Studies on Steroid Metabolism in Human Endometrial Tissue

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1. A computerized technique is described for the quantitative determination of radiometabolites from incubation studies. 2. Seven steroid substrates have been incubated with human endometrial tissue. The principal radiometabolites were identified and determined after 2 hr. incubation without the addition of cofactors and after 4 hr. incubation with cofactors. 3. The main products from progesterone were 20 α -dihydroprogesterone and 5 α -pregnanedione with lower yields of 5 β -pregnanedione and 20 β -dihydroprogesterone. There was no evidence for 17 α -hydroxylase activity. 4. 17 α -Hydroxyprogesterone was transformed into small yields of 17 α ,20 α - and 17 α ,20 β -dihydroxypregn-4-en-3-one. In one incubation there was evidence for conversion into androstenedione. 5. Dehydroepiandrosterone was transformed into small amounts of androstenedione, 5 α -androstenedione and androsterone. 6. Androstenedione and testosterone were interconvertible, the reaction favouring the formation of androstenedione. 5 α -Androstenedione and androsterone were formed from both substrates. There was no evidence for the formation of phenolic steroids. 7. Oestrone and oestradiol-17 β were interconvertible, the reaction favouring the formation of oestrone.

The human endometrium undergoes cyclical changes in morphology and function that have been shown to be dependent on the biosynthesis and secretion of ovarian hormones. However, little information is available on the concentration and transformation of the principal ovarian hormones and their metabolites in this tissue.

Sweat, Bryson & Young (1967) reported on the transformation of oestradiol-17 β [oestra-1,3,5(10)-triene-3,17 β -diol] by human proliferative endometrium and subsequently Bryson & Sweat (1967) reported on the transformation of progesterone (pregn-4-ene-3,20-dione) in tissue from the same phase of the cycle. The principal findings were a high formation of oestrone [3-hydroxyoestra-1,3,5(10)-trien-17-one] from oestradiol-17 β , and significant yields of 20 α -dihydroprogesterone (20 α -hydroxypregn-4-en-3-one), 5 α -pregnane-3,20-dione and an unidentified (dihydroxy) compound from progesterone. In addition, these authors suggested that the human endometrium has minimal capacity for the side-chain cleavage of 17 α -hydroxyprogesterone (17 α -hydroxypregn-4-ene-3,20-dione) or for the formation of oestrogens from neutral steroid intermediaries.

The present paper concerns the transformation of isotopically labelled progesterone, 17 α -hydroxyprogesterone, dehydroepiandrosterone (3 β -hydroxyandrost-5-en-17-one), androstenedione (androst-4-

ene-3,17-dione), testosterone (17 β -hydroxyandrost-4-en-3-one), oestrone and oestradiol-17 β by human secretory endometrium and illustrates the application of a computerized technique for the quantitative determination of radiochemically pure metabolites.

EXPERIMENTAL

Solvents and reagents. All solvents were of A.R. grade and redistilled before use. Pyridine and acetic anhydride were redistilled under reduced pressure and stored over CaCl₂.

Silica gel (Camag DSF-5) with binding agent and fluorescent indicator was used for t.l.c. The plates were prepared with 60 g. of silica gel plus 100 ml. of water and activated at 120° for 1 hr. When cool the plates were pre-washed with methanol and reactivated at 120° for 30 min.

Cofactors. ATP, NAD⁺, NADP⁺, NADH and NADPH were obtained from Boehringer Corporation (London) Ltd., London W.5. A standard solution containing 1 μ mole of each compound in 100 μ l. of Krebs-Ringer bicarbonate buffer (Krebs, 1950) was prepared immediately before incubation.

Steroids. Authentic steroid standards for use as chromatographic markers and for recrystallization studies were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Androsterone acetate (3 α -acetoxy-5 α -androstan-17-one) and androstanolone acetate (17 β -acetoxy-5 α -androstan-3-one) were purchased from Steraloids Ltd., Croydon, Surrey.

Substrates. The following substrates were obtained from The Radiochemical Centre, Amersham, Bucks. (specific

radioactivities in mc/m-mole): [4-¹⁴C]progesterone, 58.5; 17 α -hydroxy[4-¹⁴C]progesterone, 35.9; dehydro[4-¹⁴C]epiandrosterone, 57.1; [4-¹⁴C]androstenedione, 56.6; [4-¹⁴C]testosterone, 55.2; [4-¹⁴C]oestrone, 53.4; [4-¹⁴C]oestradiol-17 β , 53.7. A sample from each batch was tested for radiochemical purity by t.l.c. followed by radioautography and liquid-scintillation counting.

Endometrium. The tissue was obtained either by curettage or from uteri removed at total hysterectomy.

Preparation of tissue and incubation conditions. The radioactive substrates (2 μ g. in 10 μ l.) were added to 25 ml. incubation flasks and evaporated to dryness under a stream of N₂. Fresh tissue was used in all studies, the incubations starting 10–20 min. after surgery. The components of the uterus were dissected under a magnifying lens-lamp and the endometrium was cut into small pieces (approx. 1 mm.³) with scissors; thus many of the cells were left intact. The tissue mince was divided into a number of portions, weighed on water-repellent paper and washed into the incubation flasks with 3 ml. of Krebs–Ringer bicarbonate buffer, pH 7.4. Then 1 mg. of benzylpenicillin was added to each flask. The tissues were incubated at 37° for 2 or 4 hr. under air in a metabolic shaker. The reactions were terminated by the addition of 10 ml. of redistilled acetone and the flasks stored in the refrigerator at 4° overnight.

Extraction of steroids. The incubation mixture was decanted through filter paper into a 100 ml. round-bottomed flask and the residual tissue extracted twice more with 12 ml. of hot acetone. The pooled acetone extracts were evaporated under reduced pressure with a flash evaporator until only the buffer remained. The volume was made up to 10 ml. with water and the pH adjusted to 10.0 with 0.3 M-NaOH. The steroids were extracted again from the aqueous medium with 2 $\times$ 50 ml. of diethyl ether and the combined ether extracts washed twice with 20 ml. of water. The ether extract was evaporated to dryness in a 100 ml. round-bottomed flask. The dry residue was dissolved in methanol-chloroform (1:1, v/v), transferred to a pointed test tube and evaporated under N₂ at 60°.

Thin-layer chromatography. The dried extract was transferred with 3 $\times$ 0.5 ml. of chloroform-methanol (1:1, v/v) to a glass plate coated with silica gel to a thickness of 250 μ m. The chromatogram was developed in chloroform-methanol (99:1, v/v) and the spectrum of metabolites examined by radioautography with overnight exposure of the plates to Kodirex No-Screen X-ray film (Kodak Ltd., London W.C.1).

The areas of radioactivity were aspirated on to a filter disk (no. 3 grade) and eluted with ethanol (3 $\times$ 0.5 ml.). The eluates were evaporated, redissolved to a known volume and 5% portions removed for liquid-scintillation counting. The residual liquid was evaporated again. The remaining silica gel along the path of the chromatogram was also aspirated on to a filter disk, eluted with ethanol and the radioactivity determined.

Formation of derivatives. The dry steroid residues from the first t.l.c. plate were acetylated by the addition of 0.1 ml. of pyridine and 0.1 ml. of acetic anhydride followed by heating at 60° for 30 min. in a constant-temperature water bath. The reagent was removed under N₂ in a water bath at 60° after addition of a small amount of methanol to assist evaporation.

Thin-layer chromatography of derivatives. The acetylated steroid extracts were further fractionated on silica gel

DSF-5 together with side and central standards. For standards that were u.v.-absorbing (235.7 nm.) 2 μ g. of steroid was used and 5 μ g. for those staining with phosphomolybdic acid (5%, w/v, in ethanol). Sufficient radioactive standard was applied to give a dark spot from an overnight exposure (5000 d.p.m.). The plates were developed in benzene-ethyl acetate (4:1, v/v) and radioautographed overnight. The steroid spots were eluted with ethanol, as was the remaining silica gel containing the residual radioactivity from each run. The solvent was evaporated to dryness under N₂ in a water bath and a portion (5%) taken for liquid-scintillation counting. The eluate from the silica gel on the chromatogram was transferred to a counting vial and the radioactivity determined.

Recrystallization to constant specific radioactivity. The extract containing the radiometabolite was evaporated to dryness in a pointed tube and the authentic non-labelled steroid added so that when possible 1 mg. corresponded to approx. 1000 d.p.m. of the product. The mixture was completely dissolved in methanol-chloroform (1:1, v/v), and a fraction transferred to a small glass container that had been previously weighed. The vessel was placed in a metal holder and the steroid residue was dried by heating in an oven for 1 hr. at 60°. The vessel was then allowed to cool and equilibrate. Each vessel was weighed and reweighed after further drying until successive weights of the residue were within 0.5%. All weighings were made on a Mettler-M5 balance (E. Mettler Ltd., Zurich, Switzerland). The glass vessel was transferred to a vial containing 8 ml. of scintillation fluid maintained at approx. 18° and shaken until the steroid had completely dissolved. The radioactivity was determined by liquid-scintillation counting to an accuracy of better than 2% and the result corrected for quenching. The d.p.m./mg. constitutes the initial specific radioactivity. The first crystallization was performed with the minimal volume of an appropriate solvent depending on the solubility of the steroid (acetone, methanol or ethanol at 50–60°). The tube was stoppered and left in a refrigerator at 4° until crystals had formed. If appropriate solvents were used the crystals adhered to the bottom of the tube and there was a clear separation between the mother liquor and the crystals. The mother liquor was transferred to a weighed vessel and dried in an oven at 60° for 1 hr. A portion of the crystalline residue was treated in the same way, and both fractions were carefully weighed. In this manner the d.p.m. for both mother liquor and crystals were obtained. Successive recrystallizations were performed from different solvents, e.g. tetrahydrofuran, methanol-water, acetone-pentane. A mathematical analysis of the errors inherent in this approach (Axelrod, Matthijssen, Goldzieher & Pulliam, 1965) indicates that three successive crystallizations of a pure radioactive compound should yield values for the specific radioactivity that are within $\pm$ 5% of the average of the three values.

Measurements of radioactivity. A Nuclear-Chicago model 6860 (mark 1) liquid-scintillation counting system was used for the determination of radioactivity. The scintillation fluid contained 3 g. of 2,5-diphenyloxazole/l. of toluene; 8 ml. was used for counting samples containing ¹⁴C and 10 ml. for those containing tritium. The counting efficiency for each sample was determined by reference to a calibration curve, which was plotted from a set of quenched standards by using an external ¹³³Ba source and a channels-ratio method.

Calculation of results. A generalized scheme for calculating

Scheme 1. Scheme for the quantitative determination of radiometabolites. See the Experimental section for details. LSC, Liquid-scintillation counting.

the percentage of initial radioactivity in any metabolite is illustrated in Scheme 1. A quantity of radioactive substrate (X d.p.m.) was incubated with the minced tissue. After extraction, a portion, α , was taken for liquid-scintillation counting, giving a radioactivity X_0 d.p.m. in the sample. Hence the total activity recovered was X_0/α d.p.m. and the correction factor for losses is $(X/X_0)/\alpha$, i.e. $X\alpha/X_0$.

Assume that n clearly defined radioactive areas were observed on the first t.l.c. plate. After elution of the i th area, a portion, α_i , was taken for liquid-scintillation counting, giving a radioactivity X_i d.p.m. and X_i/α_i d.p.m. in the complete eluate. Similarly, the radioactivity of all n eluates was determined, as was the total radioactivity of the residue on the plate, X_R d.p.m. Hence the total radioactivity accounted for on the first t.l.c. plate was:

$$X_R + \sum_{i=1}^n \frac{X_i}{\alpha_i} \text{ d.p.m.}$$

As the radioactivity accounted for after extraction was X_0/α d.p.m., the correction factor for losses on the first t.l.c. plate and in the transfer and liquid-scintillation counting of the portion, α , is:

$$\frac{X_0/\alpha}{X_R + \sum_{i=1}^n \frac{X_i}{\alpha_i}}$$

Similarly, if m clearly defined radioactive areas were observed when the i th eluate was transferred to the second t.l.c. plate, and the radioactivity of the residue was $X_{R'}$, the total radioactivity accounted for on the second t.l.c. plate was:

$$X_{R'} + \sum_{j=1}^m \frac{X_{ij}}{\alpha_{ij}} \text{ d.p.m.}$$

As the radioactivity accounted for on the first t.l.c. plate was X_i/α_i d.p.m., the correction factor for losses on the second t.l.c. plate, and in the liquid-scintillation counting of the portion α_i , is:

$$\frac{X_i/\alpha_i}{X_{R'} + \sum_{j=1}^m \frac{X_{ij}}{\alpha_{ij}}}$$

Hence the total correction factor for losses in the extraction, purification and liquid-scintillation counting processes is the product of these individual factors, i.e.:

$$\frac{X\alpha}{X_0} \cdot \frac{X_0/\alpha}{X_R + \sum_{i=1}^n \frac{X_i}{\alpha_i}} \cdot \frac{X_i/\alpha_i}{X_{R'} + \sum_{j=1}^m \frac{X_{ij}}{\alpha_{ij}}}$$

The remainder of the ij th metabolite from the second t.l.c. plate was recrystallized to constant specific radioactivity to determine its purity, β_{ij} , and hence the measured radioactivity of the ij th metabolite, $\beta_{ij}X_{ij}/\alpha_{ij}$ d.p.m.

The corrected radioactivity of the ij th metabolite, M_{ij} , expressed as a percentage of the radioactivity of the substrate added, X d.p.m., is therefore given by:

$$\beta_{ij} \cdot \frac{X_{ij}}{\alpha_{ij}} \cdot \frac{X\alpha}{X_0} \cdot \frac{X_0/\alpha}{X_R + \sum_{i=1}^n \frac{X_i}{\alpha_i}} \cdot \frac{X_i/\alpha_i}{X_{R'} + \sum_{j=1}^m \frac{X_{ij}}{\alpha_{ij}}} \cdot \frac{100}{X}$$

which reduces to:

$$M_{ij}(\%) = \beta_{ij} \cdot \frac{X_{ij}}{\alpha_{ij}} \cdot \frac{X_i}{\alpha_i \left(X_{R'} + \sum_{j=1}^m \frac{X_{ij}}{\alpha_{ij}} \right)} \cdot \frac{100}{\left[X_R + \sum_{i=1}^n \frac{X_i}{\alpha_i} \right]} \quad (1)$$

A general programme using this expression, and suitable for any number of radiometabolites, has been written for the Olivetti Programma 101.

Additional evidence of identification. The second t.l.c. plate does not completely separate the acetylated derivatives of dehydroepiandrosterone, androstanolone, androsterone, epiandrosterone and aetiocholanolone. If recrystallization studies indicated the presence of one of these compounds other than the one suspected, a separation could be achieved and the percentage of each in the total determined by g.l.c. with a Pye 104 model 84 chromatograph and ^{63}Ni electron-capture detection. Glass columns (144 cm. $\times$ 4 mm. internal diam.) were packed with 3% XE-60 (cyanoethyl methyl silicone) on Gas Chrom Q and operated at 190°. The carrier gas was argon-methane (95:5) at 50 ml./min. The detector was maintained at 220°, and every 150 $\mu\text{sec.}$ a 47 v pulse of 0.75 $\mu\text{sec.}$ duration was applied.

The heptafluorobutyrate derivatives were prepared (Collins, Sisterson, Koullapis, Mansfield & Sommerville, 1968), either after the first t.l.c. or after hydrolysis of the acetylated derivatives with methanolic NaOH (Bush, 1961). An appropriate portion of the sample was transferred to a solid injection syringe (SS60; 3½ in. needle; Hamilton Co. Inc.) and applied directly to the top of the column. The detector response was displayed on a chart recorder and the

amount of each compound determined by electronic digital integration (Infotronics, model CRS-10HB) of the standard and unknown. The retention times of the heptafluorobutyrate derivatives relative to dehydroepiandrosterone heptafluorobutyrate (7.1 min. = 1.0) were: androsterone, 0.78; aetiocholanolone (3 α -hydroxy-5 β -androstan-17-one), 0.87; epiandrosterone (3 β -hydroxy-5 α -androstan-17-one), 1.13; androstanolone, 1.37.

EVALUATION OF METHOD

The procedure for the quantitative determination of radiometabolites has been evaluated from a theoretical approach and in practice by replicate analysis of a known mixture of labelled steroids.

Theoretical assessment. In an attempt to determine the random error on the percentage of initial radioactivity in metabolite M_{ij} the random errors on each term in eqn. (1) were assessed. The overall percentage error on M_{ij} [$\Delta M_{ij} = (\text{s.d.} \times 100)/M_{ij}$] was then determined from eqn. (2) below, derived from the usual laws for the combination of additive and multiplicative independent normal errors:

$$\Delta^2 M_{ij} = \Delta^2 \beta_{ij} + \Delta^2 X_{ij} + \Delta^2 X_i + \Delta^2 \alpha_{ij} + \Delta^2 \alpha_i$$

$$\begin{aligned} & + \frac{\Delta^2 X_{R'} \cdot X_{R'} + \sum_{j=1}^m \left(\frac{X_{ij}}{\alpha_{ij}} \right)^2 \cdot (\Delta^2 X_{ij} + \Delta^2 \alpha_{ij})}{\left(X_{R'} + \sum_{j=1}^m \frac{X_{ij}}{\alpha_{ij}} \right)^2} \\ & + \frac{\Delta^2 X_R X_{R'} + \sum_{i=1}^n \left(\frac{X_i}{\alpha_i} \right)^2 \cdot (\Delta^2 X_i + \Delta^2 \alpha_i)}{\left(X_R + \sum_{i=1}^n \frac{X_i}{\alpha_i} \right)^2} \end{aligned} \quad (2)$$

where $\Delta\beta_{ij}$ is the overall percentage error on β_{ij} , and similarly for other terms.

The maximum error on each term in eqn. (1) was evaluated as follows.

α_{ij} , α_i . There are two errors in each term, one from diluting the extract to 1 ml. and one from removing 50 $\mu\text{l.}$ for counting. These two errors were then combined to give:

$$\Delta = (\Delta_1^2 + \Delta_2^2)^{\frac{1}{2}} = (2.0^2 + 1.0^2)^{\frac{1}{2}} = 2.2\%$$

X_{ij} , X_i , $X_{R'}$, X_R . Similarly there are two errors in each of these terms, one from the counting error assuming Poisson statistics, and one from the determination of counting efficiency by an external standard channels-ratio method. As 20 000 counts were recorded for each sample the counting error was about 0.7%, and the error on efficiency was estimated to be 3%. These combine to give an overall error of 3.1%.

β_{ij} . There are four errors contributing to this term: the weights of the portion of initial sample and crystals after the third crystallization, and the determination of d.p.m. in both samples. All samples were weighed to within 0.5% and the d.p.m. measured to within 3.1%. These values combine to give an overall error of 4.5%.

If these values are incorporated into eqn. (2) then the

total random error on M_d will be approx. 6%, and if the portions taken at each stage are the same and counted under identical conditions then the percentage error will be similar for all products.

Practical assessment. The theoretical evaluation of the procedure assumes equal extraction and losses of steroids within the polarity range under investigation. To test these assumptions two experiments were undertaken. In the first ^{14}C -labelled progesterone, androstenedione, dehydroepiandrosterone, 17α -hydroxyprogesterone and testosterone were added to different flasks containing 300mg. of endometrial tissue. The tissues were extracted with acetone and ether and 5% portions of the extracts removed for liquid-scintillation counting. The recovery of all steroids (corrected for quenching) was 90–95%. In the second series of experiments a known mixture of these five steroids was added to 300mg. of endometrial tissue. The mixture was analysed and the radioactivity associated with each component (corrected for quenching and experimental losses) was expressed as a percentage of the total radioactivity added. The results from six replicate determinations are shown in Table 1. The mean recovery of the five steroids was 88% and, as the theoretical error was 6% (1σ), the mean recovery found in practice is within 2σ of the amount added.

RESULTS

The endometrial tissue used in this series of experiments was removed from six premenopausal subjects (aged 15–42 years). Histological examination of all tissues indicated a secretory pattern. The tissues were incubated with five neutral substrates for 2 hr. (without the addition of cofactors) and for 4 hr. (with cofactors). In addition, the metabolism of oestrone and oestradiol- 17β was investigated over a period of 2 hr. (without cofactors).

Control incubations. Seven incubation flasks were prepared each with 300mg. of tissue, 3ml. of buffer and 10ml. of acetone. Then $2\mu\text{g.}$ of a different substrate was added to each of the flasks, which were agitated by hand, and extracted after standing overnight at 4° . No identifiable radiometabolites were visible on the first radioautogram and the percentage of each substrate recovered (corrected for losses and purity) ranged from 82% to 95%.

Incubation with $[4\text{-}^{14}\text{C}]$ progesterone. (a) For 2 hr. without cofactors. A 370mg. portion of the minced tissue was incubated with $2\mu\text{g.}$ of progesterone.

After extraction of the radiometabolites and radioautography of the first t.l.c. plate, eight principal products were visible. After further t.l.c. and recrystallization studies, 71% of the progesterone was recovered untransformed. The principal product, 5α -pregnanedione, was available in sufficient quantity (3% of initial radioactivity) for recrystallization to constant specific radioactivity. 5β -Pregnanedione, 20α -dihydroprogesterone, 20β -dihydroprogesterone and 20α - and 20β -hydroxy- 5α -pregnan-3-one were tentatively identified on the basis of their chromatographic properties.

(b) For 4 hr. with cofactors. A 230mg. portion of tissue was incubated with $2\mu\text{g.}$ of progesterone and $1\mu\text{mole}$ of each cofactor. In this instance ten radioactive areas were visible on the first radioautogram. Of the progesterone added 47% was recovered untransformed, and 5α - and 5β -pregnanedione were identified by recrystallization to constant specific radioactivity (Table 2). Under these experimental conditions 20α - and 20β -dihydroprogesterone were produced in slightly higher yield and were both identified by recrystallization studies after oxidation to progesterone with chromic acid (Bush, 1961). 20α - and 20β -Hydroxy- 5α -pregnan-3-one were again identified on the basis of their chromatographic properties, but there was insufficient of the radiometabolites and authentic steroids for more positive identification.

Only 64.5% of the initial radioactivity was accounted for, but there was no evidence for the formation of identifiable amounts of 17α -hydroxyprogesterone, or 3-hydroxylated compounds.

Incubation with 17α -hydroxy $[4\text{-}^{14}\text{C}]$ progesterone.

(a) For 2 hr. without cofactors. A 175mg. portion of tissue was incubated with $2\mu\text{g.}$ of 17α -hydroxyprogesterone. Six principal products were visible on the first t.l.c. plate. Of the 17α -hydroxyprogesterone added 99% was recovered untransformed, and the two principal products were identified on the basis of their chromatographic properties as $17\alpha, 20\alpha$ - and $17\alpha, 20\beta$ -dihydroxypregn-4-en-3-one (0.34% and 0.22% respectively). There was no evidence for the formation of androstenedione.

(b) For 4 hr. with cofactors. Another portion of

Table 1. Replicate analyses of labelled steroids added to endometrial tissue

Mixture	Amount added (%)	Recovery from six replicate analyses (% mean $\pm$ S.D.)	Recovery of mean (%)
Progesterone	20.71	18.6 ± 0.70	90
Androstenedione	19.60	17.0 ± 0.70	87
Dehydroepiandrosterone	24.97	23.55 ± 2.30	94
17α -Hydroxyprogesterone	11.10	10.06 ± 1.46	91
Testosterone	23.61	18.43 ± 2.75	78
Total	100	—	—

Table 2. *Percentage conversion in vitro of [4-¹⁴C]progesterone into various metabolites by human endometrial tissue*

Abbreviations: C, crystals; ML, mother liquor.

Metabolite	Initial	Specific radioactivity (d.p.m./mg.)			Purity (%)	Conversion (%)
		First recrystallization	Second recrystallization	Third recrystallization		
5 α -Pregnanedione	231 { C ML }	215 451	199 343	198 210	86	4.57
5 β -Pregnanedione	120 { C ML }	91 321	89 302	100 122	83	1.31
20 α -Dihydroprogesterone*	61 { C ML }	56 141	54 93	49 51	80	7.33
20 β -Dihydroprogesterone*	32 { C ML }	28 131	28 42	27 31	84	2.07
20 α -Hydroxy-5 α -pregnan-3-one		Chromatographic evidence for identity				1.48
20 β -Hydroxy-5 α -pregnan-3-one		Chromatographic evidence for identity				0.45
Untransformed progesterone	238 { C ML }	232 297	225 266	227 191	95	47.29

% of radioactivity accounted for: 64.5

* Recrystallized after oxidation to progesterone.

Table 3. *Percentage conversion in vitro of 17 α -hydroxy[4-¹⁴C]progesterone into various metabolites by human endometrial tissue*

Abbreviations: C, crystals; ML, mother liquor.

Metabolite	Initial	Specific radioactivity (d.p.m./mg.)			Purity (%)	Conversion (%)
		First re-crystallization	Second re-crystallization	Third re-crystallization		
Androstenedione	16 { C ML }	14 26	14 26	14 18	88	1.32
17 α -Hydroxy-5 α -pregnan-3-one		Chromatographic evidence for identity				1.79
17 α ,20 α -Dihydroxypregn-4-en-3-one		Chromatographic evidence for identity				13.38
17 α ,20 β -Dihydroxypregn-4-en-3-one		Chromatographic evidence for identity				12.62
Untransformed 17 α -hydroxy-progesterone	1523 { C ML }	1229 1588	1379 1607	1353 1638	89	48.06

% of radioactivity accounted for: 77.17

tissue weighing 507 mg. was incubated with 2 μ g. of 17 α -hydroxyprogesterone and 1 μ mole of each cofactor. Again six principal radioactive areas were observed on the first radioautogram. Under these conditions 48% of the 17 α -hydroxyprogesterone was recovered untransformed (Table 3), and 17 α ,20 α - and 17 α ,20 β -dihydroxypregn-4-en-3-one were obtained in good yield. In addition, a small yield of androstenedione was identified on the basis of recrystallization studies, indicating a low lyase activity, and there was chromatographic evidence for the formation of 17 α -hydroxy-5 α -pregnan-3-one.

Incubation with dehydro[4-¹⁴C]epiandrosterone.

(a) For 2 hr. without cofactors. A 400 mg. portion of tissue was incubated with 2 μ g. of dehydroepiandrosterone. Four main radioactive areas were

visible on the first radioautogram. The identification of a small yield of androstenedione (1.6%) by recrystallization to constant specific radioactivity indicated a low 3 β -ol dehydrogenase plus $\Delta^5 \rightarrow \Delta^4$ -isomerase activity. The fraction containing the untransformed substrate was subdivided and shown by recrystallization studies to contain approx. 60% of substrate and 15% of androsterone. Further evidence that these were the principal compounds in this fraction was obtained by deacetylation with methanolic sodium hydroxide (Bush, 1961), formation of the monoheptafluorobutyrate derivatives and g.l.c. The extract was found to contain 594 ng. of dehydroepiandrosterone and 34 ng. of androsterone. There was also g.l.c. evidence for the presence of a small amount of androstanolone but

Table 6. *Percentage conversion in vitro of [4-¹⁴C]testosterone into various metabolites by human endometrial tissue*

Abbreviations: C, crystals; ML, mother liquor.

Specific radioactivity (d.p.m./mg.)

Metabolite	Initial	First recrystallization	Second recrystallization	Third recrystallization	Purity (%)	Conversion (%)
5 α -Androstanedione	181 { C ML }	178 192	187 205	179 201	99	20.75
Androsterone	70 { C ML }	52 204	40 73	39 57	56	2.25
Androstenedione	4821 { C ML }	4849 5153	4834 4880	5052 4605	100	50.08
Untransformed testosterone	19 { C ML }	18 32	17 23	17 21	90	1.52

% of radioactivity accounted for: 74.60

Table 7. *Percentage conversion in vitro of [4-¹⁴C]oestrone and [4-¹⁴C]oestradiol-17 β by human endometrial tissue*

Abbreviations: C, crystals; ML, mother liquor.

Specific radioactivity (d.p.m./mg.)

	Initial	First recrystallization	Second recrystallization	Third recrystallization	Purity (%)	% of initial radioactivity
Expt. 1						
Oestrone*	3218 { C ML }	2634 4081	2805 2879	2826 2604	88	75.30
Oestradiol-17 β	484 { C ML }	586 541	497 501	476 498	98	2.25
Oestradiol-17 β *	385 { C ML }	378 429	369 401	335 379	87	8.80
Oestrone	2146 { C ML }	1935 3190	1924 1824	1838 1877	86	63.90
Expt. 2						
Oestrone*	8374 { C ML }	9165 10002	7475 9076	7535 9202	90	85.54
Oestradiol-17 β	184 { C ML }	179 201	166 198	168 176	91	1.45
Oestradiol-17 β *	201 { C ML }	189 221	184 218	187 198	93	1.75
Oestrone	10181 { C ML }	10533 12986	9851 9868	10432 10208	100	92.90

* Substrate.

accumulated to a sufficient extent to give a visible peak on g.l.c. (i.e. < 250 pg.), but there was a small peak corresponding to androstanolone.

Incubation with [4-¹⁴C]testosterone. (a) For 2 hr. without cofactors. A 362 mg. portion of tissue from the same endometrium as in the short-time experiment with androstenedione was incubated with 2 μ g. of testosterone. The same five compounds were isolated. However, in this instance 99% of the testosterone was transformed and 90%

of the initial radioactivity was recovered as androstenedione, 2.1% as 5 α -androstanedione, 0.65% as androsterone and 0.39% as epiandrosterone; 94.31% of the radioactivity was accounted for and there was again no evidence for the formation of significant amounts of C₁₈ steroids.

(b) For 4 hr. with cofactors. A 360 mg. portion of tissue was incubated with 2 μ g. of testosterone and 1 μ mole of each cofactor. A spectrum of products was obtained similar to that in the short-time

experiment (Table 6). G.l.c. studies on the androsterone fraction again suggested the presence of a small amount of androstanolone.

Incubation with [4-¹⁴C]oestrone and [4-¹⁴C]-oestradiol-17 β . Two pieces of endometrial tissue from different subjects were subdivided and incubated with oestrone and oestradiol-17 β . The first incubations contained 2 μ g. of substrate and 290 mg. of tissue, and the second 1 μ g. of substrate and 150 mg. of tissue. In these preliminary studies no cofactors were added and the tissues were incubated for 2 hr. The results of both incubations are shown (Table 7).

DISCUSSION

The degree of modification of steroid hormones by a particular piece of human endometrial tissue *in vitro* is influenced by many factors, and although this approach demonstrates potential transformations it does not preclude the occurrence of others that may not be revealed under the experimental conditions. Important factors that influence the metabolism *in vitro* are the state of the tissue (slice, mince or homogenate), the mass of the substrate, the ratio of substrate to tissue, the addition of cofactors and the time of incubation. The present study was primarily designed to investigate the ability of endometrial tissue to transform certain steroids under what may be described as basal conditions, namely incubation without cofactors for 2 hr. In addition, the same tissue was incubated with each substrate for 4 hr. after the addition of cofactors to the medium so that minor metabolites and the products of multiple enzyme activities could be determined.

The principal finding was that, in the particular tissues studied under these conditions, progesterone was not transformed into 17 α -hydroxyprogesterone; the latter was not converted in appreciable amount into androstenedione, nor were phenolic steroids isolated. However, all substrates were extensively metabolized to steroids containing the same number of carbon atoms; most transformations were to reduced forms and the analysis of radiometabolites was complicated by the possible formation of stereoisomers at C-3, C-5, C-17 and C-20. Progesterone was transformed in good yield into 5 α -pregnanedione and 20 α -dihydroprogesterone and, to a much smaller extent, into 5 β -pregnanedione and 20 β -dihydroprogesterone. In addition, there was chromatographic evidence for the formation of 20 α - and 20 β -hydroxy-5 α - and -5 β -pregnan-3-one, but the limited availability of authentic steroids prevented further identification of these products. Further evidence for 20 α - and 20 β -hydrogenase activity was obtained from the isolation of 17 α ,20 α - and 17 α ,20 β -dihydroxypregn-

4-en-3-one from the incubation of 17 α -hydroxyprogesterone. There was no evidence for reduction of the oxo group at C-3.

A relatively high proportion of dehydroepiandrosterone was transformed and the isolation of radiochemically pure androstenedione demonstrated 3 β -hydroxy dehydrogenase and $\Delta^5 \rightarrow \Delta^4$ -isomerase activity. There was no evidence for an accumulation of androst-5-ene-3 β -17 β -diol or testosterone, suggesting low 17 β -hydrogenase activity. There was a small conversion of androstenedione into testosterone and a high yield of androstenedione from testosterone in the same tissue, again indicating that these basal conditions tend to favour formation of products with an oxo group at C-17.

There was a good yield of 5 α -androstanedione and androsterone from both androstenedione and testosterone; significant yields of androstanolone or androstane-3 α ,17 β -diol were not obtained with any of the substrates.

The incubations with oestrone and oestradiol-17 β provide further evidence that endometrial tissue tends to oxidize steroids with a hydroxyl group at C-17 to the corresponding ketone and are in agreement with the findings of Sweat *et al.* (1967).

This study of the metabolism of seven substrates by secretory endometrium has confirmed and extended the observations of Bryson & Sweat (1967) on the metabolism of progesterone by proliferative endometrium. The principal transformation of progesterone was towards the 20 α -hydrogenated and 5 α -reduced metabolites, and the C₁₉ and C₁₈ steroids tended to retain an oxo group at C-17 or to form a 5 α -reduced metabolite with this configuration. If the steroid metabolism observed in this series of experiments occurs in the intact endometrium, where there is probably far less substrate, the problem remaining is whether the conversions are associated with the action of ovarian hormones on endometrial growth and activity or whether they represent a process employed by the tissue to remove excessive hormonal stimulation.

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REFERENCES

- Axelrod, L. R., Matthijssen, C., Goldzieher, J. W. & Pulliam, J. E. (1965). *Acta endocr., Kbh.*, Suppl. 99.
- Bryson, M. J. & Sweat, M. L. (1967). *Endocrinology*, **81**, 729.
- Bush, I. E. (1961). *The Chromatography of Steroids*, pp. 360, 363. Oxford: Pergamon Press Ltd.
- Collins, W. P., Sisterson, J. M., Koullapis, E. N., Mansfield, M. D. & Somerville, I. F. (1968). *J. Chromat.* **37**, 33.
- Krebs, H. A. (1950). *Biochim. biophys. Acta*, **4**, 249.
- Sweat, M. L., Bryson, M. J. & Young, R. B. (1967). *Endocrinology*, **81**, 167.