Journal of Steroid Biochemistry, Vol. 9, pp. 657–665

0022-4731/78/0701-0657$02.00/0

# CORTICOSTEROID PRODUCTION *IN VITRO* BY ADRENAL TISSUE FROM RATS WITH INHERITED HYPOTHALAMIC DIABETES INSIPIDUS (BRATTLEBORO STRAIN)

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(*Received* 16 *August* 1977)

## SUMMARY

Steroid profiles formed by adrenal tissue from rats of the Brattleboro strain homozygous for the recessive gene causing diabetes insipidus (DI rats) were compared with those formed by glands from heterozygotes (non-diabetic: non-DI) during incubation *in vitro*. The most striking observation was that glands from DI rats showed a greatly reduced capacity to produce certain steroids with mineralocorticoid activity, particularly deoxycorticosterone (DOC) and 18-hydroxy-DOC (18-OH-DOC), and to a lesser degree, aldosterone (ald). Corticosterone (B) and 18-hydroxy-B were produced in similar amounts by glands from DI and non-DI animals. The impairment of DOC and 18-OH-DOC production in adrenals from DI animals was a feature of both capsule (mostly zona glomerulosa) and inner zone incubations. In incubations of tissue from DI animals, the addition of ACTH, or a low concentration of angiotensin amide to the incubation media stimulated corticosterone alone in inner zone incubations, but was without effect on other steroids, or on capsule incubations: with non-DI tissue, ACTH (but not the low concentration of angiotensin) stimulated corticosterone production, but again there was no effect on other steroids, or on the glomerulosa. Higher concentrations of angiotensin, or the addition of LH had only marginally significant effects. Addition of a "physiological" concentration of ADH to adrenal tissue from normal Wistar rats had no effect on the steroid profile. The results suggest the existence of as yet unidentified adrenocortical stimulators or inhibitors which exert effects on production of specific steroids, especially DOC and 18-OH-DOC, and which are effective both on the zona glomerulosa and on the inner adrenocortical zones.

## INTRODUCTION

In the Brattleboro strain of rats with inherited hypothalamic diabetes insipidus (DI) first described by Valtin and Schroeder[1, 2], production of antidiuretic hormone (ADH) is minimal, although the production of oxytocin is less affected. This condition is governed by an autosomal recessive gene at a single locus pair and heterozygous animals are able to produce ADH[3]. Diabetic animals have a high water turnover, and concentrate the urine only minimally in response to dehydration, hypertonic saline, nicotine or ether stress[2, 4–7]. Serum osmolarities and sodium concentrations may be higher than in normal rats, while plasma potassium concentrations are low; heterozygotes (non-DI) show intermediate conditions between normal Long Evans rats and DI rats in these respects,[6, 7, but see ref 8 also]. Plasma renin activity and angiotensin II concentrations are higher in DI than in non-DI animals[6, 9, 10].

Adrenal function in these animals has not been studied extensively. However, the effects of adrenalectomy, and of hormone replacement suggest a physiological role for both glucocorticoids and mineralocorticoids in DI animals[8, 11]. On the other hand plasma levels of corticosterone and aldosterone are lower than in non-DI animals[6], and stress raises plasma corticosterone to a lesser extent in DI than in non-DI animals, according to some authors[12, 14] but not others[15]. There may be an impaired capacity to secrete ACTH since the adrenals respond to ACTH itself[12, 14].

Development of suitable methods for measuring five steroids: corticosterone (B), deoxycorticosterone (DOC), 18-hydroxydeoxycorticosterone (18-OH-DOC), 18-hydroxycorticosterone (18-OH-B) and aldosterone (Ald), and access to a colony of Brattleboro rats afforded the opportunity of examining the steroid profile formed by adrenal tissue from these animals *in vitro*, and of measuring the response to *in vitro* stimulants.

## MATERIALS AND METHODS

### *Animals*

Homozygous (DI) and heterozygous (non-DI) female rats from the Brattleboro colony maintained in the Department of Zoology, University of Sheffield were despatched by rail to London, where they were maintained in the animal house at St. Bartholomew's Medical College for a stabilisation period of three

to four days before use. During this time they were fed on Spratts laboratory diet No. 1, with a plentiful supply of tap water. When required for adrenal incubations, the animals were killed by cervical dislocation, and the adrenals were quickly excised and cleaned, and stored on ice before incubation.

*Incubations*

Glands were decapsulated, and the capsules, consisting largely of zona glomerulosa, and inner zones were incubated separately in 5 ml Krebs–bicarbonate Ringer with glucose, with tissue from two glands in each flask containing 5 ml medium. Incubations were for 2 h at 37°C. Two batches of incubations were performed. In the first, five control flasks for capsules and inner zones, were incubated at the same time as similar numbers of flasks receiving ACTH (Synacthen 20 mU/ml Ciba-Geigy) or a "physiological" dose of angiotensin II (Hypertensin Ciba-Geigy 20 ng/ml).

In the second batch of incubations, control flasks were incubated at the same time as flasks containing 1 μg/ml luteinising hormone (LH; NIH batch S 16) or a high dose of angiotensin II (1 μg per ml).

To test the effects of ADH directly, whole adrenals from normal Wistar female rats were minced and incubated as above for 2 h, under control conditions and in the presence of 20 μU/ml arginine vasopressin (Sigma).

*Extraction and quantitation of steroids*

The method for extraction and quantitation of steroids has been developed from g.l.c. methods described previously by us, and by other authors[38, 39]. The essential features in which this present method differs from those previously employed[38] are (i) initial extraction of incubation media with hexane to give a "DOC fraction", (ii) use of the 3-enol-heptafluorobutyrates of DOC and B acetates, and of the γ-lactones of ald, 18-OH-DOC and 18-OH-B instead of the simple acetates and lactones as previously used[38] (iii) omission of paper chromatographic steps, with resolution and quantitation of the compounds of interest by direct application of samples to the g.l.c. column after derivative formation.

*Procedure.* In more detail, the following procedures were adopted. Incubation media, each of 5 ml, were first extracted with 2 ml hexane (for DOC; fraction 1), and then with 2 × 2.5 ml ethyl acetate. The dried residues of fraction 1, and an aliquot (usually 30%) of the ethyl acetate extracts (for corticosterone, fraction 2) were treated with acetic anhydride/pyridine to form the steroid acetates as before[38]. The remaining aliquot of the ethyl acetate extracts (for 18-oxygenated steroids, fraction 3) were oxidised with periodic acid overnight, and the γ-lactones of 18-OH-DOC, ald and 18-OH-B were extracted and washed according to established procedures[38]. All fractions were then similarly treated with 0.2 ml 20% heptafluorobutyric anhydride (Pierce) in acetone for 30 min at 60°C. At the end of this period the excess reagent was evaporated off under a stream of air. The samples were taken up in ethyl acetate, and suitable aliquots, usually 1/500th for glomerulosa incubation extracts, and 1/2500 for inner zone incubation extracts of each of fractions 1–3 were applied sequentially to the glc column.

*Chromatographs.* The gas chromatographs used were (1) Packard model 7839, equipped with a tritium foil electron capture detector (voltage 130 V, not pulsed). (2) Pye series 104, equipped with a $^{63}$Ni electron capture detector (Voltage 47–60 V pulsed, pulse space 150 μs, pulse width 0.75 ± 0.25 μs. In both cases, the column packing used was XE-60 on Gas Chrom Q support, at a loading of the stationary phase of about 0.3%. This packing was prepared according to the method of Rapp and Eik-Nes[40].

Columns (60 cm long, 4 mm i.d.) were maintained at a temperature of 220°C, and $N_2$ was used as carrier gas, at a flow rate of about 100 ml min$^{-1}$. Under these conditions the retention time for the 3-enol-heptafluorobutyrate of DOC-acetate was about 4 min (varying by up to 20 s from column to column), and the retention times of the other compounds measured relative to heptafluoro-DOC-acetate were: heptafluorocorticosterone-acetate 0.9; heptafluoro-18-OH-B-γ-lactone 1.2; heptafluoro-18-OH-DOC-γ-lactone 1.5; heptafluoroaldosterone-γ-lactone 2.7. Under these conditions there is therefore a completely satisfactory separation of the derivatives of the three 18-oxygenated steroids (fraction 3) to permit their determination in a single chromatographic step. While the two steroids estimated as derivatives of the acetates (i.e. DOC and B) have retention times similar to other steroids, they are contained in separate fractions (1 and 2 respectively) following the extraction procedure, and are thus chromatographed separately.

*Sensitivity.* The derivatives used are very sensitively detected by the electron capture detector under these conditions [cf. ref. 39] and for all compounds and under standard attenuator settings used routinely, 50 pg gives a peak of about 1 cm height with linearity of detector response up to at least 1 μg. In practice of course it is difficult to put a very large aliquot of material from a biological sample directly on to the g.l.c. column to take full advantage of this sensitivity because the amounts of other lipids above a certain level cannot be adequately resolved by standard g.l.c. columns, and hence swamp the peaks of interest. Nevertheless application of aliquots up to about 1/250th of the total sample, and resolution and quantitation of the steroid peaks is entirely feasible and satisfactory for incubation media. This means that the effective limit of sensitivity of the whole method, i.e. the lowest amount of steroid extracted from incubation media which can be measured, is 12–15 ng. For most incubations, in which a pair of glands is incubated, this is quite acceptable.

*Recovery, accuracy, precision.* To test these essential parameters, steroids were added to water in the following concentrations B 500 ng/5 ml; DOC 500 ng/5 ml; 18-OH-DOC 250 ng/5 ml; 18-OH-B 360 ng/ml and ald 150 ng/5 ml. Twenty replicate extractions were performed according to the procedures set out above, and the steroids were estimated by g.l.c. In addition, small amounts (~5000 c.p.m.) of recently purified $^3$H-labelled steroids were added and taken through the extraction and derivative formation procedures, and their recovery was estimated up to the point of g.l.c. Corrected by the appropriate factor for aliquot sampling, recovery of DOC in fraction 1 was 71%, B in fraction 2 was 100%; in fraction 3 recoveries were 18-OH-B 97%; 18-OH-DOC 87%; ald 70%.

When g.l.c. values were corrected for these losses, calculated values for the replicate extractions from water were (means ± S.D.) DOC 479 ± 93 ng (coefficient of variation 19.4); B = 552 ± 95 ng (coefficient of variation 17.3); 18-OH-DOC 251 ± 41 ng (coefficient of variation 16.6); 18-OH-B 352 ± 63 ng (coefficient of variation 17.8) aldo 150 ± 29 ng (coefficient of variation 19.7%). It is clear that even with the application of a standard correction for recovery, rather than an individual correction factor for each compound in each sample, the accuracy and precision of this method are at least as good as many other assay systems, e.g. radioimmunoassay.

For further estimation of the accuracy of the assays for the 18-oxygenated compounds, radioimmunoassay (RIA) methods were used. For 18-OH-B and 18-OH-DOC these are based on antisera to the γ-lactones of 18-OH-B and 18-OH-DOC which were raised and characterised by Drs. V. Martin and C. W. Edwards [cf. ref 41]. In brief, neither show significant cross reactivity to a variety of steroids, with the exception that the antiserum to 18-OH-B-γ-lactone give 100% cross reactivity to 18-OH-DOC-γ-lactone. In direct assays from incubation media values for 18-OH-DOC can be subtracted from those for 18-OH-B. For aldosterone, the antiserum distributed by NIH was used. This has been characterised by the same methods as described by other authors, by whom also its use in a direct assay on rat adrenal capsule incubation extracts has been validated[42].

Extracts were made of media in which separated capsules and inner zones of adrenocortical tissue from normal and from sodium deplete rats were assayed by the g.l.c. method and by RIA. Correlation parameters were: 18-OH-DOC $r = 0.94$, slope 0.7; 18-OH-B $r = 0.91$, slope 0.6; ald $r = 0.90$, slope = 0.7 ($n = 10$ throughout). This represents good correlation between the two methods: in fact the slopes (which are less than 1) reflect the fact that the RIA values were systematically somewhat higher than the g.l.c. values, and appear to indicate a slightly lower specificity for the radioimmunoassay.

*Specificity* In this method use is made of the fact that some prior fractionation partly by the use of specific extraction techniques, and partly through formation of suitable derivatives, imparts some grouping of the compounds before chromatography. Thus deoxycorticosterone is preferentially extracted into hexane (fraction 1), while the other steroids are extracted into ethyl acetate (fractions 2 and 3). In addition, the oxidation step includes a wash with sodium bicarbonate, thus eliminating 18-deoxy-21-hydroxysteroids (which form aetioacids on oxidation with periodate) from the oxidised fraction 3.

By far the greater part of the specificity is afforded by the powers of resolution of the g.l.c. system itself. In all cases peaks corresponding to the steroids are obtained which are symmetrical and show no evidence of contamination. It is remarkable that very few peaks are seen on chromatograms of incubation media extracts in addition to those for the compounds under study. Two exceptions are; (i) in fraction 2 another product regularly appears with a retention time relative to the DOC derivative of 1.4. (ii) In fraction 3 another compound is frequently detected having a retention time relative to the DOC derivative of 2.3. Some evidence for the specificity of the methods lies also in the comparison with RIA (above). It should further be noted that the values routinely obtained with these methods, as in the present paper are entirely consistent with those obtained from similar incubation experiments using other previously described methods[38]. In addition, blank extracts taken through these methods give zero interference peaks.

## RESULTS

### (a) *Nature of steroid formed*

The most striking feature of the results shown in Figs 1–4 is that adrenal tissue from the DI rats has a grossly impaired capacity to form three steroids which can somewhat loosely be classified together as mineralocorticoids, i.e. DOC, aldo, 18-OH-DOC. The production of B and 18-OH-B, on the other hand is in the normal range. The impairment of the ability to form the mineralocorticoids *in vitro* is a feature of both glomerulosa and inner zones. The steroid profile produced by capsules and inner zones from heterozygotes on the other hand is scarcely distinguishable from that from tissue of Wistar rats, routinely studied in this laboratory, with the possible exception that aldosterone values are somewhat low.

### (b) *Effects of stimulation*

The overall steroid profile is little affected by stimulation by the factors used in these experiments, in that the range of products from DI rat adrenals is at all times impaired (as specified above) when compared with the heterozygote controls. Some minor changes in individual compounds are apparent, but offer no reversal of the DI condition.

In the presence of ACTH (Fig. 1a and b) steroid production was little affected in the capsule incuba-

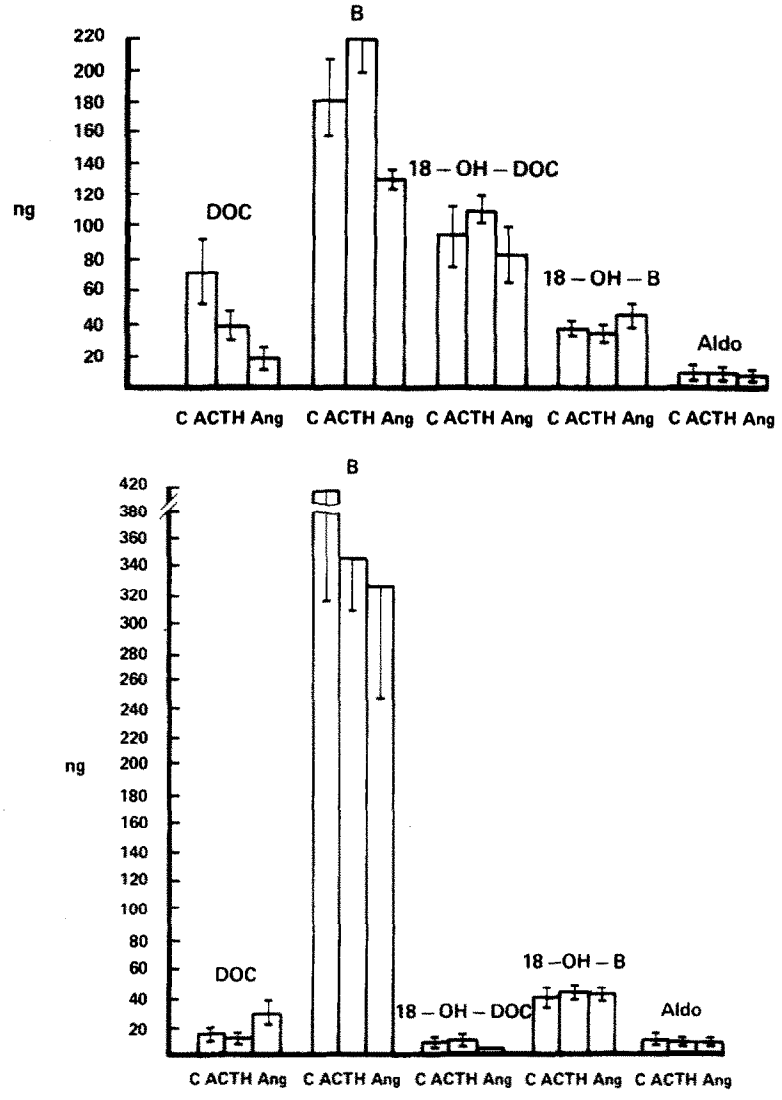


Fig. 1. Steroids produced by incubated adrenal capsules from rats heterozygous (non-DI) (upper) and homozygous (DI) (lower) for a recessive gene causing diabetes insipidus. Under control conditions (C) the production of DOC and 18-OH-DOC is significantly lower in DI than in non-DI adrenal incubations ($P < 0.05$; 0.001 respectively). Addition of ACTH or angiotensin amide (ang) gave no clear effects (means ± S.E., $n = 5$ throughout).

tions, but stimulation of corticosterone occurred in inner zones incubations (Fig. 2). DOC and 18-OH-DOC were not significantly affected. There was no difference between DI and non-DI rats in these parameters. Hypertensin, at a concentration of 20 ng per ml, gave no increase of any steroid in the capsules in either DI or non-DI rats (Fig. 1a and b), or in the inner zones of non-DI rats (Fig. 2). In DI rats however, inner zone production of corticosterone was significantly increased by Hypertensin, to an extent as great as seen with 20 mU per ml ACTH. Once again, neither DOC nor 18-OH-DOC were affected (Fig. 2).

In the second series of incubations, the stimulants used were LH (1 μg per ml) and a higher dose of Hypertensin (1 μg/ml). In this case there was considerably greater variance in the control incubations, and the seeming stimulation of corticosterone production by Hypertensin in the capsules and inner zones of non-DI animals, and by LH in the inner zones is not significant (Figs 3 and 4). No changes were seen at all in non-DI rats. One change with LH stimulation is significant, this is the production of 18-OH-B by capsules in non-DI rats (Fig. 3a).

*Effects of ADH on Wistar rat adrenals*

In control whole adrenal tissue incubations, production of corticosterone, 18-OH-DOC and 18-OH-B was (ng per adrenal ± S.E.M.) 1784 ± 387, 820 ± 158, 103 ± 33 respectively. In the presence of 20 μU per ml arginine vasopressin the values were 1590 ± 383, 1054 ± 166 and 150 ± 19 respectively.

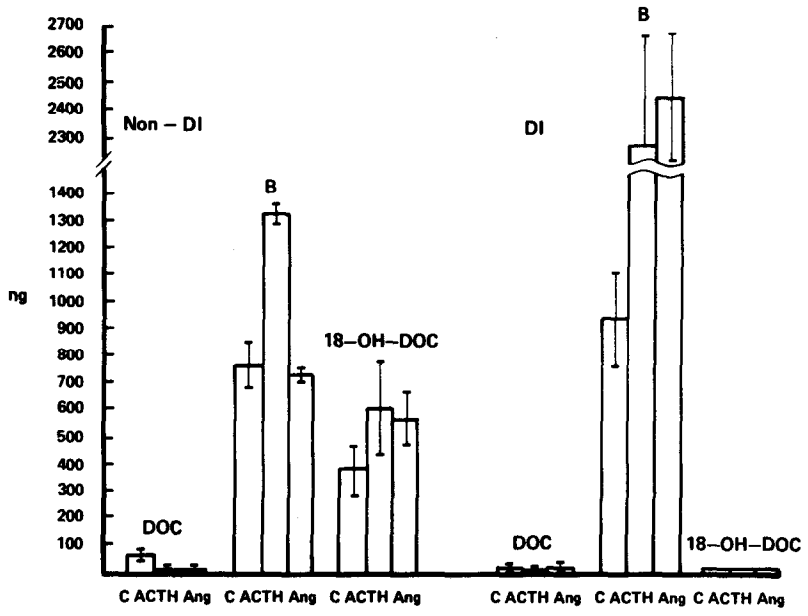


Fig. 2. Steroids formed by incubated inner adrenocortical zones from non-DI and DI animals as for Fig. 1. Under control conditions DOC and 18-OH-DOC production were again significantly lower in DI than in non-DI incubations ($P < 0.01$ for each compound). Addition of ACTH (20 mU/ml) significantly stimulated corticosterone production in both DI and non-DI incubations ($P < 0.025$; 0.001 respectively), angiotensin amide (20 ng/ml) stimulated corticosterone production in DI incubations ($P < 0.001$), (means ± S.E., $n = 5$ throughout).

and were thus not significantly different from control values.

## DISCUSSION

### *Stimulation of corticosterone secretion*

Studies on adrenocortical function in DI rats of the Brattleboro strain have thus far been concerned mostly with the mode of stimulation of corticosterone in these animals. Thus McCann *et al.*[12] showed that increases in plasma corticosterone following varying degrees of stress were less in DI than non-DI rats. They interpreted their findings in terms of an action of vasopressin in potentiating the pituitary response to CRF, but did not study the response of the adrenal to ACTH directly. While McCann *et al.* found that CRF content of stalk-median eminence was similar in DI and non-DI animals this was not confirmed by Arimura *et al.*[15] who found that hypothalamic content of CRF was in DI rats only 50% of controls. In contrast again, they found that the response to stress was similar to controls at least under certain more severe stress conditions but less in others.

This variability in findings reported by different groups is also found in studies on the direct response of the adrenals to ACTH. Thus Yates *et al.*[13] extended the findings of McCann *et al.*[12] and showed that while the response of the adrenal to stress was impaired in DI rats, its response to ACTH was as good as in controls. On the other hand Wiley *et al.*[14] found that the adrenal of DI rats was less sensitive to ACTH than controls. In association with these findings it should be also noted that in some work basal circulating levels of corticosterone are lower than those of non-DI animals, whereas in other no difference was recorded [e.g. refs 6, 13].

In total the literature suggests that differences in corticosterone secretion between DI and non-DI animals in the adrenal response to stimulation by ACTH are probably only slight.

This is borne out by the results reported here. The stimulation of corticosterone by ACTH (Figs 1 and 2) is comparable in the two groups of animals, although it should be noted that this is greater than seen in similar circumstances in glands from female Wistar rats[33]. The relative lack of effects on 18-OH-DOC in the non-DI group is consistent with results obtained with normal Wistar animals[33].

The lack of effect of a low concentration of Hypertensin in non-DI animals (Figs 1 and 2), is again compatible with the literature, which has thus far not delineated an unequivocal physiological role for angiotensin II in this species[16, 17, 18]. However, the large increase in corticosterone production by the inner zones in the DI animals is unexpected (Fig. 2), and may indicate a degree of sensitisation to Hypertensin, caused by the high circulating levels of angiotensin II in these animals[6, 9, 10]. The higher concentration of Hypertensin (Figs 3 and 4) gave no clear cut effects in either zone; although mean values for corticosterone were higher, the results were not statistically significant. Stimulation of corticosterone in both capsule and inner zones from normal animals

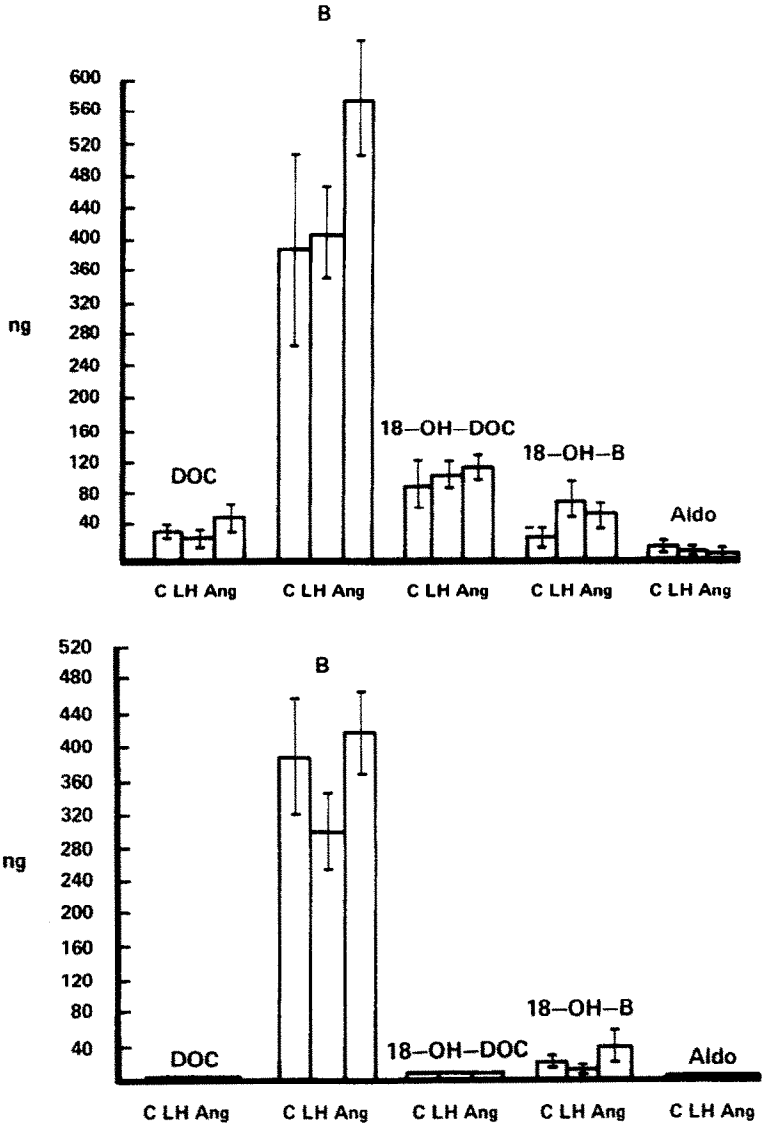


Fig. 3. Steroid profiles from incubated capsules from non-DI (upper) and DI (lower) animals. Control output of DOC, 18-OH-DOC and aldosterone were significantly lower in DI than in non-DI ($P < 0.01$ for all three compounds). In non-DI capsule incubations LH (1 μg/ml) stimulated 18-OH-B ($P < 0.05$), other effects of LH and angiotensin amide (1 μg/ml) were not significant (means ± S.E., $n = 5$ throughout).

by this concentration of angiotensin has been shown by other authors[17]. There was no effect in the DI animals. LH, which has been shown to stimulate corticosteroid production in adrenals from normal female Wistar rats[19] had little effect in these experiments on either group of animals, with the possible exception of the effect on capsule 18-OH-B production in non-DI glands. In recent experiments with Wistar rats 18-OH-B has been found to be more stimulated by LH than other steroids[34].

*Steroid profile*

In these experiments the most significant results were the impaired production of DOC, 18-OH-DOC, and to a lesser extent aldosterone by adrenal tissue from DI animals despite normal B and 18-OH-B output (Figs 1–4). It is interesting that this group of steroids can be classified together, albeit somewhat loosely as mineralocorticoids [20–25]. In addition, two of them, DOC and 18-OH-DOC are suspected as having some role in raising blood pressure, both in experimental animals[25] and in man[26–29]. In this connection, it may be noted that DI animals tend to have lower blood pressures than non-DI[35]. Although this cannot be attributed directly to a lack of the steroids concerned, it is at least conceivable that together with anti-diuretic hormone these hormones form a functional group of agents controlling blood pressure in normal animals.

The interesting problem which this raises is to iden-

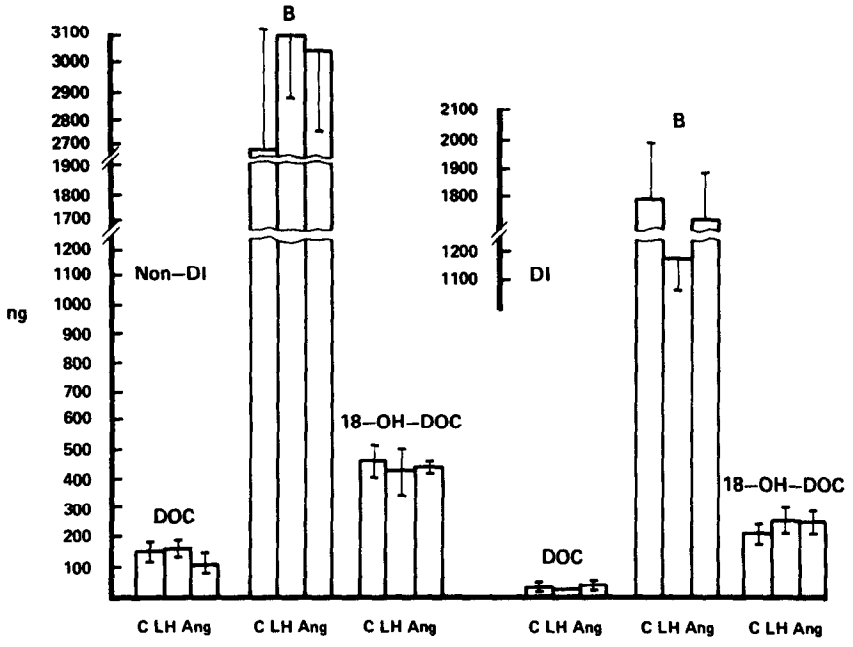


Fig. 4. Steroid profiles from adrenal inner zones from non-DI and DI animals incubated as Fig. 3. Production of DOC and 18-OH-DOC was significantly lower in control DI than in control non-DI incubations ($P < 0.001$ for each compound). Effects of LH (1 μg/ml) or angiotensin amide (1 μg/ml) were not significant (means ± S.E., $n = 5$ throughout).

tify the factor(s) which specifically maintain DOC and 18-OH-DOC in normal rats. The major objective in adding the various potential stimulants, ACTH, angiotensin II and LH to incubations of adrenals from Brattleboro rats was therefore to investigate whether any of them could reverse the differences in steroid profiles produced by non DI and DI adrenals. Previously they have not been studied with a view to determine whether they may have greater effects on 18-OH-DOC and DOC than on aldosterone or corticosterone in these animals. From the present results it appears that they do not. Indeed the literature generally gives few clues on this topic: certainly among the mineralocorticoids aldosterone has been by far the most widely studied. In the glomerulosa, which is the unique source of aldosterone, various factors can stimulate steroid output, including sodium depletion, potassium, angiotensin and serotonin[30]. Two points can be mentioned in this connection. One is that in any conditions in which aldosterone production from endogenous precursors has been reported to be increased by addition of stimulants *in vitro*, it is usually accompanied by an increase in corticosterone, although this may not necessarily be as great[31]. In sodium depletion, on the other hand, corticosterone values may be depleted, in association with its increased conversion to aldosterone[31]. In the present experiments however, differences in corticosterone and aldosterone production between DI and non-DI animals are quite slight compared with differences in DOC and 18-OH-DOC. The other critical point is that most factors which stimulate aldosterone in the glomerulosa, including sodium depletion, are generally considered to have little effect on inner adrenocortical zones, which are controlled by ACTH alone[31, 32]. As seen here, ACTH affects corticosterone more than 18-oxygenated compounds (Figs 1 and 2) as does the low dose of angiotensin. No factors have yet been identified which stimulate 18-OH-DOC and DOC specifically without effect on B and aldosterone production in the glomerulosa, or on B in the inner zones. The possibility that potassium may have a role in stimulating 18-OH-DOC production has not been examined. It is true that DI animals show lower circulating potassium levels than non DI[6, 8], however the difference is only quite slight[8]. In any case potassium has thus far only been shown to be an effective stimulator in the glomerulosa [e.g. ref. 32], whereas an explanation for the differences between DI and non-DI rats requires a systemic factor of some kind acting on other zones equally. It is also possible that other features of the incubation conditions, for example lack of a pre-incubation period [see ref. 33], or inappropriate concentrations of stimulants might account for the lack of an effect on 18-OH-DOC. However this once again only points to the differences in the control of 18-OH-DOC and corticosterone, since corticosterone responds well under these conditions. From these results it also appears that ADH, at least as judged by its effects on addition *in vitro* cannot fill the role of DOC and 18-OH-DOC stimulator. It is still possible that for its effects to be observed, chronic treatment with ADH (or as sug-

gested by these experiments), its chronic lack is required. The results therefore suggest the existence of systemic factors, as yet unidentified, which specifically stimulate DOC and 18-OH-DOC production throughout the adrenal cortex of the rat. Alternatively it is also possible to envisage a mechanism for modulation of DOC and 18-OH-DOC production independent of B production based on actions of inhibitors rather than stimulators [see ref. 33].

The intracellular mechanism, which maintains corticosterone production, without concomitant secretion of DOC and 18-OH-DOC, is also mysterious, in view of the generally accepted theory that the major site of stimulation in the biosynthetic pathway for steroids in the adrenal cortex is at a point preceding cholesterol side chain cleavage[36, 37]. This mechanism also deserves close study.

*Acknowledgements*—We are most grateful to Professor I. Chester Jones and Dr. R. J. Balment for providing homozygous and heterozygous Brattleboro rats, to Drs V. I. Martin and C. R. W. Edwards for the antiserum to 18-OH-B and 18-OH-DOC γ-lactones, to Dr. D. M. Burley (Ciba-Geigy, Horsham) for Synacthen and to the National Institutes of Health, Bethesda, U.S.A. for providing LH.

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