Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 407–411. Pergamon Press. Printed in Great Britain

PRODUCTION OF TESTOSTERONE AND CORTICOSTEROIDS BY THE RAT ADRENAL GLAND INCUBATED *IN VITRO* AND THE EFFECTS OF STIMULATION WITH ACTH, LH AND FSH

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(*Received* 18 *September* 1975)

SUMMARY

Using techniques of *in vitro* incubation it was found that the Wistar rat adrenal has the capacity to synthesise testosterone from added radioactive precursors and endogenous precursors.

With [^{14}C]-progesterone as added precursor the formation of labelled testosterone was demonstrated by methods including chromatography in various systems, the formation of derivatives and isotope dilution. In 2 h incubations of female glands the yield of labelled testosterone was 0.01–0.04% of the added precursor, i.e. about 1% of that of corticosterone.

From endogenous precursors, testosterone production was measured by RIA. Confirmation of the values was obtained by cross checking with values obtained with the samples in another laboratory (using RIA with a different antiserum), and by comparison with a g.l.c. method based on the diheptofluorobutyrate derivative with electron capture detection.

The capacity of the female gland to produce testosterone was approximately twice that of the male (per mg tissue), and yields were approximately 1% of those of total corticosteroids as measured by CPB. Addition of FSH or LH to the incubation media in varying concentrations up to 0.6 and 0.4 μg/ml respectively had no sustained effect on testosterone output, whereas ACTH (0.2 μg/ml) tripled the output from female glands. Corticosteroid output was stimulated by all three tropic hormones similarly.

The results offer an explanation for reports in the literature showing the partial dependence of circulating testosterone levels on the presence of the adrenal and the variation of corticosteroid output with the stage of the oestrous cycle in rats.

INTRODUCTION

It has long been accepted that the mammalian adrenal cortex is a source of androgens as well as corticosteroids. In the human adrenal for example, dehydroepiandrosterone (DHA) is a prominent secretory product especially as the sulphate [1], and in the human female, androstenedione secretion by the adrenal cortex may contribute significantly through peripheral conversion to the total pool of circulating testosterone [2] and oestrogens [3]. Other C_{19} steroids which may be secreted by the adrenal cortex in man and other species include 11β-hydroxyandrostenedione and, in lower yields than the others, testosterone [4–7].

Secretion of androgens by the rat adrenal cortex has received little direct study. This may have occurred because most reports state that 17α-hydroxylation was not detected when using rat adrenal preparations [8, 9] and this process is normally regarded as essential in androgen biosynthesis [10]. However, strong circumstantial evidence for androgen production by the rat adrenal cortex stems from the studies of Kniewald *et al.* [11]. They found that circulating plasma levels of testosterone were elevated in the period immediately after castration and only later decreased to the very low values normally found. On the other hand, circulating testosterone levels were reduced rapidly after adrenalectomy. These findings suggest either that the adrenal cortex secretes testosterone directly or possibly that there may be some interaction between adrenals and testis in testosterone formation.

Studies on the production of testosterone by rat adrenal tissue from radioactive precursors have yielded conflicting evidence. In some experiments, no formation of C_{19} steroids was obtained from cholesterol, pregnenolone or 17α-hydroxyprogesterone [12] whereas in others, transformations of pregnenolone into dehydroepiandrosterone, androsterone, testosterone and 11β-hydroxyandrostenedione was reported [13, 14].

This paper re-examines the capacity of the rat adrenal gland to secrete testosterone *in vitro*, and the effect of trophic hormones on testosterone and corticosteroid production.

MATERIALS AND METHODS

Incubations. White Wistar rats (200–250 g body wt) were killed by cervical dislocation and the adrenals quickly removed, cleaned and stored on ice until

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required for incubation, which invariably followed within 1 h. Three series of incubations were performed.

Experiment 1. Conversion of [^{14}C]-progesterone. Ten pairs of female rat adrenals were minced and incubated in 10 ml Krebs bicarbonate Ringer containing [4-^{14}C]-progesterone (1 μCi: S.A. 193 μCi/mg) for 2 h at 37° C. Incubation medium and tissue were then exhaustively extracted with ethyl acetate and the products were fractionated in the systems of chromatography previously described [15]. Further procedures with this material are given in Results.

Experiment 2. Production of steroids from endogenous precursors and the effects of trophic hormones. Individual pairs of adrenals were incubated in 5 ml Krebs bicarbonate Ringer (containing 0.33 M Ca^{2+}) for 30 min at 37° C. At the end of this period the incubation medium was discarded and fresh medium added, with trophic hormones when appropriate (see below). Incubation then proceeded for a further 2 h. Tissue and medium were then homogenised and in some experiments samples were taken for the estimation of protein [16]. Steroids were exhaustively extracted with ethyl acetate and appropriate aliquots taken for assay.

Experiment 2a. Adrenals from six male rats, six control female rats and three groups of six female rats with addition to each 5 ml incubation medium of either 0.4 μg LH, 2 μg FSH, or 1 μg ACTH were incubated as described above.

Experiment 2b. Adrenals from female rats were incubated with varying doses of LH (0, 0.1, 0.2, 0.5 and 2 μg/5 ml) and FSH (0, 0.1, 0.2, 0.5, 2.0 and 3.0 μg/5 ml) (six incubations per dose).

Experiment 3. Interaction of adrenal and testis tissue. Interstitial tissue was prepared from rat testes by microdissection [17]. Six 10 mg batches were separately incubated in 5 ml Krebs bicarbonate Ringer for 2 h at 37° C. In another series of incubations six pairs of adrenal glands from the same animals were incubated separately and in a third series the two tissue types were incubated together. Steroids were extracted at the end of the incubation period with ethyl acetate.

Steroid assay. A competitive protein binding assay based on dog plasma transcortin was used to give an index of total corticosteroid output [18]. In one set of experiments (Experiment 2a) a radioimmunoassay, based on an antiserum raised to the 3-carboxymethoxime derivative of corticosterone, was used to test the validity of the competitive protein binding assay. The antiserum was generously donated by Drs. V. Martin and C. W. Edwards and is described by Gross *et al.* [34]. It shows cross reactivity with deoxycorticosterone (24%) but no significant reactivity with other steroids likely to be produced by rat adrenals. The antiserum was used at a concentration of 1:6000 and was equilibrated with the extracts and standards overnight at 4° C. Separation of free and bound steroid was performed on Sephadex minicolumns [18].

For testosterone a radioimmunoassay based on an antiserum raised to the 3-carboxymethoxime derivative of testosterone was used as previously described [18]. The antiserum was generously donated by Dr. D. Exley. It has properties exactly similar to other testosterone-3-carboxy-methoxime derived antisera [35] and has negligible cross reactivity with a wide variety of C_{21}, C_{19} and C_{18} steroids, except for a 20% reaction with 5α-dihydrotestosterone. To provide verification of the results, duplicate samples of the extracts from Experiment 3 were estimated in the laboratory of Dr. W. P. Collins using an antiserum raised to the carboxyethyl thioether derivative of 1α-hydroxytestosterone, which was characterised by the usual methodology [36], and gives negligible cross reactivity with a wide variety of other steroids, with the exception of 5α-dihydrotestosterone (10–15% cross-reactivity). In addition, total extracts in Experiment 1 were treated with heptafluorobutyric anhydride in acetone [19] and samples were subjected to g.l.c. using a Packard chromatograph (model 7839). The glass columns used were 2 ft long with a packing of 0.3% XE-60 on Gas Chrom. Q. Chromatograms were run at a temperature of 210° C. Peaks corresponding to testosterone diheptafluorobutyrate were measured and augmented without loss of symmetry by the addition of authentic material.

RESULTS

Formation of radioactive steroids (Experiment 1). In extracts of samples incubated with [4-^{14}C]-progesterone, material corresponding to testosterone on paper chromatography in the systems toluene: 70% methanol and light petroleum (B.P. 60–80°): 75% methanol was acetylated with acetic anhydride and pyridine, and rechromatographed in the light petroleum: 75% methanol system. It was then eluted, hydrolysed with NaOH, oxidised with *tert.* butyl chromate, and rechromatographed in the same system. Authentic [^{3}H]-androstenedione was added to the material corresponding to androstenedione, and the mixture rechromatographed in thin layer silica gel films (Eastman chromagram) in the systems light petroleum–benzene–ethylacetate (1:1:4 by vol.); chloroform–methanol (97:3 v/v) and toluene–methanol (96:4 v/v). Successive ^{3}H/^{14}C values were 28.3, 30.1 and 26.0, which (in view of the low yields obtained) were regarded as being sufficiently close to indicate homogeneity of the biosynthetic and authentic materials. Corticosterone was isolated as previously described [20] and both products were estimated after chromatography as the acetates.

Yields of testosterone were approximately 0.01–0.04% of the added [^{14}C]-progesterone precursor, compared with 1–4% yields for corticosterone.

Formation of testosterone from endogenous precursors (Experiments 2a–b). Using samples of incubations of female adrenals (controls and stimulated by ACTH and gonadotrophin), values obtained by the competitive protein binding for corticosteroids show

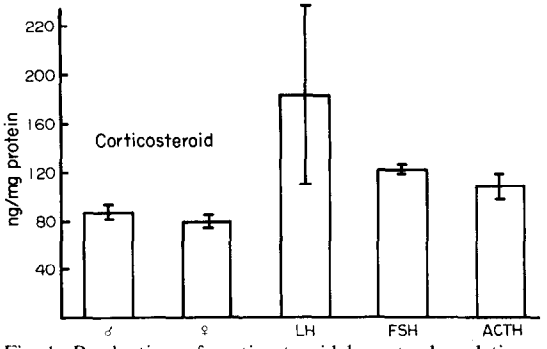

Fig. 1. Production of corticosteroid by rat adrenal tissue incubated *in vitro* for 2 h (1 pair glands/5 ml Ringer/flask). Values expressed as ng corticosteroid produced/mg protein, are means of 6 incubations ± S.E. Experiments with additions of trophic hormones were performed with female tissue only and the additions were either 1 μg ACTH, 0.5 μg LH or 2 μg FSH/flask. Comparison of ACTH addition with female controls, $P < 0.02$; comparison of FSH addition with female controls, $P < 0.001$.

satisfactory correlation ($r = 0.7$) with the radioimmunoassay for corticosterone although the CPB data were systematically higher than the RIA data by a factor of at least 1.5. This may be anticipated in view of the affinity of transcortin for many types of corticosteroid.

Values for testosterone obtained by use of the routine radioimmunoassay gave a good correlation with values obtained by Dr. W. P. Collins ($r = 0.93$; slope 0.95). Similarly, values obtained from the peak heights in the g.l.c. procedures gave a correlation coefficient of 0.7 with the RIA (the loss of correspondence here when compared with the RIA is almost certainly due to the lower accuracy of the g.l.c. method in which internal standards were not routinely used).

Both male and female adrenals produced testosterone from endogenous precursors (Fig. 2). In these experiments it should however be noted that the female glands had a greater capacity to produce testosterone than the male. When expressed per mg protein, the female capacity was approximately twice that of the male (570 pg/mg vs 210 pg/mg). It should also be borne in mind that the female gland is at least 25% larger than the male, hence the overall capacity of the female adrenal to produce testosterone *in vitro* was greater still.

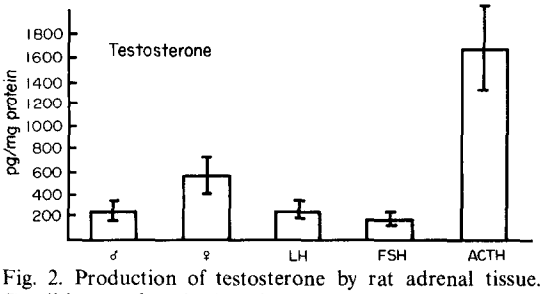

Fig. 2. Production of testosterone by rat adrenal tissue. Conditions as for Fig. 1. Values, expressed as pg testosterone produced/mg protein, are means of 6 incubations ± S.E. Comparison of male and female, $P < 0.05$; comparison of ACTH addition with female controls, $P < 0.02$.

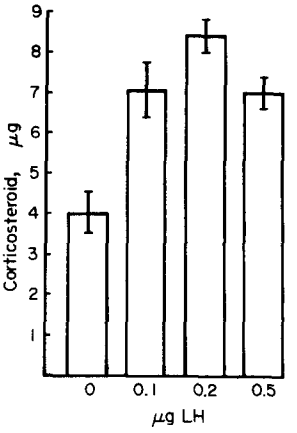

Fig. 3. Production of corticosteroid by female rat adrenal tissue. Conditions as for Fig. 1. Additions were 0.1, 0.2 or 0.5 μg LH/flask. Values expressed as total corticosteroid produced/incubation, are means of 6 incubations ± S.E. Comparison of maximum stimulation with controls, $P < 0.001$.

Effects of stimulation. From Fig. 1 (Experiment 2a) it can be seen that when expressed per mg protein the production of corticosteroid (measured by CPB) by male and female adrenals is similar. When 0.1 μg/ml LH or 0.4 μg/ml FSH or 0.2 μg/ml ACTH was added to female tissue similar stimulation of corticosteroid production was observed. (Although the mean values between control and LH stimulated incubations appear different, these differences are in this case not statistically significant, but see Fig. 3). In contrast, no effect of the gonadotrophins was seen on testosterone production (Fig. 2) which was greatly stimulated by ACTH.

Maximal output of corticosteroid production was obtained under gonadotrophin stimulation with additions of about 0.2 μg LH (Fig. 3) and 2 μg FSH (Fig. 4) (Experiment 2b). In contrast no sustained stimulation of testosterone was found with either gonadotrophin at any concentration (Figs. 5 and 6).

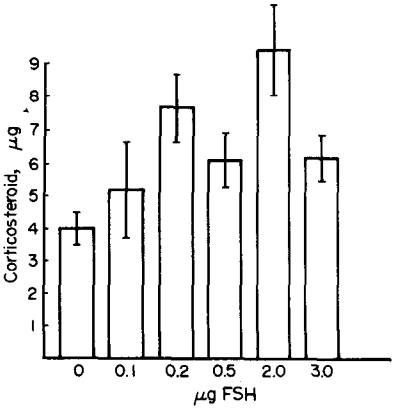

Fig. 4. Production of corticosteroid by female rat adrenal tissue. Conditions as for Fig. 1. Additions were 0.1, 0.2, 0.5, 2.0 or 3.0 μg FSH/flask. Values, expressed as total corticosteroid produced/incubation, are means of 6 incubations ± S.E. Comparison of maximum stimulation with controls, $P < 0.01$.

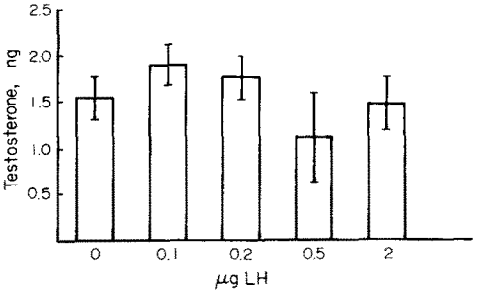

Fig. 5. Production of testosterone by female rat adrenal tissue. Conditions as for Figure 1. Additions were 0.1, 0.2, 0.5 or 2 μg LH/flask. Values, expressed as total testosterone produced/incubation, are means of 6 incubations ± S.E.

Mixed tissue incubations. (Experiment 3). In Fig. 7 it can be seen that the incubation of adrenal tissue and interstitial testis tissue together gave no increases in testosterone production beyond that expected by the addition of the products of the two tissues incubated separately.

DISCUSSION

It now seems clear that the rat adrenal cortex, like those of other mammalian species, has the capacity to synthesise and secrete testosterone *in vitro*. The results reported here not only show the formation of testosterone from radioactive progesterone but also its secretion under different conditions of stimulation from endogenous precursors. This confirms and extends the results of other workers [13, 14, 21] who have demonstrated the production of androgens from radioactive pregnenolone by rat and also mouse adrenal tissue. The pathway for formation of testosterone has not been studied and requires investigation but the yields of testosterone relative to other steroids are so small as to make this difficult. However the formation of androgens presumably proceeds via 17α-hydroxylation and it has been suggested this process may be increased when the 11β-hydroxylase system is inhibited [22], which may prove a useful experimental approach.

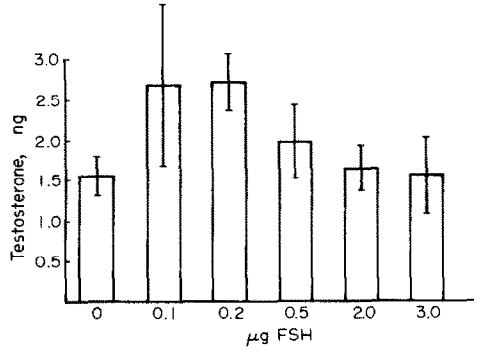

Fig. 6. Production of testosterone by female rat adrenal tissue. Conditions as for Fig. 1. Additions were 0.1, 0.2, 0.5, 2.0 and 3.0 μg FSH/flask. Values, expressed as total testosterone produced/incubation, are means of 6 incubations ± S.E.

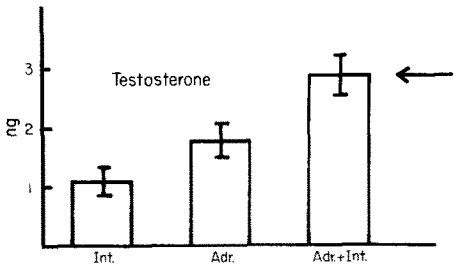

Fig. 7. Production of testosterone by rat testis interstitium (10 mg tissue/flask) and rat adrenal tissue (1 pair glands/flask) incubated *in vitro* for 2 h both separately and together. Values, expressed as total testosterone produced/incubation, are means of 6 incubations ± S.E. Arrow indicates the sum of the amounts of testosterone produced by the individual tissues incubated separately.

Effects of ACTH. The fact that testosterone production is stimulated by the addition of ACTH to the incubation medium is in agreement with the studies of other authors on the production of adrenal androgens in other species. In man for example, ACTH increased DHA and DHAS secretion [23–25] *in vivo* and *in vitro* [26]. Higher concentrations of androstenedione and testosterone (and oestrogen) were found in human adrenal vein blood than in circulating plasma [6] and these too were increased by ACTH administration. On the other hand, *decreased* levels of circulating testosterone after ACTH treatment has been shown in man [27, 28] and the guinea-pig [29]. It is possible that peripheral conversions of the androgens may complicate the issue here. However, in one marsupial species, *Trichosurus vulpecula*, ACTH administration caused a decline in circulating androgen which in this case was consistent with changes *in vitro* [18]. In another, *Didelphis virginiana*, ACTH was without effect either on circulating androgens or on androgen production *in vitro* [30].

It is therefore possible that species variation may account for some of these anomalies (see below).

The relative lack of effect of ACTH on corticosteroid production in these experiments is somewhat unexpected in view of the literature on ACTH effects *in vitro*. Studies in progress suggest this arises from the nature of the methodology (currently many authors use either superfusion or cell suspension techniques which can modify the response) and the precise source of the tissue (Vinson, unpublished observations).

Effects of gonadotrophins. The stimulation of corticosteroid output by both LH and FSH in these experiments is unexpected. While evidence based on morphological and histological studies has in the past suggested the possibility of such an effect [31, 32], direct evidence has been lacking. The phenomenon offers an explanation for the increases in corticosteroid secretion seen in prooestrous rats [33]. In experiments with *Didelphis virginiana*, stimulation of corticosteroid production by the addition of LH *in vitro* has already been reported [30]. The lack of effect of

the gonadotrophins on testosterone production by rat adrenals is consistent with the general body of literature which cites ACTH as the main controlling agent for adrenal androgen secretion (see above). However, other authors report that gonadotrophins stimulate androgen formation in human adrenal tissue [26] and in two marsupial species PMS or HCG treatment stimulated testosterone production [18, 30]. In view of the differences between these species and the rat with regard to stimulation of testosterone formation by both ACTH (see above) and gonadotrophins, it appears that there may be wide species variation in the control mechanisms.

Finally it appears that there is no indication that the adrenal and testis interact in any way in the production of testosterone *in vitro* (see Fig. 7). An interpretation of the findings of Kniewald *et al.* [11] could therefore be that after castration, testosterone is produced by the adrenal during the period of post-surgical trauma.

Acknowledgements—We are most grateful to the Wellcome Trust for a research grant (to GPV). We are also most grateful to Drs V. Martin, C. W. Edwards and D. Exley for the generous gift of antisera and to Dr D. M. Burley (Ciba-Geigy) for the Synacthen used in these studies. We are also grateful to NIH for the supply of FSH and LH. In addition we express our appreciation of the radioimmunoassay carried out in the laboratory of Dr W. P. Collins in connection with the validation of our assays.

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