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Sodium-Retaining Activity of Some Natural and Synthetic 21-Deoxysteroids

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SUMMARY

The effect of progesterone and six other C21-deoxysteroids on renal sodium retention by male adrenalectomized rats was compared with the effect exerted by the natural corticoids aldosterone, 11-deoxycorticosterone, and corticosterone. Steroids were active in the following order: aldosterone > 11,19-oxidoprogesterone > 5α*H*-3,20-pregnanedione ≥ 5β*H*-3,20-pregnanedione > progesterone = 11-ketoprogesterone > 6,19-oxidoprogesterone = 11-keto-6,19-oxidoprogesterone ≥ corticosterone. All C21-deoxysteroids, except 11,19-oxidoprogesterone, exhibited parabolic log dose-response functions, indicating an effect that opposes renal sodium retention at high doses. 11,19-Oxidoprogesterone and the natural corticoids exhibited normal, exponential, log dose-response curves. Diverse geometric parameters related to molecular planarity were calculated and their correlation with biopharmacological properties was attempted. The best linear regression was obtained for correlation of the concavity of log dose-reponse parabolas (second-order coefficients) of C21-deoxysteroids with the C3═O/ring D angle of these molecules. A good linear regression could also be obtained for correlation of the affinity of C21-deoxysteroids, except 11,19-oxidoprogesterone, for purified type I mineralocorticoid receptors with those angles. The latter correlation deteriorated upon incorporation of the affinity data for the three natural corticoids, due to similar affinities of these hormones for type I mineralocorticoid receptors, but could be restored when the binding data for the unpurified, corticosterone-binding globulin-containing stage of the receptors were considered. *In vivo* binding data followed the same trend as that for unpurified receptors.

At variance with glucocorticoids, which exhibit a slightly torsioned steroid nucleus at the A/B-ring junction, MCs seem to require an overall flat conformation for optimal activity (1–3). As a general rule, higher affinity ratios for the MR are obtained when substituents that tend to bend the A-ring towards the α face of the steroid molecule are eliminated, e.g., 19-nor- and 11-deoxysteroids (2). The same effect is obtained upon introduction of ketalic bridges, which flatten the overall structure, as in aldosterone and related 18-oxygenated analogues. In this respect, Yamakawa *et al.* (2) have proposed that for 3-ketosteroids an inverse relationship exists between the distance from the C3 oxygen to the C5/C14 plane and MR affinity. Duax *et al.* (3, 4) have summarized the conformational requirements on ring A of steroid hormones for optimal binding to different receptors; in the case of the MR the ideal conformation would be, according to those authors, a 1α-envelope to a 1α,2β-half-chair containing the 3-keto-4-ene function.

For several years we focused our attention on the relative importance of an overall planar conformation for sodium-retaining activity; the question arose as to the contribution to hormonal action of planarity on one hand and typical functional groups, such as the 21-hydroxyl and 18-carbonyl groups, on the other. Our original question was whether the planar conformation is necessary and/or sufficient for a steroid to acquire sodium-retaining activity.

Our initial approach to the problem was to confer, with minimal functional group variations, various degrees of "bending" to the A/B-ring junction of progesterone. The latter is the simplest hormone with a pregnane skeleton that lacks the aforementioned functional groups. The resulting analogues were assayed for sodium-retaining activity. The structural changes were achieved by introduction of tailored substituents aiming at or opposing planarity, based on molecular

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ABBREVIATIONS: MR, mineralocorticoid receptor(s); DOC, 21-deoxycorticosterone; CBG, corticosterone-binding globulin; MC, mineralocorticoid; RU-28362, 11,17-dihydroxy-6-methyl-17-(1-propynyl)androsta-1,4,6-trien-3-one; ANOVA, analysis of variance; LSD, least significant difference; BCD plane, plane defined by rings B, C, and D; HPLC, high performance liquid chromatography; ADX, adrenalectomized.

mechanics (MM2) and semiempirical calculations (AM1). An oxido bridge was used both to obtain the highly planar [11,19-oxidoprogesterone (**2**)] and the highly bent [6,19-oxidoprogesterone (**7**)] structures, thus minimizing the effect of variations in functional groups (see Fig. 2 for structures). Planarity versus torsion was also compared using the 5αH and 5βH reduced derivatives of progesterone. Finally, an increase in molecular flexibility was studied using 11-keto derivatives.

However, once these assays were underway, we noticed exceptions to the "planarity requirement." For example 5βH-3,20-pregnanedione (**6**), a strongly bent steroid due to the *cis*-fusion of rings A and B, exhibited almost as intense sodium-retaining properties as did the fairly flat 5αH-isomer (**5**). This made us search for alternative conformational parameters related to but not absolutely coinciding with planarity, such as the orientation of the C3 carbonyl group with respect to the BCD plane or the plane defined by ring D alone. Better correlations could be established, which led to the synthesis, assay, and inclusion in correlation attempts of another progesterone derivative, pregna-1,4-diene-3,20-dione (**9**), differing from progesterone in planarity but less so in the orientation of the C3 carbonyl.

All of the compounds were injected into ADX rats and the effect on sodium retention was measured *in vivo* by the method of Kagawa *et al.* (5). Activity was also compared *in vitro* and *in vivo* by measuring [^{3}H]aldosterone displacement from renal cytosol (6).

Materials and Methods

Animals. Male Sprague-Dawley rats weighing 250–300 g underwent adrenalectomy 48 hr before the experiments. They were fed with 0.9% NaCl solution and food (Purina Diet 1) *ad libitum* until 12 hr before the experiment; at that time food was removed.

Steroids and reagents. 11,19-Oxidoprogesterone (**2**), 6,19-oxidoprogesterone (**7**), and 11-keto-6,19-oxidoprogesterone (**8**) were synthesized as described previously (7). 5αH-3,20-Pregnanedione (**5**) was obtained by hydrogenation of pregnenolone acetate over 10% palladium/carbon, followed by saponification and Jones oxidation. Pregna-1,4-diene-3,20-dione (**9**) was prepared by oxidation of progesterone with dichlorodicyanobenzoquinone in benzene. Progesterone, 5βH-3,20-pregnanedione (**6**), aldosterone, corticosterone, and 11-deoxycorticosterone were purchased from Sigma Chemical Co. (St. Louis, MO). [1,2-$^3H_2$]Aldosterone (59 Ci/mmol) was purchased from New England Nuclear. RU-28362 was a kind gift from Roussel-Uclaf (Romainville, France). All reagents used were analytical grade.

Radioactivity counting. Samples were measured in a Tracor Analytic Marck III liquid scintillation counter with 50% efficiency for tritium counts. Scintillation cocktail contained 2,5-diphenyloxazole (4 g) and 1,4-bis-2-(4-phenyloxazolyl)benzene (0.24 g) in toluene (1000 ml).

Computational calculations. Molecular mechanics calculations were carried out with the program PCMODEL 386 4.0 (Serena Software), using the MMX force field and VESCF calculations for the conjugated systems. Semiempirical calculations were performed with HyperChem release 2.0, using the AM1 method and the Polak-Ribiere optimization algorithm. All geometries were reoptimized twice to ensure convergence. Conformational searches were conducted in each case to ensure that the most stable conformer was obtained. Best planes for groups of atoms were those defined by the primary and secondary inertial axes of the atoms involved; only carbon atoms were considered for the calculation of inertial axes.

Administration of steroids for rat bioassays. Steroids were dissolved in ethanol/propylene glycol/0.9% NaCl solution (1:2:37). The final concentration was adjusted so that the dose corresponding to 100 g of body weight was dissolved in 0.1 ml of vehicle. For controls, the same solvent mixture without steroid was used.

Rats were anesthetized with ether and their urinary bladders were emptied by suprapubic pressure. They were then given subcutaneous injections of 2.5 ml of 0.9% NaCl solution, followed by intramuscular injection of the steroid solution or control solvent. The penis was ligated immediately afterwards.

Determination of sodium-retaining activity. After 2.5 hr, rats were anesthetized as before, urine was aspirated from the bladder into a graduated syringe, and urinary volume was measured. Urinary sodium and potassium levels were determined by flame photometry. Results were expressed as the ratio of the excretion rates of the steroid-injected animals to the excretion rates of animals injected with vehicle in parallel. In all cases, steroid treatment did not affect creatinine clearance, compared with nontreated rats [creatinine was measured by the method of Yatzidis (8)]. Control experiments with 10 μg/100 g 11-deoxycorticosterone were carried out with each series. Differences were evaluated by multifactorial ANOVA, with multiple-range analysis using LSD intervals (STATGRAPH 4.0).

Binding assays *in vitro*. A modification of the method described by Koshida *et al.* (6) was followed. Briefly, ADX rats were anesthetized with ether and bled to death by heart puncture. NaCl (0.9%) was injected through the aorta until the kidneys were completely bleached. Kidneys were perfused through the renal artery with additional cold 0.9% NaCl, dissected, decapsulated, and homogenized (with a Teflon/glass homogenizer) in cold buffer (0.1 M Tris·HCl, pH 7.4, containing 10 mM EDTA, 10 mM 2-mercaptoethanol, 20 mM Na_2MoO_4, 23%, v/v, glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 2.0 trypsin-inhibitory units/ml aprotinin). Homogenates were centrifuged at 37,000 × *g* for 20 min at 0° and pellets were discarded. In experiments in which CBG contamination had to be avoided, cytosols were first adsorbed on hydroxylapatite (9). Fractions (500 μl) were incubated at 0° for 12 hr with 5.0 nM [^{3}H]aldosterone; a 1000-fold excess of radioinert aldosterone was used to determine nonspecific binding, which amounted to 15–25% of total binding. RU-28362 (1.0 μM) was added to block the binding to type II receptors (10). The steroid-receptor complex was separated from free steroid by addition of 1 incubation volume of 2% (w/v) activated charcoal/0.2% (w/v) dextran 15–20 in buffer, shaking twice for 5 sec, and maintenance in ice for 5 min, followed by centrifugation at 5000 × *g* for 5 min at 0°; radioactivity was measured in the supernatant.

Binding assays *in vivo*. Rats were given intraperitoneal injections of a solution of 2 μCi of [^{3}H]aldosterone in saline and increasing amounts of either radioinert aldosterone, DOC, 11,19-oxidoprogesterone, or 6,19-oxidoprogesterone, as indicated in Results. After 15–20 min, blood was withdrawn for the determination of [^{3}H]aldosterone (in all cases the concentration was ~5.7 nM). The rats were sacrificed immediately, and kidneys were excised, perfused (until the medulla was bleached), homogenized, and processed as described above for the *in vitro* assays. Protein was determined and binding is expressed as specific dpm-percent (mean ± standard deviation). The biological half-life of [^{3}H]aldosterone was determined to be 11.5 ± 0.7 min (five experiments).

Biological half-lives. Biological half-lives were measured for aldosterone, progesterone, corticosterone, 11,19-oxidoprogesterone, and 6,19-oxidoprogesterone according to the method of Morris *et al.* (11), using tritiated compounds in the first three cases and radioinert ones in the case of the two synthetic steroids, for which no radioactive compounds were available. In experiments with the latter steroids, plasma samples were analyzed by HPLC, using a Spectraphysics liquid chromatography system (Isochrom LC pump and Spectraseries UV1 detector with a 1.2-μl cell), a Rheodyne injector with a 1-μl internal loop, and an Ultramex C_{18} 5-μm column (1 × 250 mm). Isocratic elution used methanol/acetonitrile/water (35:28:37) at 50 μl/min.

Results

In Vivo Sodium-Retaining Activity

Sodium-retaining properties were evaluated in ADX rats as a function of the administered dose, in the large range of 0.06–100 μg/100 g of body weight. In all cases, creatinine clearance rates were not affected by steroid treatment. Only aldosterone, DOC, and the "flat" progesterone derivative 11,19-oxidoprogesterone (2) exhibited classically increasing log dose-response curves. All other steroids exhibited maximal retention at a certain dose and lost part of their activity at higher doses, indicating that a secondary effect counterbalanced the primary, sodium-retaining effect (Fig. 1).

The sodium-retaining activity of these compounds must therefore be considered according to various parameters, such as the minimal active dose, the dose at which maximal activity is attained, the magnitude of this activity, and its decrease, if any, at higher doses. Whereas one statistical method, i.e., multifactorial ANOVA with multiple-range analysis using 95% LSD intervals, allows us to deal with steroid responses *in toto*, another criterion for establishing differences between steroids takes into account the shape of the dose-response curve.

In agreement with experimental findings and as a first approximation to evaluating the shape of log dose-response curves, the data for each steroid were adjusted to a second-order polynomial. The dose range for the fitting was selected so that individual responses differed significantly from controls and the correlation coefficient was >0.85. The second-order coefficients thus obtained are a direct measure of the concavity of the polynomials and, from a biopharmacological point of view, are directly related to the secondary effect mentioned above, which opposes the primary sodium-retaining effect. For comparison purposes, and in spite of fitting with second-order coefficients close to 0, the data for 11,19-oxidoprogesterone and the natural MC aldosterone were also adjusted as indicated above. In what follows, sodium retention is therefore (a) described for each steroid, taking into account different variables, (b) represented by log dose-response curves in Fig. 1, with steroids arbitrarily grouped into four panels for the sake of clarity (and not in relation to their degree of activity), and (c) represented by the second-order coefficients of the corresponding polynomials for each steroid (Table 1).

As stated above, the most appropriate method to deal with steroid responses *in toto* was considered to be that of multifactorial ANOVA with two factors, steroid and dose. Multiple-range analysis using 95% LSD intervals was conducted for each steroid, and the results are presented in Table 2. Aldosterone is the most potent sodium-retaining steroid and forms a homogeneous group with DOC, 11,19-oxidoprogesterone, and 5α*H*-3,20-pregnanedione. 5β*H*-3,20-Pregnanedione is significantly less active than aldosterone but not clearly distinguishable, by this statistical method, from the group formed by DOC, 11,19-oxidoprogesterone, and 5α*H*-3,20-pregnanedione, although the 5α*H*/5β*H* pair clearly differ at high doses. Progesterone, 6,19-oxidoprogesterone, and the corresponding 11-keto derivatives form a homogeneous group significantly less active than the aforementioned group, and finally corticosterone is the least sodium-retaining steroid assayed, being significantly different from all of the compounds described above.

A detailed analysis reveals the following characteristics. Aldosterone, DOC, and 11,19-oxidoprogesterone are the only three steroids with sodium-retaining properties at the very low 0.06 μg/100 g level, but aldosterone is significantly more active at this dose (steroid/ADX urinary sodium ratio = 0.45 ± 0.06) than are DOC and 11,19-oxidoprogesterone (steroid/ADX urinary sodium ratio = 0.58 ± 0.07). With increasing doses this difference gradually disappears, with all three steroids reaching maximal activity at 40 μg/100 g (steroid/ADX urinary sodium ratio ~ 0.17 ± 0.05). This activity does not decrease at higher doses within the range assayed but, as mentioned above, the dose-response curves could be adjusted to second-order polynomials with second-order coefficients approaching 0, for comparison purposes.

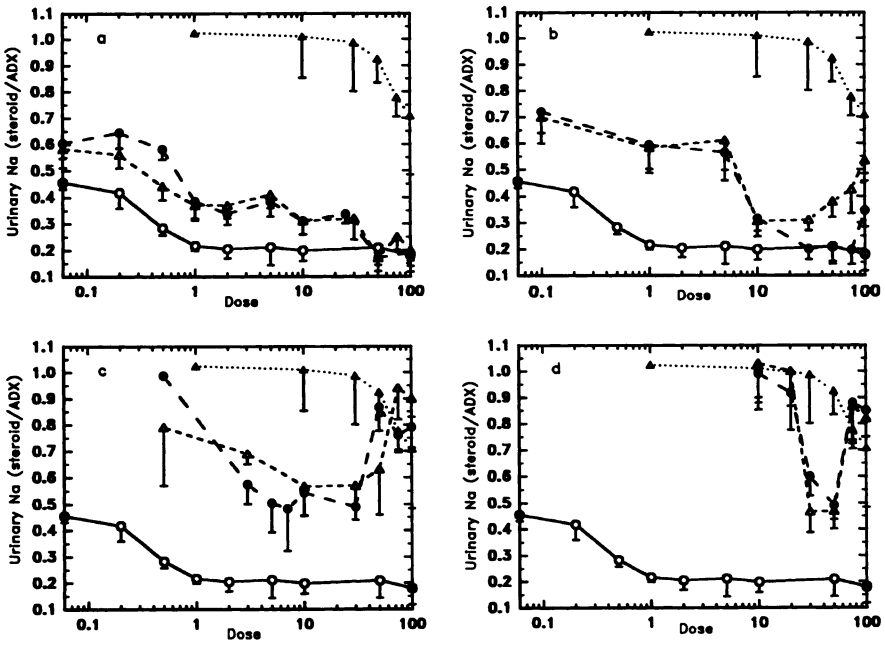

Fig. 1. Dose-response functions. Curves are distributed in a–d for the sake of clarity, and a–d do not necessarily represent categories of activity. a–d, ○, Aldosterone; ▲, corticosterone. a, ●, 11-Deoxycorticosterone; △, 11,19-oxidoprogesterone (2). b, ●, 5α*H*-3,20-Pregnanedione (5); △, 5β*H*-3,20-pregnanedione (6). c, ●, Progesterone (3); △, 11-ketoprogesterone (4). d, ●, 6,19-Oxidoprogesterone (7); △, 11-keto-6,19-oxidoprogesterone (8). Values are means ± standard errors of at least 10 rats in three experiments. Creatinine clearance rates in all cases did not differ significantly from untreated controls (35.1 ± 3.4 ml/min).

TABLE 1

Concavity of polynomial log dose-response curves

All log dose-response curves for C21-deoxysteroids were fit to second-order polynomials of the form $y = ax^2 + bx + c$ (r = correlation coefficient). For comparison, data corresponding to the natural corticoid aldosterone were also adjusted to that function. The second-order coefficient a is representative of the concavity of the function and, hence, of the relative magnitude of the counter-effect (see Results and Discussion).

Steroid[a]	a	r	Dose range
			μg/100 g
Aldosterone (1)	0.046	0.96	0.06–100
11,19-Oxidoprogesterone (2)	0.007	0.94	0.06–100
Progesterone (3)	0.308	0.91	0.5–100
11-Ketoprogesterone (4)	0.498	0.92	3–100
5αH-3,20-Pregnanedione (5)	0.577	0.97	5–100
5βH-3,20-Pregnanedione (6)	0.680	0.95	5–100
6,19-Oxidoprogesterone (7)	2.939	0.85	20–100
11-Keto-6,19-oxidoprogesterone (8)	3.713	0.85	20–100

[a] See Fig. 2 for structures.

TABLE 2

Multiple-range analysis for *in vivo* response to steroid

Multifactorial ANOVA was conducted on the data plotted in Fig. 1, with the response as a variable and the dose and steroid as factors. Multiple-range analysis was by 95% LSD intervals.

Steroid[a]	Homogeneous groups
Aldosterone (1)	b
DOC	b, c
11,19-Oxidoprogesterone (2)	b, c, d
5αH-3,20-Pregnanedione (5)	b, c, d, e
5βH-3,20-Pregnanedione (6)	c, e
Progesterone (3)	f
11-Ketoprogesterone (4)	f
6,19-Oxidoprogesterone (7)	f
11-Keto-6,19-oxidoprogesterone (8)	f
Corticosterone	g

[a] See Fig. 2 for structures.
[b–g] Same letter, homogeneous group.

5αH-3,20-Pregnanedione and 5βH-3,20-pregnanedione exhibit overlapping sodium-retaining properties from the 0.1 μg/100 g level to the 10 μg/100 g level. At the latter dose they overlap with the previous group and at higher doses the activity of 5βH-3,20-pregnanedione gradually decreases, whereas activity of the 5αH-isomer does so only slightly at the upper end of the dose range.

Progesterone and 11-ketoprogesterone start to be active at ~1 μg/100 g, and their sodium-retaining properties overlap throughout the entire dose range. Their activity is maximal in the range of 8–30 μg/100 g and then gradually diminishes, with the compounds being practically inactive above 80 μg/100 g.

The 6,19-oxido-steroids start to be active above 10 μg/100 g and their activity increases dramatically at the 30–50 μg/100 g level, being comparable to that of the previous group. Above this dose the responses of the four steroids overlap completely, with the compounds losing their activity at the higher doses.

In Vivo Potassium-Eliminating Activity

Significant increases were found in urinary potassium values when aldosterone, DOC, or 11,19-oxidoprogesterone was administered. Steroid/ADX urinary potassium ratios for aldosterone were 1.59 ± 0.10 (10 experiments) at the 1 μg/100 g level and 1.74 ± 0.05 (28 experiments) at the 0.06 μg/100 g level. DOC and 11,19-oxidoprogesterone were active at the 10 μg/100 g level and above, with ratios of 1.33 ± 0.02 (26 experiments) and 1.23 ± 0.06 (14 experiments), respectively. Dispersions were generally higher than the corresponding dispersions found in sodium retention assays. No parabolic response functions could be demonstrated for this activity.

Binding to Renal Cytosol and to Type I Receptors

In vitro. The first series of EC_{50} values listed in Table 3 refer to a preparation of "crude" type I receptors from which type II receptor sites were excluded with RU-28362. This preparation, however, still contained CBG (for the relevance of the specificity-conferring role of renal CBG, see Ref. 9). In the second series in Table 3, additional care was taken to exclude CBG by adsorption on hydroxylapatite (9); these EC_{50} values may therefore be considered to refer to CBG-free type I receptors. Indeed, the MR was cloned (12, 13) and Binart *et al.* (12) showed that the biochemical and cytochemical properties of the cloned MR are indistinguishable from those of native MR.

It can be seen that DOC and corticosterone bind to CBG, the latter, as expected, with higher affinity than the former. No other steroid assayed binds strongly to that globulin

TABLE 3

EC_{50} values for steroid-binding *in vitro* to CBG-containing (crude) and CBG-free (purified) type I receptors and *in vivo* to type I receptors

Steroid[a]	EC_{50}[b]		
	Crude	Purified	*In vivo*
		nM	
Aldosterone (1)	4.54 ± 0.09	4.55 ± 0.30	16.95 ± 3.30
11,19-Oxidoprogesterone (2)	42.35 ± 1.98	35.75 ± 2.40	52.65 ± 6.02
Progesterone (3)	36.50 ± 3.51	29.85 ± 7.60	ND[c]
11-Ketoprogesterone (4)	29.25 ± 1.71	25.17 ± 6.51	ND
5αH-3,20-Pregnanedione (5)	31.23 ± 1.89	26.25 ± 2.95	ND
5βH-3,20-Pregnanedione (6)	73.44 ± 6.48	73.50 ± 5.90	ND
6,19-Oxidoprogesterone (7)	2545 ± 374	2413 ± 451	2322 ± 734
11-Keto-6,19-oxidoprogesterone (8)	2489 ± 281	2264 ± 388	ND
DOC	10.58 ± 0.59	4.45 ± 0.13	36.67 ± 9.36
Corticosterone	131.63 ± 6.98	4.40 ± 0.40	ND

[a] See Fig. 2 for structures.
[b] Values correspond to the mean of at least six experiments.
[c] ND, not determined.

(differences between CBG-free and CBG-containing receptors are at most equal to 20% in C21-deoxysteroids).

In vivo. In addition to the purely *in vivo* effect represented by sodium retention, the *in vivo* binding of aldosterone, DOC, 11,19-oxidoprogesterone, and 6,19-oxidoprogesterone was also assayed. These affinities are shown in the third series of EC_{50} values listed in Table 3. The following features can be seen. (a) Aldosterone and DOC, the two natural MCs, exhibit a poorer binding *in vivo* than *in vitro* to unpurified type I MR. For DOC, this binding is obviously still much weaker, compared with purified MR. (b) No differences can be observed between the EC_{50} values for *in vitro* (purified or crude MR) and *in vivo* binding of the oxidosteroids. (c) The *in vivo* binding order is similar to that for crude MR *in vitro* (aldosterone > DOC > 11,19-oxidoprogesterone > 6,19-oxidoprogesterone).

Biological Half-lives

To ascertain a possible influence of biological half-lives of these compounds on their activity, this variable was measured for five of the compounds. Aldosterone exhibited a half-life of 12 min, progesterone one of 53 min, and corticosterone one of 45 min. The accuracy of HPLC measurements for the two synthetic compounds **2** and **7** was lower, but it could be established that the half-life of 11,19-oxidoprogesterone (**2**) was between 10 and 20 min and that of 6,19-oxidoprogesterone (**7**) was 28 min. It can be seen that this parameter *per se* is unrelated to the sodium-retaining activity of the steroids assayed (see also Discussion).

Conformational Analysis

The minimum energy conformations of aldosterone and the 21-deoxysteroids **2-9** obtained by AM1 calculations are shown in Fig. 2. Although molecular mechanics calculations reproduced most of the results of the semiempirical method, they failed in the case of the highly strained conjugated system of ring A of 11,19-oxidoprogesterone (**2**). The oxidosteroids **2** and **7** exhibit, despite their functional similarity, quite distinct conformations, characterized by a quasi-*trans* A/B-ring fusion for the 11,19-oxido derivative (**2**) and a quasi-*cis* fusion for the 6,19-oxidosteroid (**7**). Furthermore, the A-ring has a 1α-envelope conformation in 11,19-oxidoprogesterone but the less usual 1β-envelope conformation in 6,19-oxidoprogesterone and its 11-keto analogue (**8**); the less stable quasi-*trans*-conformers of the latter compounds (2.1 kcal/mol by AM1 calculations) have the A-ring as a 1α-envelope. It has been shown by two-dimensional NMR spectroscopy that a related 6,19-oxido-Δ^4-3-ketosteroid adopts the more stable quasi-*cis*-conformation, as predicted above by AM1 calculations (14). Table 4 shows some relevant geometric parameters for the steroids studied; the 11-keto derivatives **4** and **8** did not differ significantly from the 11-deoxy analogues **3** and **7**, although the lack of the axial 11β-hydrogen probably increases the flexibility of the molecules (15). The orientation of the carbonyl group with respect to the BCD or ring D planes approximately follows the bending of ring A, except for the 5α- and 5β-reduced steroids, which present a chair-type conformation for this ring. Included in Table 4 are the data for the bisketalic form of aldosterone (from AM1 calculations) that has been proposed as the active form of the hormone (2, 4). In this case the ketalic bridges between C18 and carbons 11 and 20 distort the molecule at

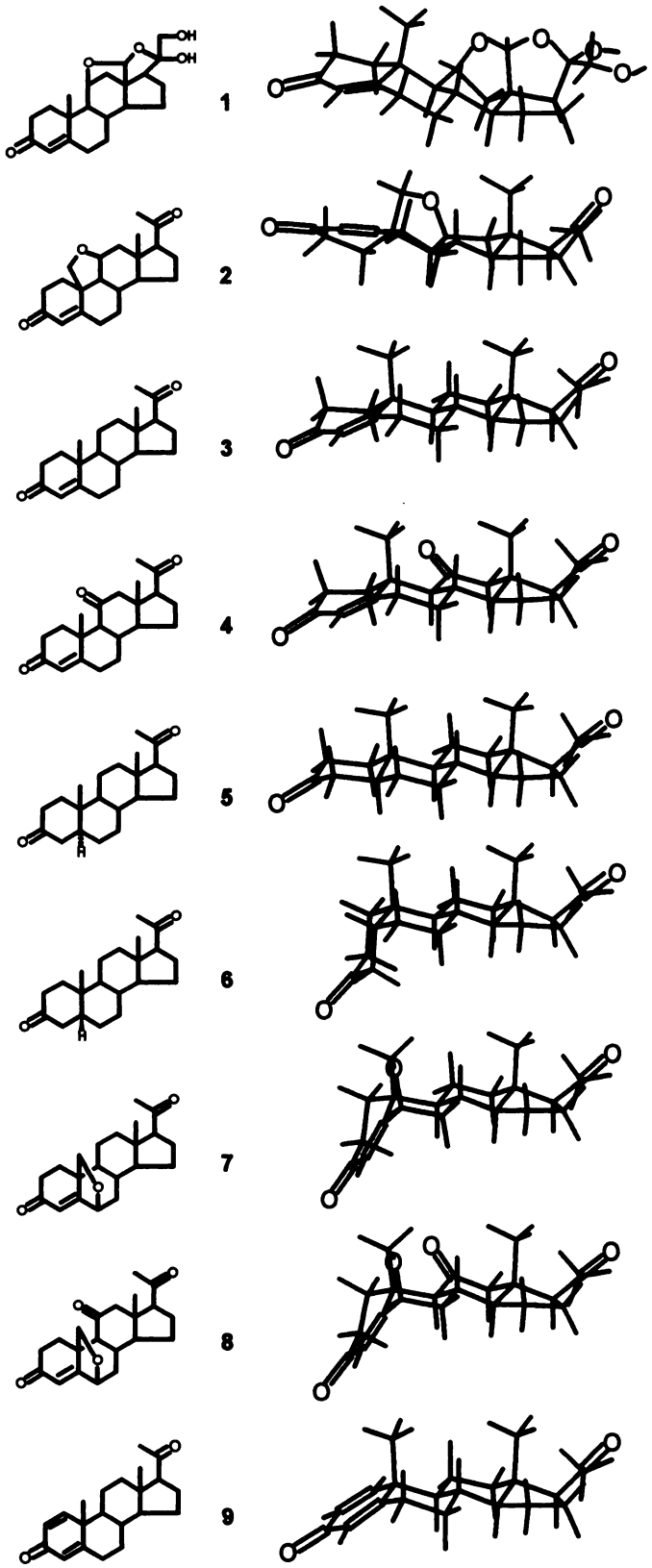

Fig. 2. Structures (*left*) and most stable conformers (*right*) of the bisketalic form of aldosterone (**1**) and the 21-deoxysteroids 11,19-oxidoprogesterone (**2**), progesterone (**3**), 11-ketoprogesterone (**4**), 5α*H*-pregnane-3,20-dione (**5**), 5β*H*-pregnane-3,20-dione (**6**), 6,19-oxidoprogesterone (**7**), 11-keto-6,19-oxidoprogesterone (**8**), and Δ^1-progesterone (**9**), from AM1 calculations.

TABLE 4

Geometric parameters of the C21-deoxysteroids and natural corticoids

Values correspond to the most stable conformer of each steroid, obtained from AM1 calculations. All angles were calculated after projection onto a reference plane defined by the secondary and tertiary inertial axes of atoms C5 to C17.

Steroid[a]	Angle		
	A/D[b]	A/BCD[b]	C3═O/D[b]
	degrees		
Aldosterone (1)[c]	−8.7	−21.1	−15.5
11,19-Oxidoprogesterone (2)	4.4	−3.7	8.9
Progesterone (3)	−21.6	−24.4	−27.3
11-Ketoprogesterone (4)	−21.3	−24.6	−26.5
5α*H*-3,20-Pregnanedione (5)	−7.9[d]	−8.7	−32.4
5β*H*-3,20-Pregnanedione (6)	−68.6[d]	−69.8	−44.0
6,19-Oxidoprogesterone (7)	−57.8	−57.6	−55.2
11-Keto-6,19-oxidoprogesterone (8)	−57.5	−57.7	−54.6
Corticosterone	−26.2	−27.7	−33.5

[a] See Fig. 2 for structures.
[b] Planes are defined by the primary and secondary inertial axes of the corresponding carbon atoms, as follows: A, C3, C4, C5, and C10; BCD, C5 to C17; D, C14 to C17.
[c] Data for aldosterone correspond to the bisketalic form.
[d] Ring A plane is defined by C1 to C5 and C10.

the C/D-ring junction, and hence differences are observed in the angles with respect to either the BCD or ring D planes.

Discussion

The original question of whether a flat conformation would be necessary and sufficient for a pregnane steroid to acquire sodium-retaining properties could be partially answered. An example is the flat conformation of the strongly sodium-retaining 21-deoxysteroid 11,19-oxidoprogesterone (**2**) (similar in this respect to aldosterone and much flatter than DOC), indicating that such a conformation would enable the MC effect to occur even in the absence of a C21-hydroxyl group. The group of aldosterone, DOC, and 11,19-oxidoprogesterone is by all biopharmacological parameters considered the most active. These steroids exhibit classical log dose-response curves without maximal sodium retentions at any midpoint. The more active aldosterone merges the flat conformation with the 21-hydroxy group, whereas these characteristics appear separately in the other two compounds.

Difficulties arise if one tries to apply the "planarity rule" to other pregnane derivatives, especially to reduced progesterone analogues, and if one tries to compare these *in vivo* results with binding properties for type I MR. Indeed, correlations of the diverse parameters representing sodium retention versus planarity persistently deteriorated upon inclusion of 5β*H*-3,20-pregnanedione (**6**), which, in spite of its pronounced bending of ring A with respect to the BCD plane, exhibited sodium-retaining properties close to those of its flat 5α-isomer (**5**).

The alternative option of comparing biopharmacological activity and the orientation of the C3-carbonyl group was based on the similarity exhibited for the angle between the C═O bond and the BCD or ring D planes in both pregnanedione isomers (see Table 4) and the fact that a closely related parameter, i.e., the distance between the C3-oxygen and the C5/C14 plane, could be correlated with receptor affinity by Yamakawa *et al.* (2). The relevance of the D-ring for biological activity has already been demonstrated by Duax *et al.* (3, 4).

In Fig. 3 we plotted the C3═O/D (Fig. 3, *upper*) and A/D (Fig. 3, *lower*) angles against the second-order coefficients of the dose-response polynomials (Table 1), on a logarithmic scale. A highly significant linear correlation (r = 0.934) is shown in Fig. 3, *upper*. It must be emphasized that these correlations were only intended for 21-deoxysteroids, but even so we found aldosterone (but not DOC) to fit into the regression line. Fig. 3, *lower*, exhibited poorer correlations (r = 0.787), due mainly, but not exclusively, to the data for 5β*H*-3,20-pregnanedione.

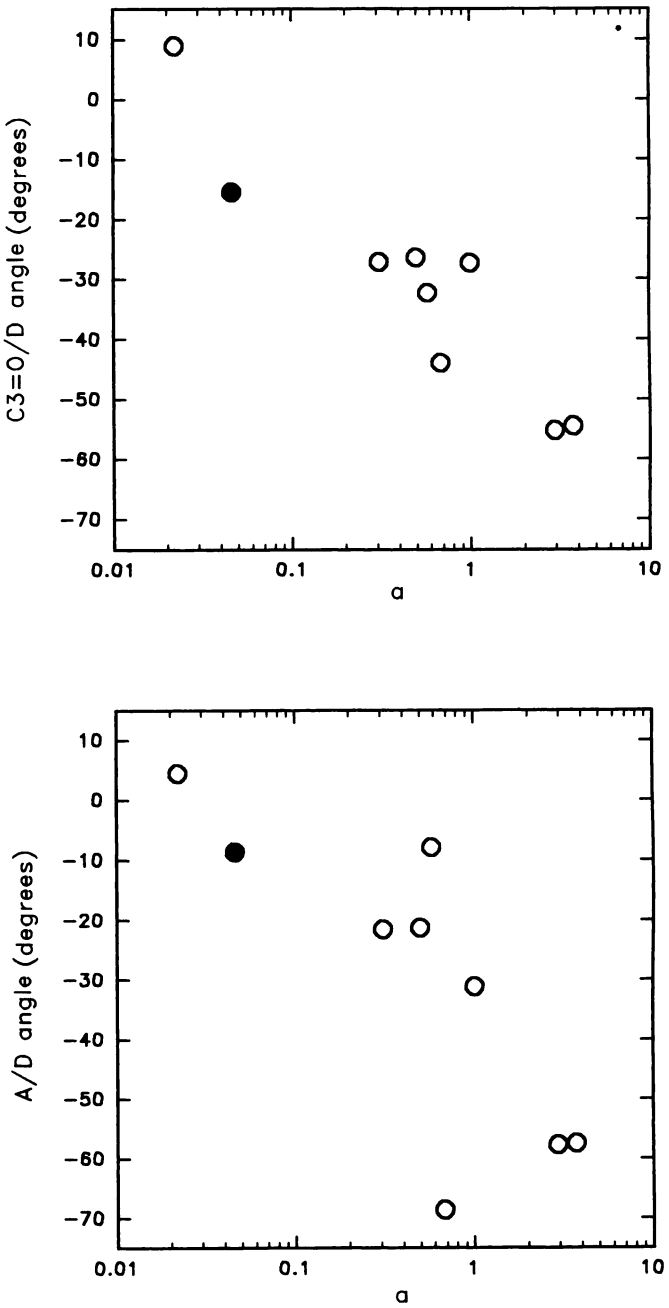

Fig. 3. Shapes of dose-response polynomials versus geometric parameters for steroids **1-9**. For the significance of second-degree coefficients (*a*), see Table 1. Geometric parameters are from Tables 4 and 5. ○, 21-Deoxysteroids; ●, aldosterone. Linear regression: C3═O/D angle versus log *a*, r = 0.934; A/D angle versus log *a*, r = 0.787.

Once we were aware of these results, we synthesized and assayed another simple progesterone derivative, in which the torsion of the A-ring was enhanced by a procedure thus far not used in this work, namely the introduction of an additional double bond in position 1, which renders a C3═O/D angle of −27.4° (close to that of progesterone). The dose-response data for Δ^1-progesterone (**9**) (Table 5) fitted into the regressions in Fig. 3. The data also fitted into those of Fig. 4 (see below).

The results described fail to take into account the flexibility of the molecules involved, a factor whose importance has been signaled by Delettré *et al.* (15). Qualitatively, reduced progesterone derivatives are quite flexible, whereas the 6,19-oxido and 11,19-oxido analogues are rigid molecules. Furthermore, whereas DOC may adopt more planar conformations, corticosterone (with an added hydroxyl group at position 11, close to the angular methyl groups) may "bend" easily but not flatten, due to unfavorable steric interactions on the β-face of the molecule. We are currently investigating whether this flexibility can improve the chances for a good correlation with the data in Fig. 3, *lower*, where planarity is represented on the *y*-axis, by including a quantitative evaluation of molecular flexibility.

In view of these good correlations, we tried similar ones relating the C3═O/D angles to affinities for purified, as well as unpurified, type I receptors (Tables 3 and 5) on a logarithmic scale. A linear correlation (r = 0.955) could be obtained for all steroids analyzed except 11,19-oxidoprogesterone, but including aldosterone, DOC, the glucocorticoid corticosterone, and Δ^1-progesterone, when their conformational parameters were plotted against binding to the crude type I preparation. The correlation deteriorated when binding to the purified, CBG-free, type I receptor was considered (r = 0.932). Not only 11,19-oxidoprogesterone but also DOC and corticosterone escaped from the correlation, with the binding of the latter two compounds being indistinguishable from that of aldosterone (Fig. 4).

The *in vivo* MR affinities of aldosterone, DOC, 11,19-oxidoprogesterone (**2**), and 6,19-oxidoprogesterone (**7**) retain the same order as the *in vitro* affinities for unpurified (but not purified) MR (Table 3). Aldosterone binds better than DOC, which binds better than 11,19-oxidoprogesterone, with the 6,19-oxido analogue exhibiting the weakest binding. Aldosterone, DOC, and 6,19-oxidoprogesterone show a strong tendency toward a correlation (even though they are only three cases), whereas 11,19-oxidoprogesterone exhibits a much weaker affinity than expected. The binding of aldosterone and DOC, in contrast to that of the two 21-deoxysteroids, undergoes a pronounced decrease. This might be due to their pronounced metabolism. Morris (16) injected physiological amounts of [^{3}H]aldosterone into rats and demonstrated significant percentages of tritiated tetrahydroaldosterone and dihydroaldosterone in their plasma during the latent period of the hormone. Interestingly, the flat 5α-dihydroaldosterone possesses 1/30 of the MC activity of aldosterone, whereas the other metabolites possess between 1/60 and 1/500 of that activity (16). However, not only free reduced derivatives but also their conjugates and other highly polar metabolites

TABLE 5

Biopharmacological and geometrical data for Δ^1-progesterone (9)

Parameter	Value
Angle (degrees)[a]	
A/D	−31.2
A/BCD	−33.6
C3═O/D	−27.4
a[b]	1.011 (r═0.85)
EC_{50} (nM)[c]	
CBG-containing MR	33.45 ± 3.80
CBG-free MR	30.27 ± 8.86

[a] See footnote to Table 4 for plane definitions and details.
[b] See footnote to Table 1 for definitions; dose range was 20–100 μg/100 g.
[c] See Table 3.

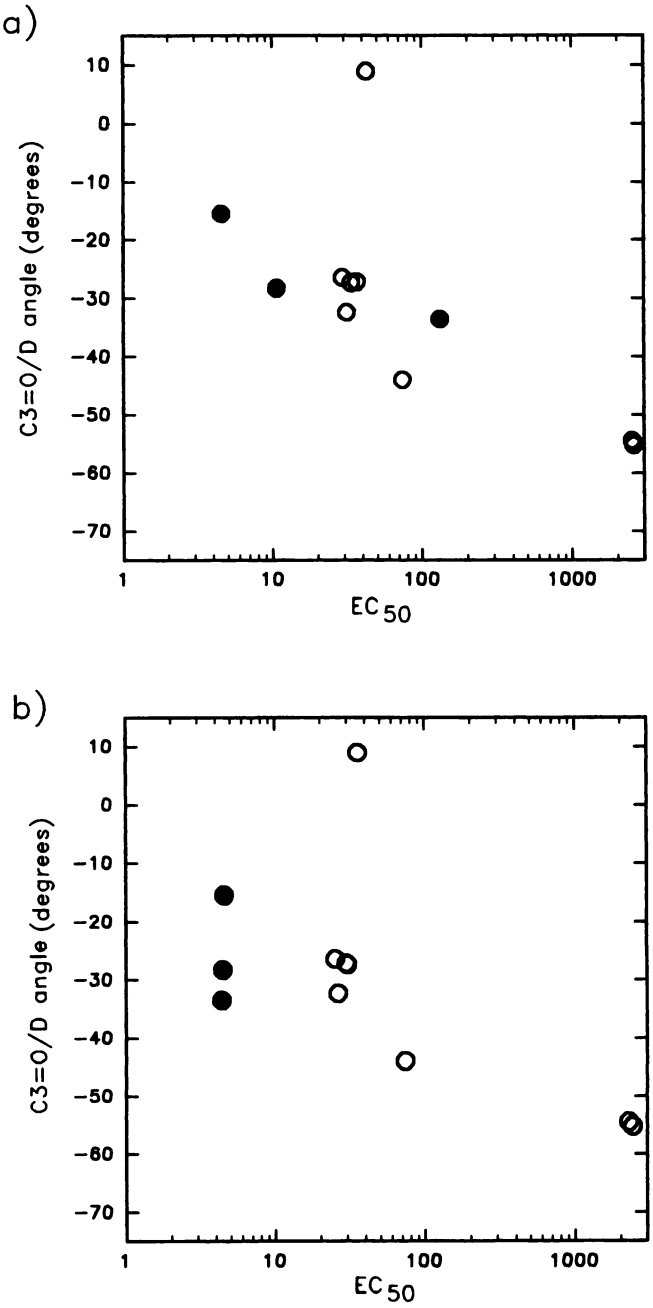

Fig. 4. Steroid binding to CBG-containing (a) and CBG-free (b) receptors versus geometric parameters for steroids **1-9**. EC_{50} values are taken from Tables 3 and 5. Geometric parameters are from Tables 4 and 5. ○, 21-Deoxysteroids; ●, natural corticoids. Linear regression for C3═O/D angle versus log EC_{50} (excluding 11,19-oxidoprogesterone): a, r = 0.955; b, r = 0.932.

("nonextractable polar derivatives") and protein complexes are present in significant amounts in rat plasma (although in minor concentrations in ADX rats). None of these water-soluble derivatives or complexes binds significantly to MR (17, 18), whereas tetrahydro derivatives do so to a minor degree. An analogous situation might be found for progesterone and its derivatives.

The reason for the failure in the correlation of geometric parameters with binding to purified type I receptors, in contrast to the good results obtained in the other comparisons, may well be found in studies from the past two decades showing intrinsic sodium-retaining specificity to be dependent not on a single receptor type but on the competitive effects of MC binding to biologically active receptors versus binding to proteins sequestering hormonal ligands or even catalyzing their transformation into inactive metabolites (see Ref. 19 for a recent review).

Of particular interest, in this sense, are early papers (9, 20), demonstrating such a sequestering action for CBG. For example, aldosterone, DOC, and corticosterone exhibit identical affinities for purified type I receptors but different affinities, decreasing in that order, for crude renal cytosolic preparations with blocked type II sites (9, 21).

More recently, the emphasis on competitive effects has been shifted toward two types of C11 dehydrogenase, one reversible and the other unidirectional, that could inactivate MCs by catalyzing the formation of their 11-keto derivatives (19). This theory is based on clinical observations of the apparent MC excess syndrome (22, 23), on experiments with brain tissue with scarce protection against glucocorticoids (9, 24), and on studies by Funder and co-workers indicating that target-tissue specificity for the MCs is enzyme mediated, rather than receptor mediated (19, 25). Interestingly, as suggested (19), the inactivating role might not be limited to C11 dehydrogenase but might include 3α,20β-steroid reductase and other "catabolyzing" enzymes.

Even so, it is difficult to explain how gradual changes in molecular conformations can lead to subtle biopharmacological changes without the intermediation of similar complementary alterations in a single receptor family. In fact, no single factor (a receptor, a metabolizing enzyme, a transport protein, or even the biological half-lives of the compounds, comprising a conjunction of various factors) can, according to these results, be held solely responsible for the observed correlation between geometry and sodium retention. A highly speculative solution to this conundrum may be found in evolutionary pressures. Indeed, the changing torsion of the C3═O group (and/or ring A) with respect to the D-plane in our series of steroids may mimic similar changing torsions that occurred in a vast family of pregnane steroids, some of them extinct and others now serving a different purpose. Such a gradual conformational change could have led to the loss or gain of sodium-retaining properties, linked only in the 21-deoxy series to the appearance or disappearance of a countereffect, through various sequential or simultaneous mechanisms evolving around the highly conserved type I receptor. This converging strategy could have been more efficient for survival than a strategy depending on conformational changes in a single family of complementary receptors.

Whatever their ultimate cause, these results allow us to classify the steroids of this work into three categories. The first is a sizeable group of natural and synthetic 21-deoxysteroids, except 11,19-oxidoprogesterone, with the following characteristics: (a) parabolic log dose-response curves, (b) a linear correlation between the second-order coefficients (concavities) of these curves and the C3═O/D angles of the molecules, and (c) similar correlations of their affinities for CBG-containing and CBG-free type I receptors with those angles. The second group contains natural (i.e., 21-hydroxy) corticoids, exhibiting normal (exponential decay) dose-response functions for sodium elimination, whose C3═O/D angles correlate with their affinity for CBG-containing but not CBG-free type I receptors. The third group contains the potent, sodium-retaining, 21-deoxysteroid 11,19-oxidoprogesterone, which exhibits a normal dose-response function but with a relatively low affinity for both crude and purified type I receptors, out of relation to its biopharmacological activity.

Still unknown are (a) the mechanism(s)[1] of the countereffect in group 1 and (b) the intermediate factors through which 11,19-oxidoprogesterone causes sodium retention (possibily different receptors, different sites on one receptor, or more than one receptor). These questions are subjects of our present investigations.

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[1] In a very recent paper (while this manuscript was under revision) McDonnell *et al.* (26) reported a new role for one of the two molecular forms of the human progesterone receptor, type A. The two receptor isoforms display distinct biological functions and show a cell- and promoter-specific ability to regulate gene transcription. Those authors demonstrated that, in cellular contexts where human progesterone receptor type A is transcriptionally inactive, it can function as a ligand-dependent inhibitor of MR transcriptional activity. The authors showed curves for MR expression as a function of increasing amounts of a progesterone receptor agonist that are strikingly similar to our parabolic curves for 21-deoxysteroid log dose-dependent sodium retention. Interestingly, they also found that progesterone receptor agonists differ in their ability to facilitate the inhibitory function of human progesterone receptor type A.

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