J. Physiol. (1954) 130, 601–614

# INHIBITION BY HEXOESTROL OF ADRENOCORTICAL SECRETION IN THE RAT

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(*Received* 23 *May* 1955)

It has been known for a long time (Korenchevsky & Dennison, 1935; Selye, Collip & Thomsen, 1935) that oestrogens cause hypertrophy and hyperplasia of the rat adrenal cortex. Selye & Collip (1936) have shown that this effect is absent in the hypophysectomized rat, even when the adrenal cortex is maintained by adrenocorticotrophic hormone (ACTH). The inference is that oestrogens cause an increase in secretion of ACTH and thus produce adrenal hypertrophy indirectly. During hexoestrol administration the pituitary hypertrophies and goes on growing for at least 6 weeks (Somers, 1948). Gemzell (1952) was able to demonstrate an increase in the amount of circulating ACTH in rats injected with oestradiol. The release of ACTH by oestrogens is considered to be an example of an unspecific response to stress (Tepperman, Engel & Long, 1943).

Loeser (1939) observed the disappearance of lipids from the adrenals of rats injected with stilboestrol. A detailed examination of the effect of hexoestrol (Vogt, 1945) showed that sudanophilic lipids disappeared almost completely from the zonae fasciculata and reticularis in the course of 5 days, and from all three layers if the treatment lasted 10 days or more. These effects, too, were absent in hypophysectomized rats not given ACTH: experiments with ACTH-maintained rats were not performed.

The functional state of lipid-depleted adrenals has been a matter of conjecture. Many histologists consider them to be hyperactive glands (Dosne & Dalton, 1941; Sarason, 1943), whereas others (Zwemer, 1936; Deane & McKibbin, 1946; Flexner & Grollman, 1939) suggest that they are in a state of exhaustion following initial hyperactivity. Both these interpretations are prompted by the observation that the early response to any stress or to an injection of ACTH is a diminution of adrenal lipids, more especially of cholesterol esters. However, this diminution is followed by recovery to normal or even by deposition of abnormally high amounts of lipids whenever treatment

with ACTH is prolonged. In fact, the contrast between adrenals of rats receiving repeated doses of ACTH and of rats injected with oestrogens suggested to Selye & Stone (1950) that adrenal changes after oestrogens were due to a combination of factors of which the release of ACTH was only one. Selye & Stone consider the response to oestrogens not to differ in this respect from that to any prolonged stress. In the author's experience, however, prolonged severe stress rarely causes a lipid depletion comparable to that following the administration of oestrogens.

Another hypothesis put forward to explain the adrenal changes caused by oestrogens was that the hypertrophy of the adrenal cortex was analogous to the goitre caused by anti-thyroid substances and a result of the failure of cortical hormone production (Vogt, 1945). This theory was compatible with the poor state of health of the animals and their short survival time after adrenalectomy, but direct support for it was not obtainable at the time. Attempts were made to measure cortical activity in oestrogen-treated animals which were large enough to permit bioassay of cortical hormone in adrenal vein blood (Somers, 1948). Cats were used, but the experiments were a failure, because doses of hexoestrol tolerated by the rat caused fatal liver damage to the cats without producing appreciable loss of lipids from the adrenals.

The development of micro-methods for the estimation of adrenal corticoids, particularly their paper-chromatographic separation (Bush, 1952), permits an estimation of the secretory performance of a single rat adrenal. It is therefore possible to determine the functional state of the lipid-depleted adrenals of a hexoestrol-treated rat, and the results of such determinations form the subject of the present paper.

## METHODS

### *Experimental procedure*

*Rats.* Adult male Wistar rats were used. Urethane (1·5 g/kg) was injected subcutaneously as a 25% solution in 0·9% NaCl. When anaesthesia was complete, the trachea and right femoral vein were cannulated. All intravenous injections were made into the femoral vein. The left renal pedicle was tied through a midline incision in the abdominal wall. The left renal vein was dissected, any tributaries not coming from the adrenal gland tied, and ligatures placed around it laterally and medially to the entry of the adrenal vein. A polythene cannula passed through the flank of the animal was introduced into the lateral part of the left renal vein, heparin (1000 i.u./kg) injected intravenously, the medial ligature on the renal vein tied and the adrenal blood drained from the cannula into a cooled siliconed centrifuge tube. Collection of blood was over periods of 15–30 min. Several intravenous injections of 1 ml. 0·9% NaCl solution were made if blood collection was carried out for longer than 15 min.

*Rabbits.* Chloralose (150 mg/kg) was injected into the ear vein as a 1% solution. Ether was given if required during the dissection. The trachea and jugular vein were cannulated and the rabbit was eviscerated. The renal pedicles and any vessels coursing in the perirenal tissue were ligated; the lumbar veins crossing the adrenal glands were tied at the lateral edge of the glands. A ligature was placed around the vena cava above the right adrenal. The aorta and vena cava were then tied below the left kidney, the vena cava was cannulated just above the ligature and heparin (500 i.u./kg) was injected intravenously. The upper ligature around the vena cava was then tied

and the blood from both adrenals drained through the cannula into cooled siliconed tubes. Samples were collected for 15 min. Whenever the flow from the cannula slowed down, a few ml. of 0·9 % NaCl solution were infused into the jugular vein.

*Extraction*

The blood was centrifuged, the plasma (volumes ranging from 0·5 to 15 ml.) weighed, diluted with an equal volume of $H_2O$ and extracted by a procedure similar to, but somewhat shorter than, that used by Bush (1953*b*). It consisted of four extractions of the diluted plasma with 1 vol. of an ethylacetate-ether mixture (2:1), separate evaporation, in the same flask, of each portion of the supernatant organic solvent, transfer of the residue into a separating funnel by washing the flask with 2·5 vol. petroleum ether (b.p. 40–60° C), followed three times by 0·5 vol. of 80 % ethanol. The smallest volumes of petroleum ether and 80 % ethanol used were 10 and 2 ml. respectively, even when the amount of diluted plasma was less than 4 ml. Each portion of alcohol was shaken vigorously with the petroleum ether, drained off and evaporated *in vacuo*. The residue from the alcoholic extracts was dissolved in a small volume of ethylene dichloride, and this solution applied to filter-paper (Whatman No. 2) for chromatography. The solution was applied on 'lanes' 6 × 1·5 cm and the steroids compressed to narrow bands by standing the paper in a trough filled with a mixture of ethylacetate and methanol (2:1) (Bush, personal communication).

*Chromatography*

The method of Bush (1952) was used. The solvent mixture consisted of benzene (1000 ml.), methanol (550 ml.) and water (450 ml.) (Bush & Sandberg, 1953), and the temperature at which the papers were equilibrated and run was 34° C.

For the estimation and identification of the steroids the soda-fluorescence and a colorimetric method using blue tetrazolium (B.D.H.) were employed. The soda fluorescence test was carried out by spraying the paper with a 14 % solution of NaOH in $H_2O$ which contained 5 mg triphenyltetrazolium chloride (B.D.H.) for each 100 ml. (Bush, 1953*a*). The paper was dried in front of a battery of infra red lamps and examined in ultraviolet light. The primrose fluorescence shown by Bush (1952) to be given by cortical steroids with an $\alpha\beta$-unsaturated ketone group permits a rough quantitative estimate of the steroids and is a reliable indicator of the number of substances present. For the quantitative estimation of the individual corticoids, the extract was applied to a paper carrying markers of pure steroids (usually corticosterone and cortisol) on each side. After the development of the chromatogram, the markers were made visible by spraying the sides of the paper with blue tetrazolium (12 mg dissolved in 5 ml. ethanol and added to 25 ml. 5 % NaOH in $H_2O$), or by scanning in front of an ultraviolet lamp emitting at 240 m$\mu$.

*Elution and colorimetry*

Under the guidance of the control spots, rectangles of the paper containing individual plasma corticoids were cut out for elution, and rectangles of the same size taken from corticoid-free parts of the paper were cut out for blanks. The rectangles were cut up into small pieces and extracted with ethanol three times for 5 min on a mechanical shaker. For a surface area of 36 cm$^2$ the volumes of ethanol used were 2·7, 1·8, and 1·8 ml. Each portion of ethanol was filtered into a tube through a small plug of washed cotton-wool pushed into the stem of a microfunnel. The alcohol was evaporated to near-dryness *in vacuo* before the next alcoholic filtrate was added. The last residue was dried completely and dissolved in 0·45 ml. 95 % ethanol. This solution was used for a colorimetric estimation of the corticoids with blue tetrazolium. A method using this compound has been devised by Mader & Buck (1952) and adapted for micro purposes by Morris & Williams (1955). The amounts of reagents used here were somewhat different from those employed by Morris & Williams, the base used was tetraethylammonium hydroxide, and the readings were carried out with a Spekker absorptiometer and Ilford filter No. 607. To each glass-stoppered tube containing the ethanolic solution of corticoids or of the paper blank, 0·04 ml. of a 5 % solution of tetraethylammonium hydroxide in 80 % ethanol was added, followed by 0·05 ml. of a freshly

prepared 0·5% ethanolic solution of blue tetrazolium. After incubation for 1 hr. at 23° C the reaction was interrupted by the addition of 0·04 ml. 10% $CH_3COOH$ in 50% ethanol. The solutions were transferred by fine pipettes into microcells of 0·5 ml. capacity. With pure steroids the readings followed Beer's Law only when the extinction did not exceed 0·3; when the extinction was greater, 1:1 or 1:2 dilutions of steroid solutions and blanks with 95% ethanol had to be used in order to obtain a straight-line relationship of extinction and steroid concentration. With this procedure, there remained small deviations of the readings from the straight line which occasionally were as high as 5% (see Fig. 1). It is hoped that with the use of a spectrophotometer these inaccuracies will disappear.

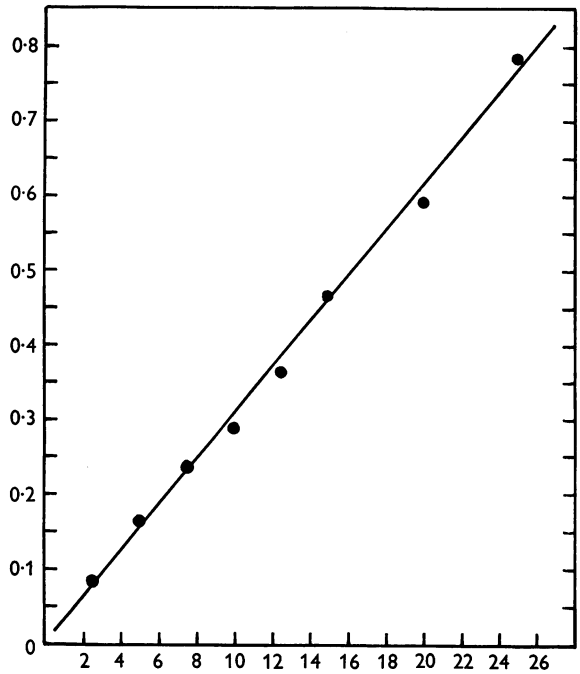


Fig. 1. Example of colour reaction of a corticoid with blue tetrazolium. Abscissa: μg cortisol per sample. Ordinate: extinction.

Another source of error was the paper blank. In order to discover abnormally high blanks which might occur occasionally, all paper blanks were read against a pure reagent blank (containing the incubated steroid-free reagents). The normal extinction of paper blanks did not exceed 0·1; washing of the paper before use with organic solvents increased the size of the blanks instead of decreasing it.

*Recoveries*

10 μg quantities of corticosterone or of cortisol were added to arterial plasma and subjected to the whole procedure outlined above. Recoveries averaged 70% with only minor variations and were the same for the two steroids. The losses of the elution process accounted for 10–15% of the total amount present. Since rat adrenal blood was never found to contain cortisol, 10 μg were added to all samples of plasma before extraction and the recoveries determined for each experiment.

*Histology*

The adrenal glands were fixed in a mixture of formalin and $K_2Cr_2O_7$, and frozen sections prepared which were stained with Sudan IV and haematoxylin.

*Drug administration*

Hexoestrol (B.D.H.) was dissolved in arachis oil and injected subcutaneously into rats as 0·2% solution and into rabbits as 2% or 5% solution. Progesterone was injected as a 2% solution in arachis oil.

*Ascorbic acid estimations*

The method of Roe & Kuether (1943) was used.

## RESULTS

### *Normal rats*

The main compound in extracts of adrenal vein plasma found to give the soda fluorescence and to reduce blue tetrazolium had the same $R_F$ value as corticosterone. Sometimes it was accompanied by traces of a slightly more polar substance. These findings confirm Bush's observations (1953*b*) that the rat adrenal does not secrete cortisol. The collection time from each rat rarely exceeded 20 min., so that the amount of plasma would not have been sufficient to detect any aldosterone (Singer & Stack-Dunne, 1955).

TABLE 1. Corticosterone secreted by the left adrenal of normal and hexoestrol-treated rats

| Normal | | | Hexoestrol-treated | | | | |
|---|---|---|---|---|---|---|---|
| No. | μg/min/g adrenal | μg/gland/hr/kg b.wt. | No. | Days of treatment | Daily dose (mg/kg) | μg/min/g adrenal | μg/gland/hr/kg b.wt.* |
| 1 | 20·8 | 65·6 | 1 | 3 | 0·37 | 5·5 | 26·1 |
| 2 | 17·1 | 65·3 | 2 | 3 | 0·37 | 6·5 | 29·2 |
| 3 | 20·1 | 64·5 | 3 | 3 | 0·37 | 8·4 | 47·5 |
| 4 | 16·4 | 55·0 | 4 | 4 | 0·50 | 5·7 | 25·1 |
| 5 | 32·3 | 132·0 | 5 | 6 | 0·43 | 5·1 | 20·6 |
| 6 | 31·5 | 114·0 | 6 | 8 | 0·46 | 7·6 | 29·0 |
| 7 | 17·8 | 59·0 | 7 | 8 | 0·50 | 9·1 | 33·5 |
| | | | 8 | 15 | 0·50 | 7·4 | 47·2 |
| Means and S.E.† of the mean | | | | | | | |
| | 22·3±2·1 | 79·3±10·8 | | | | 6·9±0·5 | 32·2±3·3 |

* Calculated per body weight at end of experiment.
† Calculated from the range (Lord, 1947).

The quantities of corticosterone produced by seven normal adult male rats are listed in Table 1. They have since been confirmed (Holzbauer & Vogt, to be published) in many more animals and with different anaesthetics. The figures are not corrected for the loss of about 30% during the processes of extraction and elution. If this fact is taken into account, the figures fall within the range found by Singer & Stack-Dunne (1955).

### *Rats injected with hexoestrol*

All eight rats injected with hexoestrol (0·37–0·5 mg/kg daily for periods ranging from 3 to 15 days) showed a greatly reduced production of corticosterone (Table 1). On the average, secretion expressed per g of gland per min was 31% and, calculated per hr per one adrenal per kg body weight, 41% of the normal. The second figure is higher than the first owing to some loss in body

weight and some increase in adrenal size. Treatment for only 2 days was sufficient to cause a fall of the corticosterone secreted per g per min to 44 % of the normal (Fig. 2). Still shorter intervals after beginning of treatment were then allowed in order to find out whether, at any time of the experiment, secretion (under conditions of operative stress) was enhanced before becoming depressed. Fig. 2 shows that this is not the case, all values between 3½ and 24 hr after the injection lying within or below the normal range.

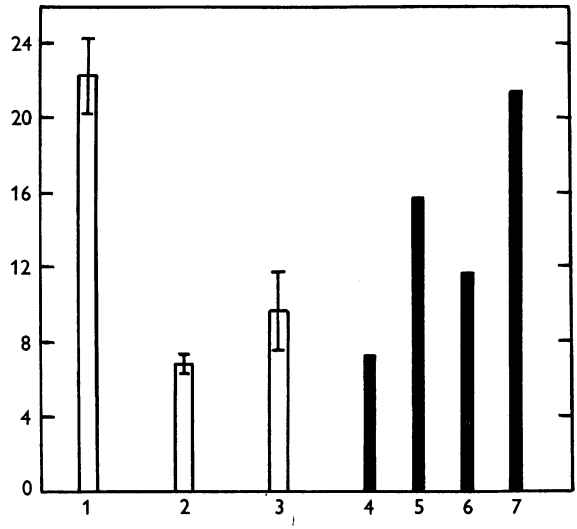


Fig. 2. Ordinate: corticosterone secreted, in $\mu$g/min/g gland, by the left adrenal of adult male rats. Column 1: mean and S.E. of the mean for seven normal rats. Column 2: mean and S.E. of the mean for eight rats given hexoestrol (0·4–0·5 mg/kg/day) for not less than 3 days. Column 3: mean and S.E. of the mean for five rats given hexoestrol (0·3–0·4 mg/kg/day) for 2 days. Columns 4–7: values for single rats given one injection of hexoestrol (0·3–0·4 mg/kg) 24, 4½, 3½ and 3½ hr before collecting the adrenal blood.

An experiment was also done to see whether the inhibition of corticosterone secretion was reversible: two rats treated with 0·5 mg/kg daily for 16 days and then allowed 7 days for recovery had the perfectly normal secretion rates of 98 and 119 $\mu$g/gland/hr/kg body weight.

An important question was whether, instead of corticosterone, another steroid was produced under the influence of hexoestrol and was replacing the normal compound. The soda-fluorescence test gives a reliable survey of very small amounts of many $\alpha\beta$-unsaturated ketosteroids. This test was carried out on part of the plasma extract of rats injected with hexoestrol, but no compound was found which was not also present in adrenal blood from normal rats.

A typical example is shown in Fig. 3. This is a chromatogram of extracts from a normal and from a hexoestrol-treated rat which are run together, both

representing plasma samples collected for 27 min. It will be seen that, in addition to corticosterone, there is, in both extracts, a smaller quantity of a less polar compound, and that in the normal rat both spots are more intense than in the hexoestrol-treated rat; the ratio of the intensities appears to be similar. This second compound was also seen by Bush (1953*b*) and is considered

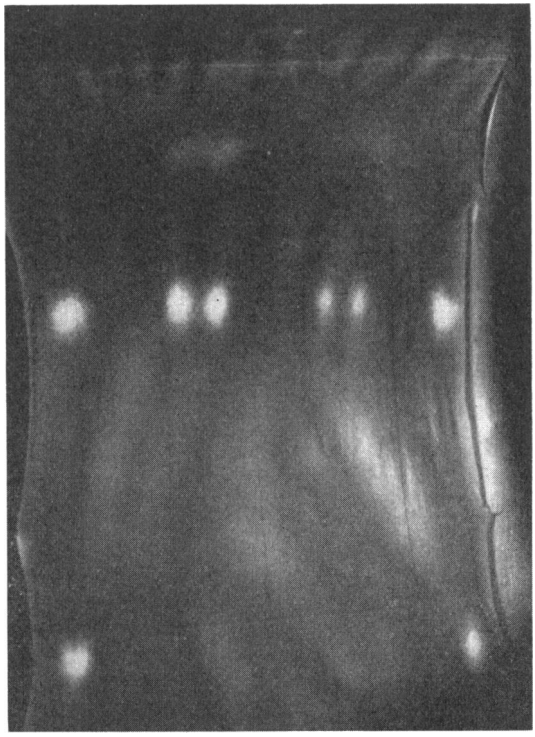

Fig. 3. Corticosterone secretion of a normal and of a hexoestrol-treated rat. Chromatogram of extracts of 27 min samples of adrenal vein plasma of 2 rats, together with markers. Origin at the bottom, front at the top of figure. Soda-fluorescence test (for details see under 'Methods'). On the left, the lower spot is a marker of 10 $\mu$g cortisol, the upper spot of 10 $\mu$g corticosterone. On the right, markers of 5 $\mu$g of the same compounds. In the region of corticosterone, the left twin spot corresponds to the extract from a normal rat, the right twin spot to the extract from a rat given twelve daily injections of 0·45 mg hexoestrol/kg body weight. The faint spots near the front are probably 11-hydroxyandrostene-dione.

by him probably to be the androgen 11-hydroxyandrostene-dione. This compound does not reduce blue tetrazolium and was found in the majority of normal rats, but only rarely after injection of hexoestrol, so that its secretion appears to be inhibited along with the production of corticosterone.

In two rats, which had received injections of hexoestrol, samples of adrenal vein blood were collected before and after the injection of corticotrophic

hormone (ACTH): a 25% increase in secretion was obtained with 5 mu. ACTH in the first, but no change was seen with 10 mu. ACTH in the second rat.

This experiment shows that it is not lack of ACTH which accounts for the low secretion rate of corticosterone. And this is what one would expect from the evidence of increased blood levels of ACTH in rats given oestrogens. The effect of ACTH on the secretion of the adrenal of the normal rat under the same experimental conditions will be dealt with in a later paper (Holzbauer & Vogt, to be published).

The adrenal cortex of all rats treated for at least 2 days with hexoestrol showed severe loss of sudanophilic lipids. A normal histological picture was only found in those three rats which were used $3\frac{1}{2}$ and $4\frac{1}{2}$ hr after the injection; Fig. 2 shows that in these rats, corticosterone secretion was hardly, if at all, depressed.

TABLE 2. Adrenal ascorbic acid concentrations (mg/100 g fresh tissue) of hexoestrol-treated rats under the stress of urethane anaesthesia

| | No. of rats | Ascorbic acid |
|---|---|---|
| Controls | 4 | 404±23* |
| Hexoestrol alone | 4 | 300±17 |
| Hexoestrol and urethane | 4 | 222±13 |

* S.E. of the mean.

An experiment was carried out by Dr M. Holzbauer in which the ascorbic acid metabolism of lipid-free adrenals was examined. The adrenal ascorbic acid of two groups of rats was estimated after four daily injections of hexoestrol (0·4 mg/kg). In one group, any stress immediately before removal of the adrenals was avoided by killing the animals by decapitation, whereas in the other group 1·5 g urethane/kg body weight were injected subcutaneously $1\frac{1}{2}$ hr before dissecting out the adrenals. This dose of urethane acts as a severe stress in normal rats and lowers their ascorbic acid by about 50%. The results of Table 2 show, first, that the hexoestrol injections by themselves lowered the adrenal ascorbic acid to $\frac{3}{4}$ of the normal figure, and, secondly, that urethane depressed it still further. Thus the mechanism by which stress causes a fall in the ascorbic acid concentration appears to be functioning in these adrenals.

The possibility will be discussed later that the effect of hexoestrol on the adrenals is produced by interference with the synthesis of cholesterol. Since progesterone is considered to be an intermediate product in the synthesis of the corticoids from cholesterol (Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker & Pincus, 1951), an attempt was made to overcome the inhibition of corticosterone synthesis by the administration of progesterone. A group of rats was given 0·4 mg hexoestrol/kg for 4 days, and their secretion of corticosterone was measured on the fifth day. Twenty-four and two hours before the collection of adrenal blood, two of the rats were injected subcutaneously with 20, and two rats with 25 mg progesterone per kg body weight. The cortico-

sterone secretion of all four rats was at, or just above, the upper limit of the secretion observed in rats given hexoestrol alone and averaged 9·2$\mu$g/min/g adrenal with a standard error of the mean of 0·15. Three control rats were injected with progesterone only. Their corticosterone secretion was perfectly normal. It is not possible to decide whether the fact that corticosterone synthesis after hexoestrol was not decisively improved by progesterone means that progesterone is not utilized by the rat adrenal under these circumstances or that the doses or timing of the injections were inadequate.

### *Rabbits*

The effect of hexoestrol on adrenocortical secretion was investigated in the rabbit in order to see whether the rat differed from related species in its response to oestrogens. As in the rat, corticosterone is the main $C_{21}$-corticoid of adrenal blood, but small quantities of dehydrocorticosterone and of cortisol were also found. The small amounts of dehydrocorticosterone were not determined separately, but the paper rectangle to be eluted was cut in such a way as to combine the regions containing corticosterone and its dehydro-derivative, and the colour formed was read off as corticosterone.

TABLE 3. Corticosterone* secreted by both adrenals of normal and hexoestrol-treated male rabbits

| Normal | | | Hexoestrol-treated | | | | |
|---|---|---|---|---|---|---|---|
| No. | Adrenal weight (g/kg b.wt.) | $\mu$g Corticosterone per min/g adrenal | No. | Adrenal weight (g/kg b.wt.) | Days of treatment | Daily dose (mg/kg) | $\mu$g Corticosterone per min/g adrenal |
| 1 | 0·28 | 2·1 | 1 | 0·24 | 5<br>6 | 0·3<br>2·0 | 6·1 |
| 2 | 0·23 | 5·1 | 2 | 0·23 | 5<br>6 | 0·3<br>2·0 | 7·8 |
| 3 | 0·16 | 3·0 | 3 | 0·22 | 15 | 5·0 | 9·0 |
| 4 | 0·24 | 8·7 | 4 | 0·27 | 14 | 5·0 | — |
| 5 | 0·32 | 1·2 | 5 | 0·21 | 14 | 5·0 | 0·84 |
| 6 | 0·16 | 1·7 | 6 | 0·17 | 7 | 10·0 | 6·4 |
| 7 | 0·31 | 2·8 | 7 | 0·25 | 7 | 10·0 | 2·0 |
| Mean | 0·24 | 3·51 | | 0·23 | | | 5·36 |

* Corticosterone and a small quantity of dehydrocorticosterone were eluted together and estimated as corticosterone.

It will be seen from Table 3 that there is much greater individual variation of cortical secretion in the rabbit than there is in the rat. This may be due to the fact that the rabbits employed were of different breeds. Another cause for variation may have been the infection with cysticerci found in some animals and not in others.

The range covered by the secretion of the normal rabbits was even exceeded by that observed in the animals injected with hexoestrol. Though the mean secretion was higher in the second group, the difference was obviously not

significant (Table 3). The doses started at a level (0·3 mg/kg) which is highly effective in the rat, and were increased to 10 mg/kg given for 7 days without causing the morphological changes in the adrenal which invariably occur in the rat. There was no adrenal hypertrophy (Table 3), no greater depletion of lipids than the occurrence of small lipid-free patches encountered equally in the controls. The only exception was rabbit 6 of the hexoestrol group, which had very little sudanophilic material in its adrenals: rabbit 7, however, treated concurrently with exactly the same dose of oestrogen, showed the usual lipid-laden glands, so that it must be assumed that the cause for the loss of lipids in rabbit 6 was not the administration of hexoestrol. Finally, the loss of weight which is observed in rats injected with hexoestrol, and which reaches about 15% of the body weight in a fortnight, either did not occur in the rabbits or did not exceed 4% of the body weight.

## DISCUSSION

It is obvious from the foregoing experiments that the administration of hexoestrol to rats inhibits secretion from the adrenal cortex during operative stress without causing a preliminary stimulation. The mechanism involved can therefore not be 'exhaustion by overactivity' and must be sought elsewhere.

The rapid and complete loss of sudanophilic material from the zona fasciculata suggests interference with steroid synthesis. Possibly, since much of the stainable material is cholesterol, and since cholesterol can act as a precursor of the steroid hormones, the inhibition may be one of cholesterol synthesis. Such a mechanism would explain the stimulation of ACTH production by a lack of corticosterone, the hypertrophy and hyperplasia of the adrenal glands, and the hyperplasia of the anterior lobe of the pituitary, if hexoestrol administration is continued for a long period of time. The resulting condition would be analogous to the response of the thyroid after the administration of thiouracil, when a goitre is produced by excessive secretion of thyrotrophic hormone.

A question of interest is whether the inhibitory effect on the adrenals is peculiar to synthetic oestrogens or also occurs with the natural products. Though corticosterone production has, so far, only been followed after hexoestrol, the histological changes reported in the literature appear to be the same after all oestrogens; assuming the histological changes to be a reliable guide, it would appear that the effect is not peculiar to hexoestrol.

In other conditions, the stainable lipids may be an ambiguous means of assessing the functional state of the adrenal cortex. Thus Skelton, Fortier & Selye (1949) have shown that the cholesterol content and the sudanophilia of the adrenal may occasionally be dissociated: by giving stilboestrol together with lyophilized anterior pituitary, they obtained glands which were depleted of cholesterol but contained sudanophilic material. There is, however, no

indication that the opposite may occur, i.e. that sudanophilia may be absent when the cholesterol content is high. Further experiments will have to be done before it can be known whether other conditions, in which severe loss of stainable lipids has been reported, also lead to a decreased secretory function of the gland. It is quite conceivable that secretion and synthesis of hormone may be so neatly balanced that the cholesterol stores in the gland may be very low when secretory rate is at its highest. Such may be the state of the lipid-depleted glands after prolonged fasting followed by glucose feeding (Eger, 1942), after 2 days of oxygen lack (Darrow & Sarason, 1944), or after exposure for 2 days to $-4°$ C (Dosne & Dalton, 1941). The gland of rabbit 6 of the hexoestrol series (Table 3) is a proven example for high secretion rate with low lipid content. There is, however, a condition in which lipid loss is likely to be indicative of inhibition of hormone synthesis, and that is pantothenic acid deficiency. This has been suggested by Winters, Schultz & Krehl (1952), who also produced evidence of functional failure of the adrenal in the deficient rat. Disturbance of extra-adrenal lipid metabolism by lack in pantothenic acid was found by Deane & McKibbin (1946); they observed that fatty livers developed in their control rats but not in their deficient rats. That extra-adrenal sterol synthesis is dependent on pantothenic acid was shown by Klein & Lipmann (1953) in experiments on cholesterol synthesis by rat liver. Thus the adrenal changes may well, in part, be the result of direct inhibition of synthetic processes rather than of 'non-specific stress' alone.

Deane & Greep (1947) have suggested that the size of the cells in a lipid-depleted adrenal will indicate whether the tissue is active or non-functional, the active cells being large. This criterion does not help in diagnosing the changes produced by hexoestrol, since the cells are enlarged as a result of the stimulation by ACTH and yet inactive.

The dosage required to produce adrenal effects is not excessive. Somers (1948) has shown that severe loss of lipid from the zona fasciculata can be obtained by giving $20\mu g$/kg hexoestrol daily for 5 days. This dose is not much above the threshold for the oestrogenic effect of this substance. In the present work, $300\mu g$/kg for 2 days were found to produce both the lipid loss and the inhibition of hormone secretion. This dose is approximately that used for therapeutic purposes in cancer in man.

This leads us to the question whether the effect of hexoestrol on adrenal cortical secretion is peculiar to the rat.

It was mentioned in the introduction that the effect was not obtained in the cat, but early damage to the liver prevented the use of high doses. This was not so in the experiments on the rabbit; they tolerated doses of hexoestrol which were 500 times as high as the smallest effective dose in the rat, but neither was the adrenal histology altered by the treatment, nor was the corticosterone secretion diminished. That hexoestrol has, however, some

effect on the pituitary of the rabbit was shown by Brown-Grant (1955) who demonstrated an inhibition of the release of $^{131}$I-labelled thyroid hormone from the thyroid by 1 mg of hexoestrol. This effect was interpreted as an inhibition of secretion of thyrotrophic hormone. The author suggested that it was due either to a non-specific stressing effect or that it was linked to the oestrogenic property of the compound. In the guinea-pig, a single injection of stilboestrol (0·75 mg/kg) has been reported to increase the excretion of formaldehydogenic steroids in the urine (Zondek & Burstein, 1952). In the rat, the same early effect may occur, but it would not be detected by the present method, which measures maximal secretion under operative stress and not submaximal increase above resting level.

Though the possibility that in man cortical secretion is inhibited by oestrogens will have to be tested directly, these observations demonstrate the magnitude of species differences and the danger of not taking them into consideration.

The theory that the mechanism by which hexoestrol inhibits corticosterone secretion is inhibition of cholesterol synthesis can be investigated directly *in vitro*. Such experiments are in progress using the incorporation of labelled acetate into cholesterol in liver slices of hexoestrol-treated rats (G.S. Boyd & W. B. McGuire, to be published) and have shown that inhibition is, indeed, produced.

## SUMMARY

1. The amounts of corticosterone secreted by the left adrenal of normal male rats and of rats injected with hexoestrol were estimated quantitatively.

2. It was found that, as soon as histological signs of depletion of sudanophilic lipids by the hexoestrol became evident in the gland, secretion of corticosterone was diminished. There was no overlap between the rate of secretion of normal and of lipid-depleted glands; expressed as $\mu$g corticosterone per min per g adrenal, the mean production of hormone after no less than 3 days of hexoestrol was 31 % of the normal.

3. The inhibition by hexoestrol was completely reversible.

4. No other compound with the chemical properties of a cortical steroid was found which might have acted as a substitute for corticosterone.

5. The inhibition of secretion was not preceded by a period of excessive secretion.

6. The hypertrophy of the adrenal which develops after prolonged administration of hexoestrol is attributed to a release of ACTH by the low level of circulating corticosterone.

7. The lipid-depleted adrenal of the rat given hexoestrol responds to stress by a fall in its ascorbic acid concentration.

8. The adrenals of rabbits, unlike those of rats, were not inhibited by hexoestrol, even when much higher doses were administered.

9. It is suggested that hexoestrol might act by inhibiting cholesterol synthesis in the rat.

My thanks are due to the Medical Research Council for defraying part of the expenses of this work, and to the Moray Endowment of Edinburgh University for a grant towards equipment. It is a pleasure to thank Mr E. Horton, B.Sc., for his help in the recovery experiments. A gift of 'blue tetrazolium' from the British Drug Houses through the courtesy of Dr V. Petrow is gratefully acknowledged. I owe the progesterone to the kindness of Prof. G. F. Marrian, F.R.S., who gave me material from a gift to him by Chemical Specialties Co. Inc., New York.

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