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The actions of ethinyloestradiol on the pituitary-adrenal system of the rat

J. D. FLACK*

Department of Pharmacology, School of Pharmacy, Brunswick Square, London W.C.1

Summary

1. Single doses of ethinyloestradiol stimulate the pituitary adrenal axis of the female rat during quiescent conditions.

2. After a stimulus which releases endogenous ACTH, there is an inhibition of the release of corticosterone from the adrenal gland, but an increase in the corticosterone stored in the gland.

3. Ethinyloestradiol (1·0 mg/kg) injected acutely or daily for 7 days does not affect the corticosterone binding capacity of plasma in female rats, or the clearance rate of an injection of corticosterone.

4. Prolonged treatment with ethinyloestradiol and ACTH inhibits the pituitary-adrenal response to a stress and to an ACTH injection, indicating a block at the adrenal and pituitary level.

5. The adrenal gland recovers more rapidly than the pituitary after cessation of the oestrogen treatment.

6. The inhibition of the pituitary-adrenal response to stress after oestrogen is probably caused by inhibition of cholesterol synthesis as suggested by others. Oestrogens can increase corticoid secretion during non-stress conditions, however, so precursor lack through exhaustion cannot be excluded.

7. It is suggested that the inhibition of the stress response after prolonged ACTH treatment is due to a decrease in sensitivity of the adrenal cortex after frequent ACTH stimulation, but the possibility that it is due to a reduction in circulating cholesterol cannot be ruled out.

Introduction

Oestrogen treatment has been reported to stimulate adrenal activity (Kitay, 1963a) and depress function (Holzbauer & Vogt, 1957a). It has been suggested that oestrogens alter the peripheral metabolism of corticosteroids (Troop, 1959) and corticosteroid binding in the blood (Sandberg & Slaunwhite, 1959). The present study was designed to investigate the relative sensitivity of the various parts of the pituitary-adrenal axis to oestrogen treatment in the rat in an attempt to explain some of the conflicting reports of the effects of oestrogen.

* Present address: The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts, U.S.A.

Methods

Female rats weighing 100–200 g of the hooded Lister strain (obtained from Fison's Pharmaceutical Ltd., Holmes Chapel, Crewe, Cheshire) were used. The conditions of animal storage, sampling of blood and assay of corticosterone were as described by Barrett & Stockham (1963).

In vitro *corticosteroid production*

This procedure was based on that described by Bakker & de Wied (1961). The left adrenal gland was removed from the rat, weighed after cleaning, quartered and transferred to a specimen pot containing Krebs bicarbonate solution at 37° C. The adrenal quarters were incubated for 1 hr at 37° C and the medium agitated by bubbling through it 95% oxygen and 5% carbon dioxide gas mixture at a flow rate of 0·3 ml/min. The corticosterone released into the medium was assayed fluorimetrically. In some experiments after the first 1 hr incubation, fresh Ringer solution was substituted containing 100 m-u ACTH and a second 1 hr incubation performed.

Plasma protein binding capacity

This was estimated by subjecting plasma equilibrated with a high concentration of corticosterone at 37° C for 30 min to gel filtration with Sephadex G-200 at 18° C using a 0·1 M Tris HCl buffer. The corticosterone found with the protein eluates was bound to a high affinity globulin. Albumin did not bind corticosterone in these experiments (Flack, 1967).

Drugs

Ethinyloestradiol powder was suspended in 0·5% w/v sodium carboxymethylcellulose containing 0·9% w/v benzoic acid as a bacteriostatic, diluted with 0·9% sodium chloride solution and injected subcutaneously. ACTH (corticotrophin, Organon, short acting or corticotrophin Zn, Organon, long acting) was diluted with 0·9% w/v saline and injected subcutaneously. Ether (Howards) was used as an anaesthetic and the application of the ether stress was as described by Stockham (1964).

Results

Effects of single doses of ethinyloestradiol

All doses of ethinyloestradiol were given 24 hr before the animals were killed. At this time all doses had induced oestrus as indicated by cornified cells in the vaginal smear. In the quiescent rat, the plasma and adrenal gland corticosterone concentrations and the *in vitro* corticosteroid production were increased above control values with all doses of ethinyloestradiol (Fig. 1). The control female rats injected with vehicle alone had a plasma corticosterone concentration of 7·6 μg/100 ml, adrenal corticosterone concentration of 22·5 μg/g and an *in vitro* corticosterone production of 25·9 μg/g/per hr. In the quiescent rat, maximum stimulation of the pituitary-adrenal system occurred at a dose of 200 μg/kg, but higher doses caused smaller rises (Fig. 1). This was true of the plasma and *in vitro* indices, but in the adrenal gland the corticosterone concentration remained high with the highest dose of ethinyloestradiol tested.

When rats treated with ethinyloestradiol were stressed by exposure to ether vapour, the plasma and *in vitro* corticosterone values were 40% lower than in the controls, whereas the adrenal corticosterone level was higher (Fig. 1). The vehicle treated rats exposed to ether vapour had plasma and adrenal corticosterone concentrations of 58·4 μg/100 ml and 65·5 μg/g respectively, and the *in vitro* corticosterone production was 142·0 μg/g per hr.

These results indicate that the dose of ethinyloestradiol injected and the state of the pituitary-adrenal axis at the time of studying its functional capacity after oestrogen are critical. In the quiescent and stressed rat, there was undoubtedly a release of ACTH from the pituitary which increased adrenal corticosterone biosynthesis, but while in the quiescent rat release of corticosterone was concomitant

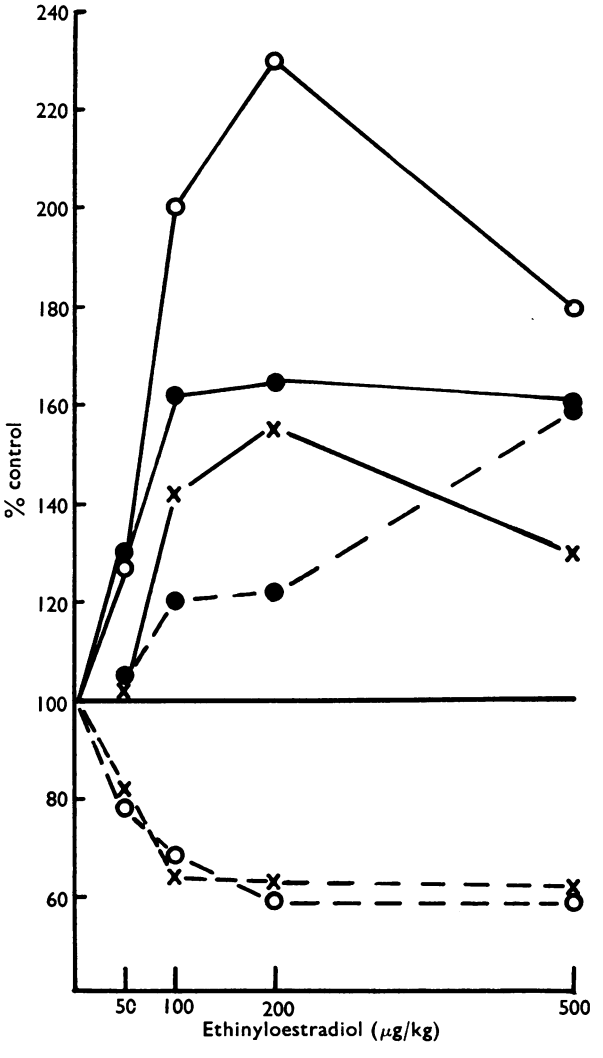

FIG. 1. Effect of single doses of ethinyloestradiol for 24 hr on the pituitary-adrenal axis in the quiescent (solid lines) and ether stressed (interrupted lines) female rat. ○, Plasma corticosterone; ●, adrenal gland corticosterone; ×, adrenal gland *in vitro* production of corticosterone.

with synthesis in the stressed rat, ethinyloestradiol caused an impairment of corticosterone release from the gland.

The increased plasma corticosterone levels after ethinyloestradiol in the quiescent rat might have been caused by an increased half-life of corticosterone in the circulation, as well as by an increase in ACTH secretion. This was examined by injecting ethinyloestradiol (1·0 mg/kg), stressing the rats 24 hr later with ether and measuring the plasma corticosterone levels at intervals after the stress. Figure 2 shows that the ether stress produced a maximum plasma corticosterone concentration of 80 μg/100 ml in the controls, but only of 50 μg/ml in the oestrogen-treated group, confirming the observations seen in Fig. 1. The rate of corticosterone disappearance was similar in the treated and control groups up to 60 min, but at that time the plasma level was still significantly higher in the control group. One hundred and twenty and 180 minutes after the stress, the plasma levels in the control animals were still decreasing, but in the oestrogen-treated rats the slightly elevated corticosterone levels did not decrease as rapidly as in the controls. As the concentration of corticosterone in the plasma was lower after an ether stress in the oestrogen treated group the rate of removal of corticosterone from the plasma may have been altered. The protein binding capacity for corticosterone was not altered by this acute oestrogen treatment—control value 47·7 μg/100 ml, oestrogen treated 43·8 μg/100 ml—suggesting that the free corticosterone may have been more rapidly metabolized in the oestrogen-treated rats. To test this, rats were adrenalectomized and injected with ethinyloestradiol (1·0 mg/kg). Twenty-four hours later these rats were injected intravenously with 12·5 μg of corticosterone, and the fall in plasma

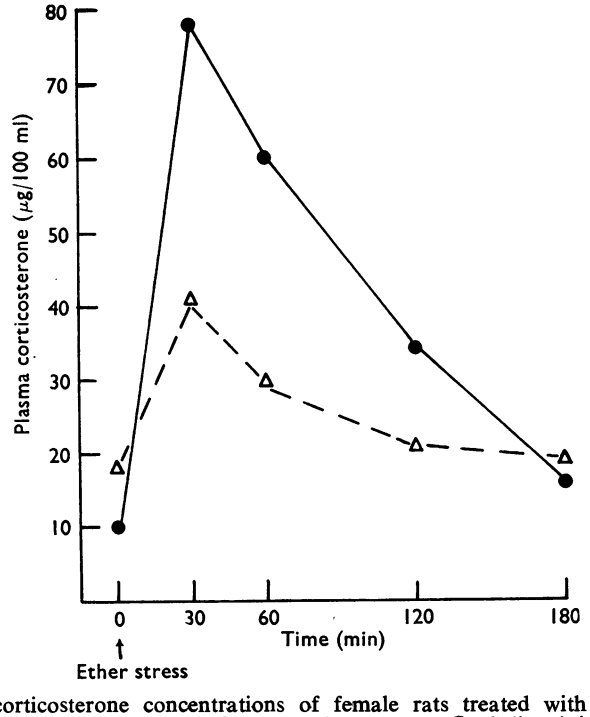

FIG. 2. Plasma corticosterone concentrations of female rats treated with a single dose of ethinyloestradiol (1·0 mg/kg) 24 hr before an ether stress. ●, Saline injected; △, ethinyloestradiol treatment. Each point represents the mean of six observations.

corticosterone monitored. The blood volume of these rats was 8·4 ml/100 g body weight, so the maximum theoretical concentration of corticosterone in the plasma was 150 μg/100 ml. In both the control and oestrogen-treated adrenalectomized rats there was a very rapid fall in plasma corticosterone concentration during the first 5 min, but at 10 and 15 min after the injection the fall was much slower (Table 1). There was no significant difference in the plasma levels of the control or ethinyloestradiol-treated rats at any time after the injection of corticosterone. The total plasma corticosterone concentration at the 5, 10, and 15 min intervals gave an indication of the rate of metabolism of corticosterone at physiological concentrations, and this was a reflection of the equilibrium between protein bound and unbound corticosterone and the rate at which both these moieties were degraded by the liver or utilized by the tissues. The results of these experiments suggested that single doses of ethinyloestradiol did not affect the peripheral metabolism of corticosterone. Thus the lower plasma corticosteroid concentration of the oestrogen-treated group in the plasma after a stress was due to altered adrenal or pituitary function.

Effects of multiple doses of ethinyloestradiol

In order to investigate further the actions of ethinyloestradiol on pituitary and adrenal function, long term treatment with 500 μg/kg per day for 7 days was undertaken. One group of rats was used 1 day, and the other group 9 days after the last injection. In the first group there was a loss of body weight and the adrenal and pituitary glands were hypertrophied and hyperaemic (Table 2). The period of 9 days without treatment in the second group enabled the rat to resume normal growth and there was a regression in adrenal but not in the pituitary size (Table 2).

TABLE 1. *Corticosterone concentrations in the plasma of adrenalectomized female rats treated with ethinyloestradiol (1 mg/kg) 24 hr before the experiment and injected with 12·5 μg corticosterone intravenously at 5, 10 and 15 min before death*

	Time after injection of corticosterone (min)					
	5		10		15	
	Control	Ethinyl-oestradiol	Control	Ethinyl-oestradiol	Control	Ethinyl-oestradiol
Plasma corticosterone, μg/100 ml ± S.E.	34·5 ± 3·2 (5)	35·0 ± 1·7 (6)	20·2 ± 3·3 (6)	22·4 ± 2·8 (6)	15·2 ± 1·8 (6)	13·7 ± 2·4 (5)

Figures in parentheses are the number of observations per group.

TABLE 2. *Effect of prolonged treatment with ethinyloestradiol (500 μg/kg per day for 7 days) on the body, adrenal and pituitary gland weights measured*

Treatment	Body weight increase (g/day)	Adrenal weight (mg/pair ± S.E.)	Pituitary weight (mg ± S.E.)
Controls	+1·5 (45)	68·4 ± 0·6 (45)	9·8 ± 0·2 (45)
Ethinyloestradiol A	−1·7 (24)	107·0 ± 3·5 (24)	15·5 ± 1·2 (24)
Ethinyloestradiol B	+0·3 (18)	75·2 ± 1·2 (18)	15·2 ± 0·8 (18)

A, 1 day after last injection; B, 9 days after last injection.

Twenty-four hours after the 7 day treatment, the plasma corticosterone concentration was unchanged and in *in vivo* steroid production reduced in the quiescent rat despite a 58% increase in adrenal weight, suggesting a marked hypersecretion of ACTH (Table 3). As the ether stress failed to alter any of the indices of pituitary-adrenal activity, there may have been a failure of either or both glands to function normally. After an ACTH injection, the plasma, *in vitro* and adrenal content changes were subnormal, which indicated a partial failure of function of the adrenal gland (Table 3). Therefore, treatment with this dose of oestrogen resulted in deranged function of both pituitary and adrenal glands. The peripheral removal of corticosterone was not altered by this dosage of oestrogen as tested by injecting corticosterone into the rat and monitoring the fall in the plasma concentration. The protein binding capacity of the plasma for corticosterone was not altered by this regimen of oestrogen treatment; 47·1 μg/100 ml in controls, 49·4 μg/100 ml ethinyloestradiol treated.

After 9 days' rest from treatment, adrenal corticosterone and *in vitro* corticosterone production in the unstressed rats were similar to those of oestrogen-treated rats killed after 24 hours, and were different from the levels in the untreated controls (Table 3). After an ether stress, all the indices of activity were still depressed when compared with untreated controls, but an ACTH injection elicited a normal *in vitro* corticosteroid production response and the plasma and adrenal content levels were raised. These results suggested that the adrenal gland recovered its ability to function more quickly than the pituitary. Further evidence of this was obtained *in vitro*. ACTH was added to the adrenal gland, which had previously been incubated for an hour with Krebs-Ringer. The results are shown in Fig. 3. The first hour's incubation reflects the amount of ACTH released endogenously in response to the ether stress (Bakker & de Wied, 1961).

After treatment for 7 days with ethinyloestradiol, an addition of ACTH to the medium did not increase the steroid production to normal levels before or after an ether stress (Fig. 3, A2 and B2). However, by injecting ACTH 30 min before killing the rats and adding ACTH to the medium, the corticosterone concentration was restored to a value not significantly different from the control level (Fig. 3, C2). When a similarly treated group of rats was left for 9 days after the last injection, and ACTH added to the medium, corticosteroid production was not impaired (Fig. 3, A3, B3, C3). These results suggest that even when the adrenal cortical cells were

TABLE 3. *Effect of ethinyloestradiol (500 μg/kg per day for 7 days) on the pituitary–adrenal axis before and after an ACTH injection (5 units/kg) or an ether stress 1 day (A) and 9 days (B) after the last injection of the oestrogen*

Treatment		Unstressed rats	Ether stressed	ACTH injection
Controls	a	11·4±2·6 (15)	91·4±1·4 (15)	80·4± 2·1 (15)
	b	22·0±2·2 (15)	48·3±5·2 (15)	62·8± 5·8 (15)
	c	46·2±9·1 (15)	90·3±2·3 (15)	90·9± 9·5 (15)
Ethinyl-oestradiol A	a	13·2±2·3 (8)	12·1±2·0 (8)***	26·4± 4·4 (8)***
	b	30·2±3·5 (8)*	22·8±2·5 (8)***	36·8± 5·4 (8)**
	c	21·8±3·0 (8)**	17·8±2·1 (8)***	47·3±11·6 (8)***
Ethinyl-oestradiol B	a	8·6±2·0 (6)	16·4±3·2 (6)***	42·4± 2·0 (6)***
	b	38·7±7·2 (6)*	28·1±4·2 (6)	52·5± 5·2 (6)
	c	23·1±5·2 (6)*	34·7±7·2 (6)***	97·5±10·2 (6)

a, Plasma corticosterone μg/100 ml; b, adrenal corticosterone μg/g; c, *in vitro* corticosterone production μg/g per hr.
Results as means±standard error. The figures in parentheses are the number of observations per group. Significant difference from control group: *$P<0·05$; **$P<0·01$; ***$P<0·001$.

functional, there was still an inhibition of release of pituitary ACTH. This may have been through lack of pituitary reserves caused by oestrogen overstimulating ACTH secretion. Under these conditions of persistently high ACTH levels, the adrenal gland may be working at maximum capacity. This led to adrenal exhaustion and hypofunction which was reversible within 9 days of the last injection of ethinyloestradiol.

Effect of ACTH treatment for 7 days

Whether the failure of the adrenal gland was due to the oestrogen treatment *per se* or to a prolonged hypersecretion of ACTH was tested by injecting long acting ACTH in a dose of 4 units/rat for 7 days.

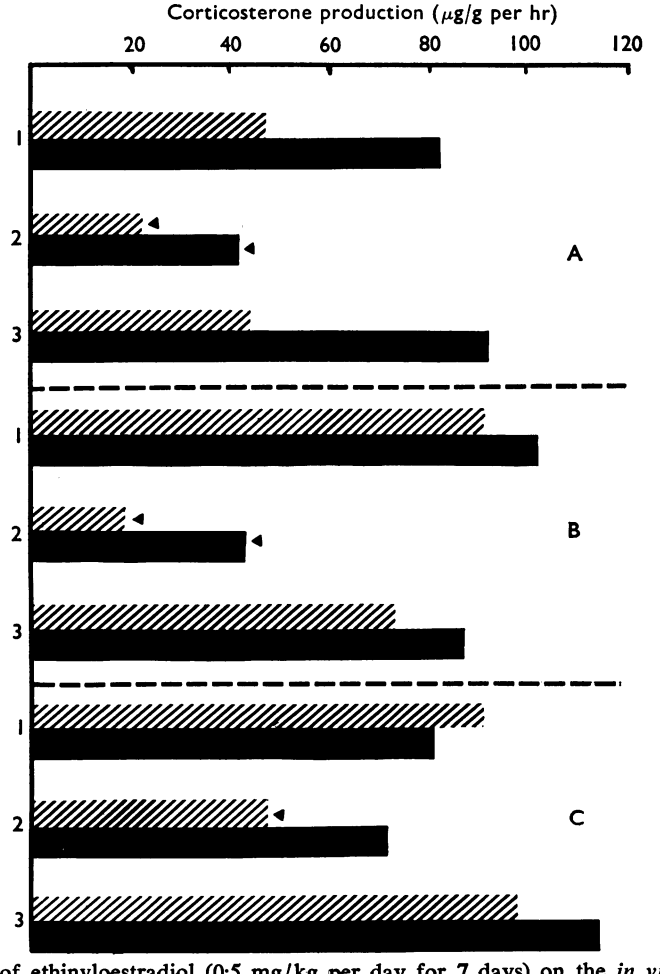

FIG. 3. Effect of ethinyloestradiol (0·5 mg/kg per day for 7 days) on the *in vitro* production of corticosterone by the adrenal gland of female rats. Hatched columns, 1 hr incubation with Krebs-Ringer; closed columns, fresh Krebs-Ringer+ACTH 100 m-u/ml. This effect was examined in the quiescent rat (A), the ether stressed rat (B), and the ACTH injected rat (C). 1, Saline injected rats; 2, rats killed 1 day after the last injection of ethinyloestradiol; 3, rats killed 9 days after the last injection of ethinyloestradiol. ◀ denotes values which are significantly different ($P<0·05$) from the control values, A1, B1, C1.

This dose caused a 56% increase in adrenal weight and no change in pituitary weight. The adrenal glands were hyperaemic and similar to those after ethinyloestradiol treatment. As in the oestrogen-treated rats, plasma and adrenal corticosterone content of unstressed rats was elevated, but there was no change in the *in vitro* index. An ether stress after such ACTH treatment failed to alter adrenal activity (Table 4), suggesting that the adrenal was insensitive to an acute release of endogenous ACTH. This possibility was confirmed by injecting short acting ACTH after the long acting ACTH and noting a very much reduced rise in adrenal activity (Table 4). Thus, part of the adrenal dysfunction after oestrogen treatment may be due solely to the continued enhanced secretion of endogenous ACTH from the pituitary.

Discussion

Single injections of ethinyloestradiol into female rats with a low basal secretion of ACTH caused increases in adrenocortical activity which were dose dependent. No alteration in the removal of corticosterone from the circulation was observed after acute or chronic administration of ethinyloestradiol. This observation is in contrast to a report by Kitay (1963b), who demonstrated an impairment of peripheral clearance of corticosterone using stilboestrol. Ethinyloestradiol did not increase the corticosterone binding capacity of the plasma ; this is in agreement with Gala & Westphal (1965). Keller, Sendelbeck, Richardson, Moore & Yates (1966), however, did observe a small increase in corticosteroid binding globulin, but only after 14 days of high doses of oestrogen given to male rats. These results are surprising in view of the many reports of an increase in corticosteroid binding activity after oestrogen treatment in man (Sandberg & Slaunwhite, 1959 ; Daughaday, Adler, Mariz, & Rasinski, 1962). In the female rat the levels of oestrogen normally circulating may result in maximal stimulation of binding activity under normal physiological conditions.

The increase in adrenal corticosterone production *in vitro* after injection of ethinyloestradiol suggested an increase in circulating ACTH. Gemzell (1952) demonstrated increased ACTH levels after oestrogen treatment, and this has been confirmed by Fonzo, Mims & Nelson (1967). Kitay (1963a), using depot oestradiol treatment for 1 week, noted that high doses of oestrogen inhibited pituitary-adrenal function, whereas low doses stimulated in the male but not in the female rat. The present results using a different dose regimen and oestrogen shows that doses of ethinyloestradiol of 50 μg/kg onwards stimulated pituitary ACTH release and adrenocortical activity. These results are in agreement with early work which

TABLE 4. *Effect of long-acting ACTH (4 units/rat for 7 days), followed by an ether stress or short-acting ACTH (5 units/kg) on the day after ceasing long-term treatment*

Treatment		Unstressed	Ether stress	ACTH (acute) (5 units/kg)
Control	a	8·6±1·2 (8)	58·5±2·6 (8)	59·7± 3·7 (4)
	b	15·1±1·4 (8)	55·0±3·6 (8)	72·5± 9·8 (4)
	c	15·7±3·8 (8)	75·0±5·7 (8)	70·0± 5·4 (4)
ACTH for 7 days	a	18·9±2·1 (6)*	22·2±3·2 (6)***	37·9±11·2 (4)
	b	26·3±4·0 (6)*	18·9±6·5 (6)**	36·6± 9·1 (4)**
	c	20·1±4·0 (6)	22·5±7·4 (6)**	40·7± 4·4 (4)***

Results as mean±standard error. The figures in parentheses are the number of observations. a, Plasma corticosterone μg/100 ml; b, adrenal corticosterone μg/g; c, *in vitro* steroidogenesis μg/g per hr. Significant differences from control group: $*P<0.05$; $**P<0.01$; $***P<0.001$.

demonstrated that after hypophysectomy oestrogens no longer cause adrenal hypertrophy and lipid depletion of the adrenal gland (Selye, Collip & Thomson, 1935 ; Selye & Collip, 1936). However, Fukui, Takeuchi, Watanabe, Kumagai, Yano & Nishino (1961) found that oestradiol added to adrenal glands *in vitro* stimulated corticosterone production, suggesting a direct action on the adrenal cortex. Other workers have demonstrated increased adrenal gland corticosterone concentrations after *in vivo* oestrogen treatment (Holzbauer, 1957b ; Llaurado, Claus & Trunnell, 1962 ; Cushman, Pulido & Hilton, 1965). Holzbauer (1957b) observed that stimulation occurred only with low initial ACTH levels and that with high circulating ACTH there was an inhibition of corticosterone secretion. These observations have been confirmed in the present paper when single doses of ethinyloestradiol were used. With prolonged administration of high doses of oestrogens, however, there was either no effect or inhibition of corticosterone secretion during basal ACTH secretion, confirming the observations of others (Kitay, 1963b ; Saba & Hoet, 1963).

There have been many reports demonstrating that oestrogens can inhibit corticosterone secretion, and in most cases the experimental procedures applied were known to cause near maximal secretion of ACTH (Vogt, 1955 ; Holzbauer, 1957b ; Holzbauer & Vogt, 1957a ; Kitay, 1963b ; Saba & Hoet, 1963 ; Pincus & Hirai, 1964).

One interesting aspect of this work has been the disassociation of adrenal gland concentrations of corticosterone and plasma concentration or *in vitro* release. Holzbauer (1957a) demonstrated that the hormone stores of the adrenal cortex reflect the secretion rate at the time of death. The present data with the unstressed oestrogen-treated rat confirmed this observation, but after a stress the concentration of corticosterone in the gland increased whereas the secretion rate was decreased. A similar phenomenon was demonstrated by Pincus & Hirai (1964) with progesterone and corticosterone treatments. These results suggest that exogenously administered steroids might interfere with the release of corticosterone from the adrenal without affecting corticosterone biosynthesis.

Prolonged treatment with ethinyloestradiol had little or no effect on corticosterone secretion in unstressed rats but there was a greatly diminished adrenocortical response to ACTH or an ether stress. Analogous results were obtained by Vogt (1955) and Holzbauer (1957b) using hexoestrol. They suggested that the inhibition seen in stressed rats was caused by an inhibition of cholesterol synthesis ; however, there was sufficient precursor to maintain normal basal secretion and elevations of this level by about 100% (Holzbauer, 1957b). Other experiments suggested strongly that this was the case (Boyd & McGuire, 1956 ; Holzbauer & Vogt, 1957a). Since stimulation of corticosterone production with oestrogen can occur, however, an alternative explanation might be that exhaustion of the precursor pool occurs due to prolonged stimulation by ACTH. Moreover it has been shown that no lipid depletion occurs in the adrenal with oestrogens after hypophysectomy (Selye *et al.*, 1935). The experiments reported here demonstrate that prolonged ACTH treatment (4 i.u. daily for 7 days), like prolonged ethinyloestradiol, inhibited the corticosterone response to a stress and an injection of ACTH, indicating hypofunction of the adrenal gland. Holzbauer & Vogt (1957b), however, demonstrated that prolonged ACTH treatment (2 i.u. daily for at least 4 days or one i.u. daily for at least 11 days) caused an increase in lipid droplets and no inhibition of corticosterone secretion. It would therefore seem unlikely that with the ACTH treatment used in this study there was lipid droplet depletion ; although it is well known

that high doses of ACTH can cause an initial depletion of lipids and adrenal cholesterol (Holzbauer & Vogt, 1957b ; Goodman, 1965). Assuming, however, that lipid droplets, and thus cholesterol esters (Moses, Davis, Rosenthal & Garren, 1969), are in abundance in the adrenal gland the possibility arises that during an acute stress this precursor pool is not available for corticosteroid biosynthesis. It is now well established in the rat (Morris & Chaikoff, 1959) and in man (Chobanian & Hollander, 1962 ; Field, Swell, Schools & Treadwell, 1960) that the cholesterol present in the adrenal glands comes mostly from the plasma. Moreover in man (Borkowski, Levin, Delcroix, Mahler & Verhas, 1967) and in the guinea-pig (Werbin & Chaikoff, 1961) it was demonstrated that the proportion of cholesterol derived from the plasma at rest, 80% in man and 60% in the guinea-pig, was not altered by administration of ACTH. Oestrogen treatment does decrease plasma cholesterol concentrations (Boyd & McGuire, 1956) and the resulting hypofunction of the adrenal during stress can be instantaneously restored to normal by intravenous infusion of plasma from normal but not from hexoestrol treated rats (Holzbauer & Vogt, 1957a). To the author's knowledge there are no reports on the effect of ACTH on plasma cholesterol levels, but since ACTH has been shown to accelerate the metabolism of plasma and adrenal cholesterol into hydrocortisone (Borkowski *et al.*, 1967) and not to stimulate cholesterol synthesis, a decrease in plasma cholesterol might be expected.

The present results with prolonged ACTH treatment can also be explained by a decreased sensitivity of the adrenal to ACTH. This explanation is supported by the work of Jones and Stockham (1966), who demonstrated a tachyphylactic effect due to frequent stimulation of the adrenal cortex with ACTH. Moreover the hypersecretion of ACTH after oestrogen administration could lead not only to an ACTH insensitive adrenal cortex but also to an ACTH depleted pituitary. Whatever the mechanism of the inhibition by oestrogen and ACTH it appears that adrenal function returns to normal more rapidly than pituitary function.

I wish to thank Dr. W. J. Tindall, of Organon Laboratories, for generously supplying corticosterone, ethinyloestradiol, and ACTH used in these studies, and the Medical Research Council for the award of a grant. The advice of Dr. M. A. Stockham during the course of this work is gratefully acknowledged.

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