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MECHANISM OF ACTION OF STEROID HORMONES

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SUMMARY

Despite the diversity in the target sites and physiological actions of the steroids, an impressive body of evidence has been assembled in support of a unitary theory of the mechanism of action of these hormones in vertebrates. The steroids set in motion a train of events, as follows: (a) penetration into the target cell, (b) stereospecific binding to high affinity receptors, (c) temperature-sensitive activation of the steroid–receptor complex, (d) attachment of the active complex to chromatin, (e) induction of RNA and protein synthesis, and (f) physiological expression of the induced protein. Although the overall sequence is well-defined, our knowledge of the molecular processes involved in each of these steps is still quite incomplete. Two of the major efforts now underway to elucidate these molecular processes involve, (a) purification and characterization, in terms of structure-function relations, of the putative receptors, and (b) studies on the nature of the interaction between steroid–receptor complexes and the genome. It is now apparent that steroids induce *de novo* synthesis of both messenger RNA (mRNA) and ribosomal RNA (rRNA); the role of the former in directing the synthesis of specific proteins is reasonably clear but that of the latter remains to be elucidated. The mechanism of induction is also under scrutiny since the observed increases in mRNA synthesis could arise in a variety of ways, *e.g.* negative or positive regulation of chromatin template (gene) activity, changes in processing of heterogeneous RNA to mRNA, effects on RNA polymerase or ribonuclease activities. Although steroidal regulation of RNA and protein synthesis is a dominant pathway, the possibility of direct actions on membranes or regulatory enzymes in some circumstances can not be excluded at the present time.

INTRODUCTION

In vertebrates, circulating hormones dominate the morphogenetic and physiological states of almost all tissues, throughout the life span. In the last 15 years, two unifying theories have emerged that account for most of these diverse cellular responses to hormones; the mediating roles of cyclic nucleotides [cyclic adenosine monophosphate and cyclic guanosine monophosphate] in the actions of peptide and catecholamine hormones, and induction of protein synthesis in the actions of steroid and thyroid hormones. The interrelationships between these two primary mechanisms are also under active study.

A wealth of evidence has led to widespread agreement on the sequence but not the details of events set in motion by the steroids in many well-defined target tissues [1–5]. An outline of this theory is shown in Fig. 1. The steroid enters the target cells, combines with a high affinity receptor forming an active complex that then binds to selective sites in the chromatin. The interaction between the hormone–receptor complex and the genome activates or derepresses transcription or post-transcriptional regulation of RNA synthesis. The products, mRNA and rRNA, dictate the synthesis of specific proteins whose properties determine the morphogenetic and physiological responses to the hormones. Although this receptor-effector system mediates many of the actions of steroid hormones, other basic mechanisms may also play a role, especially at very high concentrations of the hormones. This possibility will be alluded to briefly in relation to possible direct effects of steroids on the permeability properties of plasma and organelle membranes, or on enzymes (see below). The primary focus of attention in this report, however, will be on the induction mechanism (Fig. 1).

Steroid receptors

The mode of steroid penetration into target cells has received little attention. In one system, however, uptake of estrogen by uterine cells appears to involve facilitated rather than simple diffusion [6].

The target cells responsive to a given steroid contain high affinity, saturable cytoplasmic binding proteins (mol. wt. $\cong$ 100,000 Daltons) and the uptake of the steroid precedes the appearance of the physiological effect. These and other findings, including (1) the stereospecificity of the high affinity binding proteins, (2) the close correlation between affinity of various steroids for the binding site and physiological potency, and (3) association of the steroid–protein complex with chromatin, lend credence to the inference that these high affinity binding proteins are receptors that mediate the biological response [1, 4].

The physical and biological characteristics of all of the steroid receptors, including that of 1,25 dihydrocholecalciferol (Vitamin D derivative) are remarkably homologous [1, 4]. The main features of these homologies will be illustrated for the most part by reference to studies with aldosterone, since this steroid has been the focus of my work for more than a decade [7].

The association of steroids with target cell nuclei was first revealed by autoradiography [7, 8]. ^{3}H-

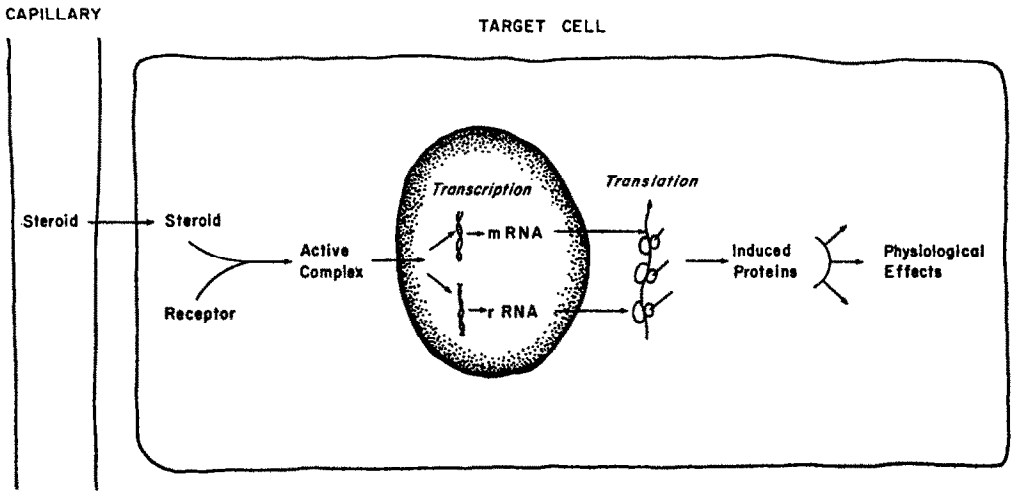

Fig. 1. General model of the receptor-induction mechanism of steroid hormone action. Modified from Feldman *et al.*[2].

Aldosterone was localized to the nuclear and perinuclear areas of toad bladder epithelium. In contrast, ^{3}H-progesterone, an inactive steroid at low concentrations compared to aldosterone, was randomly distributed. Bogoroch (cited in Edelman [9]) explored the specificity of the observed binding in competition studies (Table 1). Excess estradiol-17β, which is inactive with respect to Na^+ transport in the toad bladder, had no effect on the distribution of ^{3}H-aldosterone but 9α-fluorocortisol, an active mineralocorticoid, significantly diminished both nuclear and cytoplasmic localization. These findings imply the existence of cytoplasmic and nuclear receptors, *in vivo*.

The receptors appear to reside in the cytoplasm pending availability of their respective steroids and then bind to chromatin sites after formation of the cytoplasmic complex. This "two-step" mechanism was independently proposed by Gorski *et al.*[10] and by Jensen *et al.*[11]. An important feature of this mechanism is the requirement for temperature activation of the complex for binding to the nucleus.

The cytoplasmic-nuclear transfer process and other features of the steroid–receptor system have been incorporated into the model shown in Fig. 2. The receptor is assumed to exist in an inactive and an active conformation in equilibrium, as formulated by Rubin and Changeux[12] for allosteric enzymes. This allosteric equilibrium model was applied to steroid receptors by Samuels and Tomkins[13] in order to account for the behaviour of steroids with mixed agonist and antagonist properties [suboptimal inducers—in their nomenclature]. The phenomenon of partial agonist-antagonist behaviour is illustrated in Table 2. In the isolated toad bladder system, 11-deoxycortisol elicited

Table 1. Quantitative distribution of ^{3}H-aldosterone between cytoplasm and nucleus of toad bladder epithelium by autoradiography

Competitive steroid (100:1)	Grains/nucleus	Grains/cytoplasm
None	2·66	0·79
17β-estradiol	2·75	0·96
9α-fluorocortisol	0·84	0·45

Toad bladders were exposed to ^{3}H-aldosterone ($5{\cdot}3 \times 10^{-8}$ M) for 30 min with or without added steroid ($5{\cdot}3 \times 10^{-6}$ M). Average of 200 cells counted per section. [Bogoroch, R. & Edelman, I. S., cited in [9]].

Table 2. Agonist/antagonist activity of 11-deoxycortisol on Na^+ transport in the isolated toad bladder

No. of pairs	Steroid	Increase in SCC (6 h)* (%)	Agonist/antagonist activity (%)
8	Aldosterone (7×10^{-8} M) 11-Deoxycortisol (5×10^{-6} M)	219 ± 28 66 ± 16	+30
8	Aldosterone (7×10^{-8} M) Aldosterone (7×10^{-8} M) + 11-Deoxycortisol (5×10^{-6} M)	88 ± 21 26 ± 10	−71

* SCC denotes short-circuit current. Mean ± S.E.M. Edelman, I. S.: Unpublished results.

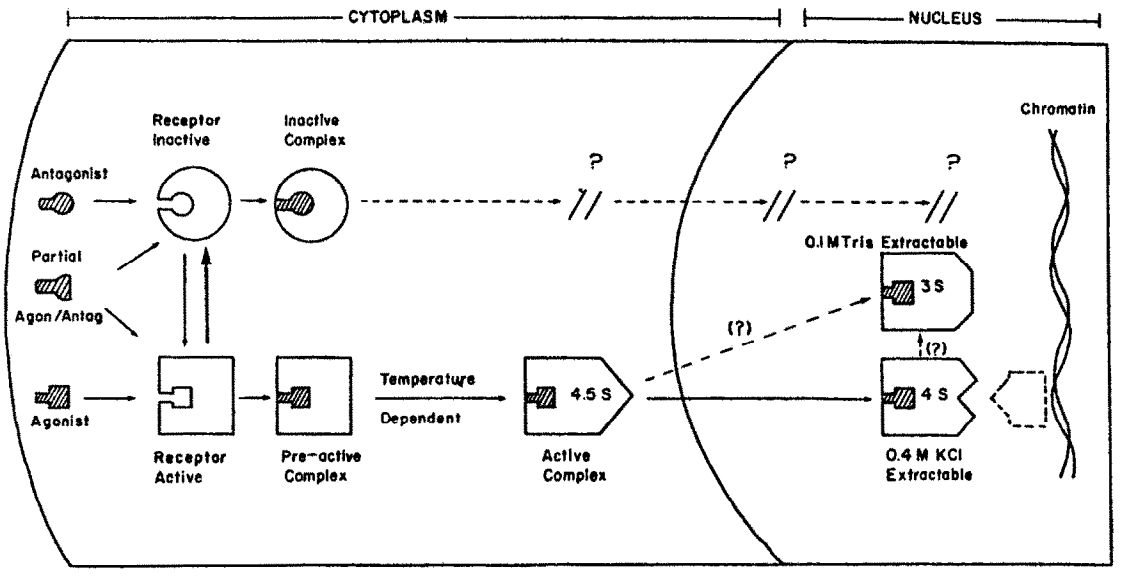

Fig. 2. Allosteric-equilibrium model of the steroid–receptor system. Modified from Feldman *et al.*[2].

an increase in active Na^+ transport, measured by the short-circuit current (SCC) technique, that was 30% of that caused by maximum doses of aldosterone and in combination with aldosterone inhibited the response by 71%. Thus total activity accounts for 100% occupancy of the putative receptors and can be rationalized as indicating that the affinity of 11-deoxycortisol for the two conformations is in the ratio of 30:70, active vs inactive. Primary agonists (e.g., aldosterone) would presumably have a high affinity for the active conformation and negligible affinity for the inactive conformation. The converse should hold for primary antagonists. Support for this model was obtained in studies with steroid antagonists. Baxter *et al.*[14] found that in hepatoma cells in tissue culture, progesterone inhibited induction of tyrosine amino-transferase by glucocorticoids and occupied cytoplasmic glucocorticoid receptor sites but failed to generate intra-nuclear complexes. Kaiser *et al.*[15, 16] reported that in rat thymocytes, the anti-glucocorticoid, cortexolone bound to the receptor but failed to transfer to nuclear binding sites. In our studies, the anti-mineralocorticoid, spirolactone–mineralocorticoid–receptor complexes (SC-26304) did not bind to nuclear acceptor sites *in vivo* (see Table 3), nor in reconstitution experiments with isolated nuclear or chromatin fractions [17]. These results are consistent with the allosteric-equilibrium model. Selective affinity for the inactive form of the receptor, however, need not be the only mechanism of action of an antagonist. Clark *et al.*[18] reported that the anti-estrogen nafoxidine–HCl, acted by causing prolonged nuclear retention of the estrogen receptor, and depletion of cytoplasmic receptor content, without inducing commensurate uterine stimulation.

Table 3. 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 i.v. with either (1) $0{\cdot}7 \times 10^{-9}$ mol ^{3}H-aldosterone + 10 × dexamethasone, or (2) $10{\cdot}4 \times 10^{-9}$ mol ^{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 non-specific labeling based on injections in paired rats with 100 × d-aldosterone. The tissue results are expressed in units of $\times 10^{-14}$ mol/mg protein. The negative values indicate that the non-specific quantities exceeded the total bound in the absence of excess cold steroid. The serum concentrations ($\times 10^{-9}$ M) of the dichloromethane extractable ^{3}H-steroid are given below the line. Mean of two experiments. From Marver *et al.*[17].

Numerous investigators have explored the physical characteristics of the cytoplasmic and nuclear forms of the steroid receptors. In high salt density gradients, the cytoplasmic steroid–receptor complexes migrate at 4 to 5S and in low salt at 7 to 9S (Table 4). Some antagonist–receptor complexes do not aggregate in low salt solutions; ^{3}H-SC-26304-mineralocorticoid receptor complexes and ^{3}H-cortexolone–glucocorticoid–receptor complexes migrated at 3S to 4S in both low and high salt density gradients [15, 17]. These findings are consistent with the inference that binding of the agonist (but not these antagonists) activates the receptor by inducing a change in receptor conformation or sub-unit interactions (Fig. 2). Sherman and co-workers [25, 26] recently proposed a subunit model for the progesterone receptor consisting of a globular subunit that binds the steroid and an associated asymmetric subunit(s) that may control specific binding of the complex to chromatin. The steroid-binding subunit was obtained by treatment of par-

Table 4. Sedimentation of cytoplasmic steroid receptor–complex in density gradients

Authors	Steroid	Tissue	Low salt	High salt
Marver *et al.*[19]	Aldosterone	Kidney	8·5S/4S	4·5S
Kaiser *et al.*[15]	Triamcinoloneacetonide	Thymus	7S/3·5S	4S
Baxter and Tomkins[20]	Dexamethasone	Hepatoma	8S	4S
Baulieu *et al.*[21] Mainwaring and Mangan[22]	Dihydrotestosterone	Ventral prostate	8S/4–5S	4–5S
Jensen *et al.*[23]	Estradiol	Uterus	8S/4S	4·5S
Sherman *et al.*[24]	Progesterone	Oviduct	8S/5S	3·7S
Marver *et al.*[17]	Spirolactone	Kidney	3S	4S
Kaiser *et al.*[15]	Cortexolone	Thymus	3·5S	3·5S

tially purified receptors with Ca^{2+} or other divalent cations and is a low molecular weight, compact, basic protein. In contrast, the intact receptor is a high molecular weight, asymmetric, acidic protein, implying an association of dissimilar subunits.

Impressive evidence that the steroid receptors are bi-functional (or multi-functional) has been obtained in studies of genetic variants of lymphoma cells in tissue culture [27]. Cell selection was accomplished by resistance to the killing action of dexamethasone in the medium. As shown in Table 5, three variants have been isolated and characterized; r^- lacks demonstrable high affinity glucocorticoid binding sites, nt^- is deficient in nuclear transfer of the complex to chromatin and d^- fails to respond despite formation of the chromatin-bound complex. The existence of the nt^- variant indicates that the steroid-binding site is distinct from the determinant for binding of the complex to chromatin and is consistent with the Sherman model of dissimilar "subunits" and her speculation that steroid-binding is the province of one subunit and binding to chromatin the province of the other.

Characterization of the primary and higher order structure of the cytoplasmic and nuclear forms of the steroid receptors will be possible when sufficient amounts of pure material are available for analysis. At the present time, a number of serious efforts to purify these receptors are underway. Significant progress has been reported in the purification of cytoplasmic estrogen receptors by affinity chromatography [28, 29] and to some extent in the purification of cytoplasmic progesterone receptors by DEAE–cellulose chromatography [30]. Promising attempts to purify and characterize the receptors with the aid of photo-affinity labels are also in progress [31, 32]. Recently, Gorell *et al.*[33] reported purification of an estradiol–receptor complex extracted from calf uterine nuclei and obtained sufficient yields for determination of molecular weight (~72,000 Daltons) and amino acid composition. More detailed information on these and related studies may answer many of the fundamental questions on structure-function determinants in the expression of receptor activity.

Nuclear binding of steroid–receptor complexes

Nuclear steroid–receptor complexes appear to be derived from the cytoplasmic pool. *In vivo*, formation of the cytoplasmic complex precedes appearance of the nuclear bound form and cytoplasmic receptor content is depleted as nuclear-binding proceeds [10, 11]. In the excised uterus, generation of the 5S nuclear ^{3}H-estradiol complex and depletion of the cytoplasmic 8S complex is temperature-dependent and these shifts are in phase. Similar findings have been obtained in reconstitution experiments with steroid-labeled cytosol fractions and unlabeled nuclear or chromatin fractions from many tissues [1]. One such experiment is shown in Fig. 3. Renal cytoplasmic fractions from adrenalectomized rats were labeled with ^{3}H-aldosterone and incubated with washed renal nuclei at 25°C. On a quantitative basis nuclear uptake of ^{3}H-aldosterone complexes (recovered by 0·1 M Tris–HCl and 0·4 M KCl elution) accounted for 60% of the receptor content lost from the cytoplasmic fraction during incubation [19]. Moreover, dissociation of the cytoplasmic complex by

Table 5. Genetic variants in glucocorticoid responsive lymphoma cells in tissue culture

Genetic type	Cytoplasmic reactants		Cytoplasmic product		Nuclear product	Physiological response
Wild	Receptor + Steroid	→	Complex	→	Chromatin-bound complex →	↓ Transport and growth death
r^-	−*		−		−	−
nt^-	+		+		−	−
d^-	+		+		+	−

* The r^- cells may either lack receptors entirely or the receptor may be present but defective in the binding reaction. From Sibley *et al.*[27].

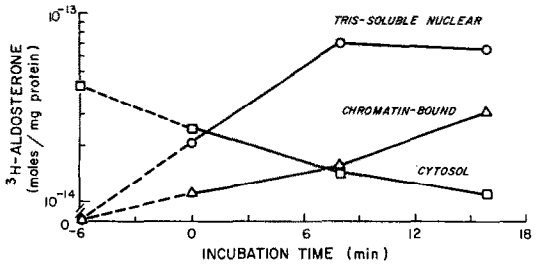

Fig. 3. Formation of nuclear ^{3}H-aldosterone–receptor complexes in reconstituted mixtures of cytosol and nuclear fractions of kidneys from adrenalectomized rats. Cytosol was pre-labeled with ^{3}H-aldosterone ($1{\cdot}3 \times 10^{-8}$ M) ± 9α-fluorocortisol ($1{\cdot}3 \times 10^{-6}$ M) and incubated with washed nuclei at 25°C. From Marver *et al.*[19].

Table 6. Intranuclear distribution of ^{3}H-aldosterone

Fraction	% Total	% Bound
Tris–$CaCl_2$ Extract	27·6 ± 0·9	63*
Chromatin	55·1 ± 2·1	76†
Residual	17·3 ± 0·3	

Mean ± S.E.M. Bound refers to the proportion of the total ^{3}H-Aldosterone in the particular fraction that was bound to a macromolecule.

* Determined by precipitation with 50% saturated $(NH_4)_2SO_4$ and G-50 Sephadex gel filtration.

† The chromatin was sheared and the resultant soluble-nucleohistone was passed through G-50 Sephadex columns. From Swaneck *et al.*[34].

heating to 37°C eliminated nuclear uptake; in contrast pre-heating the nuclear fraction did not impair uptake.

Direct association of the steroid–receptor complexes derived from the cytoplasm with chromatin accounts for much of the nuclear uptake process. Thus, in nuclei prepared from adrenalectomized rats, after injection of the steroid *in vivo*, binding of ^{3}H-aldosterone–receptor complexes to chromatin accounted for at least 55% of total nuclear accumulation (Table 6) [34]. Moreover, chromatin and intact nuclei manifest very similar acceptor activity in reconstitution experiments. For example, Mainwaring and Peterken[35] showed that undenatured cytoplasmic receptor proteins (from rat prostate) were needed to transfer ^{3}H-5α-dihydrotestosterone to isolated chromatin and that extraction of the labeled chromatin with KCl yielded a 4·5S complex indistinguishable from that found in intact prostate.

The relevance of chromatin binding of these complexes to physiological action is indicated by numerous findings: (1) In well-characterized target tissues, the ability of various steroids to block formation of the chromatin-bound specific steroid–receptor complexes correlates closely with their potencies as agonist or antagonists [1–4]. This phenomenon is illustrated in Table 7 with competition experiments on the binding of ^{3}H-aldosterone complexes to renal chromatin [34]. In addition, as noted above, steroids that form cytoplasmic receptor complexes with little or no binding to chromatin, inhibit steroid action [15, 17], and nuclear transfer negative (nt^-) lymphoma variants are resistant to cytolysis by glucocorticoids [27]. Recently, Gehring and Tomkins[36] found that temperature-activated dexamethasone–receptor complexes from glucocorticoid-sensitive lymphoma cells bound to isolated nuclei from both sensitive and resistant cells, and to homologous and heterologous DNA. The cytoplasmic complex from resistant cells, however, was deficient in binding to either nuclear or DNA fractions.

The molecular reactions involved in the association of steroid–receptor complexes with chromatin and the mechanism of modulation of gene expression by this event are, for the most part, still undefined. Chromatin acceptor activity apparently has some degree of specificity in recognizing steroid–receptor complexes, at least in some systems. Lippman and Thompson[37] found that HTC cell nuclei bound glucocorticoid–receptor complexes from L cells after saturation with HTC cell complexes and vice versa. In many systems, however, tissue specificity with respect to nuclear or chromatin acceptor activity is not demonstrable. The significance of binding of steroid–receptor complexes to chromatin in reconstitution experiments, however, has been called into question by Chamness *et al.*[38] who found no evidence of saturation of nuclear binding of estrogen-receptor complexes even when nuclear uptake was several-fold greater than that obtained with maximal physiological doses *in vivo*. These results raise the possibility

Table 7. Steroidal specificity for ^{3}H-aldosterone-binding sites in renal chromatin

No.	Competing steroid*	Relative specific activity %
24	None	100
9	Progesterone (100:1)	106 ± 18
6	17β-Estradiol (100:1)	89 ± 12
10	Cortisol (100:1)	51 ± 10
6	9α-Fluoro-cortisol (100:1)	11 ± 0·9
8	Spirolactone (10,000:1)	21 ± 3

Mean ± S.E.M. No. denotes the number of experiments.

* Molar ratio of competing steroid to ^{3}H-aldosterone is given in parentheses. From Swaneck *et al.*[34].

that only a fraction, possibly a small fraction, of binding to chromatin is involved in regulation of RNA metabolism. The Williams–Gorski model[39] emphasizes the kinetic equilibrium features of the system and posits that the amount of complex bound to nuclei *in vivo* bears a constant ratio to the initial binding of steroid to cytoplasmic receptor, *i.e.*, chromatin binding sites are always in excess. Although the problem of distinguishing between binding to sites that mediate biological actions and those that do not, remains unresolved, in a number of studies the physiological response correlated closely with the quantity of steroid–receptor complex bound to the nuclear fraction [40, 41]. Moreover, the evidence that modulation of RNA metabolism mediates virtually all of the biological actions of the steroids lends credence to the supposition that *in vivo*, binding of the complex to chromatin or to a sub-set of sites in chromatin is a part of the pathway that regulates the response.

Considerable attention has been given recently to attempts to define the components in chromatin responsible for acceptor activity. Three views have emerged: (1) Puca *et al.*[42] found that DNA-agarose had little acceptor activity for estradiol–receptor complexes and that the acceptor sites appeared to be associated with basic nuclear proteins which were extractable with high salt. The significance of these results, however, remains to be determined since this DNA may not be 'native' with respect to determinants for binding steroid–receptor complexes and many basic proteins (*e.g.*, histones, protamine) will aggregate with steroid–receptor complexes [43]. (2) Schrader *et al.*[44] isolated two ^{3}H-progesterone–protein complexes from chick oviduct cytosol by DEAE-cellulose chromatography. Component A binds to DNA but component B does not. Conversely, component B binds to purified chromatin but component A does not. They suggested that these two components may play complementary roles *in vivo* with specific binding of component B to nuclear acceptor proteins and of component A to DNA sites. (3) In many studies, pretreatment of nuclei or chromatin with DNase virtually eliminated acceptor activity in admixtures with competent steroid–receptor complexes [19, 45, 46]. Shyamala-Harris[47] found that addition of DNase released ^{3}H-estradiol–receptor complexes from prelabeled nuclei. In addition, Baxter *et al.*[46] found that agonist–receptor complexes bound to DNA and antagonist–receptor complexes failed to do so. In the study by Marver *et al.*[19], pre-heating of nuclei or subjecting them to osmotic shock or pre-treatment with RNase did not diminish acceptor activity. These results implied that DNA of chromatin was directly involved in acceptor activity. Spelsberg *et al.*[48] examined the role of other components in chromatin that may be involved in acceptor activity and concluded that an acidic nuclear protein in association with DNA determines acceptor activity.

Recently, Marver and I have explored the role of DNA in acceptor activity further, with the aid of reagents that react with DNA without disrupting nuclear or chromatin structure. Some of the properties of the reconstitution system we exploited are summarized in Table 8. Uptake of ^{3}H-aldosterone–receptor complexes from *in vitro* labeled renal cytosol by kidney chromatin is temperature dependent and reduced by 75% after destruction of 50% of the DNA by DNase. The EDTA control indicates that this effect is not ascribable to contaminating proteases since DNase is specifically divalent cation dependent. Moreover, preextraction of chromatin proteins with 0·4 M KCl does not affect acceptor activity at all. These results do not, however, exclude the possibility that a bound protein participates in acceptor activity which may be resistant to salt extraction but released by degradation of DNA. To probe the role of DNA in this process further, we chose reagents that appear to be selective in their sites of attachment to chromatin. Ethidium bromide and proflavine SO_4 intercalate between DNA base pairs with no nucleotide specificity and portions of the dyes appear to project into the major groove of the DNA helix [49, 50]. In contrast, actinomycin D intercalates specifically between the GpC base paired dinucleotide sequence and the peptide subunits lie in the minor groove of the helix [51]. A fourth reagent, netropsin, is ApT specific, does not intercalate but elongates the contour length of the helix and apparently binds primarily in the minor groove [52, 53]. All four reagents inhibit RNA polymerase activity, presumably by effects on the interaction between the enzyme and the DNA template.

Table 8. Effect of temperature, salt extraction and DNase I on acceptor activity of rat kidney chromatin*

Pretreatment Experimental/Control	DNA/RNA Ratio (% of Control)	Acceptor activity (% of Control)
0°C/25°C	100	4
+0·4 M KCl/ − KCl	110	108
+DNase/−DNase	53	27
+DNase		
+EDTA/−DNase-EDTA	96	90

* Kidney cytosol from adrenalectomized rats was labeled with ^{3}H-aldosterone at 5×10^{-9} M with or without excess cold d-aldosterone and 10×-dexamethasone (in the DNase experiments). The cytosol fractions were incubated with renal chromatin for 15 min at 25°C and the chromatin was then assayed for specific binding of ^{3}H-aldosterone. From Marver, D. and Edelman, I. S.: Unpublished observations.

Table 9. Effect of ethidium bromide on the stability and donor activity of renal cytoplasmic ^{3}H-aldosterone complexes

Cytosol pretreatment	Cytosol receptor $\times 10^{-15}$ mol/mg protein	Chromatin-bound $\times 10^{-15}$ mol/mg DNA
Control	11·8	5·2
+Ethidium bromide	12·9	5·3

Cytosol was preincubated in ^{3}H-aldosterone (5×10^{-9} M) + 10 × dexamethasone ± 200 × d-aldosterone ± 100 μg/ml ethidium bromide for 10 min at 25°C (20% glycerol). The cytosol was cleared of free steroid and free ethidium bromide by passage through a G-50 Sephadex column just before incubation with renal chromatin. From Marver, D. and Edelman, I. S.: Unpublished observations.

Accordingly, we compared the effects of these reagents on template for *E. coli* RNA polymerase activity and acceptor activity of rat kidney nuclei. ^{3}H-aldosterone–receptor complexes from rat kidney cytosol fractions were used as the donor. Pretreatment of nuclei with ethidium bromide or proflavine SO_4 reduced acceptor activity by 80 to 90% at concentrations that inhibited 70% (proflavine sulfate) to 100% (ethidium bromide) of *E. coli* RNA polymerase-template activity. It was remarkable that nuclear acceptor activity was even more sensitive than polymerase activity to proflavine SO_4. In contrast, actinomycin D impaired acceptor activity only 20% at concentrations that inhibited RNA synthesis 100%, and similar results were obtained with netropsin. We also determined that pre-incubation with ethidium bromide (100 μg/ml) had no effect on the stability of ^{3}H-aldosterone–receptor complexes as determined by Sephadex G-50 chromatography and transfer of the complexes cleared of ethidium bromide to renal chromatin (Table 9). These results imply either that the local DNA sites, probably in the major groove, play a direct role in high affinity binding of steroid–receptor complexes to the genome or that macromolecules (protein, RNA or both) closely associated with these DNA sites are so involved. In further control studies, we found no detectable increase in supernatant proteins released from chromatin after incubation with ethidium bromide (150 μg/ml) (Table 10). Thus, no evidence of protein release by the dye was obtained. These results indicate that acceptor activity is sensitive to the intimate structure of chromatin. Modulations in RNA synthesis may then be a consequence of the binding of steroid–receptor complexes to the major groove of the helix or to regulatory proteins located at this surface or both.

Effects of steroid hormones on RNA synthesis

Documentation of the early effects of steroids on RNA synthesis is now extensive and has been summarized in a recent review by O'Malley and Means[5]. Some of the salient features of these responses are as follows: Within minutes there is an early increase in the labeling of heterogeneous RNA (presumably precursor to mRNA), in the amount of DNA-like RNA, and in transcription of unique-sequence DNA. These results imply steroidal regulation of mRNA synthesis. Increased production of precursors of rRNA or enhanced labeling of rRNA and of tRNA usually follows the effects on rapidly labeled RNA and may be secondary to prior induction of mRNA synthesis.

Until recently the inference that mRNA synthesis plays a key role in steroid regulated protein synthesis was based on indirect evidence, for the most part, using inhibitors of RNA synthesis. An example of the use of inhibitors in analyzing the role of RNA synthesis in steroidal regulation of a discrete physiological process is shown in Fig. 4. Cordycepin (3′-deoxyadenosine) is an inhibitor of RNA polymerase I (nucleolar in localization and regulates transcription of genes coded for rRNA precursor), and of polyadenylation of mRNA precursor; the formation of poly-A-rich RNA provides active mRNA. As shown in Fig. 4, cordycepin had minimal effects on the baseline rate

Table 10. Effect of ethidium bromide on release of protein from renal chromatin*

Chromatin pretreatment	Supernatant protein μg/mg DNA	Chromatin protein μg/mg DNA
Control	<7·5	530
Ethidium bromide	<7·5	530

* Renal chromatin was incubated ± ethidium bromide (150 μg/ml) for 15 min at 25°C. The chromatin was sedimented by centrifugation at 10,000 *g* for 10 min and the supernatant was then centrifuged at 405,000 *g* for 12 min. These supernatants were cleared of ethidium bromide by filtration through G-50 Sephadex and the protein content of the void volume was determined by the Lowry method. From Marver, D. and Edelman, I. S.: Unpublished observations.

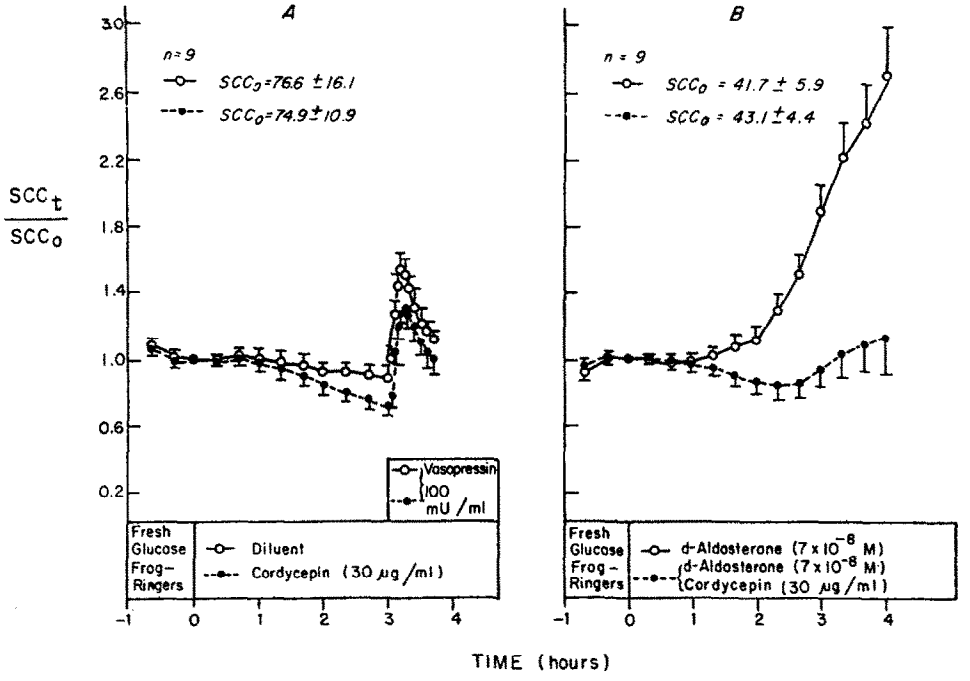

Fig. 4. Effect of cordycepin on aldosterone stimulation of Na transport in the toad bladder. Pairs of hemibladders were pre-incubated in steroid-free glucose (10 mM)-frog Ringers for 15 h. The media were then exchanged for fresh glucose (5 mM)-frog Ringers solution. In the experiments shown in Panel A, cordycepin (30 μg/ml) was added to the serosal and mucosal media of one of each pair (--●--) and vasopressin (100 mU/ml) was added to the serosal media of both hemibladders at 3 h. In the experiments shown in panel B, aldosterone (7×10^{-8} M) was added to the serosal and mucosal media of both hemibladders and cordycepin (30 μg/ml) was added to the serosal and mucosal media of one of each pair (--●--). SCC_t/SCC_0 denotes the short-circuit current at time "t" divided by that at time zero. Each point and vertical line represents the mean ± S.E.M. "n" denotes the number of pairs of hemibladders. SCC_0 denotes the absolute short-circuit current at time zero and is given as the mean ± S.E.M. From Chu and Edelman[54].

of Na^+ transport (measured as the short-circuit current) or on the response to vasopressin but virtually eliminated the aldosterone dependent increase in Na^+ transport [54]. Similar experiments with α-amanitin (an inhibitor of nucleoplasmic RNA polymerase II activity that regulates synthesis of mRNA precursor have also implicated mRNA accumulation in steroid action. Thus, Raynaud-Jammet *et al.*[55] noted that α-amanitin inhibited the estradiol-induced increase in α-amanitin-insensitive RNA polymerase (presumably polymerase I) activity in the rat uterus. The range and reproducibility of the experiments with a wide variety of inhibitors in many different systems lent credence to the idea that RNA synthesis in general and mRNA in particular was important in steroid action.

Steroids have also been shown to stimulate the synthesis of rapidly labeled nuclear RNA, to increase RNA polymerase activity and template capacity of nuclear chromatin [5]. Nevertheless, the inference that accumulation of mRNA was a key event was only recently put on a solid basis.

In the last four years, noteworthy success has been achieved in obtaining direct evidence that cellular accumulation of specific mRNA's are rate-limiting for steroid regulation of the synthesis of specific proteins. Comstock *et al.*[56] reported that the estradiol-dependent increase in ovalbumin content of the chick oviduct is preceded by an increase in mRNA (coded for ovalbumin) content and quantitatively correlated with this increase. Rhoads *et al.*[57] extended these observations by improving the recovery of specific ovalbumin mRNA and confirmed the quantitative relationship between the accumulation of ovalbumin-mRNA and of ovalbumin in the chick oviduct. This process was analyzed in some detail by Palmiter[58] who estimated that ovalbumin-mRNA rose from undetectable levels to ~70,000 molecules/oviduct gland cell in 5 days of treatment with estradiol-17β. Continued exposure to the steroid was required to maintain these high concentrations of the specific mRNA. On the basis of a half-life of degradation of ovalbumin-mRNA of ~24 h, Palmiter concluded that estrogen activation of 1 haploid gene/genome was sufficient to maintain the calculated rate of synthesis of the ovalbumin-mRNA. O'Malley and co-workers[5] obtained evidence that estradiol stimulated production of numerous copies of ovalbumin from a single gene/haploid genome by preparing fragments of ^3H-DNA complements of ovalbumin-mRNA and measuring subsequent hybridization with sheared whole DNA from the chick. The results were consistent with

DNA hybridization to unique-sequence or single copy DNA.

The likelihood that accumulation of mRNA mediates the action of most, if not all, steroid hormones was supported by findings in other systems. Schutz *et al.*[59] partially purified poly-A-rich RNA from rat liver and found that glucocorticoids increased the recoverable quantity of mRNA coded for tryptophan oxygenase and the synthesis of this enzyme. They concluded that steroidal enhancement of hepatic mRNA (tryptophan oxygenase specific) was responsible for the observed increase in synthesis of tryptophan oxygenase. Chan *et al.*[60] have also provided evidence that avidin-mRNA accumulates in chick oviduct in response to progesterone and that the time-course of this increase is consistent with the time-course of increase in avidin content.

The studies on the role of mRNA in the action of estrogens, progesterone and glucocorticoids were facilitated by the availability of antibodies to purified proteins induced by the steroid, *i.e.*, ovalbumin, avidin and tryptophan oxygenase. Aldosterone produces discrete physiological effects on Na^+ and K^+ transport without measurable effects on tissue content of RNA and protein. More than a decade ago, my colleagues and I [7] proposed that induction of mRNA synthesis mediated the action of mineralocorticoids on Na^+ transport. Since then results obtained with a variety of methods have been consistent with this proposal, including: 1. The presence of nuclear "receptors" in target tissues [34, 40, 61], 2. effects of inhibitors of RNA and protein synthesis [54, 62, 63], 3. enhancement of the activities of mitochondrial enzymes [64], 4. increase in RNA polymerase activity [54, 65], 5. increase in chromatin template activity [66], and 6. increases in the incorporation of precursors into total or nuclear RNA [8, 68, 69]. Nevertheless, positive evidence of a dependence of the action of aldosterone on mRNA synthesis was lacking until recently. In fact, some attempts to demonstrate effects of aldosterone on the synthesis of discrete classes of RNA were unsuccessful [68, 70].

Recently, Rossier, Wilce and I[71] used the isolated toad bladder sac preparation to determine the time relationships between the effects of aldosterone on Na^+ transport (measured as the short-circuit current-SCC) and incorporation of ^{3}H-uridine and ^{14}C-methyl methionine into RNA resolved by density gradient sedimentation analysis. Aldosterone increased incorporation of ^{3}H-uridine into 9 to 18S, non-methylated RNA (characteristics of mRNA) during the latent period (30 min labeling time) by 26 to 120% in a series of four separate time-course experiments. The relevance of this finding to the effect on Na^+ transport was explored with the antagonist, spirolactone (SC 9420). As shown in Fig. 5, (left hand panel), aldosterone (3×10^{-8} M) elicited the usual increase in Na^+ transport (SCC) and a significant increase in incorporation of ^{3}H-uridine into 4–18S RNA with a peak at 9–11S (30 min pulse-150 min chase). Spirolactone alone had no effect on the labeling pattern or on the SCC (middle panel) but markedly reduced the effects of aldosterone on Na^+ transport and on incorporation of ^{3}H-uridine into 9–12S RNA (right hand panel).

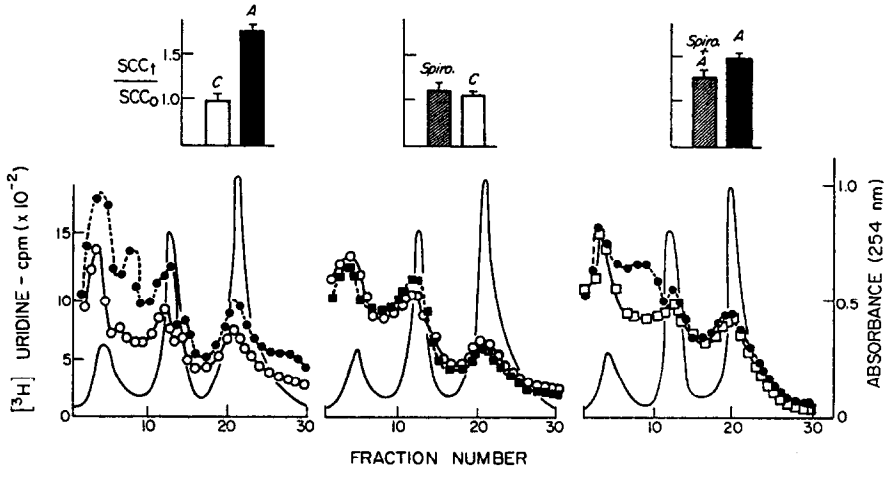

Fig. 5. The effect of spirolactone (SC 9420) on [^{3}H]-uridine incorporation. Thirty min pulse (from t = 10 min) – 150 min chase. *Left panel*: Aldosterone ($3{\cdot}5 \times 10^{-8}$ M) is denoted by closed symbols (–●– = ^{3}H-activity) and "A" (SCC_t/SCC_0) and the controls by open symbols (–○– = ^{3}H-activity) and "C" (SCC_t/SCC_0). The SCC_0 values were 229 ± 26 μA/hemibladder (aldosterone) and 228 ± 16 μA/hemibladder (controls). N = 9 pairs of hemibladders. *Middle panel*: SC 9420 ($3{\cdot}5 \times 10^{-6}$ M) is denoted by closed symbols (–■– = ^{3}H-activity) and "Spiro" (SCC_t/SCC_0), and controls by open symbols (–○– = ^{3}H-activity) and "C" (SCC_t/SCC_0). SCC_0 values were 291 ± 72 μA/hemibladder (aldosterone) and 289 ± 57 μA/hemibladder (controls). N = 10 pairs of hemibladders. *Right panel*: SC 9420 ($3{\cdot}5 \times 10^{-6}$ M) was added 15 min before aldosterone ($3{\cdot}5 \times 10^{-8}$ M) to both; aldosterone alone is denoted by (–●– = ^{3}H-activity) and "A" (SCC_t/SCC_0), and the SC 9420-treated (+ aldosterone) hemibladders by (–□– = ^{3}H-activity) and "Spiro" (SCC_t/SCC_0). SCC_0 values were 343 ± 53 μA/hemibladder (SC 9420 + aldosterone) and 273 ± 45 μA/hemibladder (aldosterone). N = 10 pairs of hemibladders. From Rossier *et al.*[71].

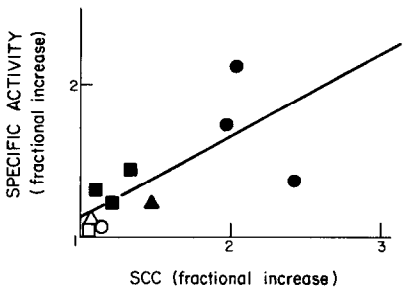

Fig. 6. The relationship between the fractional change in SCC and [^{3}H]-uridine incorporation. Each symbol represents mean of 5 to 10 pairs of hemibladders and was computed from results shown in Figs. 2 to 5. [^{3}H]-uridine specific activities computed from the average c.p.m. at 9S to 12S (normalized to area under the absorbance curve) and expressed as experimental/control ratios, are plotted on the ordinate. Mean SCC_t/SCC_0 ratios of the experimental hemibladders divided by that of the controls are plotted on the abscissa. ● = aldosterone (7×10^{-8} M) vs controls. ■ = aldosterone ($3{\cdot}5 \times 10^{-8}$ M) + SC 9420 ($3{\cdot}5 \times 10^{-6}$ M). ▲ = aldosterone (7×10^{-8} M) + cortisol (7×10^{-8} M) vs cortisol (7×10^{-8} M). □ = SC 9420 ($3{\cdot}5 \times 10^{-6}$ M) vs controls. △ = cortisol (7×10^{-8} M) vs controls. ○ = 17-α-isoaldosterone (7×10^{-8} M) vs controls. From Rossier *et al.*[71].

The physiological significance of enhanced labeling with ^{3}H-uridine was also tested with cortisol, a potent glucocorticoid. Cortisol (7×10^{-8} M) had no effect on Na^+ transport or on incorporation of ^{3}H-uridine into RNA. The inactive stereoisomer, 17α-isoaldosterone, was similarly inactive with respect to Na^+ transport and to uridine incorporation into discrete classes of RNA. In control experiments, we found that aldosterone had minimal effects (+17%) on uptake of ^{3}H-uridine into the acid-soluble pool and had no effect on ribonuclease activity in homogenates of toad bladder epithelium.

The physiological significance of the effect of aldosterone on labeling of 9–12S RNA was also supported by the finding of a significant correlation between the fractional change in ^{3}H-uridine incorporation and the increase in SCC (Fig. 6).

Additional evidence was obtained that the 9–12S, non-methylated RNA had the properties of mRNA with the aid of oligo-deoxythymidylate chromatography. Hemibladders were labeled with ^{3}H-uridine and ^{3}H-adenosine (30 min pulse, 150 min chase) and with and without aldosterone in the medium [72]. The steroid increased Na^+ transport by 131%, labeling of total cytoplasmic RNA by 19% and poly-A-rich RNA by 85% (Table 11). In control experiments the poly-A-rich RNA fraction was non-methylated after equivalent pulse-chase labeling intervals with ^{14}C-methyl-methionine. These results support the inference that augmentation of mRNA synthesis mediates the action of aldosterone on Na^+ transport.

The validity of the inference that steroidal regulation of the quantity of specific mRNA's in target cells is central to the action of the steroids is now almost beyond dispute. The molecular mechanisms involved in this process, however, are by no means well-defined. Augmentation of mRNA can be ascribed to a number of effects, including: (1) gene activation or derepression, (2) selective rescue of precursors of specific mRNA's from the pool of rapidly labeled heterogeneous RNA, (3) augmentation of poly-adenylation of specific mRNA's, (4) facilitated transport of poly-A-rich RNA to the cytoplasmic compartment, and (5) decreased rate of catabolism of the mature mRNA's. Moreover, at some point it will be necessary to define the relationship between the mechanism of attachment of steroid–receptor complexes to specific intranuclear sites and the process by which mRNA is accumulated.

Many, perhaps even all, steroid-target cell interactions result in augmentation of labeling of precursors and final products in rRNA synthesis and in RNA polymerase I activity, the enzymatic regulator of the synthesis of rRNA precursor [5]. Observed increases in chromatin template activity after treatment with steroids probably represent activation of gene sites coded for rRNA precursor rather than template activity of unique sequences [66, 73, 74]. With respect to steroids that regulate cell growth and differentiation, activation of rRNA synthesis and increases in translational capacity probably play an important role in the overall process. The contribution of this pathway to physiological action of steroids, such as aldosterone, is still obscure, however. Liew *et al.*[65] found that aldosterone increased endogenous RNA polymerase (predominantly polymerase I) activity in rat kidney and heart nuclei, with a latent period of about 1 h. Similarly, Chu and Edelman[54] noted that

Table 11. Effect of aldosterone on incorporation of nucleotides of poly-A-rich-RNA in toad bladder

Aldosterone molar	SCC_t/SCC_0 Fractional increase	Specific activity of ^{3}H-uridine + ^{3}H-adenosine (c.p.m./μg) Total RNA	Cytoplasmic RNA	Poly-A-rich RNA
0	0·57 ± 0·08	1640	1955	13,388
7×10^{-8}	1·32 ± 0·10	1870	2330	24,765
A/C	2·31 ($p < 0{\cdot}001$)	1·14	1·19	1·85

Hemibladders were incubated in ^{3}H-uridine and ^{3}H-adenosine for 30 min, followed by a 150 min chase. Epithelial layer was then analyzed for incorporation into total RNA, cytoplasmic RNA and the tris-fraction eluted from an oligo-dT-cellulose column (Poly-A-rich RNA).

N = 10 pairs of hemibladders. From Wilce, P. A., Rossier, B. C. and Edelman, I. S.: Unpublished observations.

Table 12. Effects of aldosterone and actinomycin D on electrolyte excretion in the adrenalectomized dog*

Treatment	$U_{Na}V$ (μEq/min) Control	% Change	U_KV (μEq/min) Control	% Change	U_HV (μEq/min) Control	% Change
None	114 ± 36	−21	41 ± 11	−12	56 ± 7	+5
Aldosterone (100 μg)	108 ± 47	−69†	40 ± 5	+95†	56 ± 15	+23†
Aldosterone (100 μg) + Actinomycin D (250 μg)	110 ± 37	−1	37 ± 8	+92†	47 ± 14	+55†
Actinomycin D (250 μg)	126 ± 19	−2	55 ± 13	+38†	40 ± 11	+3

* The values are the mean ± S.E.M. of separate studies in four dogs.
† Statistically significant ($p < 0.05$). From Lifschitz *et al.*[80].
$U_{Na}V$, U_KV and U_HV denote the products of the urinary concentrations of the respective ions (μEq/ml) and urinary flow rates (ml/min).

aldosterone increased the polymerase I/II ratio in rat kidney. Recently, Wilce *et al.*[72] found that aldosterone increased methylation of 18S, 28S and 40S RNA in the nuclear fraction of toad bladder epithelium, in 30 to 90 min. A corresponding increase in labeling of 18S and 28S cytoplasmic RNA was obtained in 90 to 240 min. The role of enhanced rRNA synthesis in the action of aldosterone on Na^+ transport was evaluated with a selective inhibitor of rRNA synthesis, 3′-deoxycytidine [75]. Isolated toad bladders were pre-incubated with 3′-deoxycytidine or the diluent for 1 h, treated with aldosterone (7×10^{-8} M) and continuously labeled with [^{14}C] methyl methionine for 3 h [76]. 3′-Deoxycytidine effectively inhibited nuclear synthesis of rRNA (i.e., methylation of 18S, 28S and 40S RNA) but had no effect on stimulation of Na^+ transport by aldosterone. As noted above 3′-deoxyadenosine, an inhibitor of polyadenylation of nucleoplasmic RNA as well as the synthesis of nucleolar RNA, inhibited the action of aldosterone on Na^+ transport (Fig. 4). These results lend further support to the conclusion that regulation of mRNA synthesis is necessary to physiological action and imply that rRNA synthesis is not involved in the early response. There are, of course, a number of ways by which increases in rRNA synthesis could affect mineralocorticoid action *in vivo*, *e.g.*, increasing translational capacity during long-term stimulation with aldosterone.

Possibility of non-induction mechanisms in steroid action

The evidence that the principal actions of steroid hormones are mediated by a receptor-induction mechanism as described above, is both extensive and persuasive. Nevertheless, the possibility that some effects may be produced by direct interactions between steroids and specific enzymes or organelles remains open; particularly at very high concentrations of the hormones. Steroids have been noted to modify the stability of lysosomes and the properties of enzymes [77, 78]. The effects of high concentrations of steroids on the glutamate-alanine substrate specificity of crystalline glutamate dehydrogenase has been well-documented [78]. Effects of this kind *in vivo* may account for the progressively more toxic effects of steroids when present in considerable excess. Even at physiological concentrations, however, direct action of steroids on membranes or enzymes may have escaped notice. A possible example is the finding that in the rat, actinomycin D in doses sufficient to abolish the anti-natriuretic effect of aldosterone had no effect on the kaliuretic response [67, 79]. Similar results in the dog are shown in Table 12 [80]. Actinomycin D inhibited the anti-natriuretic but not the kaliuretic or acid excretion effects of aldosterone. Thus, the effects of aldosterone on K^+ or H^+ excretion may be independent of synthesis of RNA; although induction via a pathway resistant to actinomycin D can not be ruled out on the basis of the present evidence.

The presence or absence of high affinity receptors in a given tissue may provide a clue to the possible existence of non-inductive pathways in the response to steroid hormones but this approach is complicated by the evidence of multiple high affinity receptors in many organs and tissues, such as mineralocorticoid and glucocorticoid receptors in the kidney [2]. The

Table 13. Corticosteroid receptors in rat kidney: affinities and relative binding potencies of various steroids

	Type I	Type II	Type III
Optimal steroid	Aldosterone	Dexamethasone	Corticosterone
K_{diss}*	5×10^{-10} M, 37°C	5×10^{-9} M, 25°C	3×10^{-8} M, 25°C
% Relative potency†			
aldosterone	100	20	<1
DOC	85	20	25
corticosterone	2	40	100
dexamethasone	2	100	<1

* Determined by Scatchard analysis for the optimal steroid.
† Determined by competitive binding assay against tritiated optimal steroid. The potency of unlabelled optimal steroid is taken as 100%. From Feldman *et al.*[2].

affinities of a steroid for these receptors may not vary widely. As shown in Table 13, aldosterone has a moderate affinity for the Type II (glucocorticoid receptor) and corticosterone has a similar affinity for the aldosterone receptor. Thus, at higher and higher circulating steroid levels, new effects may be recruited owing to cross-over in receptor occupancy. Numerous reports have appeared of effects of aldosterone on the mammalian heart. Funder *et al.*[81] recently demonstrated the existence of high affinity glucocorticoid binding proteins in the heart that also bind aldosterone but with a lower affinity than for corticosterone. The cardiac effects of aldosterone may be expressed by trespassing on the "glucocorticoid" receptor system. These and related questions on such diverse topics as the role of the receptor-induction mechanism and non-inductive pathways in steroid action in growth and development, neoplasia, metabolic regulation and in neurobiological systems are now receiving more and more attention but are beyond the scope of this limited review.

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