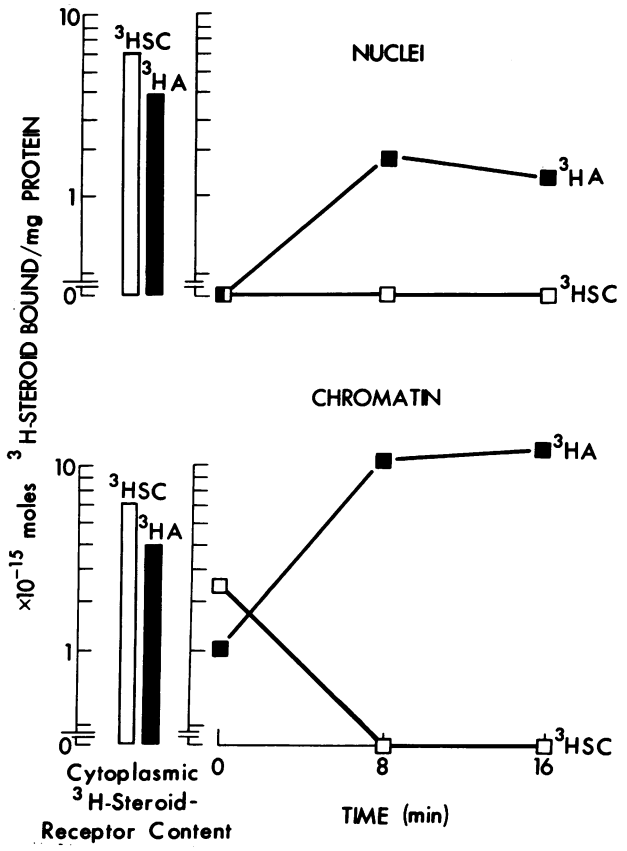

FIG. 3. Transfer of [^{3}H]aldosterone and [^{3}H]SC-26304 receptor complexes from cytoplasm to nuclei (*upper panel*) or to chromatin (*lower panel*). Cytoplasmic fractions from adrenalectomized rats were pre-labeled with 5.2×10^{-9} M [^{3}H]SC-26304 (^{3}HSC) or 1.3×10^{-9} M [^{3}H]aldosterone (^{3}HA) (+ 10× concentration of dexamethasone each). The bars on the *left* indicate the specifically bound quantities in the cytoplasmic fractions. Binding to nuclei and chromatin was determined by extraction with 0.4 M KCl. These data are corrected for nonspecific binding by subtraction of the residual quantities in parallel incubations with 1000× concentration of *d*-aldosterone. Each point is the mean of four experiments.

patterns (Table 3). In view of the inability of the [^{3}H]SC-26304 complexes to bind to specific chromatin sites in reconstitution studies, we are now exploring the possible relationship between the capacity of cytoplasmic complexes to form the 8.5 S species in a low concentration of salt and the ability to bind to chromatin specific sites.

DISCUSSION

Our results indicate that SC-26304 occupies aldosterone-specific renal cytoplasmic receptor sites but this complex fails to bind to chromatin acceptor sites either *in vivo*, or in slices, or in reconstitution studies with nuclei or chromatin, which presumably accounts for its antagonist properties. Similarly, in rat thymocytes, cortexolone (an antiglucocorticoid) competed for triamcinolone acetonide receptor sites, blocked 2-deoxyglucose uptake, and bound to the cytoplasmic receptor but failed to transfer to nuclear binding sites (12). In gradients of low salt concentration the [^{3}H]cortexolone-receptor complex migrated at 3.5 S, whereas the [^{3}H]triamcinolone acetonide–receptor complex migrated at 7 S and 3.5 S. Thus, both SC-26304 and cortexolone did not yield 7 S–8 S complexes in low salt concentrations and did not bind to nuclear acceptor sites. These results imply that the agonists generate an "active" receptor complex as defined by the ability to bind to specific chromatin sites.

The allosteric-steroid-receptor model of Samuels and Tomkins (13) based on the formulations of Rubin and Changeux (14) has been applied to the aldosterone system (5). The receptor is assumed to exist both in an inactive and active conformation, in equilibrium. Agonists presumably have a high affinity for the active form and antagonists for the inactive form. Alternatively, the agonist may induce a conformational change in the receptor that renders it capable of binding to chromatin, but the antagonist binds to the site without effecting the necessary change in conformation. In any case, the agonist–receptor complex in cytoplasm displays two properties—the formation of a 7 S–8 S complex in gradients of low salt concentration, and donor activity (which is temperature dependent) in transferring to nuclear acceptor sites.

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