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BIOLOGICAL CONSEQUENCES OF 18-HYDROXYLATION

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SUMMARY

Insertion of a hydroxyl group at C-18 modifies the biological properties of some pregnane derivatives as follows: Inserted into deoxycorticosterone (DOC) it (1) reduces or does not affect its sodium-retaining action in rats, (2) impairs or abolishes its potassium-excretory effect, (3) enhances its ability to cause the retention of urine, (4) does not appear to modify the magnitude of the hypertensive response of DOC but could alter the manner by which this response is exerted, (5) invests DOC with anti-inflammatory activity that is more pronounced in the presence than in the absence of the adrenal gland, and (6) invests it with thymolytic potency. 18-Hydroxylation, furthermore, (7) abolishes the inhibitory effect of DOC, and greatly impairs that of corticosterone (B), upon pituitary-adrenal function, (8) appears to confer upon DOC but not upon B, the capacity to inhibit the peripheral metabolism of B, and (9) obliterates the glycolytic effects of DOC, progesterone and B in the adrenal gland. Oxygenation at C-18 to an aldehyde blocks the glycolytic action of B as well, imparts upon B renowned effects on electrolyte metabolism and, also, a greatly increased ability to attenuate pituitary-adrenal function. The chemical consequences of 18-hydroxylation which include the potential for dimerization and cyclization may confer specificity upon the steroid molecule by masking functional groups attractive to some receptors, or by introducing functional groups attractive to others.

INTRODUCTION

The chemical and biological consequences of 18-hydroxylation have yet to be fully assessed and understood. Examples for the chemist are the unidentified nature of the chromogen slowly formed from 18-hydroxylated steroids bearing an α-ketol sidechain, upon exposure to the Porter–Silber reagent (phenylhydrazine dissolved in aqueous ethanolic sulfuric acid); the reason for the interference of the C-11-hydroxyl—but not the oxo—group with this reaction; the uncertain nature of the interconvertible forms of 18-hydroxydeoxycorticosterone (18-OH-DOC), and 18-hydroxycorticosterone (18-OH-B); the mechanisms controlling the interconversions and the prevalence of that reaction among other 18-hydroxylated C-21 steroids. Examples for the biologist are the potential relevance of this polymorphism which includes cyclization and dimerization, and the dearth of information regarding biological activities of 18-hydroxylated compounds in general. This paper addresses itself to the latter point and reports on some biological consequences of the 18-hydroxylation of deoxycorticosterone (DOC) and corticosterone (B).

EXPERIMENTAL

Anti-inflammatory activity of the steroids was assessed by their effects on carrageenin-induced edema in the hind paws of rats, as described by Winter *et al.* [1]. Male Sprague–Dawley rats weighing between 113–163 g were randomly divided into 5 groups containing 8 rats each. The animals were starved 16 h prior to use, but were not deprived of water. Both hind paws of each rat were measured prior to treatment. The paws of the unanesthetized rats were immersed to the level of the lateral malleous in a mercury bath. The bath was connected with a Statham pressure transducer P23BB. The output from the transducer was led through a Gross Polygraph (7P1A) preamplifier to a Honeywell digital 500 voltmeter. Voltmeter readings were calibrated in terms of millilitres displacement of mercury. One half hour after the subcutaneous administration of the compounds, 0·05 ml of carrageenin (Viscorin 402, Marine Colloids, Inc.) prepared as a 1% suspension in distilled water was injected into the subplantar area of both hind paws of all the rats. Measurements of the hind paws were taken at hourly intervals after injection of the phlogistic agent. Control rats received the vehicle which consisted of distilled water and Tween 80.

The effects of steroids on stress-induced increases in adrenal steroid production was examined as follows. The steroids were injected subcutaneously at 10 a.m. for 3 consecutive days or for 1 day only, as specified in the legends to the figures and tables. Four hours

after the last injection the rats were stressed by transfer to a new environment and decapitated 5 min later. Circulating corticosterone and the steroid production by the excised glands was estimated as described previously[2].

The effect of steroids on adrenal aerobic lactic acid production was assessed on intact mouse adrenal glands by preincubating the adrenal glands and then reincubating them in the absence or presence of steroids[3]. Lactic acid production was measured by the method of Barker and Summerson[4].

The effects of 18-OH-DOC and DOC on salt and water excretion, depicted in Fig. 1 are based on an earlier experiment[5] but the data have been re-calculated to indicate the relation between the retention of sodium and the volume of urine. The assay was performed by Dr. J. G. Rochefort of Ayerst Research Laboratories on adrenalectomized rats maintained on 1% dextrose, 0·25% NaCl and 0·025% KCl. The rats were intubated with 10 ml of 0·25% NaCl and 0·025% KCl and the urine was collected for 5 h. The steroids were injected s.c. immediately before intubation. Urinary sodium and potassium was analyzed by flame photometry.

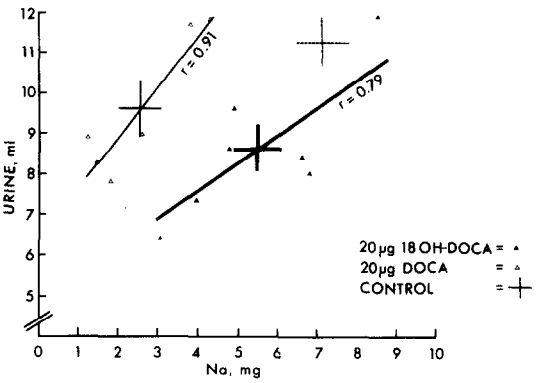

Fig. 1. Relation between urinary sodium and water retention in adrenalectomized rats injected with 20 μg of either deoxycorticosterone acetate (DOCA) or 18-hydroxydeoxycorticosterone acetate (18-OH-DOCA). Large crosses denote the mean and S. E. of sodium and water.

RESULTS

1. *Antidiuretic action of* 18-*OH-DOC*

18-OH-DOC is capable of exerting an antidiuretic effect in the adrenalectomized rat in very low physiological dosages. In Fig. 1, the data from a previously described experiment[5] have been re-evaluated to disclose the relation between water retention and sodium retention in rats injected with 20 μg of either DOCA or 18-OH-DOCA. A highly significant correlation exists between water retention and sodium retention in both groups but it will be noted that the drop in urine volume was greater in the 18-OH-DOCA treated than in the DOCA-treated rats even though the latter animals retained twice as much sodium.

Table 1. Effect of rat adrenal steroids on carrageenin-induced rat paw edema

Compound	mg/kg s.c.	No. of rats	Increase in paw volume (ml) 1 h	2 h	3 h	4 h	5 h
(a) Intact rats							
Control (vehicle)	—	8	0·72 ± 0·08	1·19 ± 0·09	1·28 ± 0·07	1·22 ± 0·10	1·21 ± 0·08
Corticosterone	30	8	0·55 ± 0·08	0·80 ± 0·11	0·79 ± 0·10	0·87 ± 0·08	0·92 ± 0·07
DOC	45	8	0·60 ± 0·09	1·12 ± 0·16	1·27 ± 0·13	1·22 ± 0·13	1·21 ± 0·14
18-OH-DOC	5	8	0·59 ± 0·06	1·07 ± 0·09	1·13 ± 0·12	1·18 ± 0·11	1·18 ± 0·15
18-OH-DOC	15	8	0·50 ± 0·07	0·99 ± 0·11	1·16 ± 0·11	1·10 ± 0·12	1·10 ± 0·09
(b) Adrenalectomized rats							
Control	—	6	1·03 ± 0·06	1·85 ± 0·07	2·03 ± 0·05	2·06 ± 0·09	—
Corticosterone	30	6	0·55 ± 0·06	1·07 ± 0·04	1·14 ± 0·05	1·07 ± 0·09	—
18-OH-DOC	15	6	0·81 ± 0·05	1·86 ± 0·11	1·91 ± 0·06	1·78 ± 0·10	—

Compound	mg/kg s.c.	No. of rats	% Inhibition 1 h	2 h	3 h	4 h	5 h
(a) Intact rats							
Corticosterone	30	8	23·6	32·7	38·3	28·7	24·0
DOC	45	8	16·7	5·9	0·8	0	0
18-OH-DOC	5	8	19·4	10·1	11·7	3·3	2·5
18-OH-DOC	15	8	30·6	16·8	9·4	9·8	9·1
(b) Adrenalectomized rats							
Corticosterone	30	6	46·6	42·2	43·8	48·0	—
18-OH-DOC	15	6	21·3	0	5·9	13·6	—

2. *Anti-inflammatory action of* 18-*OH-DOC*

The effects of 18-OH-DOC on carrageenin-induced rat paw edema were compared with those of DOC and B, although it should be noted that, because of limited amounts, even the highest dose of 18-OH-DOC was only half that used for B and one third that of DOC (Table 1). At 0·5 mg/100 g body weight, s.c., 18-OH-DOC elicited a statistically not significant reduction in rat-paw edema of 19%. At 1·5 mg/100 g the reduction was significant for the first hour amounting to 31%. At both dose-levels the inhibitory effect decreased during the following hours in contrast to that of B which was maximal 3 h after injection. DOC, tested at a dose of 4·5 mg/100 g body weight, was ineffective at all times. Table 1b gives the results of the same assay carried out on adrenalectomized rats. 18-OH-DOC, tested only at the higher dose level, still exhibited its maximal effect at the first hour ($p < 0{\cdot}02$), but the inhibition, which in intact rats at that time point had exceeded the effect of twice the dose of corticosterone, was only half as marked. In contrast to the findings with intact rats, corticosterone was equally effective at all time periods tested.

3. *Effects of* 18-*OH-B and* 18-*OH-DOC on stress-induced increases in adrenal steroid production (Table 2 and Fig.* 2)

As opposed to aldosterone, which we found, per unit mass, to be the most effective of all the steroids native to the rat examined so far in suppressing the rise in plasma B evoked by moderate stress[6, 7], 18-OH-B caused only a slight, statistically not significant, decrease in the rise of circulating B. 18-OH-DOC not only did not suppress, but it raised B levels above those found in the stressed controls, assessed by, both, circulating B levels and the steroid production of the excised glands[2, 7]. Furthermore, 18-OH-DOC, injected in combination with B, counteracted to a significant extent the suppression of circulating B. *In vitro*, however, the steroid output of the glands excised from rats that had been injected with a combination of B and 18-OH-DOC, was suppressed as much as that of the rats injected with B only.

DOC, in contrast to its 18-hydroxylated derivative, reduced the levels of circulating B. Additive effects were obtained with a combination of aldosterone, B and DOC in doses ineffective when one steroid only was injected (Table 2).

Reduction of the double bond in DOC abolished (5β-isomer) or nearly abolished (5α-isomer) the negative feedback effect.

4. *Comparison of feedback and adrenal glycolytic effects (Figs.* 2 *and* 3)

ACTH-induced stimulation of adrenal glycolysis appears to be mediated to a significant extent by corticosterone accumulating in the tissue, since exogenous corticosterone in concentrations eliciting a rise in tissue corticosterone comparable to that evoked by ACTH promotes glycolysis as well[3, 8]. As in the case of feedback action, the glycolyic effect is not confined to corticosterone, the major glucocorticoid of the rat, but is also exhibited by deoxycorticosterone and 11β-hydroxyprogesterone. In further analogy, 18-OH-DOC is not only inactive, but may even have an opposite effect, i.e., suppress glycolysis[3]. By contrast, aldosterone, 11-dehydrocorticosterone, and progesterone do not have congruent actions in the two assays; the first two steroids suppress the rise in circulating B evoked by stress, but fail to activate adrenal glycolysis *in vitro*, whereas progesterone promotes adrenal glycolysis *in vitro*, without impairing the stress-enhanced secretion of B.

DISCUSSION

Our results indicate that 18-hydroxylation can install biological activities, leave them unaltered, and abolish them. To wit, the antiphlogistic and thymolytic [2, 9] action of 18-OH-DOC; the similarities in hypertensive potency of 18-OH-DOC and DOC[9]; the

Table 2. Effects of steroid mixtures on the adrenal-pituitary axis of the stressed rat

Treatment	Dose	Serum B (μg/100 ml)	Adrenal incubation (μg/100 mg/h)		
			B	PS	uV
Control	—	27·35 ± 3·68	5·51 ± 0·55	2·73 ± 0·41	17·69 ± 1·24
ALDO	0·1 mg/100 g	23·46 ± 4·52	4·67 ± 0·76	2·14 ± 0·37	13·60 ± 1·76
B	0·1 mg/100 g	22·32 ± 4·92	5·83 ± 0·16	2·55 ± 0·20	15·66 ± 1·84
DOC	0·1 mg/100 g	23·25 ± 3·02	5·52 ± 0·67	2·59 ± 0·29	15·61 ± 1·44
ALDO + B + DOC	0·1 mg each/100 g	13·31 ± 6·00	2·98 ± 0·58	1·72 ± 0·32	10·39 ± 1·78
B	0·7 mg/100 g	4·04 ± 0·67	1·45 ± 0·09	1·05 ± 0·01	7·01 ± 0·46
B + 18-OH-DOC	0·7 mg each/100 g	10·25 ± 0·98	1·36 ± 0·24	1·10 ± 0·06	6·91 ± 0·86

Steroids were administered in 0·1 ml propylene glycol/100 g in the doses specified for 3 days. Rats were killed 4 h after the last injection.

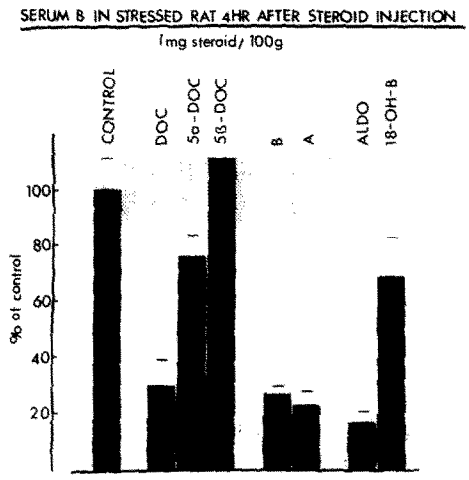

Fig. 2. Serum B levels in the stressed rat 4 h after a single injection of steroid, 1 mg/0·1 ml propylene glycol/100 g. Control animals received propylene glycol only.

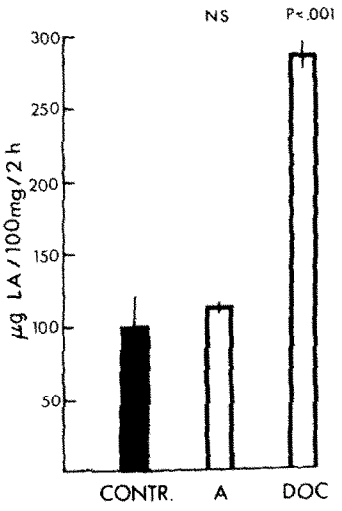

Fig. 3. Effects of 11-dehydrocorticosterone (A) and deoxycorticosterone (DOC) on aerobic lactic acid production by intact mouse adrenal glands *in vitro*.

failure of 18-OH-DOC, and the impaired ability of 18-OH-B, to suppress pituitary-adrenal function. The manner by which a glucocorticoid type action is bestowed upon, or removed from, DOC remains to be established. The fact that the anti-inflammatory effect of 18-OH-DOC was more pronounced in intact than in adrenalectomized rats, but was still significant in the absence of the adrenal, suggests that 18-OH-DOC may act through direct, as well as adrenal-mediated mechanisms, in preventing paw edema, in causing thymus involution, and in evoking effects associated with mineralocorticoid activity. This would seem plausible also from the exacerbation of the stress-induced rise in plasma corticosterone levels obtained with 18-OH-DOC, suggesting ACTH-release, and from the disproportionate effects of 18-OH-DOC on sodium and water retention, suggesting ADH-release.

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DISCUSSION

Vinson:
I believe you said at the beginning of your talk that in some circumstances you can actually get, in vivo, greater amounts of 18-hydroxy-DOC than corticosterone being secreted. (cf. Oliver *et al.*, *Science*, **182**, (1973) 1249). Is that right?

Birmingham:
Yes, that may occur at times in the stressed rat. Also, with respect to feedback control, 18-OH-DOC unlike corticosterone (unlike most other adrenal steroids studied, in fact) does not exert a negative feedback effect on adrenal-pituitary function. A colleague here, Miss Tiptaft, can confirm us on this and would like to show some slides if possible. Her observations are similar to ours, namely that 18-OH-DOC not only fails to inhibit but may in fact enhance adrenal–pituitary responsiveness to stress. Our experiments also show considerably higher plasma corticosterone levels in

animals treated simultaneously with corticosterone and 18-OH-DOC than in animals treated with corticosterone only, suggesting that 18-OH-DOC may in part counteract the negative feedback effect of B. However, the in vitro adrenocortical function was inhibited to the same extent in both groups and this may well imply a peripheral effect of 18-OH-DOC on e.g. corticosterone metabolism or plasma half-life rather than a centrally mediated effect on ACTH secretion.

Tiptaft:

ACTH secretion is influenced by two corticosteroid feedback mechanisms. The first period of inhibition of stress-induced release of ACTH is of brief duration occurring immediately after the administration of corticosteroids while their level in the plasma is rising. This has been designated fast feedback. The second period of inhibition of ACTH release requires larger doses of steroids and is of much longer duration, not being present until an hour or more after steroid administration and independent of corticosteroid levels at the time of stress. This has been called slow or delayed feedback. We became particularly interested in 18-OH-DOC when considering the structure-activity relationship of various steroids in the feedback control of ACTH secretion. This work was done in collaboration with Dr. U. T. Jones.

Male rats (140–180 g) were injected s.c. with 2 mg/100 g of a steroid 4 h before exposure to a 2 min ether stress, and their effect on stress-induced release of ACTH was assessed by the plasma corticosterone concentration 30 min after stress. Pregnenolone, 17α-OH-Pregnenolone and Progesterone had no effect on stress-induced release of ACTH when compared with vehicle control group. 11β-OH Progesterone, 11β, 17α-diOH Progesterone, 11-Deoxycorticosterone and Corticosterone all had the ability to reduce the stress-induced release of ACTH. However, 17α-OH Progesterone and 18-OH-DOC caused an exaggerated ACTH release (P 0·01). (Fig. 1.)

Various steroids were tested for an effect on the fast feedback mechanism, and they were administered 10 min before exposure to ether stress. The majority of steroids had no effect on ACTH release, but 18-OH-DOC (100 μg/100g), 11-Deoxycorticosterone (2mg) and 11-Deoxycortisol (10mg) all caused an exaggerated ACTH release ($P < 0{\cdot}01$) in response to the stress (Fig. 2).

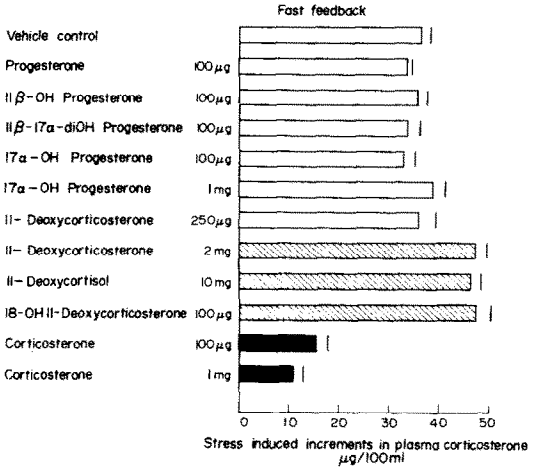

Fig. 2. (Tiptaft).

Therefore, we confirm the observation of Dr. Birmingham that 18-OH DOC causes exaggerated stress-induced ACTH release on the slow feedback mechanism (Kraulis, Traikov, Li & Birmingham, *J. Steroid Biochem.* **4** (1973) 129), and we add that this effect is also seen on the fast feedback mechanism.

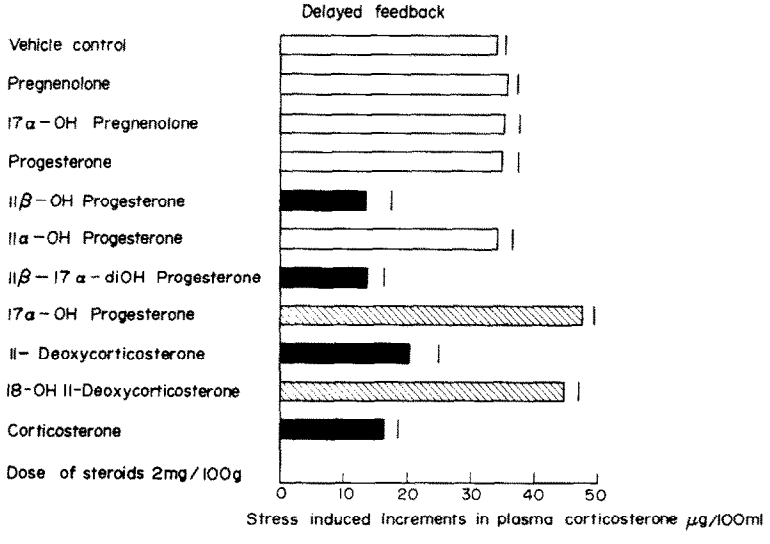

Fig. 1. (Tiptaft).

Munck:

What can you tell us about the metabolism of 18-hydroxy DOC? Has anyone tried binding 18-hydroxy-DOC to mineralocorticoid receptors?

Birmingham:

We don't know. We are looking for mineralocorticoid receptors. 18-Hydroxy-DOC does not bind to CBG which may be one of the reasons why it might be more active. As far as the metabolism is concerned, the ancient work in the rat showed that it is not excreted in the form of a Porter-Silber chromogen because when Porter and Silber first developed this reaction they looked for it in rat urine. Of course, one must remember that in the rat, steroids are excreted mainly via the bile. I know that when you look at it in an unfriendly fashion 18-hydroxy-DOC decides to do all sorts of things. It may dimerize, it forms a lactone, it forms also polar compounds. So it is not a very stable thing to work with, to say the least.

Ungar:

In the mouse, 18-hydroxy-B seems to be produced in larger quantities than 18-hydroxy-DOC. Do you know if the 18-hydroxy-B is hypertensive in the mouse?

Birmingham:

I haven't tried, and I don't find any 18-hydroxy-DOC in the mouse at all. We haven't tried 18-hydroxy-B because it's too expensive. In order to show hypertension you have to be able to inject, at least in the rat, 200 μg a day. We were very lucky to be able to establish the effect in 3 weeks, using 10 rats.

Ungar:

You can probably obtain more 18-hydroxy-B if you use the mouse and isolated this compound and then use it for your experiments.

Birmingham:

May I make a comment? I just want to point out that I think hypertensive potency is certainly not necessarily related to sodium-retaining potency, there must be something else to it.

Crabbé:

Dr. Birmingham, although I concur with your last statement, I'd like to know whether there is any evidence for or against a sodium retaining effect exerted by this steroid compound on extra renal structures, let's say, on the G.I. tract or other possible sites of excretion of sodium for this animal.

Birmingham:

Well, it's a very good question. I don't know yet. I'm hampered by the fact that there is never enough of the material around. We have synthesized it but it took us a very long time and one has to decide what to do with it. It certainly should be tried in all sorts of vertebrates.