THE EFFECT OF OESTRADIOL ON THE RESPONSE OF HYPOPHYSECTOMIZED GUINEA-PIGS TO ADRENOCORTICOTROPHIN

By R. V. BROOKS, BARBARA E. CLAYTON* and JANET M. WORDEN
From the Department of Chemical Pathology, St Thomas's Hospital Medical School, London, S.E. 1

(Received 15 May 1959)

SUMMARY

1. A method is described for the preparation and maintenance of hypophysectomized guinea-pigs.

2. Following hypophysectomy the response of female guinea-pigs to adrenocorticotrophin (ACTH) as measured by the urinary excretion of 17-ketosteroids (KS) and 17-ketogenic steroids (KGS) was reduced by 53% for KS and 59% for KGS. The decreased response was seen as early as 1 week after operation.

3. In the absence of the pituitary gland, oestradiol benzoate did not modify the response to administered ACTH as measured by the urinary excretion of KS and KGS.

It has been shown previously that following a standard dose of adrenocorticotrophin (ACTH), the urinary excretion of 17-ketosteroids (KS) and 17-ketogenic steroids (KGS) was the same in intact female or ovariectomized guinea-pigs [Clayton & Hammant, 1957]. The excretion of KGS in response to ACTH was, however, less if the guinea-pigs had been treated with oestradiol prior to receiving ACTH. The KS response was not affected by the administration of oestradiol. It was suggested that there might be a direct inhibition of adrenocortical hormone production in response to ACTH as a result of treatment with oestrogens.

It is generally believed that oestrogens do not affect adrenal function in the absence of the pituitary gland. Thus Bourne & Zuckerman [1940] found that the adrenals of hypophysectomized rats atrophied, even though oestrogen was given. Oestrogen causes depletion of lipids in the adrenals of intact rats, but this does not occur in hypophysectomized rats [Selye, Collip & Thomson, 1935; Selye & Collip, 1936]. Gemzell [1952] found increased secretion and formation of ACTH by the pituitary glands of rats treated with oestrogen, and considered that there was a direct effect of oestrogen on the pituitary. Diethylstilboestrol causes an increase in liver glycogen in the intact rat but not in the adrenalectomized [Janes & Nelson, 1942; Long, 1942] or hypophysectomized one [Janes & Nelson, 1942].

The purpose of the studies reported in this paper was to discover whether oestradiol could affect the excretion of urinary steroids in response to administered ACTH in the absence of the pituitary gland. For reasons already given [Clayton & Hammant, 1957], the guinea-pig was the species of choice.

* Present address: The Hospital for Sick Children, Great Ormond Street, London, W.C. 2.
MATERIALS AND METHODS

Animals

A mixed strain of female guinea-pigs obtained from one M.R.C. accredited breeder was used. At the time of hypophysectomy they weighed 248–413 g, were growing rapidly but were capable of reproducing. When injected with ACTH for the first time, hypophysectomized guinea-pigs weighed 358–460 g and the corresponding intact animals weighed 358–453 g. Intact guinea-pigs previously treated with cortisol (see Experiments and Results, section 4) weighed 417–584 g when injected with ACTH. In order to avoid variations due to stress, animals were placed singly in their metabolism cages for 5 days before ACTH tests were begun, and were handled by the same two people throughout each experiment. The animal laboratory was maintained at a temperature of 70°F.

Diet

All guinea-pigs were fed ad lib. on Rowett cubes, cabbage and tomato juice; intact animals were supplied ad lib. with tap water and hypophysectomized ones with a solution containing 8 % glucose and 1 % sodium chloride (w/v). The tomato juice was made by mixing approx. 50 g condensed tomato purée (Cirio brand) with 1 l. tap water. When the animals were in metabolism cages the cubes were crushed to a paste with tap water to avoid contamination of the cages.

In addition, during the first 2 weeks after hypophysectomy, each guinea-pig was given 0·25 g glucose dissolved in 0·5 ml. distilled water twice daily by mouth from a pipette. Following operation, hypophysectomized guinea-pigs would frequently not eat well, and had to be fed individually with tomato juice from a pipette.

Collection of urine and determination of urinary steroids

All 24 hr collections of urine were begun at about 10 a.m. Urine was collected into flasks containing a few drops of chloroform and standing in vacuum flasks containing solid carbon dioxide. After completion, urine collections were stored at −15°C until required, when they were thawed under cold running water and used immediately. Urinary KS and KGS were determined by a slight modification [to be published] of the method of Norymberski, Stubbs & West [1953] and Gibson & Norymberski [1954]. Androsterone was used as a standard.

Chromatography of urinary corticosteroids

The pH of the urine was adjusted to 6 with concentrated HCl. The urine was then filtered and the filtrate extracted three times with 2 vol. ethyl acetate. Aliquots of this extract were chromatographed in the C system of Bush [1952] for 4 hr and for 16 hr. Quantitative estimation of steroids was made by measuring the soda-fluorescence on the paper chromatogram in a fluorimeter.

Technique of hypophysectomy

Operation was performed under ether anaesthesia, following 0·65 mg atropine sulphate i.p. about 15 min previously. The pituitary was removed by the parapharyngeal approach. The hole in the pituitary fossa was made with a no. 12 round dental
burr, and the gland was sucked out under direct vision at a pressure of 650–750 mm Hg. The burr hole was closed with a small piece of gelatin sponge. It was unnecessary to cannulate the trachea.

Maintenance of hypophysectomized guinea-pigs

It was very difficult to obtain good survival rates without using maintenance therapy with ACTH or cortisol postoperatively.

The two hypophysectomized guinea-pigs in section 2 received no maintenance therapy at all, and the first ACTH test was performed 1 week after operation.

For all other hypophysectomized guinea-pigs, one of two methods of maintenance has been used. In the first method, which was used for ten of the guinea-pigs in section 3, ACTH (various batches of h.p. Acthar gel, Armour Laboratories Ltd) was administered s.c. twice daily in a dosage of 5–20 Armour units for 1 week. (Armour h.p. Acthar gel is standardized so that the average human physiological response to 1 Armour u. is equivalent to that obtained from 1 U.S.P. u. or 1 i.u. depending on the state of the animal.)

By trial and error, the second method of maintenance was elaborated. It has been used for all other animals in experiments described in this paper, and is considered the method of choice. Hypophysectomies were always performed on a Monday or Tuesday. In the late afternoon of the day of operation, the guinea-pigs were given 6 mg cortisol acetate suspension by mouth. This treatment was repeated twice daily for 2 weeks, except that the second dose on each Saturday was given i.p. and no dose was given on Sundays. Occasionally treatment was continued for a further 3–4 days if the clinical state of the animal appeared to warrant it. Thereafter, the guinea-pigs survived without further maintenance therapy for varying periods, frequently for many months. Ultimate death was probably due to hypoglycaemia, and could sometimes be prevented by the administration of cortisol acetate and glucose again for 2 or 3 days.

No hypophysectomized guinea-pigs used in the experiments reported in this paper had received any treatment with cortisol acetate or ACTH for at least 2 weeks before an ACTH test was performed.

Criteria for the completeness of hypophysectomy

Hypophysectomized guinea-pigs were chosen for the experiments if they showed: (a) A smaller weight gain than intact animals. (The guinea-pig is peculiar in exhibiting growth following hypophysectomy [Clayton, 1959; Knobil & Greep, 1959],)

(b) Cessation of oestrous cycles, atrophy of the nipples and external genital organs.

(c) Maintenance of a soft fur as compared with the coarse fur developing in intact guinea-pigs as they become older.

At varying intervals after completion of the experiments the animals were killed and the pituitary fossae were examined macroscopically to check the completeness of hypophysectomy. In addition, the pituitary fossae of five animals from section 3 were selected at random and serially sectioned at a thickness of 4 µ to confirm the absence of pituitary tissue. The ovaries and uteri of all animals were checked.
for atrophy; the right adrenal glands were weighed, since the adrenal should not be enlarged above its initial weight at the time of hypophysectomy, i.e. 98 ± 21 mg. In practice, an incompletely hypophysectomized guinea-pig is easily detected.

Other treatments

ACTH test: 10 Armour u. of H.P. Aerthar gel were given s.c. twice daily at 10 a.m. and 4.30 p.m. for 2 days. The batch numbers of the ACTH preparations were 22586 for all tests in sections 1, 3, 4 and 6, and P 36102 for all tests in section 2.

Oestradiol monobenzoate: 0.25 mg oestradiol monobenzoate in oil (British Drug Houses Ltd) was given s.c. once daily at 10 a.m. except on Sundays.

The days on which ACTH and oestrogen were given are noted in the experimental sections.

Statistical analysis

Tests for significance were made by standard statistical procedures [Finney, 1952].

EXPERIMENTS AND RESULTS

Section 1. Response of intact female guinea-pigs to ACTH

ACTH was administered for 48 hr to nine intact guinea-pigs. Urine was collected from each guinea-pig during the 48 hr of administration of ACTH, and KS and KGS were determined on each collection.

The mean individual response to ACTH was 1.11 ± 0.25 mg KS and 1.09 ± 0.67 mg KGS/24 hr.

Section 2. Effect of length of time since hypophysectomy on the response to ACTH

ACTH tests were performed on two hypophysectomized guinea-pigs (which had received no ACTH or cortisol) at 1, 2, 3 and 7 weeks after operation. Two intact guinea-pigs of approximately the same initial age and weight were also tested repeatedly at the same times.

The results are shown in Fig. 1. The smaller response of the hypophysectomized guinea-pigs compared with the intact ones was apparent after 1 week. Control animal Xg temporarily had a mild infection (probably Salmonella enteriditis), and this probably accounted for the poor response to the ACTH test performed after the 2nd week.

It was concluded that 5–7 weeks after hypophysectomy would be a satisfactory time to test the influence of oestradiol on the response to ACTH.

Section 3. Effect of oestrogen on response of hypophysectomized guinea-pigs to administered ACTH

Fourteen separate similar experiments employing pairs of guinea-pigs were carried out and each was planned as shown in Table 1. Day 1 was the day on which the experiment was begun and was in fact 23–40 days after operation, i.e. the ACTH test was performed on the 35th to 52nd day after operation. The mean interval between stopping cortisol and commencing oestradiol was 23 days. Twenty-eight hypophysectomized guinea-pigs were used in all.
Urine was collected from each guinea-pig during the 24 hr prior to the ACTH test and during the 48 hr of administration of ACTH. KS and KGS were determined on each 48 hr collection, and on some of the 24 hr collections (see section 5).

Oestradiol was very toxic to hypophysectomized guinea-pigs and it was observed that those treated with it ate less food. Four experiments had to be abandoned as the oestrogen-treated guinea-pig of each pair died. Ten experiments were completed.

The results are given in Table 2. They show that there was no significant difference between the response to ACTH only, and ACTH after treatment with oestradiol. Therefore oestradiol did not modify the response to ACTH in the absence of the pituitary.

Table 1. Plan of each experiment in section 3

<table>
<thead>
<tr>
<th>Day of expt.</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-11</td>
<td>ACTH only</td>
</tr>
<tr>
<td>12</td>
<td>ACTH</td>
</tr>
<tr>
<td>13</td>
<td>ACTH + oestradiol</td>
</tr>
<tr>
<td>12</td>
<td>No treatment</td>
</tr>
<tr>
<td>13</td>
<td>Oestradiol</td>
</tr>
<tr>
<td>12-13</td>
<td>ACTH</td>
</tr>
<tr>
<td>13</td>
<td>ACTH</td>
</tr>
</tbody>
</table>

Hypophysectomized guinea-pigs excreted less steroids compared with the intact ones (section 1), the response being reduced by 53 and 59% for KS and KGS, respectively. These decreases in excretion were highly significant ($P < 0.001$ for KS; $P = 0.001$ for KGS). There was no indication that the response to ACTH varied in magnitude during the interval of 35–52 days after hypophysectomy.

In one further experiment a pair of hypophysectomized guinea-pigs was treated as shown in Table 1, and the urinary corticosteroids were examined by paper chro-
matography. The guinea-pig receiving oestrogen excreted 5 µg cortisol/day and the untreated one 9 µg. When ACTH was administered to both, the oestrogen-treated guinea-pig excreted 50 µg, and the animal receiving ACTH alone 130 µg cortisol. The chromatograms on the 2 days during which ACTH was given showed, in addition, a spot less mobile than cortisol which also reduced blue tetrazolium and gave the yellow soda-fluorescence characteristic of the Δ4–3 ketone grouping. Increasing the time of running of the chromatogram from 4 to 16 hr resulted in the resolution of this polar zone into five separate spots. Two of these spots gave both the reducing and soda-fluorescence reactions and had \( R_f \) values agreeing with those of \( \beta \)-hydroxy- and 2α-hydroxycortisol. The three remaining spots gave only the soda-fluorescence reaction.

Table 2. Response of hypophysectomized guinea-pigs to ACTH, and effect of pretreatment with oestradiol

(All excretions are means ± s.d.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Excretion of steroid (mg/guinea-pig/24 hr)</th>
<th>Mean difference in response to ACTH only and ACTH + oestradiol and probabilities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KS</td>
<td>KGS</td>
</tr>
<tr>
<td>ACTH</td>
<td>0.49 ± 0.22</td>
<td>0.42 ± 0.19</td>
</tr>
<tr>
<td>ACTH + oestradiol</td>
<td>0.35 ± 0.18</td>
<td>0.37 ± 0.31</td>
</tr>
</tbody>
</table>

Ten guinea-pigs received ACTH and ten received ACTH + oestradiol.

Section 4. Response of intact guinea-pigs previously treated with cortisol to ACTH and to ACTH + oestradiol

In section 3, the hypophysectomized guinea-pigs had previously received cortisol. It was conceivable that it was the administration of cortisol for 2 weeks after hypophysectomy and not the absence of the pituitary which had abolished the ability of the guinea-pig to respond differently to ACTH + oestradiol than to ACTH alone.

Table 3. Plan of eleven similar experiments described in section 4

<table>
<thead>
<tr>
<th>Day of expt.</th>
<th>ACTH only</th>
<th>ACTH + oestradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–14</td>
<td>Cortisol</td>
<td>Cortisol</td>
</tr>
<tr>
<td>15–38</td>
<td>No treatment</td>
<td>No treatment</td>
</tr>
<tr>
<td>39–49</td>
<td>No treatment</td>
<td>Oestradiol</td>
</tr>
<tr>
<td>50</td>
<td>ACTH</td>
<td>ACTH</td>
</tr>
<tr>
<td>51</td>
<td>ACTH</td>
<td>ACTH</td>
</tr>
</tbody>
</table>

Eleven experiments employing pairs of intact guinea-pigs were performed as shown in Table 3. A total of twenty-two guinea-pigs was used. The interval of 15–38 days (see Table 3) was the mean time between stopping cortisol and starting oestradiol in the hypophysectomized guinea-pigs used in the experiments in section 3. Urinary KS and KGS were determined for each guinea-pig during each 48 hr of ACTH administration.

The results are given in Table 4. They show that the response to ACTH + oestradiol
HYPOPHYSECTOMY AND ADRENAL RESPONSE

was significantly different from that in response to ACTH alone. Therefore oestradiol modified the response to ACTH, even though the guinea-pigs had previously received cortisol.

Table 4. *Response of intact guinea-pigs, previously treated with cortisol, to ACTH and ACTH + oestradiol*

(All excretions are means ± s.d.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Excretion of steroid (mg/guinea-pig/24 hr)</th>
<th>Mean difference in response to ACTH only and ACTH + oestradiol and probabilities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KS</td>
<td>KGS</td>
</tr>
<tr>
<td>ACTH</td>
<td>0.83 ± 0.25</td>
<td>0.81 ± 0.28</td>
</tr>
<tr>
<td>ACTH + oestradiol</td>
<td>0.82 ± 0.39</td>
<td>0.58 ± 0.34</td>
</tr>
</tbody>
</table>

Eleven guinea-pigs received ACTH, and eleven received ACTH + oestradiol.

*Section 5. Urinary excretion of steroids by hypophysectomized guinea-pigs prior to receiving ACTH*

In five of the experiments described in section 3, urinary steroids were determined on day 11 immediately before treatment with ACTH.

The mean excretion of KS and KGS by guinea-pigs which had received no oestrogen was 0.21 ± 0.08 and 0.15 ± 0.08 mg/24 hr, respectively. In those which had received oestrogen the corresponding excretions were 0.13 ± 0.06 and 0.14 ± 0.10 mg/24 hr for KS and KGS, respectively.

The mean excretion of KS and KGS by normal intact guinea-pigs from the same colony is 0.25 ± 0.02 and 0.13 ± 0.02 mg/24 hr [unpublished observation]. Urinary extracts from baseline urines are impure, and it would be unwise when dealing with these small amounts to conclude more than that no great change in resting excretion has followed hypophysectomy. The excretion of KS has in fact apparently been lowered by oestradiol ($P = 0.04$), but at these low levels of excretion it is doubtful whether any significance should be attached to this finding.

Table 5. *Time since hypophysectomy and response to ACTH*

<table>
<thead>
<tr>
<th>Guinea-pig</th>
<th>Interval between beginning of experiment and ACTH test (weeks)</th>
<th>Excretion of steroid in response to ACTH (mg/guinea-pig/24 hr)</th>
<th>Body weight at time of ACTH test (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Hypox.</td>
<td>7¼</td>
<td>KS</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>KGS</td>
<td>0.54</td>
</tr>
<tr>
<td>B. Hypox.</td>
<td>7¼</td>
<td>KS</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>KGS</td>
<td>0.73</td>
</tr>
<tr>
<td>C. Intact</td>
<td>7¼</td>
<td>KS</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>KGS</td>
<td>1.77</td>
</tr>
<tr>
<td>D. Intact</td>
<td>7¼</td>
<td>KS</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>KGS</td>
<td>1.60</td>
</tr>
</tbody>
</table>

(At the beginning of the experiment, two guinea-pigs were hypophysectomized (hypox.) and two were left intact.)
Section 6. Further experiment on the effect of time since hypophysectomy on the response to ACTH

Two guinea-pigs from section 3, which had received no oestrogen and had had an ACTH test 52 days after hypophysectomy, were given a further ACTH test 14½ weeks later (i.e. a total of 22 weeks after hypophysectomy). Similarly, two intact guinea-pigs from section 1 were given a further ACTH test 14½ weeks after the first test.

The results show that whereas with increasing age the response of the intact guinea-pigs to ACTH was greater, that of hypophysectomized guinea-pigs was slightly less (Table 5).

DISCUSSION

When investigating the interrelation between two glands or one gland and a specific hormone there is always the possibility that the picture may be confused by the fact that the observed effects might be mediated via the pituitary gland. The experiments previously reported by Clayton & Hammant [1957] and Nadel, Young, Hilgar & Burstein [1958] on the modification of response to ACTH by oestradiol and stilboestrol, respectively, were performed in intact guinea-pigs. It has now been shown that in the absence of the pituitary gland, oestradiol does not affect the response to ACTH as measured by the urinary output of steroids. The administration of oestradiol to intact guinea-pigs previously treated with cortisol did not inhibit the ability of the oestrogen to modify the response to ACTH. The lack of effect of oestradiol in hypophysectomized guinea-pigs, therefore, would appear to be due to the absence of the pituitary and not to previous treatment with cortisol. A similar result was observed by McKerns [1957] in rats; during in vitro studies, adrenal secretion was not inhibited by oestradiol and he concluded that the effect must normally be mediated via the pituitary gland. He found, on the other hand, that ethinyl oestradiol and stilboestrol strongly inhibited adrenocorticoid secretion directly.

Chromatographic studies have shown no qualitative differences in the excretion of corticoids in response to ACTH and to ACTH + oestradiol. This is in agreement with the findings of Nadel et al. [1958] in intact guinea-pigs. The chromatograms performed in the one experiment in section 3 showed less cortisol in the oestrogen-treated guinea-pig; this may be accounted for by the fact that the level of excretion of KGS after ACTH + oestradiol has a large standard deviation.

Observations have been made in man and the rat [Vogt, 1955; Holzauer, 1957] on the effect of oestrogen on adrenal function, the pituitaries being intact. In human subjects, it was shown by Taliaferro, Cobey & Leone [1956] and Wallace, Silverberg & Carter [1957] that the administration of stilboestrol and oestradiol, respectively, led to a rise in the levels of plasma 17-hydroxycorticoids. Daughaday [1956, 1958] described the binding of corticosteroids and related hormones by human plasma and plasma protein fractions, and Chen, Mills & Bartter [1958] have suggested that oestrogen increases the quantity of specific binding protein for cortisol. Whether or not the lowered urinary excretion of KGS in response to ACTH and oestradiol in the intact guinea-pig is the result of a similar mechanism must await further experiments. If it is, then it would appear that the production of specific binding protein may depend on the pituitary.
Although the rat and guinea-pig are both rodents, it is not even certain that the mechanism of inhibition of adrenal function [Vogt, 1955] is the same in the two species [Clayton & Hammant, 1959], since the histological changes observed in the adrenals of rats and guinea-pigs after oestrogen treatment are very different.

The lowered response of the adrenal of the hypophysectomized guinea-pig to ACTH was to be expected, since following hypophysectomy in other species the adrenal loses much of its sensitivity to ACTH. For example, in hypophysectomized rats relatively large amounts of ACTH are required to produce adrenal repair after allowing atrophy to occur for 2 weeks [Greenspan, Li, Simpson & Evans, 1950]. Similarly, Nelson [1956] found that a single i.v. injection of ACTH to a dog hypophysectomized for some weeks failed to produce a corticoid response. When, however, such an injection had been preceded by 9 days treatment with ACTH, a response was obtained. These observations in the rat and dog are dependent, too, on the fact that the adrenals atrophy following hypophysectomy. In contrast, atrophy of the adrenals is small or absent in the guinea-pig [Schweizer & Long, 1950; Clayton, 1959], and this may account for the retained, albeit reduced, ability to respond to ACTH.

The determination of urinary steroids as a measure of adrenal function is always open to the criticism that the metabolites in the urine do not necessarily directly represent the secretion by the adrenals. In the guinea-pig it is not yet known what proportion of the daily production of steroids by the adrenals appears as metabolites in the urine.

The toxicity of oestrogen in hypophysectomized guinea-pigs was noticed previously by Hill & Stalker [1942], and death of treated animals was associated with hypoglycaemic coma. It was suggested that the disturbance in blood sugar might result from a direct effect of oestrogen on metabolism. Since, however, the animals were free to eat at will, and since oestrogen leads to a reduction in food intake [e.g. Ingle, 1949], this might explain the observed fall in blood sugar. The toxic effect even in intact animals is well known and has been demonstrated, for example, by Campbell, Bern & Deome [1956], who studied the effect of oestrogen on the resistance of mice to stress. The marked tendency of newly hypophysectomized guinea-pigs to develop severe hypoglycaemia leading to coma and death has been commented on also by Hill & Stalker [1942]. By careful postoperative care, particularly with reference to diet and maintenance therapy with ACTH or cortisol, it has been possible to overcome this difficulty.

We are grateful to Miss P. M. Jacobs for her technical assistance with these experiments.

The ACTH and cortisol used in these experiments were a gift from The Medical Research Council.

REFERENCES