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# STUDIES ON 5α-REDUCTASE ACTIVITY IN HUMAN OVARIAN TISSUE

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## SUMMARY

Wedge resections of ovarian tissue from two patients with micropolycystic ovaries were macrodissected into two components, one containing principally stromal tissue, and the other microcysts. The stromal tissue from one patient was subdivided into equal fractions and incubated with 17α-hydroxyprogesterone, dehydroepiandrosterone, androstenedione and testosterone. Equal weights of stromal tissue and microcysts from the second patient were incubated with 17α-hydroxyprogesterone, dehydroepiandrosterone and androstenedione. 5α-Androstanedione, androsterone and epiandrosterone were isolated from all incubations and identified on the basis of their chromatographic properties and by recrystallisation to constant specific activity. The percentage of the initial radioactivity in these three products, after correction for experimental losses, did not exceed 10 per cent. Androstenedione was the best substrate for the formation of 5α-androstanedione, androsterone and epiandrosterone, and in most instances androsterone was formed in slightly higher yield than the 3β-isomer. Both stromal and micropolycystic tissue appeared to possess similar 5α-reductase activity. No radiometabolites with the 5β-configuration were identified.

## INTRODUCTION

RESEARCH based upon the '*in vitro*' incubation of human ovarian tissue with isotopically labelled compounds has provided indirect information on the numerous enzymes involved in the biosynthesis of ovarian steroid hormones. In addition, attempts have been made to relate the potential activity of the more important enzymes to ovarian morphology [1–3].

During the course of these studies evidence has also been obtained for the presence of 5α-reductase activity in samples of normal human ovaries. Sweat *et al.* [4] have suggested the formation of 5α-pregnanedione* from progesterone; Ingiulla *et al.* [3], Galli *et al.* [5] and Forleo *et al.* [6] have demonstrated the transformation of androstenedione to 5α-androstanedione. Other studies have suggested that 5α-reductase activity is particularly evident in stromal tissue and tissues resected from micropolycystic ovaries [3, 7].

The present report is concerned with the formation of 5α-androstanedione, androsterone and epiandrosterone from four different substrates, and from both stromal tissue and microcysts dissected from wedges of micropolycystic ovaries.

*The following abbreviations and trivial names are used: A (androstenedione): 4-androstene-3,17-dione; T (testosterone): 17β-hydroxy-4-androstene-3-one; DHA (dehydroepiandrosterone): 3β-hydroxy-5-androstene-17-one: 17-OH-P (17α-hydroxyprogesterone): 17α-hydroxy-4-pregnene-3,20-dione; A-diol (androstenediol): 5-androstene-3β,17β-diol; 17α-OH-20α-DH-P (17α-hydroxy-20α-dihydroprogesterone): 17α,20α-dihydroxy-4-pregnene-3-one; 5α-A (5α-androstanedione): 5α-androstane-3,17-dione; An (androsterone): 3α-hydroxy-5α-androstane-17-one; Ep (epiandrosterone): 3β-hydroxy-5α-androstan-17-one; (pregnenolone): 3β-hydroxy-5-pregnene-20-one; (progesterone): 4-pregnene-3,20-dione; (5α-pregnanedione): 5α-pregnane-3,20-dione; (5α-dihydrotestosterone): 17β-hydroxy-5α-androstane-3-one; $E_1$(estrone):3-hydroxy-1,3,5(10)-estratrien-17-one; $E_2$(estradiol): 1,3,5(10)-estratrien-3,17β-diol; $E_3$ (estriol): 1,3,5(10)-estratriene-3,16α,17β-triol.

## MATERIALS AND METHODS

### (a) *Solvents and reagents*

All solvents were purified by standard techniques and redistilled prior to use. Camag silica gel DSF-5 was used for thin layer chromatography. The plates were prepared with 50 g silica gel plus 105 ml water to a thickness of 0·25 mm and activated at 110°C for 1 hr. When cool the plates were pre-washed with ethanol and re-activated at 100°C for 30 min.

### (b) *Substrates*

Radioactive substrates were supplied by The Radiochemical Centre, Amersham, Bucks, England: 17$\alpha$-hydroxyprogesterone-4-$^{14}$C (s.a.: 35·9 mCi/mM), dehydroepiandrosterone-4-$^{14}$C (s.a.: 27·2 mCi/mM), androstenedione-4-$^{14}$C (s.a.: 34·8 mCi/mM), testosterone-4-$^{14}$C (s.a.: 55·2 mCi/mM).

All steroids were chromatographed on paper before being used as substrates.

### (c) *Coenzymes*

A standard solution containing ATP, NAD, NADP, NADH and NADPH (1 $\mu$mol of each in 100 $\mu$l of Krebs Ringer phosphate buffer) was prepared immediately before incubation, and 100 $\mu$l were added to each flask.

### (d) *Tissues*

The ovarian tissue was obtained by bilateral wedge resection from two women with micropolycystic ovaries. In both instances an attempt was made to separate the microcysts from the surrounding stromal tissue, so that the enzymatic activity of both components could be investigated. The tissues were placed under a magnifying lamp and all visible microcysts removed with fine surgical instruments. Subsequent histological examination of both fractions showed that many of the follicles were surrounded by stromal cells, and furthermore small follicles – either atretic or in the first stages of maturation – could be identified in the stromal tissue. However, there was an effective separation of the tissue into one fraction containing predominantly follicular tissue and another containing predominantly stromal tissue.

### (e) *Apparatus*

A radiochromatogram scanner, Packard Model 7201, was used for the detection of carbon-14 after both paper and thin-layer chromatography. Liquid scintillation counting was performed with a Packard Tricarb Liquid Scintillation Counter, model 3375. The liquid scintillator contained 5 g of 2,5-diphenyloxazole (PPO) and 300 mg of 1,4-di(4-methyl,5-phenyloxazole)-benzene (dimethyl POPOP) dissolved in one litre of redistilled toluene. 10 ml of this solution was used for each determination. The weighings for the recrystallisation studies were made on a Mettler M-5 balance maintained in a room at 25°C with humidity control and minimal vibration.

## METHOD FOR THE ANALYSIS OF RADIOMETABOLITES

### 1. *Incubation*

Fresh tissue was used in all studies, the incubations starting twenty to thirty minutes after surgery. After macrodissection under a magnifying lamp, the tissues were minced into pieces approx. 1 mm$^3$, subdivided into a number of portions and

weighed. The radioactive substrates prepared in ethanol were added to 25 ml incubation flasks so that the ratio between tissue and substrate was 50,000:1 (w/w). After the ethanol was dried under a stream of nitrogen, the tissue minces were washed into the incubation flasks with Krebs Ringer phosphate buffer until the tissue buffer ratio was 1:5 (w/v). Portions of the residual stromal tissue were examined histologically and found to be virtually free of microcysts. The flasks were placed in a water bath at 37°C and shaken mechanically (120 vibrations/min). The reactions were terminated after 4 hr by the addition of 20 ml acetone, and the flasks stored in a refrigerator at 4°C overnight.

2. *Extraction*

The general procedure for the isolation and quantitative determination of radiometabolites has been described in detail by Forleo *et al.*[8]. Briefly, each incubation mixture was extracted five times with 20 ml of acetone at 50°C and the extract filtered into a R.B. flask. The acetone was then evaporated on a rotary evaporator until only the buffer remained. 20 ml of sodium hydroxide pH 9·0 was added to each flask and the mixture re-extracted twice with methylene dichloride: ether (1:4 v/v). The washed extracts were evaporated to dryness, redissolved to a known volume and 1 per cent removed for liquid scintillation counting.

3. *Separation of neutral and phenolic fractions*

1 ml of ethanol was added to each extract and this was transferred with 25 ml benzene to a separating funnel containing 25 ml petroleum ether (b.p. 40°–60°). The mixture was then extracted twice with 25 ml of 1·6 per cent sodium hydroxide [9]. After extraction, the petroleum ether: benzene was washed twice with distilled water, and evaporated to dryness. The extract was then redissolved to a known volume and 1 per cent removed for liquid scintillation counting. The 1·6 per cent sodium hydroxide was discarded.

4. *Analysis of neutral fraction*

The extract was quantitatively transferred with chloroform: methanol: ethyl acetate 1:1:1 (by vol.) to Whatman No. 2 chromatography paper using a glass capillary. The first chromatogram was performed with a Bush A system (hexane: methanol: water 100:89:15, by vol.) at 37°C. The chromatogram was scanned for radioactivity and divided into three parts (A – B – C). Depending on the substrate, fraction A contained radioactivity at the origin plus 17α-hydroxyprogesterone and testosterone.

Fraction B contained the partially resolved peaks of androstenedione, epiandrosterone, androsterone and dehydroepiandrosterone. This fraction was eluted from the paper using the elution apparatus described by Zander [10] with 25 ml of chloroform: methanol: ethyl acetate 1:1:1 (by vol.) into a conical tube. An aliquot was taken for liquid scintillation counting and the remainder transferred to a thin-layer plate of silica gel and run twice in the system chloroform: methanol 99:1 (v/v). Two distinct peaks of radioactivity were obtained. The less polar corresponded to authentic androstenedione and the other to dehydroepiandrosterone, androsterone and epiandrosterone. The more polar fraction was eluted, an aliquot removed for liquid scintillation counting, and the remainder rechromatographed on paper (Whatman No. 2) for 18 hr in the system isooctane: methanol: water 10:9:1 (by vol.) (11). This system completely resolved the extract into three peaks

which corresponded to authentic androsterone, epiandrosterone and dehydroepiandrosterone. Further evidence for the identification of androsterone and epiandrosterone was obtained by adding 50 mg of authentic steroid to the extracts and recrystallising the mixtures until constant specific activity was achieved between the crystals and mother liquor.

*Additional evidence of identity*

In addition to the procedure described above, samples of androsterone and epiandrosterone isolated from the incubation media were rechromatographed on paper (15 × 103 cm) for 24 hr in the system methylcyclohexane : propylene glycol : methanol (1 : 1 : 2, by vol.). Two distinct peaks of radioactivity were observed with migration velocities corresponding to authentic androsterone and epiandrosterone. The results of five successive recrystallisations of these products are shown in Table 1.

Fraction C contained 5α-androstanedione. After elution, an aliquot was removed for liquid scintillation counting and the remainder chromatographed on a

Table 1. Recrystallisation data of epiandrosterone and androsterone following extended paper chromatography

| Metabolite | $C^{14}$ (dpm/mg) Crystals | Mother liquor |
|---|---|---|
| Epiandrosterone | 3246 | 11479 |
| | 2769 | 5049 |
| | 2650 | 2743 |
| | 2816 | 2342 |
| | 2768 | 2678 |
| Androsterone | 8143 | 6935 |
| | 8106 | 6847 |
| | 7748 | 7986 |
| | 7594 | 7166 |
| | 7176 | 7457 |

thin-layer plate of silica gel. Only one peak of radioactivity was observed with an $R_F$ value corresponding to that of the authentic material. The extract was eluted, an aliquot removed for counting, authentic steroid added, and the mixture recrystallised as above.

## RESULTS

The total number of counts was determined at each successive step in the procedure and corrected for counting efficiency. At each step, the recovery of steroid was corrected to 100 per cent in terms of loss of total counts and the radioactivity in each recrystallised metabolite was expressed as the percentage of the initial radioactivity added in the form of substrate.

*Control incubations*

Four incubation flasks were prepared with 250 mg of tissue, 2·5 ml of buffer and 10 ml of acetone; 5 μg of a different substrate were added to each flask. The incubation mixtures were agitated by hand and extracted with warm acetone, then

Table 2. Percentage distribution of radiometabolites obtained from various labelled substrates by stromal tissue and microcysts of cases 1 and 2

| Tissue | Substrate incubated | Radiometabolites recovered (% of total radioactivity) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 17 OH-P | 17α-OH-20α-DH-P | DHA | A | T | A-diol | 5α-A | An | Ep |
| Case 1 | | | | | | | | | | |
| Stromal | 17-OH-P | 35·9 | (11·4)* | | 18·4 | 2·8 | | 3·4 | 2·1 | 1·3 |
| Stromal | DHA | | | 1·2 | 46·5 | 13·2 | 2·0 | 2·8 | 2·0 | 1·7 |
| Stromal | A | | | | 44·9 | 18·2 | | 2·2 | 4·4 | 3·1 |
| Stromal | T | | | | 12·4 | 65·1 | | 3·1 | 2·3 | 2·1 |
| Case 2 | | | | | | | | | | |
| Stromal | 17-OH-P | 31·4 | (14·6)* | | 13·4 | 2·5 | | 1·8 | 2·3 | 2·5 |
| Microcysts | 17-OH-P | 10·1 | (13·3)* | | 36·7 | 2·2 | | 1·5 | 1·0 | (0·8)* |
| Stromal | DHA | | | (0·9)* | 51·1 | 9·6 | 0·9 | 2·9 | 1·6 | (0·6)* |
| Microcysts | DHA | | | — | 62·5 | 3·3 | — | 3·1 | (0·8)* | 1·1 |
| Stromal | A | | | | 52·2 | 9·1 | | 2·5 | 3·5 | 2·2 |
| Microcysts | A | | | | 57·1 | 4·8 | | 4·0 | 2·0 | 1·4 |

*Not crystallized.

left overnight at 4°C. No identifiable radiometabolites were visible on scanning the first paper chromatogram, and the percentage of each substrate recovered (corrected for losses and purity) ranged from 90 to 96 per cent.

*Case* 1

A wedge of tissue from both ovaries was removed from a 22 year old patient with the classical symptoms of the Stein–Leventhal syndrome. The microcysts were removed and the stromal tissue subdivided into four portions each weighing approx. 250 mg. These portions were incubated independently with 17α-hydroxyprogesterone, dehydroepiandrosterone, androstenedione and testosterone. The percentages of distribution of the radiometabolites recovered at the end of the incubation, corrected for losses, are reported in Table 2. No compounds were detected in the phenolic fraction, confirming previous studies regarding the lack of formation of oestrogens in incubations with tissue from micropolycystic ovaries [12–14]. The results of the recrystallisation studies for 5α-androstanedione, androsterone and epiandrosterone are shown in Table 3.

Table 3. Identification by recrystallisation to constant specific activity (d.p.m./mg) of 5α-androstanedione (5α-A), androsterone (An) and epiandrosterone (Ep) obtained from various labelled substrates

| | Initial specific activity | | Recrystallisation 1 | 2 | 3 | % of total radioactivity |
|---|---|---|---|---|---|---|
| 17α-hydroxyprogesterone | | | | | | |
| 5α-A | 587 | C | 560 | 575 | 569 | 3·4 |
| | | ML | 597 | 581 | 564 | |
| An | 492 | C | 469 | 451 | 464 | 2·1 |
| | | ML | 535 | 504 | 476 | |
| Ep | 413 | C | 398 | 391 | 401 | 1·3 |
| | | ML | 445 | 406 | 388 | |
| Dehydroepiandrosterone | | | | | | |
| 5α-A | 611 | C | 602 | 610 | 606 | 2·8 |
| | | ML | 600 | 614 | 616 | |
| An | 521 | C | 511 | 518 | 506 | 2·0 |
| | | ML | 533 | 510 | 504 | |
| Ep | 321 | C | 311 | 318 | — | 1·7 |
| | | ML | 319 | 302 | — | |
| Androstenedione | | | | | | |
| 5α-A | 766 | C | 754 | 763 | — | 2·2 |
| | | ML | 769 | 757 | — | |
| An | 623 | C | 602 | 596 | 581 | 4·4 |
| | | ML | 684 | 616 | 580 | |
| Ep | 901 | C | 874 | 885 | 863 | 3·1 |
| | | ML | 937 | 903 | 880 | |
| Testosterone | | | | | | |
| 5α-A | 1210 | C | 1184 | 1175 | 1193 | 3·1 |
| | | ML | 1205 | 1198 | 1160 | |
| An | 510 | C | 484 | 479 | 475 | 2·3 |
| | | ML | 574 | 496 | 480 | |
| Ep | 874 | C | 849 | 831 | 839 | 2·1 |
| | | ML | 907 | 860 | 834 | |

*Case 2*

Two large wedges were removed from the ovaries of a 23-year old patient with the symptoms of the Stein–Leventhal syndrome. Both wedges were dissected into microcysts and stromal tissue and equal weights of both components (approx. 250 mg) incubated with 17α-hydroxyprogesterone, dehydroepiandrosterone and androstenedione. The results from this study are shown in Table 2; once again no estrogens were detectable. Data from the recrystallisation studies for 5α-androstanedione, androsterone, and epiandrosterone are shown in Tables 4, 5 and 6.

Table 4. Identification by recrystallisation to constant specific activity (d.p.m./mg) of 5α-androstanedione (5α-A), androsterone (An) and epiandrosterone (Ep) obtained from incubations with 17α-hydroxyprogesterone

| | Initial specific activity | | Recrystallisation 1 | 2 | 3 | % of total radioactivity |
|---|---|---|---|---|---|---|
| Stromal tissue | | | | | | |
| 5α-A | 752 | C | 737 | 758 | 746 | 1·8 |
| | | ML | 719 | 729 | 730 | |
| An | 314 | C | 284 | 295 | 278 | 2·3 |
| | | ML | 369 | 296 | 285 | |
| Ep | 768 | C | 732 | 729 | 737 | 2·5 |
| | | ML | 814 | 743 | 722 | |
| Microcysts | | | | | | |
| 5α-A | 423 | C | 416 | 422 | — | 1·5 |
| | | ML | 431 | 416 | — | |
| An | 639 | C | 627 | 640 | 631 | 1·0 |
| | | ML | 618 | 628 | 686 | |
| Ep | | | Insufficient material for recrystallisation | | | (0·8) |

## DISCUSSION

This study confirms and extends previous work on the isolation and quantitative determination of $C_{19}$-steroids with a 5α configuration. For although 5α-androstanedione had been isolated from incubations of ovarian components with a variety of $C_{19}$ substrates [5, 6], little was known of any further metabolism of this compound in ovarian tissue. However, similar studies with liver had demonstrated the possible role of 5α-androstanedione as an intermediary in the formation of androsterone [15–18]. The results from the present series of incubations demonstrate the potential formation of 5α-androstanedione, androsterone, and epiandrosterone in both stromal tissue and microcysts from two patients with micropolycystic ovaries. However, in all incubations the total radioactivity recovered in these three products did not exceed 10 per cent of that added as substrate. In addition, both stromal and micropolycystic tissue appeared to possess similar 5α-reductase, 3α-hydrogenase, and 3β-hydrogenase activity. Androstenedione was the best substrate for the formation of androsterone and epiandrosterone and the yields of androsterone were always slightly higher.

At the present time, using pregnenolone-4-$^{14}$C and progesterone-4-$^{14}$C as substrates, we have not been able to find sufficient labelled 5α-androstanedione,

Table 5. Identification by recrystallisation to constant specific activity (d.p.m./mg) of 5α-androstanedione (5α-A), androsterone (An) and epiandrosterone (Ep) obtained from incubations with dehydroepiandrosterone

| | Initial specific activity | | Recrystallisation 1 | 2 | 3 | % of total radioactivity |
|---|---|---|---|---|---|---|
| Stromal tissue | | | | | | |
| 5α-A | 623 | C | 552 | 523 | 562 | 2·9 |
| | | ML | 788 | 675 | 501 | |
| An | 873 | C | 821 | 835 | 820 | 1·6 |
| | | ML | 912 | 850 | 813 | |
| Ep | | | Insufficient material for recrystallisation | | | (0·6) |
| Microcysts | | | | | | |
| 5α-A | 512 | C | 506 | 498 | — | 3·1 |
| | | ML | 531 | 510 | — | |
| An | | | Insufficient material for recrystallisation | | | (0·8) |
| Ep | 380 | C | 350 | 364 | 349 | 1·1 |
| | | ML | 411 | 373 | 356 | |

Table 6. Identification by recrystallisation to constant specific activity of 5α-androstanedione (5α-A), androsterone (An) and epiandrosterone (Ep) obtained from incubations with androstenedione

| | Initial specific activity | | Recrystallisation 1 | 2 | 3 | % of total radioactivity |
|---|---|---|---|---|---|---|
| Stromal tissue | | | | | | |
| 5α-A | 1985 | C | 1899 | 1950 | 1920 | 2·5 |
| | | ML | 1956 | 1952 | 1941 | |
| An | 1221 | C | 885 | 854 | 863 | 3·5 |
| | | ML | 1445 | 952 | 874 | |
| Ep | 510 | C | 514 | 494 | — | 2·2 |
| | | ML | 502 | 490 | — | |
| Microcysts | | | | | | |
| 5α-A | 982 | C | 974 | 950 | 963 | 4·0 |
| | | ML | 969 | 964 | 953 | |
| An | 425 | C | 364 | 342 | 351 | 2·0 |
| | | ML | 462 | 386 | 360 | |
| Ep | 609 | C | 581 | 594 | 577 | 1·4 |
| | | ML | 598 | 570 | 584 | |

androsterone or epiandrosterone to allow their positive identification. Furthermore, it has not been possible to demonstrate the formation of any metabolites with a 5β configuration. In these experiments no attempt was made to identify 5α-dihydrotestosterone, which is now under study. However, if the 5α-reductase, and the 3α- and 3β-hydrogenase activity observed in this series of experiments also occurs in the intact ovary where there is probably far less substrate, the ques-

tion arises as to whether the conversions are to secretory products, or whether they are associated with an action of the ovarian hormones themselves on some aspect of ovarian growth or activity.

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