Journal of Steroid Biochemistry, Vol. 9, pp. 677–683

0022-4731/78/0701-0677$02.00/0

STEROID 17-HYDROXYLATION AND ANDROGEN PRODUCTION BY INCUBATED RAT ADRENAL TISSUE

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(*Received* 6 *October* 1977)

SUMMARY

Rat adrenal tissue was decapsulated, and capsules (considered to consist largely of zona glomerulosa) and inner zones were separately incubated with [4-^{14}C]-progesterone. Testosterone was found to be formed by both tissue types in yields at least 1% of those of corticosterone, confirming earlier observations. In addition, evidence was found for the production of radioactive 17-hydroxyprogesterone, androstenedione, 11-deoxycortisol and cortisol, in addition to the usual 17-deoxysteroids. Yields of cortisol were up to 20% of those of corticosterone, yields of the other steroids were about the same as testosterone. No significant differences in the yields of these products were found in capsule and inner zone incubations. From endogenous precursors, cortisol was again found to be formed in similar amounts by both tissue types. In some incubations, testosterone was apparently produced in larger amounts by the capsules, in others amounts were similar in capsules and inner zones. Addition of ACTH (20 mU per ml) increased the yields of testosterone in capsules but not inner zones. Sodium depletion had no effect on testosterone production. It is concluded that 17-hydroxylation can and does occur in the rat adrenal cortex, and it is likely that the pathways for the formation of cortisol and the androgens are similar to those described for other tissues. It is unlikely that 17-hydroxylation occurs in the cells of the glomerulosa, but the evidence suggests that it may be prominent in a region of the fasciculata directly underlying the glomerulosa, in the position of the zona intermedia.

INTRODUCTION

Species variation in adrenal steroidogenesis first became apparent when Bush[1] showed that the major product of rat adrenal tissue is corticosterone, and not cortisol as in other species. Since that time the nature of the steroid profile formed by the rat adrenal has been widely studied, and it is generally found that five major steroids are normally formed: corticosterone, 18-hydroxydeoxycorticosterone, deoxycorticosterone, aldosterone, and 18-hydroxycorticosterone[2–7]. The production of 17-hydroxysteroids of the pregnane series and androgens and oestrogens has not received detailed study, despite indications that the rat adrenal has some slight capacity to form steroids arising from hydroxylation at C-17[8–11]. The physiological role of such a pathway for cortisol production would generally be regarded as insignificant in the presence of large amounts of corticosterone, however the possible role of adrenal production of androgens and oestrogens may, under certain circumstances, be important physiologically[12–14].

In recent studies it was found that incubated rat adrenal tissue has the capacity to form testosterone from both endogenous precursors and from added [^{14}C]-progesterone, in amounts approximately 1% of those of corticosterone. Production of testosterone from endogenous precursors was enhanced by the addition of ACTH to the incubation medium, but LH and FSH had no effect[15].

This finding prompts several queries regarding the pathway by which testosterone is formed in rat adrenal tissue, the possibility that other steroids arising via a 17-hydroxysteroid pathway may also be produced, and whether such products are formed in any specific adrenocortical zone. This paper examines some of these questions.

MATERIALS AND METHODS

Animals. Female rats of the Wistar strain, bred in the Animal House at St. Bartholomew's Medical College were used. They were normally fed the stock diet, except that one group of animals was supplied with a wholemeal flour diet[16] with a low Na^+ content (4 mmol/Kg) for 2 weeks (experiment 3).

Incubations. Glands were fractionated by squashing between glass plates, after which the capsule remains almost intact with a layer of adhering cells and can be removed with forceps. The remainder of the gland is collected together. Light microscopy shows that the capsular portion consists largely of zona glomerulosa cells with some 10% contamination with outer zone fasiculata cells, as has been described by other authors [e.g. 28], whereas the inner zone portion con-

Table 1. Systems of chromatography

1. Toluene–70% methanol	Paper
2. Light petroleum–75% methanol	Paper
3. Light petroleum–36% tert. butanol	Paper
4. Cyclohexane benzene (1:1)–80% methanol	Paper
5. Light petroleum–benzene–ethyl acetate (1:1:4, by vol.)	t.l.c.
6. Chloroform–methanol (97:3, v/v)	t.l.c.
7. Toluene–methanol (96:4, v/v)	t.l.c.

tains no detectable zona glomerulosa contamination. The two fractions of tissue derived from one pair of glands were incubated separately in Krebs bicarbonate Ringer solution with glucose (2.0 g/l) for 2 h at 37 C under 95% O_2/5% CO_2. Individual experiments were as follows:

1. Tissue was incubated as above in 1 ml medium containing [4-^{14}C]-progesterone (61 mCi/mmol; 1 μCi per flask) with 6 flasks for each tissue type.

2. Incubations were performed in flasks containing 5 ml ringer, and the medium was discarded after 30 min. Fresh medium was added, without further additions in the 6 control flasks, but with the addition of ACTH (Synacthen, Ciba-Geigy, 20 mU per ml) to 6 experimental flasks.

3. Incubations were carried out using tissue from control animals in the presence or absence of ACTH (as above but without preincubation) and from animals subjected to a sodium deplete diet for 14 days[16]. There were 6 flasks per group.

Extraction and quantitation of steroids. Steroids were extracted from incubation media with ethyl acetate.

Radioactive steroid products. Known amounts of tritiated corticosterone, cortisol, 17-hydroxyprogesterone, androstenedione and testosterone (~10,000 c.p.m.) were added to incubation media prior to extraction for estimation of procedural losses. Tritiated androstenedione was also added to isolated fractions expected to contain 11-deoxycortisol, following their oxidation (Table 2). Following extraction, steroids were fractionated in the paper and thin layer chromatographic systems shown in Table 1. Initial fractionation was in the systems 1 (1 h) and 2 (2 h) without elution.

Non-radioactive products. Corticosterone, deoxycorticosterone, aldosterone, 18-hydroxydeoxycorticosterone and testosterone were measured by GLC and RIA methods described elsewhere[15, 17].

Further evidence for cortisol production in rat adrenal incubations was obtained when extracts were chromatographed in system 1 (Table 1), material with the mobility of cortisol was eluted and oxidised with chromic acid and rechromatographed in system 2 (Table 1). Material with the same mobility as adrenosterone was subjected to GLC using the system described previously[18]. In all cases the material showed a single symmetrical peak with same retention time as authentic adrenosterone. Routinely, cortisol was measured by a modification of this method in which acid products were extracted into 2% sodium bicarbonate following oxidation of an aliquot of the samples with periodic acid to yield the lactones of the 18-oxygenated products, and the aetioacids of the 21-hydroxy-18-deoxysteroids. This solution was then acidified, and the steroid acids, including the C_{20} aetioacid formed from cortisol, was re-extracted into ethyl acetate. Further oxidation with chromic acid then yields a mixture of C_{19} 17-oxosteroids, formed from 17,21-dihydroxysteroids, and aetioacids of 21-hydroxy-17-deoxysteroids, which do not undergo further oxidation[19]. The products are then taken

Table 2. Procedures used in isolation and identification of 17α-hydroxylated compounds obtained after incubation of rat adrenal tissue with [4-^{14}C]-progesterone

Compound	Procedure	Chromatography
Cortisol	Extraction*	1, 2
	Acetylation	4
	Hydrolysis, oxidation with chromic acid	2, 5†, 6†, 7†
11-deoxycortisol	Extraction	1, 2
	Acetylation	3
	Hydrolysis, oxidation*	2, 5†, 6†, 7†
17α-hydroxy-progesterone	Extraction*	1, 2
	Acetic anhydride/ pyridine treatment	2
	Oxidation	2, 5†, 6†, 7†
Androstenedione	Extraction*	1, 2
	Acetic anhydride/ pyridine treatment	2
	Reduction with $NaBH_4$	2†
	Acetylation	2†, 5†
Testosterone	Extraction*	1, 2
	Acetylation	2
	Hydrolysis, oxidation	2, 5†, 6†, 7†

* = Addition of authentic ^{3}H material. † = Estimation of ^{3}H/^{14}C ratio.

up in ethyl acetate, and washed with 2% sodium bicarbonate, thus giving a pure sample of the oxidation products of 17,21-dihydroxysteroids. Reaction with heptafluorobutyric anhydride[15] is then followed by gas chromatography on 0.3% XE-60-gas chrom Q at 225 C. The method is thus highly specific since potential contaminants are eliminated by the extraction procedure, and gives a coefficient of variation of 18. Recovery is about 80%, and sensitivity allows detection of cortisol in amounts down to 5 ng.

In a previous publication on the production of testosterone by adrenal tissue, the RIA used was validated by comparison with results obtained by RIA in another laboratory, and by comparison with a GLC method[15]. In further validation of the testosterone method, extracts from one incubation of capsule and inner zone tissue, in the presence of ACTH and under control conditions, were subdivided, and part was directly assayed by RIA. To the other fractions, known aliquots of [^{3}H]-testosterone were added, and they were chromatographed in system 1. Following elution, procedural losses were estimated on the basis of the recovery of the radioactive tracer, and amounts of testosterone were assayed by RIA. Results obtained by direct RIA, and assay after paper chromatography were compared.

RESULTS

Radioactive products

Identification of radioactive cortisol, 11-deoxycortisol, 17-hydroxyprogesterone, androstenedione and testosterone was carried out by the procedures indicated in Table 2. In each case, tritium/^{14}C contents were measured at the stage indicated, and further procedures were carried out on fractions pooled together from different incubations. Successive ^{3}H/^{14}C ratios for these compounds in the final stages (indicated in Table 2) are shown in Table 3. In general the consistency of the isotope ratios confirms the homogeneity of the authentic and extracted material. One exception is in the case of androstenedione, where reduction to testosterone gave an inhomogeneous mixture which was not resolved until the material was acetylated and rechromatographed. Based on these findings, the yields of the products was estimated and expressed as a percentage of the added precursor. The results are indicated in Fig. 1 and compared with yields of corticosterone [isolated by established procedures, 20]. Yields of testosterone are about 1% of those of corticosterone, confirming earlier findings[15], and those of androstenedione, 17-hydroxyprogesterone, 11-deoxycortisol are of the same order. Yields of cortisol are up to 20% of those of corticosterone. There was no significant differences between the yields of any of these compounds in the capsule and inner zone preparations.

Table 3. Final ^{3}H:^{14}C ratios of 17α-hydroxylated steroids isolated from incubations of capsules and inner zones with [4-^{14}C]-progesterone after mixture with authentic ^{3}H-labelled material (see methods)

	Capsule	Inner zone
Cortisol	0.40	0.29
	0.41	0.24
	0.38	0.32
11-deoxycortisol	25	2.6
	22	2.9
	24	2.6
17α-hydroxyprogesterone	43	28
	51	31
	52	30
Androstenedione	11.4	3.9
	17.9	8.1
	18.1	6.9
Testosterone	25	14.1
	23	15.7
	22	14.6

Products from endogenous precursors

Yields of deoxycorticosterone, corticosterone, 18-hydroxydeoxycorticosterone, 18-hydroxycorticosterone and aldosterone in capsule and inner adrenocortical zone incubations from untreated female Wistar rats are shown in Fig. 2. There are some clear differences between the steroid profiles obtained in the two tissue types. In particular, yields of corticosterone and 18-hydroxydeoxycorticosterone are very much higher in inner zone incubations than in the capsules. On the other hand the capsule is the unique source of aldosterone, and it also produces most of the 18-hydroxycorticosterone produced by the gland (some is formed by the inner zones, but in the methods used it appears in the same GLC chromatograms as 18-hydroxydeoxycorticosterone, which is in such large amounts that measurement of 18-hydroxycorticosterone becomes unreliable).

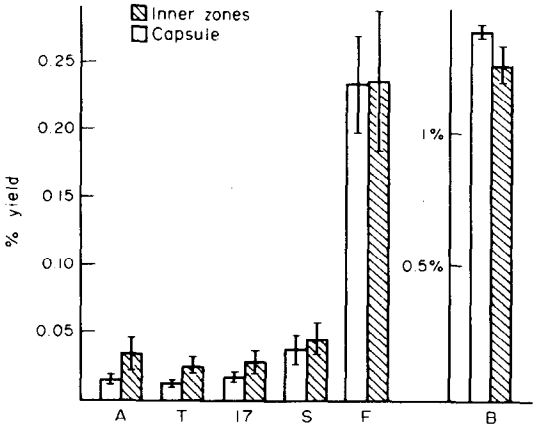

Fig. 1. Yields of labelled steroid products formed following 17-hydroxylation from 1 μCi [4-^{14}C]-progesterone during incubation with pairs of rat adrenal capsules or inner zone tissue, as described in the Methods. There are no significant differences between the yields of steroid formed by each tissue type. n = 6 throughout. A = androstenedione, T = testosterone, 17 = 17-hydroxyprogesterone, S = 11-deoxycortisol, F = cortisol, B = corticosterone.

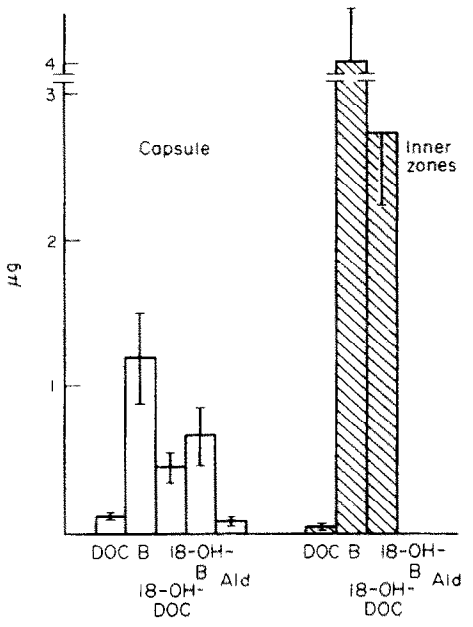

Fig. 2. Yields of 17-deoxysteroids formed from endogenous precursors following incubation of rat adrenal capsules and inner zones as for Fig. 1. DOC = deoxycorticosterone, B = corticosterone, 18-OH-DOC = 18-hydroxydeoxycorticosterone, 18-OH-B = 18-hydroxycorticosterone, ald = aldosterone.

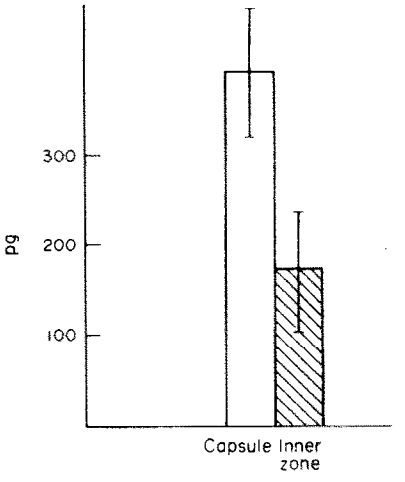

Fig. 4. Yields of testosterone from endogenous precursors following *in vitro* incubation of rat adrenal components as for Figs 1–3.

Measurement of cortisol, on the other hand, shows similar yields from both capsule and inner zone incubations. Yields of cortisol in inner zone incubations were about 5% of those of corticosterone (Fig. 3), but in the capsule incubations they were very much more, up to 30%. Yields of testosterone in some incubations were greater in capsules than in inner zones (Fig. 4). In others (Fig. 5) control yields in capsules and inner zones were similar, but testosterone production in the capsules only was stimulated by the addition of ACTH to the incubation media. Figure 5 also shows that sodium depletion had no effect on testosterone production by either tissue type.

The correlation (Fig. 6) between the assays for testosterone performed directly on aliquots of the extracts compares well with values obtained after paper chromatography, with the possible exception that under control conditions in inner zone incubations, the direct assay results may be higher. In other incubations the correlation was good. Overall, including the control inner zone results, the correlation coefficient $r = 0.85$, and the slope of the regression line was 1. Figure 6 again shows that ACTH does not stimulate testosterone production in the inner zones incubation, but in this experiment the effect in the capsules is also not statistically significant.

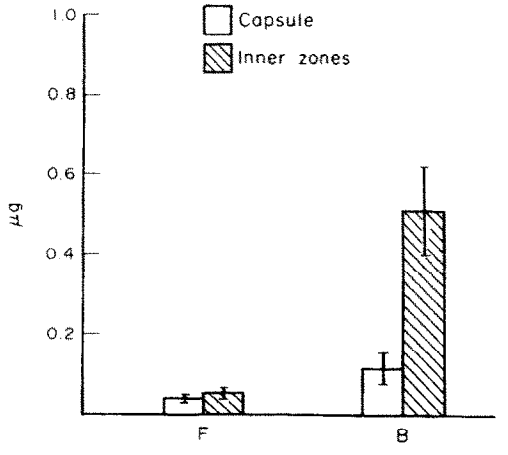

Fig. 3. Yields of cortisol and corticosterone formed from endogenous precursors during *in vitro* incubation of rat adrenal capsules and inner zones as for Figs. 1 and 2. F = cortisol, B = corticosterone.

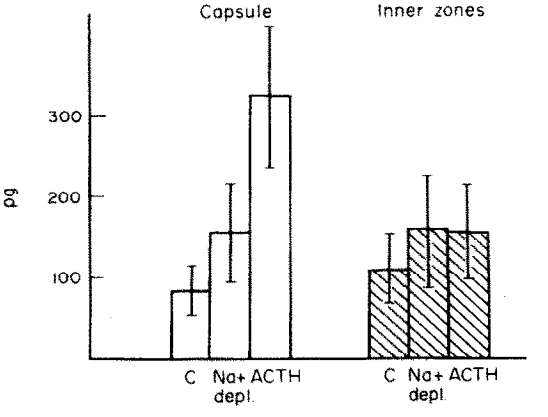

Fig. 5. Production of testosterone from endogenous precursors by rat adrenal tissue incubated as for Figs 1–4. Tissue was taken from control animals (C) or animals fed a sodium deplete diet (Na^+ depl.). Tissue from control animals was also incubated with 20 mU per ml ACTH (A). Comparison of C and A (capsules) $P < 0.01$.

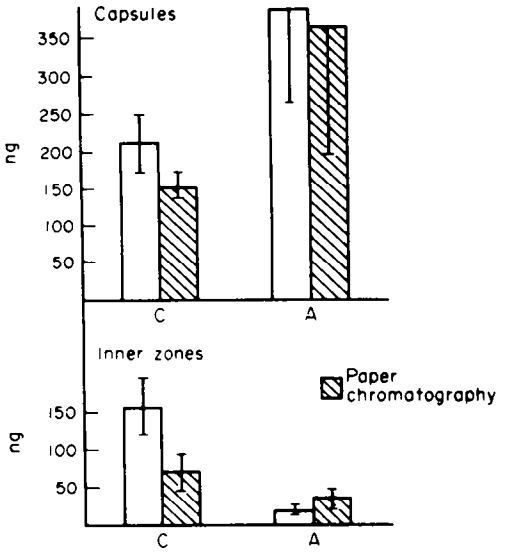

Fig. 6. Yields of testosterone from endogenous precursors following incubation of rat adrenal components as for Figs 1-4. (except that a 30' pre-incubation medium was discarded), under control conditions (C) and in the presence of 20 mU per ml ACTH (A). Clear columns are values obtained by direct assay, and shaded columns are values obtained on the same samples following paper chromatography as described in the Methods.

DISCUSSION

Nature of steroid products

The possibility that the rat adrenal cortex can synthesize steroids arising through hydroxylation at C-17 has not received detailed attention. Following the observation of Bush[1] that in contrast to other species corticosterone is predominantly produced, most authors have concentrated their studies on the 17-deoxysteroid series in this species. Although some earlier authors considered that cortisol production was possible[21], this view was largely held to be based on less than adequate fractionation procedures[22]. In view of the fact that further polar steroids which, like cortisol, give a positive Porter Silber reaction were later identified as 18-hydroxydeoxycorticosterone and 18-hydroxycorticosterone[2-5], some confusion over the nature of these products was indeed likely.

Nevertheless, the possibility that rat adrenal tissue can produce cortisol has been raised more recently from time to time, based on evidence obtained from incubated glands from normal rats[9] and from animals with regenerating adrenal hypertension in some studies[8] but not others[23]. Good evidence for the production of cortisol from normal Sprague–Dawley rat adrenals from [^{3}H]-pregnenolone and [^{3}H]-progesterone was obtained by Karaboyas and Koritz[9]. In their experiments yields of cortisol approximately 1% of those of corticosterone were obtained from both precursors. There has been a similar degree of uncertainty with regard to the adrenal production of androgens in the rat. In some experiments, no formation of C_{19} steroids was obtained from added cholesterol, pregnenolone or 17-hydroxypregnenolone[24], whereas in others transformation of pregnenolone into dehydroepiandrosterone, androsterone, testosterone and 11β-hydroxyandrostenedione was reported [10, 11]. In addition, the work of Kniewald *et al.*[14] provided strong indirect evidence for a source of testosterone other than the gonads.

The present results, which confirm and extend those previously reported[15], provide additional evidence for the view that the rat adrenal can indeed produce testosterone, both from added radioactive progesterone and from endogenous precursors, in amounts about 1% of those of corticosterone, and that in addition other products which may arise through 17-hydroxylation of progesterone are also formed from radioactive precursors in similar yields, including androstenedione, 17-hydroxyprogesterone and 11-deoxycortisol (Fig. 1). The yield of cortisol is surprisingly high, and from radioactive progesterone it is produced in amounts approaching 20% of those of corticosterone. These values are substantially higher than those reported by Karaboyas and Koritz[9]. One possibility is that since they used tritiated precursors, with much higher specific activity than the [^{14}C]-progesterone used in the present studies, different relative yields of products may be obtained owing to differential saturation effects at various points in the pathway. Alternatively, these differences may relate to variations between differing strains of rats used in the two studies. In any case, it is quite clear that exogenous precursors mix with endogenous pools to varying extents, and thus do not reflect quantitative aspects of endocrine function very accurately[20, 25]. From endogenous precursors, which may be considered a more reliable reflection of *in vivo* function, the yield of cortisol is more than 10% of that of corticosterone in inner zones, and much higher in the capsules (Fig. 3).

Cellular origin of products arising through 17-hydroxylation

Within the adrenal cortex, the possibility of separate functions for the different cell types has over the years attracted some speculation. At one time it was thought that the zona reticularis might be a site of adrenal androgen production, from histological and histochemical evidence, but this was later discounted[26]. More recently it has been found that in general the same range of steroid hormones is produced in fasciculata and reticularis cells from the rat adrenal cortex, although there are quantitative differences under basal and stimulated conditions: in particular androstenedione is formed by the two cell types[27]. In the present experiments, the data suggest that the capacity to form 17-hydroxysteroids, and C_{19} steroids extends at least up to the border between the fasciculata and the glomerulosa. While the process

of separation of glomerulosa and inner zones by decapsulation is fairly crude, resulting in a contamination of glomerulosa cells by fasciculata cells of up to 10%, there is little or no glomerulosa tissue in the inner zone preparation: certainly the steroid profiles produced by the two tissue types in terms of the conventional corticosteroids (Fig. 2) strongly support the view that the functions of very different tissue types are reflected in these incubations[28–30]. It is somewhat surprising then to find that from radioactive precursors, the production of cortisol and 11-deoxycortisol (Fig. 1) is indistinguishable in capsule and inner zones, and for testosterone, androstenedione and 17-hydroxyprogesterone, the apparently higher mean values obtained with inner zone tissue are not significantly different from capsules. From endogenous precursors too, the yield of cortisol was similar in the two tissue types, and in the case of testosterone, more was formed in the capsule preparations in some incubations (Fig. 4), and in other cases only the testosterone formed by the capsule preparation was increased by ACTH (Fig. 5). The question then arises: does 17-hydroxylation occur in the glomerulosa? The answer to this is probably no. Aldosterone is a specific marker for glomerulosa cells [28, 29, cf. Fig. 2] clearly any other product coming from glomerulosa cells (and not inner zones) would show a similar exclusive origin. Furthermore, the glomerulosa is relatively insensitive to ACTH stimulation[28, 31] while as seen here and as previously reported[15], testosterone output by incubated glands generally responds to ACTH. Again, under conditions of sodium depletion, the enhanced steroid production of aldosterone is usually seen also in glands incubated *in vitro*[33], and while this is partly associated with a "late pathway" effect[34], it is clear that testosterone production was not similarly affected. Both from the point of view of the distribution of testosterone (and the other steroids which follow 17-hydroxylation in the pathway), and the response to ACTH and sodium depletion, the data would support the view that these steroids originate from the inner zones, although there may be a marginally richer source in the tissue underlying the glomerulosa (possibly the z. intermedia) which is thus distributed between capsule and inner zones following conventional decapsulation. The inconsistent response to ACTH obtained in the experiments may simply reflect the lack of homogeneity of the preparations.

Finally it is worth repeating that there may well be some species variation in these effects: work on the marsupial *Trichosurus vulpecula* suggests that in contrast to the Eutherian adrenals which have been studied, there may well be an association of testosterone production with the innermost adrenocortical zones[26].

Acknowledgements—We are grateful to Dr. D. Exley for supplying the testosterone antiserum, and to Dr. D. M. Burley (Ciba-Geigy) for the supply of Synacthen. We are also most grateful to the MRC for project grant support (to GPV).

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