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OVARIAN STEROIDOGENESIS STUDIED BY MASS FRAGMENTOGRAPHY

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SUMMARY

A practical mass fragmentographic procedure is described which is suited to *in vitro* studies of gonadal endocrine function and which allows reliable determination of nanogram amounts of a wide range of steroids, including those formed from deuterium-labelled precursors. Steroids were isolated by chromatography using Dextran gel (Lipidex®-5000) and determined by mass fragmentography as the 3-enol trifluoroacetates or *t*-butyldimethylsilyl ethers. Application of the technique is illustrated by experiments on steroid hormones produced in organ culture by isolated sheep ovarian follicles with and without gonadotrophic (HCG) stimulation, and an analysis of steroids produced by entire ovine and human ovaries maintained *in vitro* in a perfusion apparatus.

Large ovarian follicles from sheep on Days 4–14 of the oestrous cycle produced in organ culture predominantly oestradiol-17β and testosterone, as well as small amounts of pregnenolone, androstenedione, oestrone and DHA. Addition of HCG transiently enhanced the release of oestradiol-17β, caused a marked and sustained increase in the production of pregnenolone, and initiated the synthesis of progesterone and 20α-hydroxy-4-pregnen-3-one. When [16-$^2H_1$]-pregnenolone (200–500 ng ml^{-1}) was added to the culture medium, it was transformed into oestrogens and androgens, albeit in yields which suggested a clear preference for endogenous precursors. At least nine steroids were produced by sheep and human ovaries maintained in the perfusion apparatus.

INTRODUCTION

Recent studies of the control of ovulation have emphasised the need for further information about the individual functions and inter-relationships of the various tissue elements which make up the ovary.

One approach has been to isolate the individual tissues and examine their behaviour under suitable conditions in organ culture. The potential of this technique has been illustrated by our previous studies on isolated ovarian follicles [1–4], in which changes in endocrine function were monitored by determining the steroid hormones released by cultured follicles using radioligand assays, but the restricted range of steroids which could be reliably determined by these methods was a serious limitation. The need was felt, therefore, for an analytical procedure both rigorous and capable of identifying and determining many steroids, some of which are of closely related structure, so as to obtain a more detailed picture of steroid production resulting from hormone administration.

A technique ideally suited to this purpose is mass fragmentography (MF) [5, 6] which offers the additional advantage of providing the simultaneous identification and determination of isotopically-labelled steroids formed in tissue after administration of labelled precursors. The aim of the present study was to explore the usefulness of MF in the analysis of steroid hormone production by ovarian follicles maintained in organ culture, and by entire ovaries maintained *in vitro* in a perfusion apparatus.

EXPERIMENTAL

Materials. HCG (Pregnyl®) was the product of Organon Laboratories Ltd., Morden, Surrey, U.K. The unlabelled steroids used were obtained from Steraloids Inc., Pawling, New York, U.S.A. or synthesized by standard procedures. Sodium borodeuteride (>96% of ^2H) was supplied by Sharpe, Mercke and Dohme, Kirkland, Quebec, Canada. Radioactively-labelled steroids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

[16-$^2H_1$]-Pregnenolone. A mixture of 3β-hydroxy-5,16-pregnadien-20-one (200 mg) and sodium borodeuteride (60 mg) in pyridine (5 ml) was maintained at room temperature for 20 h. At the end of this period, the whole mixture was poured into water, acidified and extracted with diethyl ether. The ethereal solution was washed with water, brine, and then dried. Removal of the solvent gave a white solid which was chromatographed on alumina (6 g). Elution with benzene:ethyl acetate (25:1, v/v) gave [16-$^2H_1$]-pregnenolone (120 mg, 60%) m.p. 186–190° (lit. [7] 193°), mass spectrum, M/z 317 (M^+), 299 ($M-H_2O$), 96% $^2H_1$ as estimated by mass spectroscopy. No loss of label occurred on base equilibration of the product, confirming that the deuterium had entered only the 16-position.

Culture of ovarian follicles. Ovaries were removed within 40 min of slaughter from sheep of mixed breed, mainly merino cross, between Days 4–14 of the oestrous cycle, and transported to the laboratory in ice-

chilled Dulbecco phosphate buffered saline (Commonwealth Serum Laboratories (C.S.L.), Parkville, Victoria, Australia) containing 50 μg Kanamycin ml^{-1} (Sigma Chemical Company, St. Louis, U.S.A.). Follicles 4–6 mm in diameter representing 5–11.4 mg wet wt tissue (exclusive of follicular fluid) were dissected from the ovaries and established individually in organ culture as described by Moor[1]. The culture medium was replaced at intervals of 24 h and stored at $-15°$ for analysis.

In addition to ascertaining the range and output of steroids produced by untreated follicles maintained in culture for three days, two additional experiments were carried out.

In the first experiment the effects were investigated of including 20 iu HCG ml^{-1} medium throughout the period of culture, and in the second experiment deuterated pregnenolone (500 ng ml^{-1}) was included in the medium to determine whether the ^{2}H-label would be incorporated into the metabolic steroid pools. In both experiments, follicles were randomly allocated amongst the various groups with five follicles per group.

Ovarian perfusion. Entire sheep ovaries, obtained as described above, and human ovaries obtained from patients undergoing hysterectomy, were isolated and maintained *in vitro* in a recirculating perfusion apparatus [8, 9]. The perfusion system used Medium 199 (C.S.L.) containing 6% dextran as the perfusate. Ovaries were perfused with 50 ml medium for periods of up to 6 h; the medium was changed at hourly intervals.

Assay procedure. The assay was designed to specifically determine oestradiol-17β, estrone, testosterone, androstenedione, dehydroepiandrosterone, pregnenolone, progesterone, and 20α-hydroxy-4-pregnen-3-one, and to allow the detection of a wide range of dehydrogenated or hydrogenated derivatives, including ^{2}H-labelled metabolites.

Aliquots of medium (0.3–0.5 ml) obtained from cultures with follicles (or without follicles as controls) were equilibrated for 5 min at 25° with 200–500 ng 19-norandrostenedione and 19-nortestosterone as internal recovery and reference standards (IRS). In addition, another aliquot of the control medium was equilibrated with the IRS and 200–500 ng amounts of the eight steroids of interest as a quality control (QC). The mixtures were then extracted with 4 ml diethyl ether, freezing being used to facilitate the complete separation of phases.

A similar extraction procedure was used for analysis of blood samples (1 ml) or perfusion medium (5 ml), a minimum of 4 vol of diethyl ether being used. The ether was removed at 45° under a stream of N_2 and the residue transferred to small (80 mm × 4 mm) Lipidex®-5000 (Packard Instrument Co., Downers Grove, IL U.S.A.) columns equilibrated with a mixture of light petroleum (b.p. 60–80°) and chloroform (95:5, v/v). Extracts were transferred to the columns in 4 × 1 ml solvent mixture, and the eluant was collected (Fraction 1). Any remaining residue was transferred to the column in 3 ml $CHCl_3$, and this eluant was collected (Fraction 2). The columns were reusable after re-equilibration with the solvent mixture, and they were stored between assays in a sealed vessel of light petroleum.

Fraction 1 contained the 19-norandrostenedione, together with any androstenedione and progesterone present. Further preparation for MF analysis involved the removal of solvent under a stream of N_2, and treatment of the residue with 0.3 ml trifluoroacetic anhydride (TFAA) (Pierce Chemical Co., Rockford, IL, U.S.A.) for 20–30 min at room temperature [10]. Immediately prior to analysis the TFAA was removed under a gentle stream of N_2 at room temperature and the residue taken up in 50 μl benzene for MF.

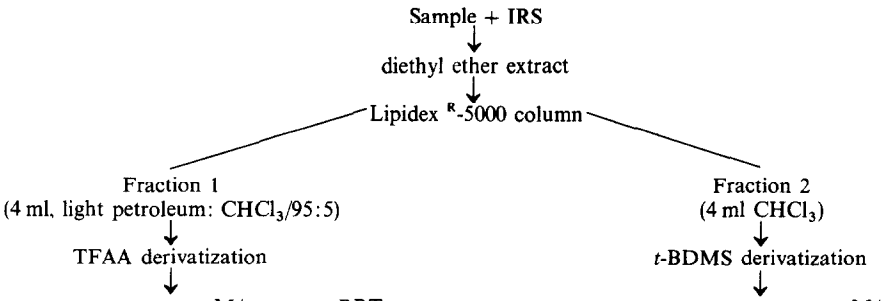
Fraction 2 contained the hydroxy steroids and was prepared for analysis by removing the solvent under a stream of N_2 and treating the residue with 0.1 ml of an N,N-dimethyl formamide solution containing imidazole (2M) and *t*-butyldimethylsilyl chloride (*t*-BDMS, 1 M; Willow Brook Labs. Inc., Waukesha, U.S.A.). After heating the reaction mixture for 2 h at 60°, 2 ml water was added and the derivatives extracted into 2 ml diethyl ether [11]. The ether was then removed under a stream of N_2 at 35–40° and the residue taken up in 50 μl of benzene for MF.

Mass fragmentography. The analytical system consisted of a Pye 104 gas chromatograph interfaced via a single-stage membrane separator to an AEI MS-30 single beam mass spectrometer equipped with a multiple peak monitor having six channels adjusted to 250 ms dwell time on each mass. The mass spectrometer was operated under the following conditions: resolution 1000, full accelerating voltage (4 kV), ionizing current 100 μA, electron energy 25 ev, ion source temperature 200° and molecular separator temperature 240°.

The g.l.c. was carried out on a glass coil column (1.5 m × 2 mm I.D.) containing either glass beads (100/120 mesh) coated with 0.1% OV-101 or Gas-Chrom Q® (100/120 mesh) coated with 1% OV-101, both packings being obtained from Applied Science Laboratories Inc., State College, PA, U.S.A.

The carrier gas flow was 40 ml min^{-1} and the column was held at 185° during analysis of the TFAA derivatives (Fraction 1), and programmed as follows for the *t*-BDMS derivatives: initially (2 min) 220°, then heated at a rate of 10° min^{-1} to a final temperature of 285°. With both fractions 1 and 2, up to one tenth of the sample could be injected without loss of chromatographic resolution.

A schematic representation of the assay procedure is shown in Fig. 1. References masses (292.9825, 330.9793) for mass fragmentography were selected from the spectrum of perfluorokerosene which was admitted into the ion source *via* an all-glass, heated, inlet system. Results were calculated as steroid concentration in culture medium (ng ml^{-1}) = NX/KYV,



	M/z	RRT
19-norandrostenedione	368.160	1.00
androstenedione	382.176	1.11
progesterone	410.207	1.72

	M/z	RRT
19-nortestosterone	331.209	1.00
oestrone	327.178	0.64
DHA	345.225	0.44
testosterone	345.225	1.13
5α-dihydrotestosterone	347.241	0.72
5β-dihydrotestosterone	347.241	0.56*
pregnenolone	373.256	0.84
20α-hydroxy-4-pregnen-3-one	373.256	2.60
3β-hydroxy-5α-pregnan-20-one	375.271	0.8
3α-hydroxy-5β-pregnan-20-one	375.271	0.5
oestradiol-17α	443.280	1.20
oestradiol-17β	443.280	1.55*
cholesterol	445.386	2.50
5-androsten-3β,17β-diol	461.327	0.96
5α-androstan-3β,17β-diol	463.342	1.17
5β-androstan-3α,17β-diol	463.342	0.75
19-hydroxytestosterone	475.306	1.52
5α-pregnan-3β,20α-diol	491.374	1.74
5β-pregnan-3β,20α-diol	491.374	1.88*

* Complete base-line separation from preceding steroid.

Fig. 1. Flow chart of extraction procedure. Included are the mass ion (M/z) monitored, and the relative retention time (RRT) of the steroid derivatives. See text for details and conditions of gas chromatography.

where N = mass (ng) IRS added to V, vol. (ml) of medium extracted, K = ratio of response of mass spectrometer detector to a standard composed of equal masses of all steroids, including IRS, and X, Y = responses of detector to a given steroid and the IRS, respectively.

Where deuterated steroids were included, allowance for natural isotopic contribution (^{13}C, ^{29}Si) arising from any unlabelled steroid was calculated using (corrected response)$_{M+1}$ = (response)$_{M+1}$ − L (response)$_M$, where L = ratio of the response of ion at mass(M + 1) to response of the same ion at mass M calculated from analysis of a standard. At least 4 determinations were made for each sample to ensure adequate precision. A simple computer program was devised for these calculations.

RESULTS

Evaluation of assay procedure

A detailed assessment was made of those steroids of particular interest, namely progesterone, andro-

Table 1. Steroid production by sheep ovarian follicles in culture; effect of HCG

	Day 1		Day 2		Day 3	
	−HCG	+HCG	−HCG	+HCG	−HCG	+HCG
C21 Pregnenolone	3 ± 1	30 ± 20	9 ± 4	32 ± 20	3 ± 2	25 ± 16
Progesterone	0	30 ± 8	0	91 ± 11	0	171 ± 28
20α-hydroxy-4-pregnen-3-one	0	30 ± 8	0	241 ± 22	0	683 ± 28
C19 DHA	0.1 ± 0.1	1.7 ± 1	0	0	0	0
Androstenedione	14 ± 2	13 ± 2	15 ± 3	0	10 ± 1	0
Testosterone	60 ± 17	13 ± 3*	64 ± 12	0	47 ± 8	0
C18 Oestrone	1.5 ± 0.7	1.0 ± 1.0	1.3 ± 0.6	0	2.4 ± 1.0	0
Oestradiol-17β	25 ± 7	44.5 ± 14*	20 ± 7	22.6 ± 6	13 ± 4	5.2 ± 3

* Indicates that the effect of HCG treatment was significant ($P < 0.05$).

Results are expressed as ng (mean ± S.E.M., n = 5) steroid released (mg follicular tissue. 24 h)$^{-1}$. Where indicated, HCG (20 iu ml^{-1}) was included in the culture medium throughout the 3-day period of culture.

stenedione, dehydroepiandrosterone, testosterone, pregnenolone, 20α-hydroxy-4-pregnen-3-one, oestrone and oestradiol. Recovery of radioactive steroids taken through the procedure was greater than 85% in the appropriate fraction. The accuracy and precision of the method were determined by analysing, on different occasions, four 0.5 ml samples of control medium to which were added 200 ng of each of the 8 steroids listed above. No steroids (<0.5 ng/ml) were found in the control medium. The mean values ± S.D. in the experimental results, and the coefficients of variation (CV) were: progesterone, 198.5 ± 20.2 (CV = 10); androstenedione, 199.2 ± 13.8 (CV = 6); DHA, 194.4 ± 30.0 (CV = 15); testosterone, 198.2 ± 14.0 (CV = 7); pregnenolone, 195.9 ± 29.5 (CV = 15); 20α-hydroxy-4-pregnen-3-one, 192.1 ± 38.6 (CV = 20); oestrone, 202.4 ± 29.2 (CV = 14); oestradiol, 186.3 ± 37.8 (CV = 20).

Estimates of the variance arising from the mass fragmentographic step alone were obtained by determining repeatedly, the steroids obtained from one of the above extracts. The results of 4 measurements, in which 1 ng of each steroid was injected, expressed as CV, were: progesterone, 9.2; androstenedione, 4.6; DHA, 1.4; testosterone, 7.2; pregnenolone, 2.0; 20α-hydroxy-4-pregnen-3-one, 3.8; oestrone, 8.4; oestradiol, 9.9.

The detection limits routinely achieved, expressed as ng per sample; were: androstenedione, 0.3 ng; progesterone, 0.9 ng; DHA, 0.07 ng; testosterone, 0.1 ng; pregnenolone, 0.4 ng; 20α-hydroxy-4-pregnen-3-one, 1 ng; estrone, 0.03 ng oestradiol, 1 ng. These limits were set not so much by the instrumental capabilities, but by the necessity to relate quantitatively the steroid peak to that of the IRS at the attenuator setting available.

With the amounts of steroid specified a peak ≥1 cm was obtained at an attenuator setting of ×1 which could be quantitatively related to a 7 cm peak obtained with the IRS at an attenuator setting ×100. A linear relationship was demonstrated between the peak height response to each steroid and the IRS, over a range extending from the levels shown above to 1 μg per sample.

In assessing the deuterated steroids formed in incubation mixtures containing [16-$^2H_1$]-pregnenolone it was found that the contribution from the natural isotopic abundance of ^{13}C and ^{29}Si in an undeuterated steroid varied as expected with the steroid under examination, ranging from 25% to over 40% for di-t-BDMS steroid ethers.

The CV of estimates of the ratios of the peak heights of M + 1 and M in 5 determinations when 1 ng of each steroid was injected were: oestradiol, 3.3; oestrone, 4.2; DHA, 4.7; testosterone, 4.1; pregnenolone, 3.8; 20α-hydroxy-4-pregnen-3-one, 5.0; androstenedione, 3.0; progesterone, 4.2.

Ovarian steroid production

Steroids released by sheep ovarian follicles in culture. Preliminary studies of medium from cultures of untreated follicles indicated the presence of oestradiol-17β (10–1000 ng/ml), testosterone (14–300 ng/ml), androstenedione (10–40 ng/ml), pregnenolone (0–60 ng/ml) and DHA (10–40 ng/ml); no progesterone or its 20α dihydro product (<1 ng/ml) were detected. Treatment with HCG (20 iu/ml) caused a transitory increase in oestradiol-17β, a drop in androgen production and a sustained increase in output of progesterone and 20α-hydroxy-4-pregnen-3-one. The results of the first experiment are summarised in Table 1, and typical traces are shown in Fig. 2.

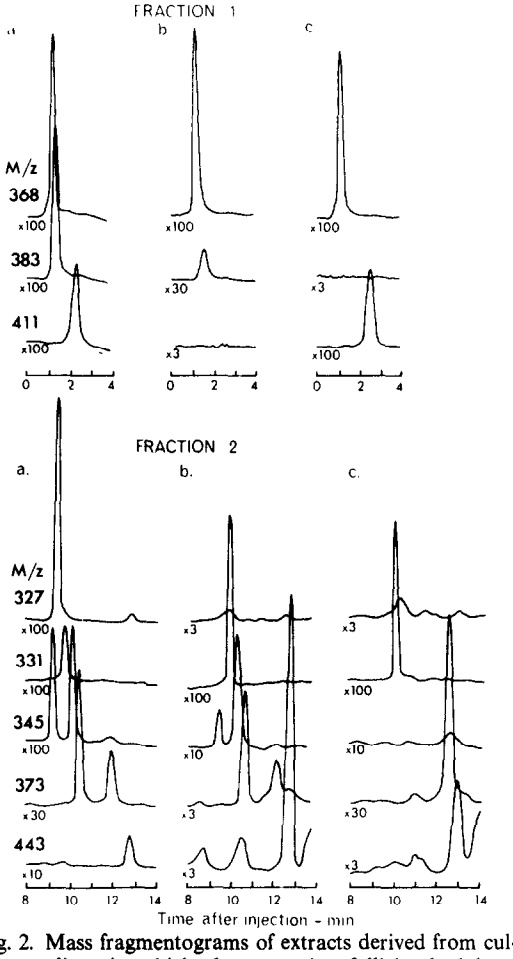


Fig. 2. Mass fragmentograms of extracts derived from culture medium in which sheep ovarian follicles had been cultured. The specific ion monitored (M/z) is shown above, and the attenuation settings below, each trace. For details of extraction, isolation, derivatization and g.l.c. procedures, see text. Mass fragmentogram (a) was with a standard mixture of equal mass (10 ng) amounts of steroid comprising, for Fraction 1, 19-norandrostenedione (IRS), androstenedione and progesterone, and, for Fraction 2, oestrone, 19-nortestosterone (IRS), DHA, testosterone, pregnenolone, 20α-hydroxy-4-pregnen-3-one and oestradiol-17β. Medium was obtained from the third day of culture of individual follicles in (b) the absence or (c) the presence of HCG (20 iu ml^{-1}). Note the effect of HCG in causing the disappearance of androstenedione (M/z = 383), DHA and testosterone (M/z = 345), a reduction in oestradiol-17β (M/z = 443), and a marked increase in 20α-hydroxy-4-pregnen-3-one (M/z = 373) and progesterone (M/z = 411) production.

Table 2. Steroid hormone production by sheep ovarian follicles in culture: incorporation of deuterated pregnenolone

	Day 1		Day 2		Day 3	
	Do	D1	Do	D1	Do	D1
C21 Pregnenolone	2.8 ± 0.6		8.9 ± 3.8		3.3 ± 0.8	
Progesterone	0.7 ± 0.3	0	0	0	0	0.1 ± 0.1
20α-hydroxy-4-pregnen-3-one	0	0	0	0	0	0.9 ± 0.4
C19 DHEA	0.1 ± 0.1	0	2.1 ± 0.7	0	0.7 ± 0.2	0
Androstenedione	13.6 ± 2.4	1.1 ± 0.2	15.5 ± 2.6	1.9 ± 0.5	10.0 ± 0.8	0.5 ± 0.2
Testosterone	36 ± 9.8	1.7 ± 0.4	59.8 ± 17.5	1.7 ± 2.9	35.6 ± 7.8	2.2 ± 1.6
C18 Oestrone	0.3 ± 0.2	0	0	0	0	0
Oestradiol	23.5 ± 6.7	4.0 ± 1.5	18.4 ± 5.5	2.7 ± 1.5	10.8 ± 4.5	1.4 ± 0.7

Five follicles were maintained individually in culture medium to which had been added 500 ng ml^{-1} [16-$^2H_1$]-pregnenolone. Results are expressed as ng (mean ± S.E.M., $n = 5$) unlabelled steroid (Do) or labelled steroid (D1) released (mg follicular tissue. 24 h)$^{-1}$ throughout the 3-day period of culture.

In addition, the extracts were examined for other known ovarian steroids by monitoring g.l.c. effluent for the characteristic $(M-57)^+$ ion (Fig. 1). No evidence was obtained for the presence of 5α- or β-dihydro testosterone, oestradiol-17β, 19-hydroxytestosterone, 5-androstene-3β,17β-diol or any androstanediols or pregnenediols (M/z = 491, M/z = 461). The pregnenolone trace (M/z = 373) contained several peaks, including one with a relative retention time similar to that of 3β-hydroxy-5α-pregnan-20-one, but these were also present in control medium.

In the second experiment, with deuterated pregnenolone, evidence of incorporation of deuterium into both androgen and oestrogen fractions was obtained (Table 2). No incorporation into the progesterone or 20α-hydroxy-4-pregnen-3-one fractions was evident.

Steroids released by sheep ovaries perfused in vitro. Typical traces obtained from the analysis of 5 ml of the perfusion medium are shown in Fig. 3. In 6 ovaries examined, the mean steroid levels (ng ml^{-1}, range in parentheses) determined in the medium after the first hour of perfusion were: DHA 0.9 (0–3.5); testosterone, 0.6 (0–2.1); androstenedione, 0.4 (0–1.0); pregnenolone, 1.6 (1.1–2.6); progesterone, 9.9 (5.7–14.5); 20α-hydroxy-4-pregnen-3-one, 7.4 (1.7–9.8); and oestradiol-17β, 5.0 (2.1–9.7). Evidence was also obtained of trace amounts of oestrone, 5α-dihydrotestosterone, 3β-hydroxy,5α-pregnen-20-one and 5-androstene-3β,17β-diol in some perfusates. No androstanediols, oestriol, or pregnanediols were found. With continued perfusion there was a decline in steroid production unless HCG (20 iu ml^{-1}) was added to the medium.

Steroids released by human ovaries perfused in vitro. Typical traces obtained from the analysis of 5 ml of the perfusion medium is shown in Fig. 3. In 6 ovaries examined, mean steroid levels (ng ml^{-1}, range in parentheses) determined in the medium after the first hour of perfusion were: DHA, 1.0 (0.4–1.6); testosterone, 1.2 (0–2.7); androstenedione, 2.9 (0.9–8.7); pregnenolone, 3.5 (0–7.4); progesterone, 23.2 (8.4–53.9); 20α-hydroxy-4-pregnen-3-one, 2.2 (0–5.8); and oestradiol-17β, 9.5 (3.3–17.4). Evidence was also obtained for the presence, in several perfusates, of oestrone,

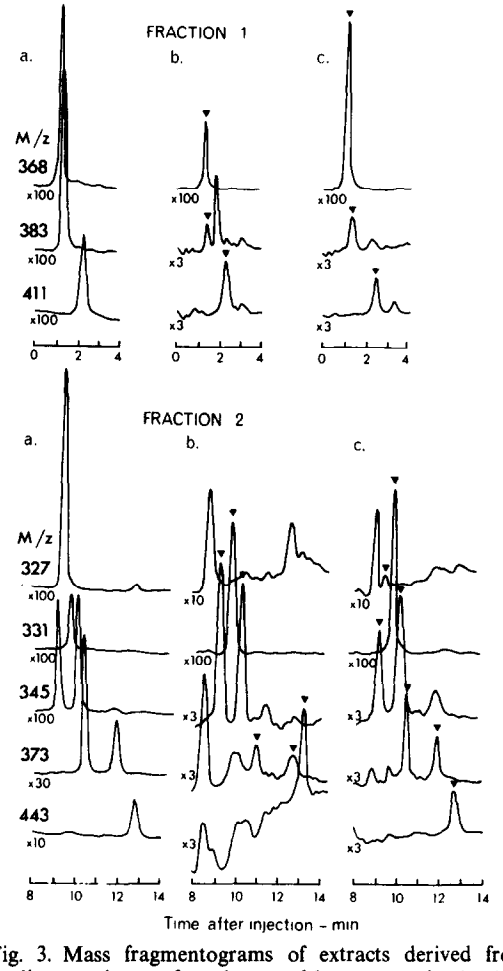


Fig. 3. Mass fragmentograms of extracts derived from medium used to perfuse sheep and human ovaries *in vitro*. The specific ion monitored (M/z) is shown above, and the attenuator setting below, each trace. For details of extraction, isolation, derivatization and g.l.c. procedures, see text. Mass fragmentogram (a) was with a standard mixture of equal mass (10 ng amounts of steroids comprising, for Fraction 1, 19-norandrostenedione (IRS) androstenedione and progesterone, and for Fraction 2, oestrone, 19-nortestosterone (IRS), DHA, testosterone, pregnenolone, 20α-hydroxy-4-pregnen-3-one and oestradiol-17β. Medium was obtained after 1 h perfusion of (b) sheep and (c) human ovaries. The extract injected was derived from approximately 2% of the total perfusate. ▼ Identifies the ion peaks corresponding to reference steroids

3β-hydroxy-5-pregnen-20-one and 19-hydroxytestosterone. No other steroids were detected.

DISCUSSION

In studies of ovarian function the development and application of comprehensive multihormone analytical procedures is necessary to gain insight into the regulation and control of ovarian steroidogenesis. The procedure described here is well suited to this purpose.

It has several advantages over the radioligand binding procedures previously employed in our studies; not only is it possible to determine a wider range of steroids with a much greater degree of confidence, but also, using suitable isotopically-labelled substrate or precursors, it allows concurrent studies on hormone metabolism and biosynthesis to be carried out without the tedious and time-consuming isolation and purification procedures essential in experiments with radioisotopes.

In the procedure described, specificity towards a particular steroid is dependent upon its behaviour during filtration on a lipophilic gel, its gas chromatographic properties and its mass spectral characteristics. In the *in vitro* studies, the simple extraction, purification and derivitization procedures employed, proved to be adequate because control medium contained no substances interfering at the sensitivity required for analysis of the amounts of hormones encountered. However, in achieving specificity the potential to measure certain steroids of interest was lost, as for example in electing to use a TFAA derivative in the analysis of Fraction 1 which, under the conditions employed, reacts selectively with α,β-unsaturated ketonic moieties, other non-polar steroids in this fraction such as the pregnane- and androstanediones could not be determined.

Without adequate chromatographic separation procedures, interference could occur in Fraction 2 between steroids with the same mass and polarity, including epimers or structural isomers. In this regard it should be noted that considerable advantages are to be gained from the use of *t*-BDMS, as this reagent exhibits a high degree of selectivity [11, 12] in etherification, and mixtures of epimers at the 3,5 or 17 positions show complete base-line separation on g.l.c. The spectral characteristics and overall stability of *t*-BDMS derivatives also made them particularly well suited to the isotope studies. The electron impact spectra of mono-t-BDMS steroid ethers are nearly all dominated by the $(M\text{-}57)^+$ ion, that is the ion formed by loss of *t*-BDMS, which may carry up to 40% of the total ion charge [11, 12, 19]. In isotope incorporation studies it is of considerable advantage to be able to utilize this ion because it is guaranteed to contain any label that was present and interpretation is much simplified.

The results obtained indicate that sheep ovarian follicles greater than 4 mm diameter produce both oestrogens and androgens in culture, as do ovarian follicles from a number of other species [2–4, 14–18]. The sustained production of androgens may in part reflect follicular immaturity or atresia, as the follicles were not obtained at a regularly specified time of the cycle. The recent findings of McNatty *et al.* [18] indicate that the fluid obtained from the follicle destined to ovulate contains mainly oestrogens with only low concentrations of androgens.

The effect of HCG on the follicle was to cause a transitory stimulation of oestrogen production, an inhibition of androgen release, and a marked and sustained rise in progestin formation. The amount of HCG added corresponds to the addition of about 2 μg/ml LH and the pattern of response is very similar to that seen in our previous studies with LH [3]. However, in the present experiment, a transitory increase in oestrogen production, rather than a decline, occurred after treatment with gonadotrophin. Whilst this changed response might have been due to differences in the gonadotrophin employed, it was more likely to be the result of our use previously of follicles obtained from sheep stimulated with pregnant mare serum gonadotrophin. Further information is required to settle this matter.

The experiments with deuterium-labelled pregnenolone illustrate the potential of this approach to studies of steroid biogenesis and metabolism, and serve to highlight the difficulties. The latter stem mainly from the abundance of naturally occurring isotopes. The coefficient of variation of estimates of the (M + 1) ion of unlabelled steroids was found to be about 5%, which means that for reliable measurements to be made, the fraction of the total (M + 1) ion which results from any isotopically-labelled steroid present must be at least 10–15%. Thus even with repeated analyses to improve the precision of the estimates, it is doubtful if the incorporation of singly-labelled precursors at levels of less than 4% can be measured reliably. Future work in this area will be dependent upon the synthesis of precursors with more than one label. Nevertheless, the results of experiments with [16-$^2H_1$]-pregnenolone are similar to those obtained in a separate study in which follicles were exposed to [4-^{14}C]-pregnenolone [20] where oestradiol was found to be the principal isotopically-labelled metabolite released by follicles throughout the period of culture. This also confirms that [16-^{2}H]-labelled compounds can be transformed into oestrogens without loss of label [13]. Interestingly, little labelling of the androgen pool occurred, and no progesterone or its 20α-dihydro product was released into the medium in either experiment.

The steroid hormones identified and determined in the perfusion experiments described here are those known to be released into the blood by the ovaries of the sheep [21] and the human [22] *in vivo*, and by human ovaries perfused *in vitro* [23–25].

No information has been available previously on the production of steroids by sheep ovaries perfused

in vitro. The rate of release of the luteal hormones *in vitro*, namely progesterone and 20α-hydroxy-4-pregnen-3-one, is several orders of magnitude less than that of the ovary *in vivo* [21], although the rates of release of the steroids presumed to stem from the follicles [4] are, with the exception of androstenedione, similar to those from the ovary *in situ* [21]. Whether the reduction in secretion of the progestins is due to lack of gonadotrophins or other blood-borne lutrotrophic factors is unknown. It is of interest to note that bovine luteal tissue appears to retain its full secretory function when the ovaries are maintained during perfusion with whole, citrated blood [26, 27].

Considering human ovaries, the amount of progesterone produced in the first hour of perfusion in this study (0.4–2.7 μg) is very similar to that found by Zander and his associates (0.1–3.2 μg, [25]). The oestradiol production rate however appears to be only about one tenth of that found by Kennedy *et al.* in their study of human ovaries perfused with non-recirculated synthetic medium [24]. As with the sheep studies, the amount of progesterone formed in perfusion is much lower than that found *in vivo* [22].

A recent preliminary report [23] on the application of GC–MS and MF for the detection, identification and quantitation of steroid hormones released by human ovaries perfused *in vitro* confirms the presence of the other steroids reported here. In addition, several other substances were detected, including 19-hydroxyandrostenedione, oestriol and oestrone, although no quantitative data were presented. It should be noted, however, that in the experiments reported by these workers, the perfusion medium contained added androstenedione (40 μg ml^{-1}), and this could have acted as a precursor for both the 19-hydroxy intermediates and the oestrogens [28].

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