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SPECIES VARIATION IN ADRENOCORTICAL FUNCTION: THE SECRETION OF CORTISOL BY NORMAL RABBIT ADRENAL TISSUE *IN VIVO* AND *IN VITRO*

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SUMMARY

Using paper, thin layer and gas chromatographic methods, it was shown that normal rabbit adrenal tissue has the capacity to form and secrete cortisol from endogenous precursors *in vitro*, and *in vivo* under pentobarbitone anaesthesia. The identity of the extracted cortisol was confirmed by gas chromatography/mass spectrometry of 4-androstene-3,11,17-trione formed from the cortisol by chromic acid oxidation. Levels of cortisol *in vivo* were 21±4μg per 100 ml of whole adrenal vein blood, compared with figures of 155±11μg/100 ml for corticosterone, 18±3μg/100 ml for deoxycorticosterone and 3±1μg/100 ml for aldosterone. Cortisol formed from endogenous precursors *in vitro* was secreted in slightly higher amounts relative to corticosterone.

These findings conflict with those of other authors who have been unable to detect cortisol as a secretion product of the normal rabbit adrenal (although it may be produced by glands maximally stimulated with ACTH).

Further experiments showed that in incubations where appreciable amounts of cortisol were formed from endogenous precursors, negligible amounts were produced from radioactive pregnenolone and progesterone. It is suggested that in unstimulated rabbit adrenal tissue incubated *in vitro*, the radioactive precursors may not penetrate the endogenous precursor pool.

INTRODUCTION

THE secretion of corticoids by rabbit adrenal tissue has been investigated by a number of authors. The original findings of Bush[1] indicated a lack of adrenal 17α-hydroxylase activity: cortisol was undetected in adrenal vein blood whereas corticosterone was readily demonstrated. Kass *et al.*[2] in following certain physiological data which suggested the secretion of 17α-hydroxycorticoids, were also unable to detect cortisol in the normal adrenal venous effluent in this species, but following treatment with adrenocorticotrophic hormone over periods of seven to twenty-eight days the secretion of cortisol into the adrenal vein became apparent and ultimately exceeded the secretion of corticosterone. Whereas similar effects of ACTH were also recorded by Krum and Glenn[3] these authors were able to show a basal cortisol secretion rate in untreated animals of 0·86μg/min/g adrenal. More recently, these phenomena have been reinvestigated by Fevold[4–5]. Using an *in vitro* technique, he in turn was unable to detect the transformation of radioactive pregnenolone or progesterone or of endogenous precursors to cortisol by normal rabbit adrenal tissue, although substantial yields were obtained following prolonged treatment with ACTH. Later he showed that the maximal response to ACTH was obtained after only two or three days. Subsequently, Fevold also found that the principal pathway for the formation of 17α-hydroxycorticoids in ACTH stimulated rabbits from pregnenolone involved

17α-hydroxypregnenolone, 17α-hydroxyprogesterone and 11-deoxycortisol as intermediates, whereas corticosterone was formed by the conventional pathway involving progesterone and deoxycorticosterone as intermediates [5].

These results seem to indicate a remarkable and unique feature of the physiology of the rabbit adrenal cortex. It is difficult to find another example of the induction of a steroid hydroxylase by a trophic hormone in endocrine tissue, which was not demonstrable at any level of activity in control animals (see Ref. [6] for review). For this reason it was decided to investigate more closely the secretion of cortisol in untreated animals.

MATERIALS AND METHODS

New Zealand white rabbits were maintained in the animal house of the Medical College of St. Bartholomew's Hospital. Adrenal vein blood was collected under pentobarbitone sodium anaesthesia (Nembutal, Abbot Laboratories Ltd., 40 mg/kg) according to the method described by Allison and Powis [13]. To the whole blood, known amounts of [^{3}H]deoxycorticosterone, [^{3}H]corticosterone, [^{3}H]aldosterone and [^{3}H]cortisol were added to correct for procedural losses. The blood was diluted with an equal volume of water and frozen overnight before extraction.

In vitro methods. Adrenal gland tissue taken from freshly killed unanaesthetised animals was sliced and incubated in Krebs–Ringer bicarbonate solution at 37°C under an atmosphere of 95% O_2; 5% CO_2, with 0·1 μCi [4-^{14}C]progesterone (S.A. 185μCi/mg) and 1·5μCi [7α-^{3}H]pregnenolone (S.A. 1·44mCi/mg). Samples (1 ml) of the incubation medium were withdrawn after specific time intervals, and were replaced by samples of fresh incubation medium without radioactive precursors.

Fractionation of steroids

Liquid phase chromatography. Steroids were extracted from blood and from incubation media in ethyl acetate, and fractionated by paper and thin layer chromatography (Table 1). The mass and isotope content of deoxycorticosterone, corticosterone and aldosterone were assayed using the paper and gas chromatographic technique previously described [7].

Cortisol was determined gas chromatographically as follows: after initial fractionation on paper in systems 1 and 2, the areas expected to contain cortisol

Table 1. Liquid phase chromatographic systems

System	Solvents	Support (Whatman No. 1 paper or thin layer silica gel)	Average time of run (hr)
1.	Light petroleum: 70% aq. methanol	Paper	2
2.	Toluene: 70% aq. methanol	Paper	2
3.	Light petroleum: 36% t-butanol	Paper	3
4.	Light petroleum/benzene/ ethyl acetate (1 : 1 : 4, by vol)	Thin layer	1
5.	Benzene/methanol (9 : 1, by vol)	Thin layer	1

were eluted with chloroform–methanol (2:1), and subjected to chromic acid oxidation[8]. The extracts were then rechromatographed in system 4. Material with the mobility of adrenosterone (4-androstene-3,11,17-trione) was eluted and subjected to gas chromatography.

Vapour phase chromatography. Gas chromatography was carried out on a Packard (model 7839) apparatus. The 150 cm columns used routinely were packed with 1% XE-60, 3% SE-30 or 1% OV-1 on chromosorb 'W', and were maintained at 210–220°C. Nitrogen was used as carrier gas and supplied at 70 ml/min. The detector was a tritium foil electron capture detector to which a voltage of 130V was applied, and which was at 225°C. Under these conditions an extremely sensitive response to authentic adrenosterone was obtained[12], and quantities as little as 1 ng could be estimated. Estimations of mass were generally performed on one-tenth aliquots, and the remaining 9/10 was used for isotope estimation.

RESULTS

In all incubations, and in all samples of adrenal vein blood, material was detected with the characteristics of cortisol. The evidence for the identity of cortisol may be summarised thus: the free material showed mobilities identical with those of authentic material in systems 1, 2, 3. Upon oxidation to adrenosterone its mobilities were identical with those of synthetic material (systems 1, 4, 5) and on gas chromatography the oxidised material showed retention times identical with those of authentic material on SE-30, XE-60 and OV-1 columns. In addition a sample of the oxidised material was subjected to combined gas chromatography/mass spectrometry by Drs. C. J. W. Brooks and B. S. Middleditch, University of Glasgow, and this furnished definitive evidence of the material's identity (Figs. 1 and 2).

While the formation of cortisol from endogenous precursors was always detectable, this was not so in the case of the transformation of the radioactive precursors. Traces of ^{3}H were detected, though in insufficient quantities to allow any procedures for characterisation, but no evidence at all for incorporation of ^{14}C into cortisol was found. This was also true of the potential intermediates 17α-hydroxyprogesterone and 11-deoxycortisol.

Figures for the concentrations of corticosterone, aldosterone, deoxycorticosterone, and cortisol in rabbit adrenal vein blood are shown in Table 2, and yields of the major compounds from [^{3}H]pregnenolone, [^{14}C]progesterone and from endogenous precursors during incubation *in vitro* are shown in Figs. 3 and 4.

Table 2. Steroid components in normal rabbit adrenal vein whole blood

Steroid	Concentration (μg/100 ml)($n = 5$; mean ± S.E.)
Cortisol	21 ± 4
Corticosterone	155 ± 11
Deoxycorticosterone	18 ± 3
Aldosterone	3 ± 1

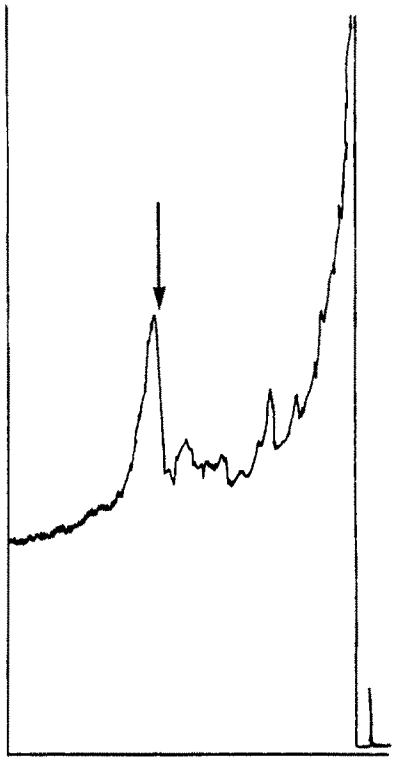

Fig. 1. Gas chromatogram of 4-androstene-3,11,17-trione derived from cortisol secreted by normal rabbit adrenal. The column was 1% OV-1; the vertical arrow indicates the point at which the mass spectrum (see Fig. 2) was taken.

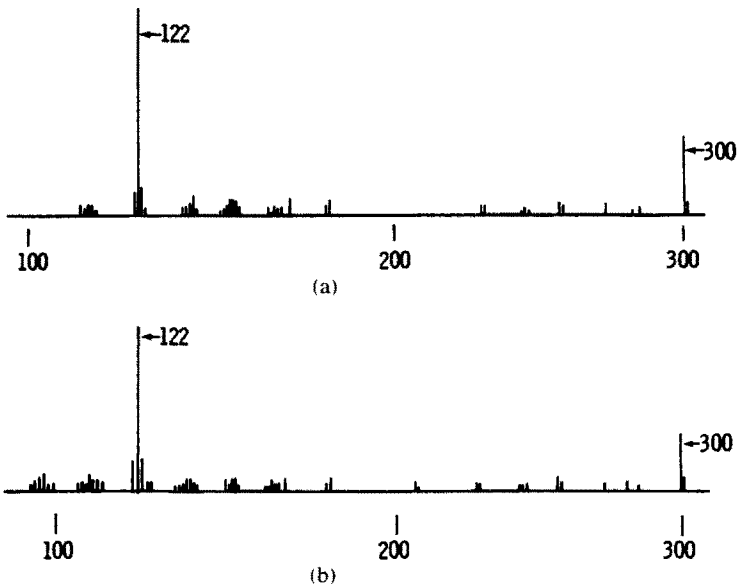

Fig. 2. Mass spectra of (a) authentic 4-androstene-3,11,17-trione, and (b) 4-androstene-3,11,17-trione derived from cortisol secreted by normal rabbit adrenal. Both spectra are characterised by the molecular ion peak (m/e 300) and the base peak (m/e 122), together with a few smaller peaks.

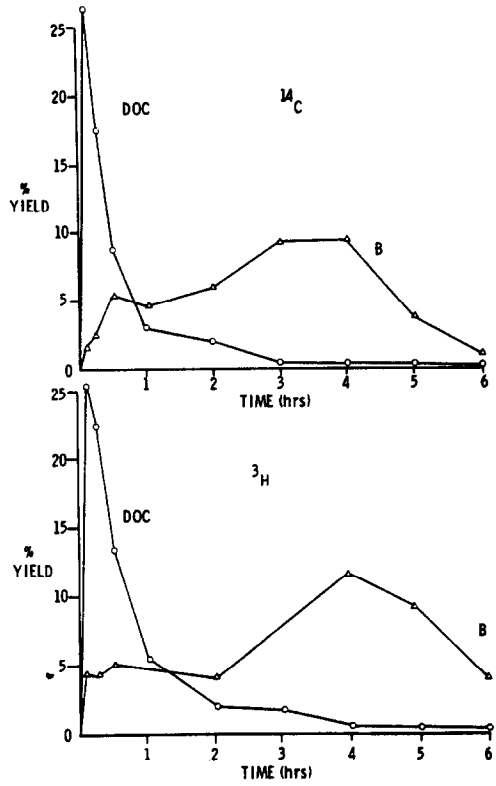

Fig. 3. Yield time curves for products isolated during incubation of normal rabbit adrenal tissue with [7α-^{3}H]pregnenolone and [4-^{14}C]progesterone. DOC = deoxycorticosterone; B = corticosterone. The yield of cortisol from [7α^{3}H] pregnenolone reached a maximum (at six h) of less than 0·1%; no ^{14}C was detectable in this fraction.

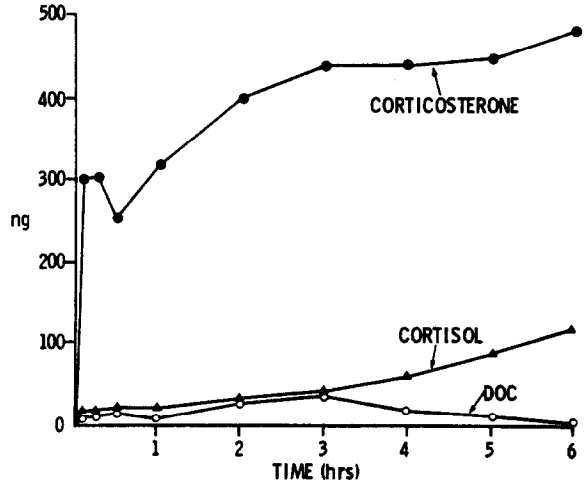

Fig. 4. Yield time curves for products from endogenous precursors during the same incubation of normal rabbit adrenal tissue as for Fig. 3. DOC = deoxycorticosterone.

DISCUSSION

The results (Table 2) show that cortisol must be considered to be a normal and significant component of the rabbit adrenocortical secretion, both *in vivo* and *in vitro*, and that a 17α-hydroxylase system is present even when the glands have not been maximally stimulated with ACTH. Previous workers[1, 3, 4] have been generally unable to show the production of cortisol from endogenous precursors: this may be a reflection of the relative lack of sensitivity of the methods used. Morosova[9] showed that some of the material gained from normal rabbit adrenals incubated with very large amounts of progesterone exhibited the same mobility as cortisol and gave a positive Porter–Silber reaction, although the amounts were slight compared with those of corticosterone. This material, however, was not clearly separated from 18-hydroxydehydrocorticosterone (18,21-dihydroxy-4-pregnene-3,11,20-trione) which is also produced by the rabbit adrenal in large amounts[10]. In addition, Fevold[4] was also unable to detect any formation of cortisol from radioactive progesterone, pregnenolone or cholesterol by normal rabbit adrenal tissue.

It is possible that much of the divergent opinion may be reconciled on the basis that the conversion of radioactive pregnenolone or progesterone does not reflect the normal capacity of the gland to secrete cortisol, and this would also seem to be suggested by our results (cf. Figs. 3, 4). There may be a number of explanations for this: for example it is not clear (without pre-incubation) whether the amount of cortisol isolated following incubation of adrenal tissue reflects the continued formation of the hormone *in vitro*, or merely the release of preformed material. It is possible to envisage for example that one way in which the lack of correlation between the formation of labelled and non-labelled cortisol might arise is in a situation in which the transformation of progesterone to cortisol occurs only slowly, whereas the pool of cortisol in the tissue may be high.

Alternatively, it might be worth considering a further possibility. In the *in vitro* incubations of rabbit adrenal tissue with radioactive precursors, described here, the yield time curves for the formation of deoxycorticosterone and corticosterone from [^{3}H]pregnenolone and [^{14}C]progesterone (Fig. 3) follow a characteristic pattern found in other species[6]. An initial very large peak in doubly labelled deoxycorticosterone was followed by its utilisation as a substrate for corticosterone formation, which proceeded at a slower rate although it eventually constituted the major steroid product detectable in the incubation medium (cf. Fevold[5]). This contrasts with the results for the formation of the same compounds from endogenous precursors, and the *mass* of DOC remained very low in amount throughout the incubation, and there was a high rate of corticosterone formation. The secretion of deoxycorticosterone was also low compared with corticosterone *in vivo* (Table 2).

There may be a number of factors which bring about this situation, depending on the dynamics of the biosynthetic system, and the contrast between conversion of precursors added at a single point in time and those fed continuously to the biosynthetic enzymes. The results do recall, however, similar findings obtained using rat adrenal tissue[7], in which it was also shown that the exogenous precursors did not penetrate the endogenous pool to the extent that they were metabolised as a homogeneous mixture.

This possibility emerges more strongly in the present experiments with regard to the formation of the 17α-hydroxylated steroids. *In vitro*, only very small traces

of [^{3}H]cortisol (insufficient for adequate identification) were found, and no evidence was found for [^{14}C]cortisol formation at all. This finding seems to be unprecedented: while it is true that a major pathway for the formation of cortisol from pregnenolone may bypass progesterone formation, it has hitherto always been possible to demonstrate the conversion of progesterone to cortisol in other species in which cortisol is known to be secreted (see Ref. 6).

It is quite possible that the relative lack of conversion of exogenous precursors to cortisol arises from the saturation of the appropriate enzyme systems by the endogenous precursors. One way in which ACTH may act, therefore, is by increasing the penetration of endogenous precursors by the exogenous precursors. There are precedents for this view [7, 11].

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