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IN VITRO METABOLISM OF DEHYDROEPIANDROSTERONE BY MAMMARY GLAND AND MAMMARY TUMOURS IN THE RAT

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SUMMARY

The *in vitro* metabolism of [4-^{14}C]-dehydroepiandrosterone (DHEA) by mammary tissues of Wistar and Sprague–Dawley rats yielded 7α- and 7β-hydroxy DHEA as major products; the epimers being formed in a ratio of 4:1. 5-Androstene-3β,17β-diol and 7-oxo DHEA were produced in lower yields. No marked differences were found in the conversion of DHEA to these products when lactating tissue, dimethylbenzanthracene-induced mammary tumours, and oestrogen-induced mammary tumours, were compared.

INTRODUCTION

Human mammary carcinoma tissue has been shown to metabolize DHEA to androstenedione[1], testosterone and oestrone[2]. A 17β-hydroxy dehydrogenase for DHEA has also been detected in such tissues[1]. Hydroxylation of DHEA is apparently limited to the 16-position[1]. Since the dimethylbenzanthracene (DMBA)-induced rat mammary tumour of Huggins has proved to be a good model for the study of the human disease, an investigation of steroid metabolism by both DMBA-induced rat mammary tumours and oestrogen-induced rat mammary tumours has been undertaken with a view to making comparisons in the two species. In this communication results of the *in vitro* metabolism of [4-^{14}C]-DHEA in rat mammary tumours and lactating rat mammary gland tissue, are reported. The major metabolites formed by each tissue were 7α- and 7β-hydroxy DHEA accompanied by 7-oxo DHEA. The presence of a 7-hydroxylase has not previously been reported in mammary tissue.

MATERIALS AND METHODS

Materials. DHEA, 5-androstene-3β,17β-diol, 5-androstene-3β,16α-diol-17-one, 5-androstene-3β,16α,17β-triol and DHEA sulphate were purchased from Ikapharm, Israel. 7α- and 7β-hydroxy DHEA were kindly supplied by the Steroid Reference Collection, Medical Research Council, London. 7α- and 7β-methoxy DHEA were prepared as described previously[3]. [4-^{14}C]-Dehydroepiandrosterone (52 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, and was purified by chromatography on alumina immediately prior to use. 7,12 Dimethylbenzanthracene (DMBA) was a Sigma product. Amphenone B was a gift kindly supplied by Professor Roy Hertz, George Washington University Medical Centre.

All solvents were of A.R. grade and were redistilled before use.

Tissues

Lactating tissue. This was obtained from both Wistar and Sprague–Dawley female rats.

DMBA tumours. Tumours were induced in 50-day-old female Sprague–Dawley rats by a single oral dose of 20 mg of DMBA in peanut oil. These tumours were removed for experiments some 70 days after administration of carcinogen.

Oestrogen-induced tumours. Oophorectomized female Wistar rats were subjected to a continuous oestrogen environment by means of a pellet (35 mg) composed of 95% cholesterol and 5% oestradiol-17β implanted subcutaneously into the neck region. Mammary tumours first developed after some 240 days and were diagnosed histologically as fibroadenomas. In experiments reported here, tumours were taken from rats aged 340–360 days.

Methods

Metabolism of [4-^{14}C]-*DHEA by mammary tissue.* Lactating mammary tissue, or mammary tumour, was minced with scissors in 3 vol. of Krebs–Ringer phosphate, pH 7.4. After addition of NADPH (final concentration 1.2 mM), the preparation was transferred to a flask containing [4-^{14}C]-DHEA (1 μCi) in 0.05 ml of propylene glycol and incubated in a shaking water bath at 37° in an atmosphere of 95% oxygen and 5% carbon dioxide. A parallel incubation was carried out in some cases in the presence of 0.5 mM Amphenone B. Control incubations were performed in the absence of tissue. After 2 h, 10 μg of the following carrier steroids were added: androstenedione, 16α-hydroxy DHEA, 5-androstene-3β,16α,17β-triol, 5-androstene-3β,17β-diol and DHEA sulphate. Reaction was stopped by addition of 5 vol. of acetone and the mixture homogenized, filtered and the residue

washed (×2) with acetone. The filtrate was concentrated to near-dryness under reduced pressure and partitioned between methanol–water (70:30 v/v) and petroleum ether (60–80°). The latter was back-extracted with aq. methanol and the steroid metabolites present in the combined aq. methanol were partitioned into neutral, phenolic and conjugate fractions by the method of Fahmy *et al.*[4]. Counts in the phenolic and conjugate fractions were generally low and not significantly different from the controls. These fractions were not investigated further. The neutral fraction was dissolved in ethanol (0.2 ml) and aliquots taken for analysis by t.l.c. followed by radioactivity scanning.

Separation and quantitation of metabolites. Metabolites obtained by incubation of [^{14}C]-DHEA with mammary tissue were separated by t.l.c. on foil-backed 0.2 mm silica gel sheets (Merck) following two developments in solvent system I and two developments in solvent system II. Standards were run in parallel and detected by spraying with $SbCl_3$ in chloroform. 7α-Hydroxy DHEA and 7β-hydroxy DHEA gave characteristic blue colours immediately. Other 3β-hydroxy-5-androstenes gave pink colours on warming. The unstained strip containing the labelled metabolites was scanned on a Nuclear Chicago Actigraph III instrument (Model 1002). Yields were determined by weighing the areas under the curves and expressing them as a percentage of the total area.

t.l.c. Solvent systems. Proportions in the following systems are v/v. I, cyclohexane–ethyl acetate (1:1); II, chloroform–ethanol (95:5); III, benzene–ethyl acetate (9:1); IV, benzene–acetone (1:1); V, cyclohexane–ethyl acetate–ethanol (45:45:10); VI, benzene–ethanol (4:1); VII, chloroform–ethanol (9:1).

RESULTS

Metabolism of [4-^{14}C]-DHEA by mammary tissues. In Fig. 1 the products derived from the metabolism of [4-^{14}C]-DHEA by lactating mammary tissue of Wistar rats are shown. These results are typical of those found with the other mammary tissues and mammary tumours examined. The metabolite having chromatographic mobility corresponding to authentic 5-androstene-3β,17β-diol was eluted and proved to be identical to this steroid by comparison of chromatographic properties in other solvent systems, both as the free compound (solvent systems I, II, III, IV & V), and as the diacetate (solvent systems IV, V & VII).

The major metabolite had chromatographic properties identical to 7α-hydroxy DHEA and was accompanied by lesser amounts of the 7β-epimer (Fig. 1). Identical chromatographic behaviour was also observed when the metabolites were eluted and compared with authentic substances in other solvent systems (Systems I, II, V, VI and VII). Proof of identity was obtained by taking advantage of the unique and facile alkylation which 7α- and 7β-hydroxy DHEA undergo upon heating with alcohols in mildly acidic media[3]. When the labelled metabolite corresponding to 7α-hydroxy DHEA was eluted, mixed with authentic material and refluxed in aq. methanol containing a few drops of acetic acid, formation of the 7α-methoxy derivative occurred. Some slight epimerization to 7β-hydroxy DHEA was observed together with formation of a small amount of an unknown product (Fig. 2). It can be seen that the pattern of labelled and unlabelled products, revealed after staining with $SbCl_3$, is identical. The radioactive zone corresponding to 7α-methoxy DHEA was eluted from unstained t.l.c. plates. This was then chromatographed along with the radioactive metabolite corresponding to 7α-hydroxy DHEA and the specific activities compared after quantitative estimation by means of densitometry. The values agreed to within 10%. Characterization of 7β-hydroxy DHEA was

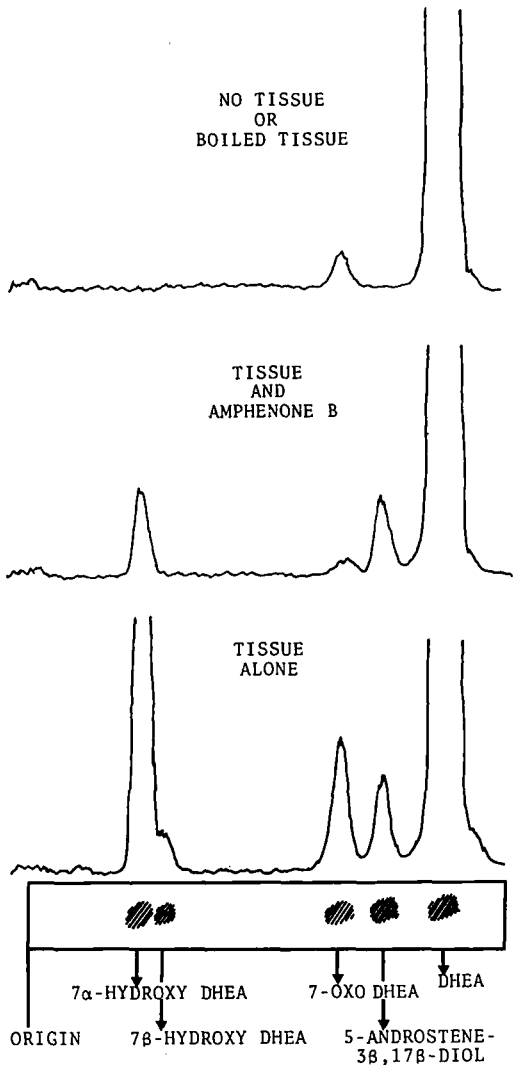

Fig. 1. Metabolism of [4-^{14}C]-DHEA by lactating rat mammary tissue minces. The components of the neutral fraction (see Table 1) were separated by t.l.c. on silica gel and subsequently scanned for radioactivity.

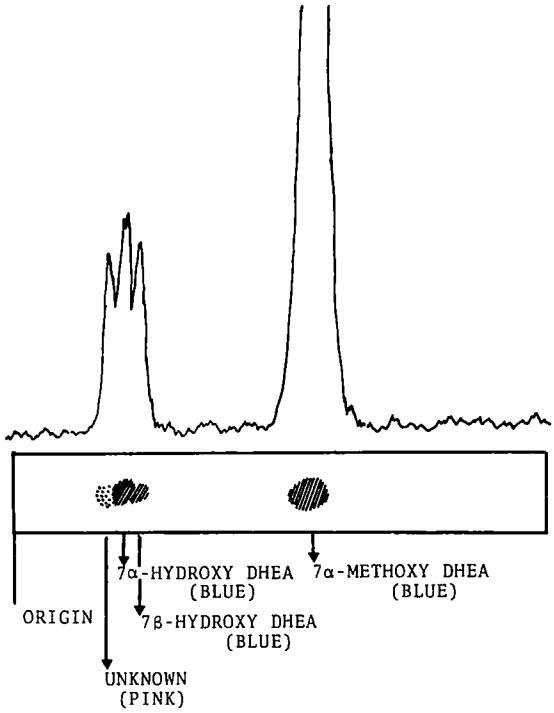

Fig. 2. Identification of [4-^{14}C]-7α-hydroxy DHEA by its facile conversion to 7α-methoxy DHEA. The radioactive metabolite corresponding to 7α-hydroxy DHEA in Fig. 1 was eluted, mixed with authentic material and refluxed in aq. methanol containing some acetic acid. Products were separated by t.l.c. on silica gel. Radioactive zones were measured by scanning and unlabelled steroids revealed by staining with $SbCl_3$.

similarly achieved by its facile conversion to 7β-methoxy DHEA upon refluxing with acidified aq. methanol. 7-Oxo DHEA (Fig. 1) was identified by chromatographic comparison with authentic material (solvent systems I, II, V, VI and VII) and by conversion to 7α- and 7β-hydroxy DHEA by reduction with sodium borohydride.

Due to the influence of the 5–6 double bond on the chemical reactivity at the 7-position in DHEA, the possibility arises that formation of 7-hydroxylated derivatives may occur by non-enzymic means. Controls, carried out without tissue, i.e. in which the [^{14}C]-DHEA was incubated and processed throughout the entire working up procedure, yielded only small amounts of 7-oxo DHEA (Fig. 1). Boiled tissue controls yielded similar results. It should be noted that [^{14}C]-DHEA, which was not purified on alumina prior to incubation, did show the presence of very small amounts of 7α- and 7β-hydroxy DHEA, in addition to 7-oxo DHEA. Of significance was the fact that these epimers were present in about equal amounts—unlike the ratios observed employing tissue incubations. In a number of experiments, a marked decrease in the yield of 7-hydroxylated products occurred in the presence of the steroid hydroxylase inhibitor Amphenone B, without any change occurring in the formation of 5-androstene-3β,17β-diol catalysed by the 17β-hydroxy dehydrogenase. The results of one such experiment are shown in Fig. 1. Table 1 summarizes yields of metabolites obtained with mammary gland and mammary tumours. [4-^{14}C]-Cholesterol (S.A. 56 mCi/mmol) did not yield detectable levels of 7-hydroxy-cholesterol when incubated with lactating mammary gland minces or homogenates. It is possible that dilution of the labelled substrate by endogenous cholesterol may have prevented detection of metabolites in such experiments.

DISCUSSION

The main metabolite of [^{14}C]-DHEA formed by incubation with normal mammary gland and mammary tumours, was 7α-hydroxy DHEA. This was accompanied by lesser amounts of the 7β-epimer and 7-oxo DHEA in every case. 5-Androstene-3β,17β-diol was the only other metabolite detected and it also was formed by each of the tissues examined (Table 1). The conversion of DHEA to 7-hydroxy DHEA averaged 8.6 ± 3.7 (S.D.)% and conversion to 5-androstene-3β,17β-diol 3.0 ± 1.0 (S.D.)%. These conversions represent percentages of the actual radioactivity in the neutral fraction (see Table 1). Conversion to 7-oxo DHEA as shown in Table 1 would be overestimated due to some formation of this substance by non-enzymic means.

A 7-hydroxylase has not previously been reported in mammary tissue. Demonstration that 7-hydroxy DHEA was formed enzymically, and not by chemical oxidation at the reactive 7-position, was based on the following facts:

(i) Freshly purified [4-^{14}C]-DHEA on incubation without tissue, or with boiled tissue, did not yield 7-hydroxylated products, but did produce small amounts (0.63%) of 7-oxo DHEA.

(ii) Unpurified [4-^{14}C]-DHEA was found to contain 7α- and 7β-hydroxy DHEA (1.1%), but these epimers were present in approximately equal amounts in contrast to the situation resulting from incubations using fresh tissue, in which the 7α:7β ratio averaged 4:1.

(iii) In the presence of the hydroxylase inhibitor Amphenone B, conversion of [4-^{14}C]-DHEA to oxygenated products by mammary tissue was reduced to very low levels, whereas formation of 5-androstene-3β,17β-diol by 17β-hydroxy dehydrogenase was unaffected (Fig. 1). Conversion to 7α-hydroxy DHEA (13.4% in the control) was reduced in the presence of Amphenone B (1.4%). 7β-Hydroxy DHEA formation was not detectable in the presence of Amphenone B, and 7-oxo DHEA formation was reduced from 2.4 to 0.5%, which suggested that 7-oxo DHEA was formed from 7-hydroxylated intermediates. By contrast, the yields of 5-androstene-3β,17β-diol were 1.8% with tissue alone, and 1.9% in the presence of Amphenone B (Fig. 1).

Table 1. Yield of products derived from the metabolism of [4-^{14}C]-DHEA by rat mammary gland and mammary tumours

Metabolite	% of radioactivity in the neutral fraction*						
	Lactating gland				DMBA	DMBA	Oestradiol-17β
	I 0.15g	II 0.15g	III 0.05g	IV 0.5g	Tumour I 0.4g	Tumour II 1.0g	induced tumour 1.4g
DHEA	85.3	83.6	80.6	91.4	86.0	77.1	86.4
5-androstene-3β,17β-diol	2.6	2.0	1.8	2.6	3.3	4.6	3.9
7α-hydroxy DHEA	5.5	8.3	13.4	2.3	6.4	5.7	6.2
7β-hydroxy DHEA	1.5	2.2	1.8	0.8	1.1	3.4	1.3
7-oxo DHEA	4.4	3.6	2.4	2.9	3.1	6.6	1.8

* The neutral fraction contained some 95% of the radioactivity recovered. The latter averaged 79.2 ± 11.3 (S.D.)%.

Formation of 5-androstene-3β,17β-diol is of interest since it is an active androgen[5] and can compete effectively with oestradiol-17β and 5α-dihydrotestosterone for binding to their respective receptors[6]. No other hydroxylated products other than 7-hydroxy DHEA were detected in the present experiments. Miller, Forrest and Hamilton[7] demonstrated the presence of low levels of a 16-hydroxylase for testosterone in DMBA-induced rat mammary tumours, but formation of 16α-hydroxy DHEA was not detected using [^{3}H]-DHEA as substrate. In contrast to rat mammary tissue, 16-hydroxylated 5-androstenes are major metabolites of DHEA employing human mammary carcinoma tissue in experiments *in vitro*[8]. In male rat liver, DHEA is hydroxylated predominantly in the 7α- and 16α-positions[9, 10]. Interestingly, recent investigations have also shown that 7-oxygenated products are the major metabolites of DHEA by human liver microsomal preparations[11]. In contrast to the male, female rat liver contains very low levels of steroid hydroxylases-steroid metabolism in this case being mainly via a sulphurylation pathway[12]. One single exception, as regards the steroid hydroxylases, occurs: female rat liver contains relatively high levels of 7-hydroxylases acting on DHEA[13]. Considerable evidence has accumulated which suggests the 7α- and 16α-hydroxylases are distinct enzymes. For example, in male rat liver both these enzymes exhibit daily rhythms in which a reciprocal relationship exists[14]. Again, the 7α- and 16α-hydroxylases for testosterone in rat liver are differently affected by drugs[15]. In liver of immature male rats, the 7α-hydroxylase for testosterone is present in the same concentration as the adult. This contrasts with the 16α-hydroxylase which is absent in the liver of the immature rat[15].

It is conceivable that the 7-hydroxylase in the rat mammary gland may play a role in the control of the level of active androgens. 7-Hydroxylated C_{19}-steroids such as 7α-hydroxytestosterone and 7α-hydroxyandrostenedione possess no androgenic activity and the presence of the enzyme in rat testis has been suggested to regulate the biosynthesis of testosterone by competing with 17β-hydroxysteroid dehydrogenase for the substrate androstenedione[16].

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Note added in proof. Since submitting this paper, in *in vitro* synthesis of 7-hydroxy DHEA by human mammary tissues has been described (Couch R. A. F., Skinner S. J. M., Tobler C. J. P. and Doouss T. W.: *Steroids* **26** (1975) 1–15). This confirms our more recent experience; formation of this derivative occurring in all of 5 human tumours examined to date.

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