IDENTIFICATION OF FOUR TYPES OF STEROID BY THEIR INTERACTION WITH MINERALOCORTICOID RECEPTORS IN THE TOAD BLADDER

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SUMMARY

The effects of various steroids on sodium transport across the toad bladder were examined in vitro. Cortisone, alone, had no effect on sodium transport but was an antagonist of aldosterone. Cortisone was shown to bind to the two non-specific binding sites for steroids in the nucleus of the toad bladder epithelial cells when present at a concentration of 10⁻⁷ M. At higher concentrations it interacted with the mineralocorticoid receptors and displaced aldosterone from these sites. Cortexolone had a weak stimulating effect on sodium transport and also antagonized the effect of aldosterone. Cortexolone, when present in excess, displaced aldosterone from its receptor sites.

Four types of steroid could be differentiated by their interaction with the mineralocorticoid receptor sites and the subsequent biological response: (a) steroids that bind to the receptors and produce the full biological effect on sodium transport; (b) steroids that do not bind and therefore are incapable of producing a response; (c) steroids that bind to the receptor sites, have no effect alone but inhibit the action of aldosterone; (d) steroids that bind to the receptor sites, produce a partial response on sodium transport and subsequently antagonize the action of aldosterone.

Multiple points of attachment are postulated for the steroid–receptor interaction.

INTRODUCTION

In previous studies of the effects of steroid hormones on sodium transport in the toad bladder, three classes of steroid have been described. First, aldosterone, deoxycorticosterone, 9α-fluorocortisol and certain less potent steroids such as cortisol, stimulate sodium transport after a latent period of about 1 h (Crabbé, 1961; Sharp & Leaf, 1964; Porter & Edelman, 1964). Secondly, progesterone and the steroid-like spirolactones have no effect upon sodium transport alone but prevent the stimulation of transport by aldosterone (Sharp & Leaf, 1964; Porter, 1968). Third, steroids such

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as testosterone, 17β-oestradiol and iso-aldosterone which are inactive, neither affecting sodium transport nor inhibiting the action of aldosterone (Edelman & Fimognari, 1968; Alberti & Sharp, 1969).

Receptor sites have been located in the nuclei of cells of both toad bladder (Ausiello & Sharp, 1968) and kidney (Fanestil & Edelman, 1966) and steroid-receptor complexes have been isolated (Edelman & Fimognari, 1968; Herman, Fimognari & Edelman, 1968; Alberti & Sharp, 1969). Thus direct investigations on the receptor-steroid interaction are possible. In the toad bladder it has been shown that compounds in the first class, e.g. aldosterone, deoxycorticosterone and cortisol, can displace [3H]d-aldosterone from the receptors. This has been demonstrated by measurement of [3H]d-aldosterone in the whole tissue (Sharp, Komack & Leaf, 1966), in purified preparations of nuclei (Ausiello & Sharp, 1968), and in receptor-complexes isolated from the nuclear preparations (Alberti & Sharp, 1969). Similarly, steroids in the second class have been shown to displace [3H]d-aldosterone thus confirming that they act as competitive inhibitors (Porter, 1968; Ausiello & Sharp, 1968; Alberti & Sharp, 1969). The third class of steroids do not displace [3H]d-aldosterone from the receptor sites, a finding which is in accord with their inability to interfere with the stimulation of sodium transport by aldosterone and which demonstrates the specificity of the steroid-receptor interaction (Sharp et al. 1966; Alberti & Sharp, 1969).

Using the four criteria: (1) effect on sodium transport, (2) effect on the stimulation of sodium transport by aldosterone, (3) ability to displace [3H]d-aldosterone from its receptors and (4) binding ability of the steroid under test, it is possible to define steroid action in the nucleus in terms of the three classes outlined, and an additional class which is typified by cortexolone.

METHODS

Toads (Bufo marinus) were obtained from National Reagents Inc., Bridgeport, Connecticut. They were maintained partially immersed in 0·6% NaCl solution for at least 2 days before use in order to reduce endogenous aldosterone secretion. Sodium transport across the toad bladder was measured as the short-circuit current in double chambers as described previously (Sharp & Leaf, 1964). One half-bladder is mounted across a double chamber allowing one quarter to be used as test tissue and the adjacent quarter as the control. Bladders were discarded if they had a starting potential of less than 5 mV. All experiments were carried out in a Ringer solution of the following composition (mm): NaCl, 113·5; KCl, 3·5; NaHCO₃, 2·4; CaCl₂, 0·89; pH in air 7·9–8·0; total solute concentration 220 millimoles/kg water. All Ringer solutions contained also 5·5 mm-glucose, 0·1 mg penicillin/ml and 0·05 mg streptomycin/ml. In the inhibitor studies, cortisone and spirilactone were added 1 h before aldosterone. Cortexolone was added 3 h before aldosterone in order to demonstrate the weak stimulation of sodium transport in the same tissues in which inhibition was demonstrated subsequently.

Incubation of toad bladders for binding studies

After pithing and exsanguinating the toads, half-bladders were incubated in Ringer solution containing [3H]d-aldosterone or [3H]cortisol at room temperature with constant shaking for 1½–2½ h. In displacement experiments paired half-bladders
Steroid hormone interactions with nuclear receptors

were incubated with 10^-8 M- or 10^-7 M-[3H]d-aldosterone, 10^-7 M-[3H]hydrocortisone or 10^-7 M-[3H] cortisone. After a 60-min incubation, the displacing unlabelled steroid, 10^-5 M or 10^-6 M final concentration, was added to one set of half-bladders and the incubation continued for a further 90 min. Further procedures on the two sets of hemibladders were performed in parallel. The reduction in bound steroid by unlabelled steroid represents displacement of [3H]d-aldosterone or [3H]cortisone from binding sites in the cells (Sharp et al. 1966; Alberti & Sharp, 1969).

Mucosal cell preparation and fractionation

These were performed as described previously (Alberti & Sharp, 1969). After incubation of the half-bladders, mucosal cells were scraped off with a glass slide into 0.1 M-tris, pH 7.4, containing 0.003 M-CaCl_2. All subsequent steps were carried out in this buffer at 0–4 °C. The mucosal cells were separated from connective tissue by two strokes in a Ten Broeck homogenizer; by this treatment the cells rise to the upper chamber of the homogenizer and the connective tissue is trapped between the pestle and the outer wall. The cells were then transferred to a Dounce homogenizer and disrupted with 40 strokes to release the nuclei. The homogenate was centrifuged at 600 g for 8 min, the pellet made up to the required volume with the tris buffer mixture, resuspended and sonicated three times for 3 s at 15–20 W output (Sonifier Cells Disrupter W 185 C, Heat Systems Co., Melville, Long Island, New York).

The pellet, before sonication, was examined microscopically and contained mainly nuclei, some membrane debris and only a few whole cells. It is thus referred to as the ‘crude nuclear fraction’. The sonicate was centrifuged for 20 min at 22,000 g and the supernatant used for estimation of bound hormone.

Estimation of bound aldosterone and other steroids

Sephadex G-25 column chromatography was used for the separation of bound from free labelled steroid as described previously (Alberti & Sharp, 1969). The tris buffer mixture was used as eluant. All fractions were assayed for radioactivity and protein content. Sepharose 6B (agarose) column chromatography was also used. Column elutions were performed at 0–4 °C in a cold room.

When time-course studies were required, zero time was taken as the time when the supernatant from the sonicated crude nuclear fraction was finally prepared.

Measurements of radioactivity

Liquid scintillation counting was used to estimate the tritium content of the fractions. Samples of 0.2 ml were counted in 10 or 15 ml scintillation fluid of the following composition: p-bis-(2-(5-phenyloxazolyl))-benzene (POPOP), 0.13 g; 2,5-diphenyl oxazole (PPO), 6.5 g; naphthalene, 104 g; methyl alcohol, 300 ml; dioxane, 500 ml and toluene, 500 ml. Counting efficiency was determined by the channels ratio method for each sample using ^131I as the external source in a Nuclear–Chicago Mark I scintillation counter and counts were corrected to 100% efficiency.

DNA assay. Pellets after centrifugation at 22,000 g were assayed for DNA using diphenylamine as described previously (Schneider, 1945; Lowry & Bessey, 1946; Ausiello & Sharp, 1968).
Protein estimation. Protein was estimated in the column eluates by measurement of absorption at 280 and 260 nm (Warburg & Christian, 1942).

Chemicals. [3H]d-aldosterone, [3H]hydrocortisone and [3H]cortisone were purchased from the New England Nuclear Corporation. Purity was better than 95% in each lot. D-Aldosterone was a gift from Dr M. M. Pechet. Cortexolone was obtained from Mann Research Laboratories Inc., New York, N.Y. Sephadex and agarose (Sephrose 6B) were obtained from Pharmacia Chemicals Inc., Uppsala, Sweden. Spirolactone (SC 14266) was a gift from G. D. Searle and Co.

RESULTS

The effect of cortisone on the stimulation of sodium transport by aldosterone

In Fig. 1 the mean results of ten experiments, on paired quarter bladders, are reported. Cortisone was added to the serosal fluid bathing one quarter-bladder 1 h before d-aldosterone was added to the fluids bathing both quarter bladders. The mean currents at time zero were 38 µA for the quarter-bladders to be treated with aldosterone and 45 µA for those treated with cortisone as well. The tissues treated with aldosterone only responded with increased sodium transport which reached maximal values 6 h after the addition of hormone. The tissue exposed to cortisone and aldosterone showed a much smaller rise in sodium transport, demonstrating that cortisone was inhibiting the effect of aldosterone. This is similar to the inhibitory effect of spirolactone (SC14266) on the stimulation of sodium transport by aldosterone as shown in Fig. 2. In these experiments the mean initial currents at time zero were 24 µA for the quarter-bladders to be treated with aldosterone and 23 µA for those treated with spirolactone as well. The competition ratio of 500:1 for both cortisone and spirolactone, relative to aldosterone, causes a significant but partial inhibition of the effects of the latter. Cortisone and spirolactone (SC14266) have been shown previously to have no effect on sodium transport when administered alone at these concentrations (Sharp & Leaf, 1964; Porter, 1968).

The effect of cortexolone on sodium transport and on the stimulation of sodium transport by aldosterone

Figure 3 shows the results of an experiment on paired quarter-bladders exposed to cortexolone and aldosterone. Cortexolone at the high concentration of 1 × 10⁻⁵ M stimulated sodium transport after a latent period of 1 h, and subsequently inhibited the response to aldosterone. Therefore, a series of 16 hemi-bladders was examined for cortexolone inhibition of aldosterone responses. In these experiments the cortexolone was added 3 h before aldosterone in order that the stimulation of sodium transport by cortexolone could be documented before its ability to inhibit aldosterone was observed. Mean initial currents were 41 µA for the tissues treated with aldosterone and 34 µA for those treated with cortexolone as well. At 4 h sodium transport in the presence of cortexolone was significantly greater than in the controls (P < 0.01). Figure 4 shows the inhibitory effect of cortexolone on the aldosterone response; it was significant 4 to 7 h after aldosterone. Thus cortexolone can both stimulate sodium transport and inhibit the effects of aldosterone.
Steroid hormone interactions with nuclear receptors

Binding of [3H]D-aldosterone, [3H]hydrocortisone and [3H]cortisone by the crude nuclear fraction of toad bladder mucosal cells

Previous work has shown that bound [3H]D-aldosterone or [3H]hydrocortisone can be extracted from the crude nuclear fraction of toad bladder mucosal cells (Alberti & Sharp, 1969). Figure 5 shows an experiment in which the binding of [3H]D-aldosterone was compared with that of [3H]hydrocortisone and [3H]cortisone in paired tissues when each was present in the incubation medium at 10⁻⁷M. At this concentration aldosterone stimulated sodium transport maximally while hydrocortisone had little effect and cortisone none. [3H]Cortisone at zero time showed

![Graph](image-url)

Fig. 1. The effect of cortisone on the stimulation of sodium transport by aldosterone. Sodium transport is expressed on the ordinate as the ratio of the short-circuit current at any time (t) to the short-circuit current (SCC) at the time of addition of aldosterone (time zero). Cortisone (5 x 10⁻⁶M) was added to the serosal fluid bathing the quarter bladders represented by the continuous line 1 h before time zero. At time zero, D-aldosterone (O---O) (1 x 10⁻⁸M) was added to the serosal fluid bathing both sets of quarter bladders.

Fig. 2. The effect of spirolactone (SC 14266) on the stimulation of sodium transport by aldosterone. Sodium transport is expressed on the ordinate as the ratio of the short-circuit current at any time (t) to the short-circuit current (SCC) at the time of addition of aldosterone (time zero). Spirolactone (5 x 10⁻⁶M) was added to the serosal fluid bathing the quarter bladders represented by the continuous line 1 h before time zero. At time zero D-aldosterone (1 x 10⁻⁸M) was added to the serosal fluid bathing both sets of quarter bladders.
47% of the total binding of \([^3H]d\)-aldosterone while \([^3H]\)hydrocortisone showed 49% of the binding. However, 2 and 5 h after preparation a considerable portion of the aldosterone complex had broken down while bound \([^3H]\)cortisone and \([^3H]\)hydrocortisone showed negligible breakdown. The net result is that the absolute amount

![Graph](image-url)

**Fig. 3.** The effect of cortexolone on sodium transport and on the stimulation of sodium transport by aldosterone. Sodium transport is expressed on the ordinate as the short-circuit current. Cortexolone \((1 \times 10^{-8}M)\) was added to the serosal solution bathing the quarter bladder represented by the discontinuous line. \(d\)-Aldosterone \((2 \times 10^{-8}M)\) was added to the serosal solutions bathing both quarter bladders 3 h later.

![Graph](image-url)

**Fig. 4.** The effect of cortexolone on the stimulation of sodium transport by aldosterone. Sodium transport is expressed on the ordinate as the ratio of the short-circuit current at any time \((t)\) to the short-circuit current \((SCC)\) at the time of addition of aldosterone \((time \; zero)\). Cortexolone \((1 \times 10^{-8}M)\) was added to the serosal fluid bathing the quarter bladders represented by the continuous line, 3 h before time zero. \(d\)-Aldosterone was added to the serosal fluid bathing both sets of quarter bladders.
Steroid hormone interactions with nuclear receptors

of binding was almost identical for the three steroids 2 and 5 h after isolation of the complexes. In a second experiment with matched pieces of hemibladder [3H]cortisone binding was 38% and [3H]hydrocortisone 59% of control binding at zero time. Again the bound [3H]p-aldosterone dissociated rapidly whilst the other two steroid complexes did not, so that the 2 h values were 45, 39 and 54% aldosterone binding at zero time for [3H]p-aldosterone, [3H]cortisone and [3H]hydrocortisone, respectively. Thus the rapidly dissociable complex, shown previously to be the mineralocorticoid receptor complex, does not appear to be demonstrable for [3H]cortisone and [3H]hydrocortisone when these steroids are used at a concentration of 10⁻⁷ M.

\[ \begin{align*}
\text{Time (h)} & \quad \text{Moles steroid bound} \times 10^{-14} \text{ per 100 } \mu \text{g DNA} \\
0 & \quad 4 \\
1 & \quad 3 \\
2 & \quad 2 \\
3 & \quad 1 \\
4 & \quad 0 \\
5 & \quad 0 \\
\end{align*} \]

Fig. 5. Comparison of the binding of [3H]p-aldosterone (○), [3H]cortisone (●) and [3H]hydrocortisone (△) in toad bladder mucosa when present at a concentration of 1 × 10⁻⁷ M in the incubation media. Binding of the steroids is expressed on the ordinate as moles \( \times 10^{-14} \) bound per 100 µg DNA. The time-course for breakdown of the steroid-protein complexes is shown by determination of bound steroid at 0, 2 and 5 h after isolation of the complexes.

\[ \begin{align*}
\text{Time (h)} & \quad \% \text{ control bound aldosterone} \\
0 & \quad 100 \\
1 & \quad 50 \\
2 & \quad 0 \\
\end{align*} \]

Fig. 6. The effect of cortisone (1 × 10⁻⁵ M) on the binding of [3H]p-aldosterone (1 × 10⁻⁴ M) in toad bladder mucosa. Binding is expressed as percentage of the zero time control binding for [3H]p-aldosterone.

Displacement of [3H]hydrocortisone (10⁻⁷ M) from crude nuclear fraction of toad bladder mucosal cells by excess p-aldosterone (10⁻⁵ M)

The previous experiments suggest that [3H]cortisone binding at 10⁻⁷ M is largely non-specific. The mineralocorticoid sites were largely unoccupied at that concentration. This was confirmed by the fact that a 100-fold excess of p-aldosterone did not reduce the binding of [3H]cortisone.
Effect of excess cortisone on the binding of \(^{3}H\)D-aldosterone

The effect of a 1000-fold excess of cortisone on the yield of bound aldosterone from the crude nuclear preparation of toad bladder cells is shown in Fig. 6. It can be seen that 46% of bound aldosterone was displaced (\(n = 4\)). Two hours after preparation, 39% of the zero-time control binding was still present compared with 50% in the control without cortisone. Thus the rate of breakdown of the bound aldosterone which remained in the presence of \(10^{-5}\text{M}\)-cortisone was less than that in the control. Therefore, the non-displaceable fraction is composed primarily of stable bound complex, a fraction previously shown to be non-mineralocorticoid specific, and thus cortisone at 1000-fold excess displaced \(^{3}H\)D-aldosterone from its specific receptors.

Little displacement of bound aldosterone was found at a 100-fold excess of cortisone over \(^{3}H\)D-aldosterone.

![Fig. 7. The effects of aldosterone (\(n = 4\)), cortisone (\(n = 3\)) and cortexolone (\(n = 2\)) at each of two different concentrations (\(1 \times 10^{-5}\) and \(1 \times 10^{-4}\)M) on the binding of \(^{3}H\)D-aldosterone (\(1 \times 10^{-8}\)M) in toad bladder mucosa. Binding is expressed as a percentage of the control binding for \(^{3}H\)D-aldosterone.](image-url)

Effect of excess cortexolone on the binding of \(^{3}H\)D-aldosterone

Cortexolone in 1000-fold excess displaced 69% of the bound \(^{3}H\)D-aldosterone from the crude nuclear fraction. The bound fraction that remained in the presence of cortexolone dissociated at a slower rate than the bound fraction in the absence of cortexolone. As with cortisone, little displacement occurs at 100-fold excess. Fig. 7 summarizes these findings and compares them with the displacement which occurs with excess unlabelled D-aldosterone. It is clear that cortisone and cortexolone have similar affinities for the mineralocorticoid binding protein and that the affinity of these two steroids is much less than that of aldosterone.
Steroid hormone interactions with nuclear receptors

Agarose column chromatography of bound [³H]cortisone from crude nuclear fraction of the toad bladder mucosal cells

Bound [³H]cortisone from the crude nuclear fraction was separated from free [³H]cortisone by Sephadex G-25 column chromatography. An aliquot of the bound [³H]cortisone was then applied to an agarose (sepharose 6B) column. A further aliquot was applied 3 h later. The complete elutions occupied 2½ h.

Figure 8 shows the elution pattern. The first peak represents [³H]cortisone bound in a complex of molecular weight of 400 000 or more, the second peak represents a molecular weight of 100 000-150 000, the third peak represents free steroid. In the first elution it can be seen that 27% of the bound hormone was in the first peak, 5% appeared between the first and second peaks, 47% in the second peak, and only 20% appeared as free steroid, i.e. had dissociated during the time taken for elution.

A second elution begun 3 h after the first, showed a first peak of 24%, a second peak of 33% and a third peak of 41%. Thus there is only a 14% further breakdown in 3 h, virtually all of it coming from the second peak.

Previous studies with aldosterone showed 8, 25 and 67% for the three peaks, the first two being non-mineralocorticoid specific (Alberti & Sharp, 1969). Allowing that at 10⁻⁷ M [³H]cortisone binding is only 38-47% of [³H]D-aldosterone binding the absolute magnitude of the first two peaks with the two hormones is similar. Designating the aldosterone binding as 100%, the peaks would be 12, 20 and 9%. Thus the only major disparity in absolute size is with the third peak, which was very much smaller with the [³H]cortisone. This is the rapidly dissociable mineralocorticoid-specific fraction. These results again suggests that [³H]cortisone at 10⁻⁷M binds primarily to non-mineralocorticoid sites.
DISCUSSION

It has been reported that cortisone in vitro has no effect on sodium transport across the toad bladder (Sharp & Leaf, 1964), a finding which is in accord with the suggestion that steroids possessing the 11-keto configuration are inactive until converted to the 11-hydroxy compound (Bush & Mahesh, 1959). In contrast to this lack of activity when present alone, cortisone does inhibit the stimulation of sodium transport by aldosterone. Figure 2 shows the partial inhibition of the aldosterone response produced by a 500-fold excess of cortisone. This result is similar to the inhibition caused by spirolactone (Fig. 2) and suggests that cortisone is a competitive antagonist of aldosterone. To test this possibility directly, the ability of cortisone to displace [3H]D-aldosterone from its nuclear receptor sites was determined. In a previous study (Alberti & Sharp, 1969) it was shown that three aldosterone binding complexes can be isolated from the nuclei of the mucosal cells of the toad bladder. Two of these were stable complexes apparently unrelated to the mineralocorticoid action of aldosterone. The other, however, was unstable but had the characteristics required of a mineralocorticoid receptor. That is, it bound [3H]D-aldosterone which could be displaced by other mineralocorticoids and spirolactones but not by inactive steroids such as testosterone. This steroid–receptor complex can be identified by its short half-life of approximately 2 h at 0-4 °C. A comparison of the binding of [3H]cortisone, [3H]hydrocortisone and [3H]D-aldosterone, all at a concentration of 10−7 M, demonstrated that the nuclei bind twice as much [3H]D-aldosterone as [3H]cortisone or [3H]hydrocortisone. However, the rate of breakdown of the various complexes is such that at 2 h the amounts bound are about equal: some of the aldosterone complex having broken down rapidly while only a small amount of breakdown occurs in the cases of cortisone and hydrocortisone. Thus the rapidly dissociable complex, previously identified as the mineralocorticoid receptor, was bound to a much greater extent by aldosterone than by the other steroids at this concentration. Cortisone and hydrocortisone were bound largely at the stable non-specific binding sites. This result is in accord with the known potency of hydrocortisone which has only slight effects on sodium transport at 10−7 M, and of cortisone

| Table 1. Classification of mineralocorticoids |

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<th>Binding to receptors</th>
<th>Stimulation of sodium transport</th>
<th>Inhibition of active steroids</th>
<th>Displace active steroids</th>
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<td>(1) Active</td>
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<td>+++</td>
<td>−</td>
<td>+++</td>
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<td>(2) Inhibitory</td>
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<td>+</td>
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<td>(3) Inactive</td>
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<td>−</td>
<td>−</td>
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<td>(4) Active and inhibitory</td>
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Examples

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Steroid hormone interactions with nuclear receptors

which is required in large excess (Fig. 6) for displacement of aldosterone. In order to prove that cortisone at 10^{-7}M was binding to the non-specific sites, and only slightly to the mineralocorticoid sites, the tissue was incubated with [3H]cortisone and an attempt made to displace the bound [3H]cortisone. At a concentration of 10^{-5}M, a 100 times that of the cortisone, D-aldosterone was unable to displace a significant amount of cortisone. Thus the cortisone was not bound to the mineralocorticoid binding sites, and this conclusion was further strengthened by the agarose column separation of the two stable non-specific complexes of [3H]cortisone. Finally it was found that cortisone displaced [3H]p-aldosterone from these specific mineralocorticoid binding sites, confirming that the antagonism of the effects of aldosterone by cortisone at high concentration resulted from competition for the mineralocorticoid receptor site. Thus cortisone behaves like the spirolactones and is probably a competitive antagonist of aldosterone. It appears that steroids containing the 11-keto group can interact with specific receptor sites, are unable to stimulate sodium transport, but act as antagonists to mineralocorticoid hormones. These 11-keto steroids do not act as antagonists in vivo because of the ease with which 11β-hydroxylation occurs.

It has been shown that cortexolone can stimulate sodium transport weakly and can also antagonize aldosterone. Cortexolone, like cortisone, displaces [3H]p-aldosterone from its receptor sites. Thus cortexolone represents another interesting type of steroid which can interact with the receptor site, give a small physiological response and yet antagonize the effects of more potent hormones by occupation of the sites. The results suggest different degrees of steroid–receptor interaction explicable by multiple points of attachment.

These studies show that steroids can be classified into four groups according to their interaction with receptor sites and subsequent effects (Table 1): (1) those that interact with the receptor and produce an effect; (2) steroids, like cortisone, which bind to the receptor, do not produce an effect but which antagonize other hormones; (3) steroids that do not bind to the receptor; and (4) a new class, steroids which produce a partial response by binding to the receptor but which can also antagonize the actions of the other hormones. These results are in accord with the idea of multiple points of attachment for the steroid to its receptor. Detailed analysis by the methods outlined, for several steroids, should provide information on the three dimensional configuration of the receptor sites for steroid hormones.

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