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THE EFFECT OF SEX AND STRAIN OF RATS ON THE *IN VITRO* RESPONSE OF ADRENOCORTICAL TISSUE TO ACTH STIMULATION

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SUMMARY

Various attempts to demonstrate the effects of ACTH on corticosterone production by whole adrenal tissue from female rats of the Wistar strain, using methods based on those of Saffran and co-workers, have failed despite use of a variety of incubation conditions and methods for steroid measurement. The finding that testosterone in similar incubations was nevertheless satisfactorily stimulated suggested that the lack of effect on corticosterone was not due to inaccessibility of the tissue to ACTH or metabolism of ACTH by the tissue.

Comparison of corticosterone and testosterone production in response to ACTH by adrenals from male and female rats of the Wistar, Lister and Sprague–Dawley strains show that in all cases the response of male tissue was far greater than female; indeed of the female glands, only those from Sprague-Dawleys showed a significant stimulation in corticosterone production with ACTH. Testosterone production, however, although much lower in amount, was stimulated similarly in male and female glands in Wistars and Sprague Dawleys, and was unstimulated in all Lister incubations. 18-Hydroxydeoxycorticosterone was not stimulated by ACTH in either male or female Wistar tissue, or in male Sprague-Dawley adrenals.

Use of a cell suspension prepared by collagenase treatment eliminated the difference in response of corticosterone to ACTH in male and female Wistar adrenal tissue, and also allowed stimulation of 18-hydroxydeoxycorticosterone by ACTH.

The results, which are borne out by reports in the literature, suggest that female rat glands contain substances which inhibit the response of corticosterone but not testosterone to ACTH, and that glands from both sexes contain inhibitors to stimulation of 18-hydroxydeoxycorticosterone. These inhibitors are eliminated by protease treatment.

INTRODUCTION

One of the key experimental approaches to the study of the mechanisms of control of adrenocortical secretion over the last 25 years has been the stimulation of adrenocortical tissue (particularly from the rat) by corticotrophin (ACTH) and other factors *in vitro*.

Before the more recent application of superfusion and cell suspension techniques [1–3] the method most generally favoured followed with minor variations the procedures set out by Saffran and co-workers[4–6]. In general the glands were removed from the animals, trimmed of excess fat, and cut into quarters, which were distributed into different flasks and incubated in a small volume of Krebs Ringer bicarbonate solution (with glucose) (usually about 1.5–2 ml). After incubation for $\frac{1}{2}$ to 1 hr, the medium ("preincubation medium") was discarded, and fresh medium which contained the ACTH to be tested was added. Incubation was continued for a further period, often 1 h, and then the steroids were extracted from the medium and the corticosterone produced was measured.

In this laboratory, using adult female rats of the Wistar strain, great difficulty has been experienced in obtaining satisfactory results with the simple method. From the current literature clearly there are many possible reasons why this might be so, ranging from the lack of accessibility of ACTH, Ca^{2+} or glucose [7–9] to the sites of action, to the rapid metabolism of the ACTH by the tissue itself [10]. More recent experiments however suggest that these possibilities are not responsible for the relative lack of effect. We have shown [11–13] that adult female Wistar rat adrenal tissue has the capacity to produce C_{19} steroids, including testosterone and androstenedione (albeit in comparatively small yields) both from endogenous precursors and from added [4-^{14}C]-progesterone. It was also found however, that whereas ACTH in concentrations of 20 mU/ml could stimulate corticosterone output by adrenal tissue only by about 25% (marginally significantly different from control values), the yield of testosterone from endogenous precursors on the other hand was tripled [11]. These results suggest that ACTH was indeed satisfactorily available to the tissue, and could exert stimulatory effects. For some reason however, the response of corticosteroid production was specifically inhibited.

It was decided to investigate the possibility that the lack of response of corticosterone to ACTH was

restricted to the female Wistar rats used in these experiments.

MATERIALS AND METHODS

Animals

Adult male and female rats of the Lister strain and the Sprague–Dawley strain were obtained from R. H. Tuck & Son, Chigwell, Essex. U.K., and maintained in the animal house at Queen Elizabeth College for up to ten days. Wistar rats were either obtained from the same suppliers and kept as above, or else they were taken from the Wistar colony maintained in the animal house at St. Bartholomew's Medical College. Animals were fed on Spratts laboratory diet No. 1.

Incubations

1. *Conventional.* Rats were killed by cervical dislocation, and the adrenals were quickly removed, cleaned and stored in beakers on ice until required for incubation. Glands were then minced with scissors, and tissue from two glands (not individual rats) was incubated in each flask containing 5 ml Krebs bicarbonate Ringer with glucose (200 mg/100 ml). Incubation was stopped after 30 min, and the preincubation medium was discarded. Fresh medium was added, and where required, ACTH (Synacthen, Ciba-Geigy) was added to give a final concentration of 20 mU per ml which for synthetic ACTH gives a near maximal response under these conditions. Incubation was continued for a further 2 h. In general, 6 control and 6 experimental flasks were used in each experiment. Tissue from the three strains of rats were used in this way, with male and female glands being treated separately.

2. *Cell suspensions.* Tissue from male and female Wistar rats was also treated with collagenase to give cell suspensions, following the method of Richardson and Schulster[14].

Cell counts, made with a Coulter counter, showed that the yield of cells was 600 000 to 800 000 per gland. Aliquots of suspensions equivalent to the cells from a pair of glands were incubated in 5 ml Krebs bicarbonate Ringer with albumin (0.5% w/v bovine serum albumin; Sigma fraction V) and glucose for 1 h, together with 20 mU per ml ACTH where appropriate.

Extraction and estimation of steroids. Following incubation, steroids were extracted from incubation media with ethyl acetate. Suitable aliquots of the extracts were taken for estimation of corticosteroid content by competitive protein binding, and testosterone by radioimmunoassay as previously described [11]. In addition in some extracts 18-hydroxydeoxycorticosterone was measured by a g.l.c. method [15].

RESULTS

Conventional incubations

Wistar rats (Fig. 1). With adrenals from adult female Wistar rats, the results are comparable with those previously reported [11]. The mean value for corticosterone production under ACTH stimulation was only about 30% higher than in the controls, and the values were not significantly different. The yield of testosterone however, was doubled by ACTH stimulation. Male adrenal steroid yields show an interesting contrast. Control values for corticosterone production were considerably lower than the females, but were increased at least five fold by ACTH. Control testosterone values were also lower than the females, and were approximately quadrupled by ACTH. The magnitude of the increment in testosterone produced by ACTH was roughly the same in males and females.

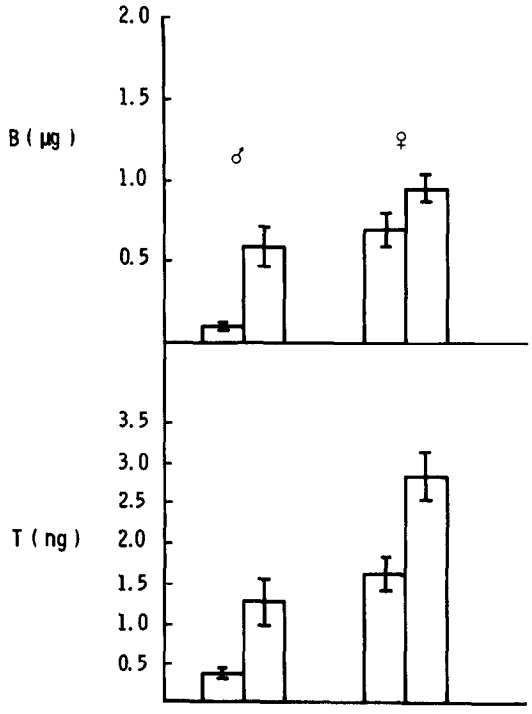

Fig. 1. Corticosterone (B) and testosterone (T) production by male and female Wistar rat whole adrenal tissue under control conditions (left hand column of each pair) and in the presence of 20 mU/ml ACTH, (right hand columns). *P* values: (comparison of control and ACTH in each case) male corticosterone, <0.005: male testosterone, <0.01: female testotosterone <0.01. ($n=6$ throughout). Amounts of steroid are expressed as means per incubation (two glands) ±S.E.M.

Lister rats (Fig. 2). Broadly similar corticosterone results were obtained with these animals (cf. Fig. 1). Male corticosteroid values were lower than the female, but were significantly increased by ACTH, whereas the female adrenal production of corticosteroid was not affected. In this case, male adrenal yields of testosterone were higher than in the female tissue, and in neither case was testosterone production affected by ACTH.

Sprague–Dawley rats (Fig. 3). In this case the control amounts of corticosterone produced by male and female glands were similar, but as in the other two

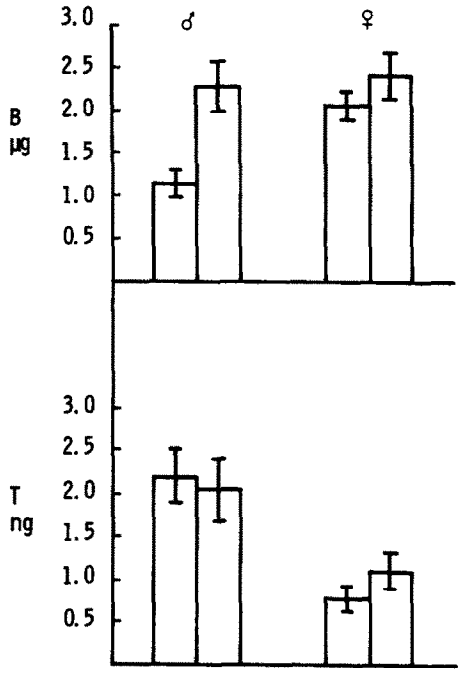

Fig. 2. Corticosterone (B) and testosterone (T) production by adrenals from male and female Lister rats, incubated as for Fig. 1. Left hand column of each pair are controls, right columns ACTH stimulated. *P* values: comparison of male corticosterone in control and ACTH incubations <0.01. ($n = 6$ throughout). Amounts are means for incubation ±S.E.M.

strains, the male glands responded much more sensitively to ACTH stimulation. In this strain alone, the response of female whole adrenal tissue to ACTH gave corticosterone values significantly different from controls. Neither the control nor the ACTH stimulated yields of testosterone were significantly different in males and females.

Cell suspension, Wistar rats (Fig. 4). Control values for corticosteroid output were somewhat lower in the male adrenal tissue incubations than in conventional incubations, and much lower in the females. Sensitivity to ACTH stimulation appears to be moderately enhanced in the male incubations, but vastly enhanced in the females, indeed in these incubations, if anything, it now appears that the adrenal tissue from the female Wistars is more sensitive than the male to ACTH stimulation.

In the cell suspensions, values obtained for testosterone was detected in ACTH treated cell suspen- 4 was taken, were low and unsatisfactory for statistical treatment. In other experiments however, testosterone was detected in ACTH treated cell suspension incubations giving values of about 200 pg per adrenal pair, for both males and females. None was detected in control incubations, but since the limit of detection was about 50 pg, no conclusion can be drawn regarding the possibility of a change in sensitivity to ACTH stimulation in this parameter following collagenase treatment.

Effect of ACTH on 18-hydroxydeoxycorticosterone (18-OH-DOC)

In whole tissue the production of 18-hydroxydeoxycorticosterone was not significantly affected by ACTH, even in incubations in which corticosterone was stimulated (Fig. 5, cf. Figs 1 and 3). However, in cell suspensions of both male and female Wistar adrenals 18-OH-DOC respond quite sensitively to ACTH stimulation (Fig. 6).

DISCUSSION

In this laboratory, over a period of about ten years, a series of unsuccessful attempts have been made to apply the methods of Saffran *et al.* [4–6] to the adrenals of adult female Wistar rats. Numerous parameters have been changed in attempting to improve the responsiveness of the glands to ACTH. These have included changing periods of incubations, from 15 minutes to four hours, and changing the period of pre-incubation, or omitting it altogether. Glands have been minced, quartered or decapsulated. ACTH has been used in amounts varying from 5 mU per ml to 1000 mU per ml, and ACTH from Armour (ACTHAR gel), Organon, Ferring, has been used as

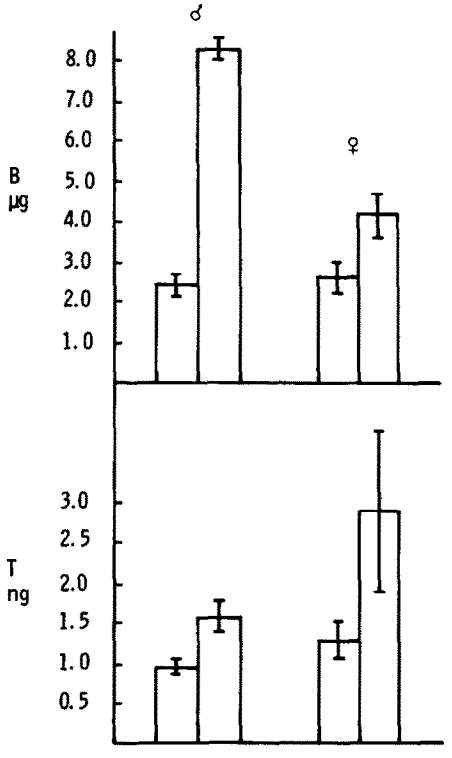

Fig. 3. Corticosterone (B) and testosterone (T) production by male and female adrenal tissue from Sprague–Dawley rats, incubated as for Figs 1 and 2. Left hand columns of each pair are controls, right hand columns ACTH stimulated. *P* values (comparison of control and ACTH stimulated in each case) male corticosterone <0.001: female corticosterone <0.05: male testosterone, <0.05 ($n = 6$ throughout). Amounts are means per incubation ±S.E.M.

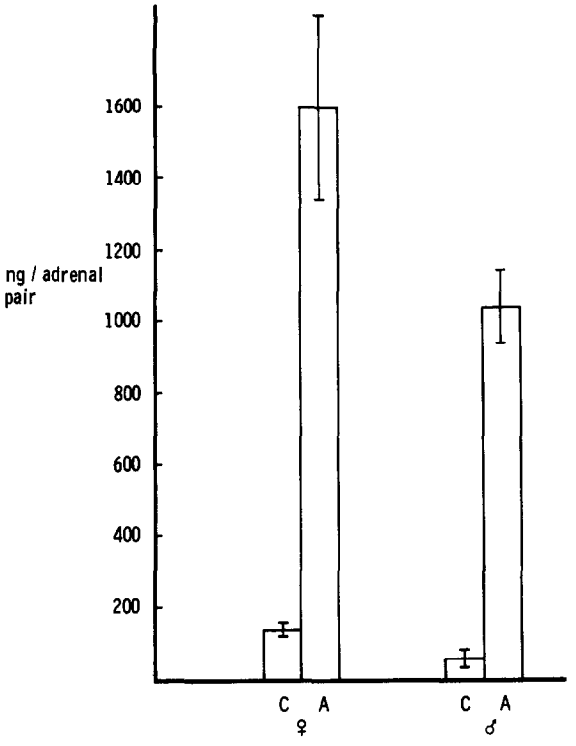

Fig. 4. Corticosterone production by cell suspensions of male and female Wistar adrenals, C = control, A = ACTH stimulated. P values (comparison of control and ACTH stimulated), males <0.001: females <0.001 ($n = 6$ throughout). Amounts are means per incubation ±S.E.M.

well as Synacthen (Ciba - Geigy). Calcium ion and glucose concentrations have been increased to double the normal values. Steroids have been measured by (1) U.V. absorption (with or without paper chromatography to separate corticosterone specifically) (2) soda fluorescence, (3) g.l.c. following paper chromatography (and on direct extracts), following formation of corticosterone acetate, or its 3-enol heptafluorobu-

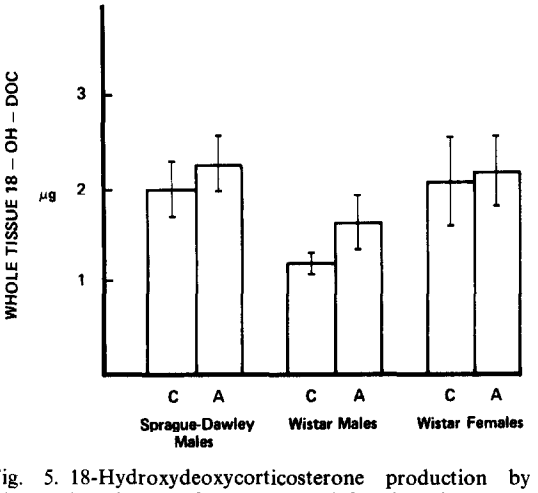

Fig. 5. 18-Hydroxydeoxycorticosterone production by whole adrenal tissue from male and female Wistar, and male Sprague–Dawley rats, incubated as for Figs 1 and 3. C = control, A = ACTH stimulated. ($n = 6$ throughout). Amounts are means per incubation ±S.E.M.

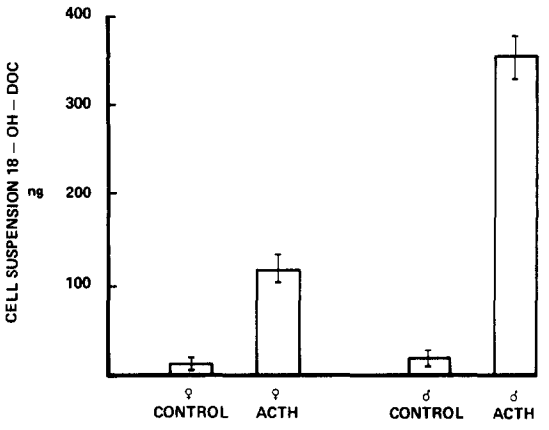

Fig. 6. 18-Hydroxydeoxycorticosterone production by cell suspensions of male and female Wistar adrenals, incubated as for Fig. 4. P values (comparison of control and ACTH) female <0.001, male <0.001 ($n = 6$ throughout). Amounts are means per incubation ±S.E.M.

tyrate, with electron capture detection, (4) competitive protein binding, and (5) radioimmunoassay. In general, the increases in corticosterone following ACTH stimulation have been similar to those described in this paper (Fig. 1).

The results of the present experiments strongly suggest that the reason for this lack of success is that glands from female rats, particularly Wistars, are hardly responsive to ACTH under these conditions, whereas male glands certainly are (Figs 1–3). It is interesting that the lack of responsiveness to ACTH is confined to corticosterone production, and that testosterone responds similarly in both sexes, although there may be some differences between strains.

Early literature

Before developing this argument further, it is clearly essential to consider the findings of earlier workers in order to understand how such a possibility can have evaded detection in the 25 years since the technique was introduced. Table 1 summarises, in a simplified form, some of the experimental parameters in 19 key papers. Two points are immediately obvious from this summary, one is the popularity of the Sprague–Dawley strain of rats, and the other is that males are clearly preferred, indeed the use of males exclusively may be inferred in 14 of the papers. In this respect it is interesting that in the present results, the response of Sprague–Dawley rat adrenals to ACTH was far greater than other strains, particularly in male tissue (Fig. 3). In another paper (Saffran and Bayliss[5]) the authors stated they used male or females, but the text does not make it clear whether or not glands from both sexes were mixed in individual experiments; from the wording it is certainly possible that they were. Saffran and Schally[6] did not state the sex of the animals, but did say that no attempt was made to analyse sex or strain (or age) differences. It seems possible that they used

Table 1.

Ref.	Authors	Sex and strain of rats (N.S = not specified)	Conc. amount of ACTH for max. effect	Increment in corticosterone yield
4	Saffran *et al.* 1952	Sprague–Dawley/ Long Evans, males	250 μg/ml of La-1-A standard	at least 200%
5	Saffran and Bayliss 1953	Sprague–Dawley, males or females	2000 mU/ml	200%
7	Birmingham *et al.* 1953	N.S. (Sprague–Dawley males?)	100 mU/ml	100%
18	Elliot *et al.* 1954	N.S. (Sprague–Dawley males?)	100 mU/ml	100%
6	Saffran and Schally 1955	Sprague–Dawley, sex N.S.	100 mU/ml	500%
8	Schönbaum *et al.* 1956	Sprague–Dawley, males	200 mU/100 mg	300%
19	Koritz *et al.* 1957	Sprague–Dawley, males	500 mU/ml	300%
10	Birmingham and Kurlents 1958	Sprague–Dawley, males	100 mU/ml	200%
9	Péron and Koritz,	N.S. (Sprague–Dawley males?)	50 mU/ml	200%
20	Birmingham *et al.* 1960	Sprague–Dawley males	300 mU/100 mg	300%
21	Bakker and DeWied 1961	Wistar, males	90 mU/ml	300%
22	Koritz 1962	N.S. (Sprague–Dawley males?)	500 mU/ml	no control values given
23	Kaplan and Bartter, 1962	Sprague–Dawley, males	50 mU/ml	400%
24	Kittinger 1964	Sprague–Dawley, females	80 mU/ml	200%
25	Müller 1975	Osborn–Mendel, males	830 mU/ml	900%
26	Halkerston *et al.* 1965	Sprague–Dawley males	250 mU/ml	140%
	Birmingham *et al.* 1965	Long Evans, males	200 mU/ml	160%
		and females	200 mU/ml	120%
28	Grahame-Smith *et al.* 1967	Hypophysectomised Holtzman males	1000 mU/ml	400%
29	Lommer *et al.* 1971	Sprague–Dawley, males	1000 mU/ml	900% (long lasting effect)
		females	1000 mU/ml	300% (very short lived effect)

mixed sexes. In a further two papers, those of Birmingham *et al.*, and Lommer *et al.*[20, 29] effects of ACTH were examined in males and females separately. In the paper by Birmingham *et al.*[2], the methods included a variation of the classical method which may be significant (c.f.[10]). Following the preincubation period, glands were incubated with ACTH for two successive periods, with fresh medium and ACTH being added for the second. It is conceivable that changing the medium in this way approximates more to the superfusion condition (see below). Even so, the female glands were less sensitive than the males, although the difference was less marked than in the present experiments (in contrast, they found that in animals with adrenal regeneration after enucleation, the female glands were more sensitive to ACTH). In the work of Lommer *et al.*[20] pooled adrenals from males were mostly used, although two figures allow the comparison with females. The response of the male glands to ACTH was sensitive, and was shown to be maximal throughout a period from 30 to 130 min of incubation. In contrast, the maximal effect of stimulation of the female glands was less than in the males, and was apparent over a very short incubation period of no more than ten min. After 45 min of incubation there was very little difference between control and ACTH stimulated corticosterone concentrations in the incubation medium. The authors themselves make no comment about possible differences between males and females. Among the papers cited in Table 1 this now leaves only Kittinger [24] who used females alone. In addition there is also a comment by Ungar[41] in which he refers to the greater sensitivity of male adrenals to ACTH when compared with females, but he gives no data.

One section of the literature, not included in Table 1 is specifically devoted to the effects of gonadal hormones on adrenocortical function, and may give the

casual reader the impression that, in contrast to the evidence presented in this paper, adrenals of female rats are *more*, not less responsive to ACTH than males. In particular the work of Kitay gives this impression. He found that ovariectomy reduced adrenal corticosterone production *in vitro* [30, 31] and this could be restored by treatment with oestrogen [32, 34]. Furthermore, *in vivo* stress or ACTH administration gave a greater increase in circulating corticosterone levels in female than in male rats [30, 31]. However, close inspection of the literature shows that, despite prolonged study of effects of gonadal hormones on hypothalamus, on the adrenals, and on liver metabolism of corticosteroids, Kitay and his coworkers have never reported any findings on what might be considered to be the key experiments, namely those to test the effects of ACTH on the adrenal production of steroids by male amd female glands *in vitro* (e.g. [35]). Indeed where glands from ovariectomised animals were incubated, and the effects of ACTH in reversing the effects of ovariectomy were investigated [36], it is interesting that ACTH was administered *in vivo*, and not added *in vitro*.

Superfusion and cell suspension experiments

It is quite clear that when the methodology is significantly changed, as in superfusion, where the products of the adrenal are not allowed to accumulate around the tissue [1, 2, 37], or in cell suspension techniques in which the tissue is treated with collagenase or trypsin [3, 14, 38], female rat adrenal tissue can become very responsive to ACTH stimulation. Indeed a combination of the two techniques leads to sensitivity to ACTH stimulation which approximates to *in vivo* sensitivity [39, 40]. In the present experiments it would seem that the enhancement of response by preparing cell suspensions (considered to be maximal here) is much greater in female than in male tissue. Inspection of the magnitude of the increment in corticosterone caused by ACTH in the male cell suspension incubation suggests it is only moderately greater than in untreated tissue incubations (cf. Figs 1 and 4) (expressed as percentage increase over the control condition it is more, because control values are smaller, however this mode of expression may be misleading). The magnitude of the female cell increment resulting from ACTH stimulation is far greater than in female untreated tissue incubations (Figs 1 and 4).

The reasons for the insensitivity of female rat adrenal tissue to ACTH stimulation *in vitro* are obviously of interest. Several possibilities present themselves. One is that ACTH may not penetrate the tissue adequately, or that if it does, it is rapidly metabolised to an inactive form [10, 42]. These possibilities are unlikely however, in view of the fact that in female tissue the testosterone response to ACTH was no different from that in male tissue (Figs 1 and 3). Another possibility is that an accumulation of an inhibitor of some kind may occur in female, but not in male adrenal tissue, which acts specifically on the corticosterone response to ACTH. The fact that even in those whole tissue incubations in which corticosterone does respond to ACTH, 18-OH-DOC may not (Fig. 5) suggest that further inhibitors affecting other steroids may also be produced, particularly since again 18-OH-DOC did respond in the cell suspension incubations (Fig. 6). The nature of such inhibitors is open to conjecture. One possibility is that steroid hormones themselves may act as inhibitors, but while this has been shown in various experimental conditions, it seems unlikely that it could account for the apparent potency of the inhibitors encountered here. Either the inhibitory effects of steroids have been found to be quite short lived [43] or, especially where the effects of corticosteroids in inhibiting the response to ACTH have been studied, the maximal inhibition observed was only of the order of 25%, and this with very high concentrations, certainly higher than normally found *in vitro* [44–47]. Because male glands are certainly at least 30% smaller than females, and thus may produce less steroid it may still be tempting to ascribe the differences in male and female response to ACTH to differences in basal steroid levels (Figs 1 and 2, cf. [30–33]). However even when basal male steroid output is the same as females (as it sometimes is, see e.g. Fig. 3) they can nevertheless respond well to ACTH.

The best explanation for these results is that female adrenals contain inhibitors to ACTH action on corticosterone, while the males do not, at least in such great concentrations. Moreover, the glands of both sexes contain inhibitors to ACTH action on 18-OH-DOC. These inhibitors (which are not steroids), are eliminated by superfusion, or by treatment with proteolytic enzymes, thus accounting for the greatly enhanced sensitivity to ACTH under these conditions.

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