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VIRILIZING LUTEOMA OF PREGNANCY: SPECIFIC STEROL AND STEROID HORMONE CONTENT

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SUMMARY

The specific sterol and steroid hormone content of a virilizing luteoma of pregnancy has been studied. The androgens, 4-androstene-3,17-dione, DHA, and testosterone, were the major free steroid hormones present in the tumor. Lanosterol, 4,4-dimethyl-8-cholesten-3β-ol, 4α-methyl-7-cholesten-3β-ol, and 7-cholesten-3β-ol were tentatively identified in the luteoma and represent the first identification of these sterols in human gonadal tissue. Three plant sterols, campesterol, stigmasterol, and β-sitosterol were also tentatively identified in trace amounts.

INTRODUCTION

WE have previously reported on the steroidogenic capacity of a typical luteoma of pregnancy[1]. The clinical and morphologic characteristics of this specific ovarian lesion have been described[2]. Although the typical luteoma of pregnancy is not associated with clinical evidence of hormone production, several reports have documented the occurrence of virilization of mother or infant in association with a luteoma of pregnancy[3–6]. We would like to report the results of an analysis of specific sterols and steroid hormones in a luteoma of pregnancy which was associated with mild virilization of mother and female infant.

EXPERIMENTAL

Case report

The patient (Charity Hospital No. T66-128242) was 26 yr old, Negro, gravida 5, para 2, abortus 2. She was admitted to Charity Hospital on June 16, 1968, after spontaneous rupture of the amniotic sac. The estimated date of confinement was July 23, 1968. Pelvic examination revealed moderate clitoral enlargement. The presenting part was high and displaced out of the pelvis by a 12 cm tumor mass which completely filled the cul-de-sac and prevented engagement of the fetal head. A Caesarean section was performed and a 7 lb, 5 oz female infant was delivered. A right ovarian tumor weighing 422 grams was removed from the cul-de-sac and a right salpingo-oophorectomy was performed. The left ovary was bisected and no gross abnormalities were present. The entire tumor was inadvertently fixed in 10% formalin. Following fixation and removal of representative portions of histologic experimentation, the formalin was poured off. The remaining tumor was frozen for 2 days before extraction was undertaken. Histologic examination showed the findings of the typical luteoma of pregnancy as has been described[2]. A corpus luteum of pregnancy was identified in the ovary separate and distinct from the luteoma of pregnancy.

The mother was carefully examined during the postpartum period. She was found to have facial acne, loss of hair in the temporal regions, and a deep voice in addition to the clitoral enlargement. All of the physical changes had occurred during the last trimester of her pregnancy. No hormonal agents had been administered during the pregnancy. The patient was discharged on June 22, 1968. During the next 6 months she had clearing of the facial acne, return of her voice to normal tonal levels, and decrease in the size of the clitoris.

The female infant was noted to have an enlarged clitoris at birth with a normal vaginal and urethral configuration. At 10 days of age a 24-h urine was collected from the infant. During the course of this collection the infant developed fever, tachypnea and listnessness. Serum electrolytes were measured at this time and showed a sodium of 120 meq/l, potassium 6 meq/l, and a CO_2 of 21 meq/l. A presumptive diagnosis of adrenal insufficiency was made and the infant was started on oral cortisone acetate plus deoxycorticosterone acetate intramuscularly. The infant did well except for excessive weight gain and the DOCA* was discontinued. All hormonal therapy was discontinued at 8 weeks of age.

Measurements of urinary 17-hydroxycorticoids, 17-ketosteroids, and pregnanetriol were carried out on 24-h urine samples obtained from the infant at 10 days, at 5 weeks while on dexamethasone, at 6 weeks while on dexamethasone plus 20 units of ACTH gel every 12 hr for 4 days, and again at 10 weeks after discontinuing all therapy for 2 weeks. The results indicated normal adrenocortical function. Pregnanetriol excretion in the urine was always 0·1 mg per 24 h or less during all manoeuvres.

At 5 months of age no advancement in bone age was detectable and there was no remaining clitoral hypertrophy. The infant has continued to do well.

The final clinical diagnosis was mild virilism in mother and female infant in association with a luteoma of pregnancy.

Materials and general procedures

All solvents were distilled before use in all glass apparatus. Thin layer silica gel HF_{254} plates were used as described for purification of steroid hormones [7]. Silica gel H plates for chromatography of sterols were made with distilled water containing 0·1% Rhodamine G-6. Sterols and 4-ene-3-keto-steroids were detected by viewing chromatograms with a short wave ultraviolet hand lamp. Sterols were eluted with dichloromethane. Steroid hormones were eluted with dichloromethane : methanol (1 : 1 v/v). The acetylation procedure, and the detection and measurement of radioactivity have been described [7]. Trimethylsilyl derivatives for gas–liquid chromatography were prepared by reacting samples with hexamethyl-disilazane, trimethylchlorosilane and pyridine (3 : 1 : 9, by vol.). Methyloxime derivative formation was carried out as described [8].

*The following trivial names and abbreviations are used: Cholesterol = 5-cholesten-3β-ol; lophenol = 4α-methyl-7-cholesten-3β-ol; 4α-methyl-7-cholestenol = 4α-methyl-7-cholesten-3β-ol; 4,4-dimethyl-7- and 8-cholestenol = 4,4-dimethyl-7- and 8-cholesten-3β-ol; 4α-methyl-8-cholestenol = 4α-methyl-8-cholesten-3β-ol; lanosterol = 4,4,14α-trimethyl-8,24-cholestadien-3β-ol; dihydrolanosterol = 4,4,14α-trimethyl-8-cholesten-3β-ol; campesterol = 24-methyl-5-cholesten-3β-ol; stigmasterol = 24-ethyl-5,22-cholestadien-3β-ol; β-sitosterol = 24-ethyl-5-cholesten-3β-ol; 17-hydroxy-progesterone = 17α-hydroxy-4-pregnene-3,20-dione; DHA = 3β-hydroxy-5-androsten-17α-one; pregnenolone-3β-hydroxy-5-pregnen-20-one; DOCA = 11-deoxycorticosterone acetate.

Gas–liquid chromatography

Six-foot coiled glass columns of 4 mm dia. were used exclusively. Siliconized columns were packed with 3% OV-1, 3% OV-17, and 3% QF-1 on 100-120 Gas Chrom Q obtained from Applied Sciences Laboratories. The 3% OV-1 and OV-17 columns were operated at 240° for steroid hormone analysis and at 250° for sterol analysis. The QF-1 column was operated at 220°. A Packard Gas Chromatograph with flame ionization detector and 10 in. recorder was used. Nitrogen pressure was 20 lb/in^2. Air and hydrogen flows were 500 ml/min and 30 ml/min, respectively. The detectors were operated at 1×10^{-10} amp at 150 V. Inlet and detector temperatures were operated at 20° above column temperature. The solvent used for gas–liquid chromatography was tetrahydrofuran. Injections were made with 10 μl Hamilton syringes containing 0·5–5 μl of solvent.

Quantitation

Mass of 4-ene-3-keto-steroids was measured after applying the Allen correction to the absorption of ultraviolet light at 240 nm on a Beckman DK-2 recording spectrophotometer. Mass of steroid hormones was also calculated following gas–liquid chromatography from the peak height after known amounts of authentic materials were injected. The detector response was linear over the range in which it was used (usually 25–250 ng). All measurements were adjusted for recovery of radioactive tracer and recorded as μg/g tissue. Sterols were measured from their peak heights on 3% OV-1 using cholesterol as a standard for an approximation of mass. Limitation on amount of pure reference compounds and impurities in many of the reference compounds made it impractical to use them for precise mass measurements. There was reasonably good agreement in the measurements when this technique was compared with measurements made using reference compounds in several instances. Recovery of sterols other than cholesterol was assumed to be the same as cholesterol.

Criteria for tentative identification for steroid hormones and sterols

1. Mobility on thin-layer and paper chromatography of free and acetylated derivatives identical to authentic reference compounds and radioactive labelled tracer (when available).
2. Absorption of ultraviolet light by 4-ene-3-keto-steroids identical to authentic reference compounds.
3. Identification of a single peak on gas–liquid chromatography with the same retention time as the authentic reference compound. Different liquid phases and chromatography of trimethylsilyl–ether derivatives were used in an attempt to show non-identity of the unknown and reference sterol compounds.

Since only small amounts of unknown material were available, the identifications should be considered tentative in the absence of infra-red or mass-spectrographic analyses.

Effect of formalin on representative steroid hormones

One hundred micrograms of progesterone and 4-androstene-3,17-dione were incubated in duplicate with 10 ml of 10% formalin for 4 days. One sample had 0·1 μCi of [^{3}H] progesterone and [^{3}H] 4-androstene-3,17-dione added before incubation with formalin. The second sample had the tracers added after the incubation period. Both samples were extracted 3 times with 50 ml of ether,

washed with 1% NaOH and then water. The solvent was removed and both samples were chromatographed on 5 × 20 cm thin-layer silica gel plates in benzene:ethyl acetate (4:1, v/v). The ultraviolet absorbing zones were eluted, their absorbance measured in a recording spectrophotometer and an aliquot taken for measurement of radioactivity. The recoveries of mass and radioactivity for the 4 steroid samples ranged from 91·7% to 100·6%, indicating that formalin apparently had no destructive effect on these particular steroids studied, under the experimental conditions employed.

Extraction and preliminary purification

An outline of the procedure is shown in Fig. 1. Tracer amounts of ^{3}H labelled progesterone, 20α-hydroxy-4-pregnen-3-one, 3β-hydroxy-5-pregnen-20-one, 4-androstene-3, 17-dione, 3β-hydroxy-5-androsten-17-one, 17α-hydroxy-4-pregnene-3,20-dione, 17β-estradiol, estrone, and ^{14}C labelled testosterone were added to 120 g of formalin fixed tissue. The tumor was homogenized in a Waring blender with distilled water and extracted 4 times with 150 ml of diethyl ether. Phenolic compounds were removed by extraction with 1N NaOH [9]. Non-polar sterols were separated from the more polar steroid hormones by a 5-transfer counter-current distribution. Tritium labelled cholesterol was added to the ligroin (c.p. 60–90°) following this separation to account for methodologic losses during the chromatographic procedures.

Sterol analysis

After saponification overnight at 80°C in 2N KOH in 50% methanol, one-tenth of the residue from the petroleum ether (c.p. 30–40°) extraction was chromatographed a distance of 15 cm on 20 × 20 cm Rhodamine 6-G plates in System A [10]. The 'C_{27}' zone would contain cholesterol, 7-cholestenol, and the C_{28} and C_{29} phytosterols—campesterol, stigmasterol, and β-sitosterol, if present. The least polar sterol zone would contain C_{30} and C_{29} sterols—lanosterol, dihydrolanosterol, and 4,4 dimethyl-7 and 8-cholesten-3β-ol. The intermediate zone would contain the C_{28} sterols—4α-methyl-7- and 8-cholesten-3β-ol. The individual zones were eluted, evaporated to dryness with nitrogen, and subjected to gas–liquid chromatography with the addition of 5α-cholestane as an internal marker. Trimethylsilylether derivatives were then formed and chromatographed on OV-1 columns.

Steroid hormone analysis

The thin-layer and paper chromatographic procedures for Method I and Method II have been described [1]. In addition, steroid hormones were measured by gas–liquid chromatography following these purification steps. Steroids with a reactive hydroxyl group were measured as acetate derivatives.

Chromatographic systems

A. Benzene:ethyl acetate (5:1, v/v).
B. Ligroin:ethyl acetate (5:2, v/v).
C. Dichloromethane:ether (5:2, v/v).
D. Cyclohexane:ethyl acetate (80:50, v/v).
E. Ligroin/propylene glycol (paper chromatography).

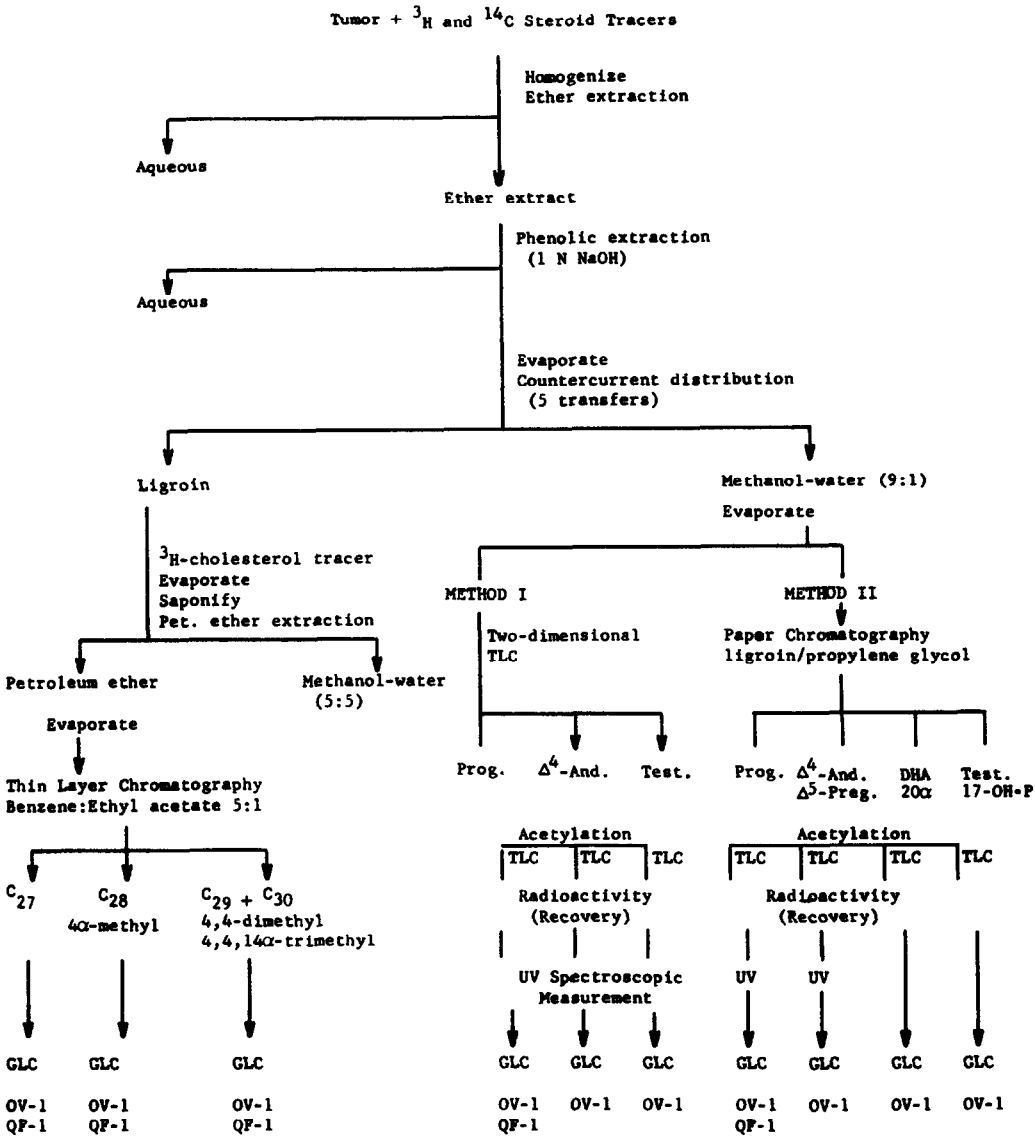

Fig. 1. Flow sheet for analysis of luteoma of pregnancy.

Reference compounds

Beta-sitosterol was obtained from a number of sources. Dr. Normal Mason, Lilly Research Laboratories, kindly supplied β-sitosterol which had only a trace of campesterol-like material present as determined by gas–liquid chromatography. For routine work, 'β-sitosterol' (Sigma) was used and appeared to contain 55% of β-sitosterol and 41% campesterol when analyzed by gas–liquid chromatography. '99% Campesterol' was purchased from Applied Science Laboratories and was homogeneous on gas–liquid chromatography. 4α-methyl-7-cholesten-3β-ol was supplied as crude lophenol by Dr. Carl Djerassi, Stanford University. The major

component on gas–liquid chromatography was assumed to be the 4α-methyl-7-ene analogue. 4α-Methyl-8-cholesten-3β-ol was supplied by Dr. Andrew Kandutsch, The Jackson Laboratory, Bar Harbor, Maine, and by Professor W. B. Whalley, University of London. The retention time of this sterol on gas–liquid chromatography was identical to the extra component in the crude lophenol supplied by Dr. Djerassi. Dr. J. L. Gaylor, Cornell University, supplied lanosterol, dihydrolanosterol, 4,4-dimethyl-7- and 8-cholesten-3β-ol. Other reference compounds were purchased from Steraloids, Pawling, New York. [7-^{3}H] Cholesterol of high specific activity was purchased from New England Nuclear Corporation, Boston, Massachusetts. Bromination[11] of the tritium labelled sterol together with carrier cholesterol and recrystallization as the dibromide suggested the presence of a small amount of radioactive impurity (probably less than 5%). The [7-^{3}H] cholesterol was used without additional purification.

Tritium and carbon-14 labelled steroid hormones used as tracers were of high specific activity. They were purchased from New England Nuclear Corporation and were periodically rechromatographed in this laboratory.

RESULTS

Method 1. One-tenth of the residue from the 90% methanol fraction was subjected to two-dimensional thin-layer chromatography on silica gel HF_{254}. Ultraviolet absorbing spots, corresponding in location to progesterone, 4-androstene-3,17-dione, and testosterone, were located and eluted from the chromatograms. Each zone was dried, treated with acetic anhydride and pyridine, and rechromatographed on 5 cm silica gel HF_{254} plates in System B. Ultraviolet absorbing silica gel zones corresponding in location to reference and radioactive tracer progesterone, 4-androstene-3,17-dione, and testosterone acetate were located and eluted. An aliquot was taken for measurement of radioactivity. The absorbance of ultraviolet light by the suspected 4-ene-3-keto-steroid was determined in methanol in a cell of 1 cm light path. The absorbance of each compound was maximal at 240 nm. Mass was calculated after application of the Allen correction, corrected for the recovery of tracer and adjusted to 1 g of tissue.

The material from the three zones was then subjected to gas–liquid chromatography on 3% OV-1 columns using 5α-cholestane as an internal standard. The testosterone acetate and 4-androstene-3,17-dione zones were each found to contain only one major peak corresponding in retention time to authentic testosterone acetate and 4-androstene-3,17-dione (Figs. 2 and 3). Mass and radioactivity were measured and showed good agreement with the spectrophotometric measurements (Table 1).

The progesterone zone was chromatographed on the 3% OV-1 liquid phase (Fig. 4) but inspection of the peak suggested the presence of 2 components and mass could not be reliably calculated. The material was chromatographed on the 3% QF-1 liquid phase and separated into 2 components – one with a retention time identical to authentic progesterone (Fig. 5). Mass was calculated from peak height using cholesterol as an internal standard. Progesterone was further characterized by the formation of the methyloxime derivative followed by gas–liquid chromatography on 3% OV-1 (Fig. 6). A single peak with a retention time identical to the authentic reference compound was seen which was separate and distinct from a larger peak with a longer retention time. The gas chromatographic

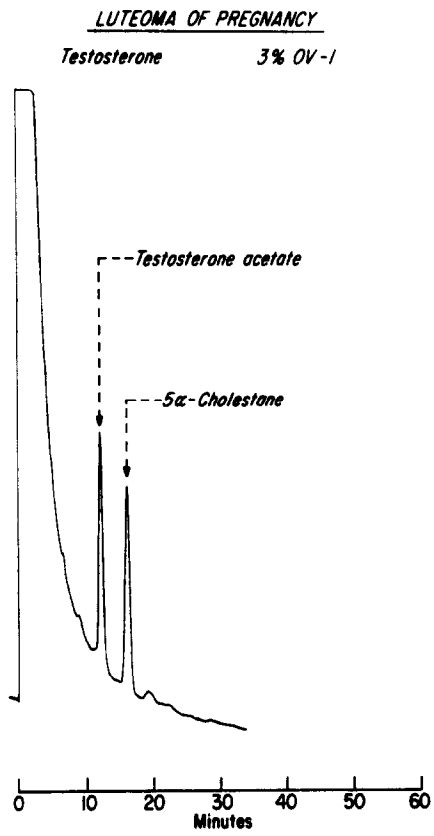

Fig. 2. Chromatographic tracing of testosterone (as the acetate derivative) from luteoma of pregnancy on 3% OV-1. 5α-Cholestane added as an internal standard.

Table 1. Steroid content of virilizing luteoma of pregnancy (micrograms per gram)

Steroid	Method I UV (240 mμ)	Method I GLC†	Method II UV (240 mμ)	Method II GLC†
3β-Hydroxy-5-pregnen-20-one*				0·96
Progesterone	0·71	0·22	0·80	0·22
3β-Hydroxy-5-androsten-17-one* (DHA)				2·4
4-Androstene-3,17-dione	1·2	1·3	1·4	1·6
Testosterone*	0·60	0·80		0·88
20α-Hydroxy-4-pregnen-3-one				trace

*Measured as the acetate derivative.
†GLC = Gas–liquid chromatography.

measurement was considerably lower than the spectrophotometric measurement (Table 1). It must be assumed that the extra unidentified component seen on gas–liquid chromatography (labelled 'a' in Figs. 4, 5 and 6) contributed to the high measurement made by ultraviolet absorbance and that the gas–liquid chromatography measurement is the correct one for progesterone.

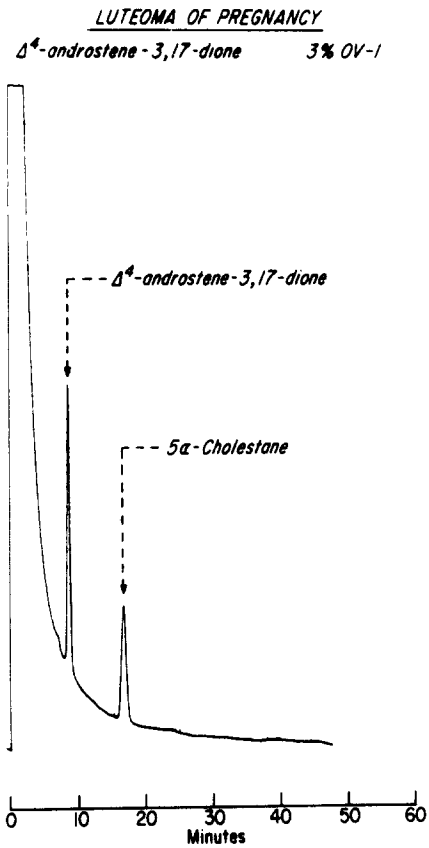

Fig. 3. Chromatographic tracing of 4-androstene-3,17-dione from luteoma of pregnancy on 3% OV-1.

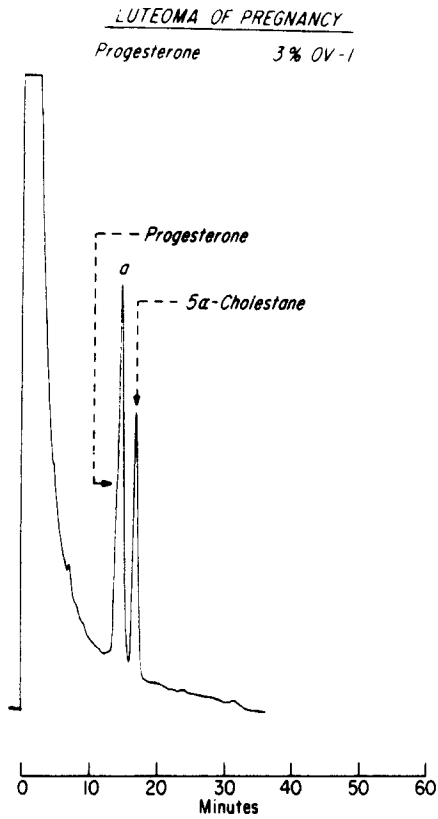

Fig. 4. Chromatographic tracing of progesterone from luteoma of pregnancy on 3% OV-1. Peak labelled 'a' is unidentified component that did not separate from free progesterone.

Method II. One-half of the residue was chromatographed on paper in System E as described[1]. The steroid zones were separated by descending paper chromatography and the chromatograms examined for absorbance of ultraviolet light and scanned for radioactivity. Three ultraviolet absorbing zones were detected corresponding to radioactive progesterone, 4-androstene-3,17-dione, and testosterone. The appropriate zones were purified by thin-layer chromatography and characterized and measured as described in Method I by the absorbance of ultraviolet light and gas–liquid chromatography. The absorbance of ultraviolet light by testosterone acetate was not measured in this instance. The results were similar to those obtained in Method I (Table 1). In addition, 3β-hydroxy-5-pregnen-20-one was measured by gas–liquid chromatography following acetylation and separation from 4-androstene-3,17-dione by thin-layer chromatography as the acetate derivative in System B. 3β-Acetoxy-5-pregnen-20-one was detected and measured as a single major peak by gas–liquid chromatography on 3% OV-1 (Fig. 7). 17α-Hydroxy-progesterone could not be detected with certainty (UV absorbance) after thin-layer chromatography of the testosterone acetate zone. The free compound is unstable on gas–liquid chromatography and could not be reliably identified with this technique.

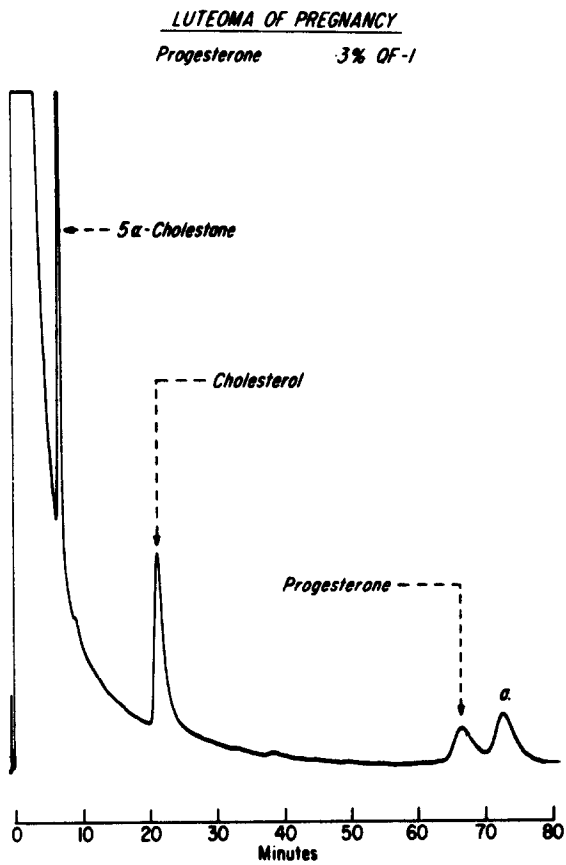

Fig. 5. Chromatographic tracing of progesterone from luteoma of pregnancy on 3% QF-1. Peak labelled 'a' is unidentified component that did not separate from free progesterone on 3% OV-1.

DHA acetate was isolated and separated from 20α-acetoxy-4-pregnen-3-one by thin-layer chromatography in System B. 20α-Acetoxy-4-pregnen-3-one was not detectable by ultraviolet spectroscopy and only a trace appeared to be present when the zone was examined by gas–liquid chromatography on 3% OV-1. DHA acetate was detected and measured as a single peak by gas–liquid chromatography on 3% OV-1 (Fig. 8). DHA appeared to be the major free steroid present in the tumor (Table 1).

Phenolic steroids

The phenolic steroid fraction was separated from the neutral fraction by shaking the ether solution with 1M sodium hydroxide according to Engel and associates[9] and purified on a 3 g silica gel column, eluting the estrogens with 200 ml of benzene:ethyl acetate (4:1). The phenolic fraction was separated by thin-layer chromatography using System D. The estrone and estradiol-17β zones were eluted, acetylated and rechromatographed in System B. The zones corresponding to the acetylated derivatives were eluted, counted, and subjected to gas–liquid chromatography on 3% OV-1. Only small amounts of radioactive estrone acetate and estradiol diacetate were recovered. Small peaks with the

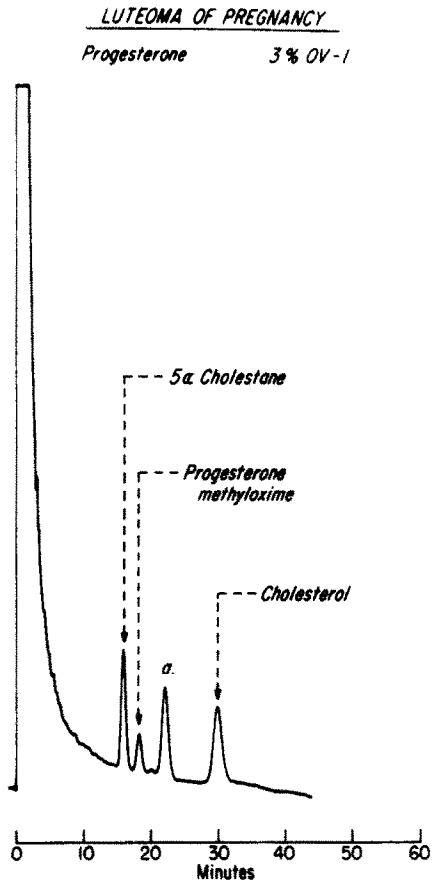

Fig. 6. Chromatographic tracing of progesterone (as the methyloxime derivative) from luteoma of pregnancy on 3% OV-1. Note presence of unidentified material 'a' which did not separate from free progesterone on 3% OV-1. Cholesterol and 5α-cholestane were added as internal standards.

appropriate retention times were noted on the chromatograms but the low recoveries made quantitation unreliable.

Sterol identification

The 'C_{27}' zone was dissolved in 500 μl of tetrahydrofuran and was found to contain materials with retention times on 3% OV-1 identical to cholesterol, 7-cholestenol, campesterol, stigmasterol, and β-sitosterol (Fig. 9). The 3% QF-1 column did not provide sufficient resolution of the compounds present in the 'C_{27}' zone to be useful. The C_{28} zone, dissolved in 100 μl, contained materials with retention times on 3% OV-1 and 3% QF-1 identical to 4α-methyl-7-cholestenol and similar to 4α-methyl-8-cholestenol (Fig. 10, Table 2). The $C_{30}+C_{29}$ zone, dissolved in 100 μl, contained materials with retention times on 3% OV-1 and 3% QF-1 identical to lanosterol and 4,4-dimethyl-8-cholestenol (Fig. 11). Dihydrolanosterol and 4,4-dimethyl-7-cholestenol could not be identified in the $C_{30}+C_{29}$ sterol zone.

Formation of trimethylsilylether (TMS) derivatives of the C_{27} zone and

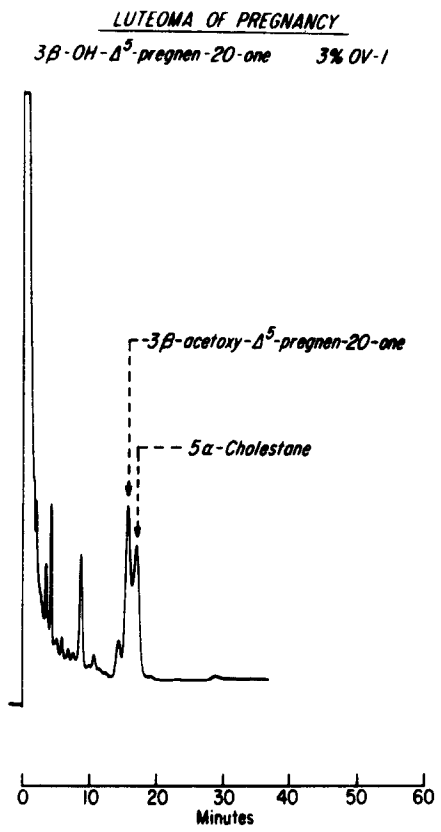

Fig. 7. Chromatographic tracing of pregnenolone (as the acetate derivative) from luteoma of pregnancy on 3% OV-1. 5α-Cholestane added as an internal standard.

chromatography on 3% OV-1 columns showed peaks with retention times identical to the TMS derivatives of cholesterol, 7-cholestenol, campesterol, β-sitosterol, and 4α-methyl-7-cholestenol (Table 2). It appeared that some 4α-methyl-7-cholestenol TMS was present in the C_{27} zone. The retention time of free 4α-methyl-7-cholestenol is close enough to that of free campesterol that they would not have separated on the 3% OV-1 column (Table 2). As the TMS derivative, 4α-methyl-7-cholestenol would chromatograph with the same retention time as stigmasterol TMS (Table 2). The mass measurements of campesterol and β-sitosterol were calculated from the peak heights of the compounds chromatographed as the TMS derivatives since both peaks appeared to have extraneous materials present in the free state. The mass of stigmasterol was calculated from peak height on chromatograms of the free sterols since there did not appear to be any overlap in the retention time of the other reference compounds with stigmasterol.

The presence of 4α-methyl-7-cholestenol was confirmed by chromatography of the free compounds on QF-1 and OV-17 columns and by chromatography of the TMS derivatives on 3% OV-1 and 3% OV-17 (Table 2). Although chromatography of the free material on QF-1 suggested the presence of 4α-methyl-8-cholestenol, chromatography as the TMS derivatives on OV-1 and OV-17 and

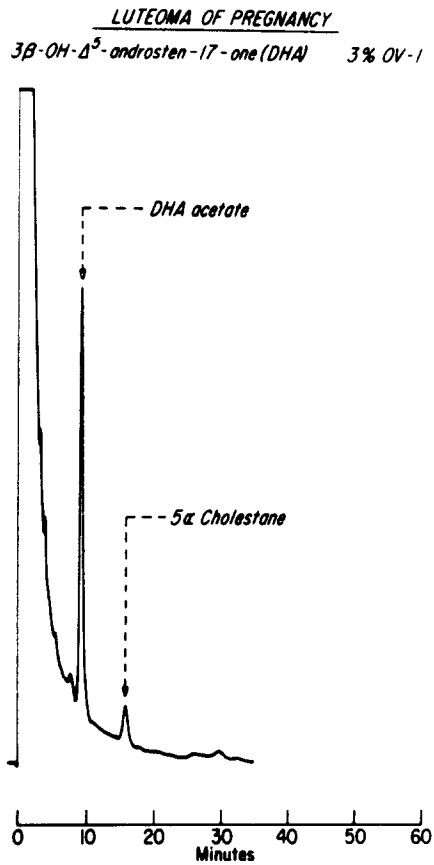

Fig. 8. Chromatographic tracing of DHA (as the acetate derivative) from luteoma of pregnancy on 3% OV-1. 5α-Cholestane added as an internal standard.

as the free compound on OV-17 suggested sufficient dissimilarity of retention times to make identification dubious (Table 2). The instability of this sterol has been noted by others (12 and personal communication from Professor W. B. Whalley). At this time insufficient evidence is available for a tentative identification of 4α-methyl-8-cholestenol.

The $C_{30}+C_{29}$ zone was examined after formation of TMS derivatives. The chromatogram was technically unsatisfactory because of probable decomposition of the sample from which solvent had evaporated several days before derivative formation was attempted. The residue of the non-saponifiable extract had been stored in a screw-cap vial in the dried state without any special precautions. When this material was subjected to further analysis by thin-layer and gas–liquid chromatography it was apparent that marked deterioration had occurred. This was most evident in the 4α-methyl and 4,4-dimethyl-8 compounds. Material stored in ligroin or tetrahydrofuran appeared to be stable for short periods of time but decomposition occurred rapidly when small amounts of these sterols were left in the dry state in air.

The procedure outlined in Fig. 1 is not designed primarily for extraction of sterol compounds and gives a relatively poor yield when compared with the

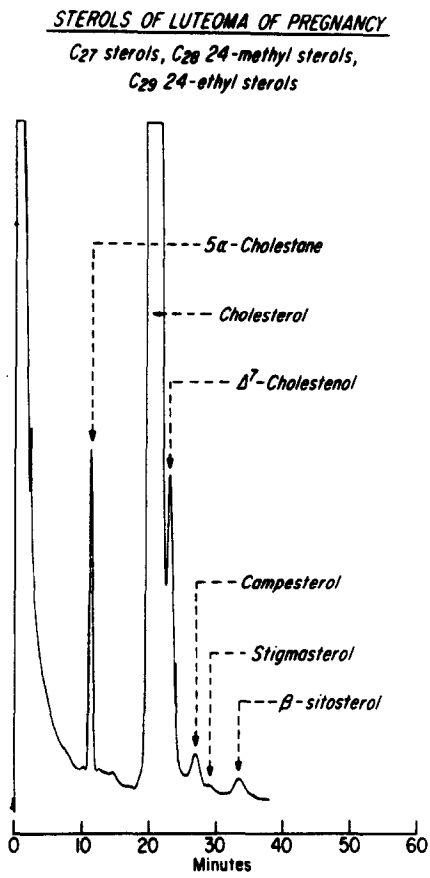

Fig. 9. Chromatographic tracing of 'C_{27}' sterol zone from luteoma of pregnancy on 3% OV-1. Retention times of authentic compounds are shown by labelled arrows.

extraction procedure of Bligh and Dyer[13]. After the completion of the described studies an additional 25 g of formalin fixed tissue were extracted by the Bligh and Dyer method. Considerably more sterol was obtained in the non-saponifiable fraction by this technique than was obtained following ether extraction. Following purification by thin-layer chromatography, the individual fractions were analyzed by gas–liquid chromatography. Essentially similar results were obtained except for the probable presence of an additional major unidentified sterol in the $C_{30} + C_{29}$ zone with a retention time between that of 4,4-dimethyl-8-cholestenol and lanosterol.

DISCUSSION

We have previously reported the isolation of 4-androstene-3,17-dione from a typical non-virilizing luteoma of pregnancy[1]. In that study fresh luteoma tissue was shown to be responsive *in vitro* to human chorionic gonadotropin and formed increased amounts of 4-androstene-3,17-dione from endogenous precursors during the course of the incubation. Incorporation of [1-^{14}C] acetate into steroid hormones could not be demonstrated and other minor steroids could not be identified since ultramicro techniques were not available to us at that time.

The luteoma of pregnancy studied in this report was histologically typical in

Table 2. Gas chromatographic characteristics of reference sterols and unknown compounds of the luteoma of pregnancy

Sterols	OV-1 Free RT*	OV-1 Free RRT†	OV-1 TMS RT	OV-1 TMS RRT	QF-1 Free RT	QF-1 Free RRT	OV-17 Free RT	OV-17 Free RRT	OV-17 TMS RT	OV-17 TMS RRT
C_{27} Zone										
Cholesterol	20·4	1·81	23·7	2·21						
A‡	20·4	1·81	23·7	2·21						
7-cholestenol	22·8	2·02	27·0	2·53						
B	23·2	2·05	27·2	2·53						
Campesterol	26·3	2·34	30·9	2·88						
C	27·0	2·39	30·8	2·87						
Stigmasterol	28·7	2·55	33·5	3·11						
D	29·0	2·57	33·4	3·11						
β-sitosterol	33·2	2·94	38·7	3·60						
E	33·4	2·96	38·5	3·58						
C_{28} Zone										
4α-methyl-8-cholestenol	25·1	2·25	30·7	2·89	23·5	3·55	35·8	3·28	30·5	2·80
F	24·1	2·17	29·6	2·75	23·5	3·58	33·5	3·05	29·3	2·68
4α-methyl-7-cholestenol	27·0	2·42	33·1	3·11	26·3	3·99	39·7	3·64	34·0	3·13
G	26·8	2·41	33·4	3·15	26·1	3·98	39·7	3·65	34·3	3·14
$C_{30} + C_{29}$ Zone										
4,4 dimethyl-8-cholestenol	31·1	2·79			28·8	4·36				
H	31·4	2·83			29·2	4·44				
Dihydrolanosterol	31·0	2·78			30·7	4·70				
Lanosterol	33·8	3·04			33·1	5·06				
I	33·5	3·02			33·1	5·03				
4,4 dimethyl-7-cholestenol	34·7	3·11								

*Retention time (min).

†Relative retention time to 5α-cholestane 5α-cholestane = 11·3 min.

‡Letters A through I refer to peaks observed on chromatograms and are listed according to the similarity of their retention times to authentic reference compounds listed above.

all respects and was associated with mild virilism of the mother and female infant. The major 4-ene-3-keto-steroid hormone identified was 4-androstene-3,17-dione, the same steroid found in the non-virilizing luteoma previously reported[1]. In addition, the androgens, testosterone and 3β-hydroxy-5-androsten-17-one (DHA), were also measured in this study with DHA being the neutral steroid present in highest concentration (Table 1). 3β-Hydroxy-5-pregnen-20-one was measured and progesterone was found to be present in small amounts (Table 1).

Among the 3 luteomas of pregnancy which have now been carefully studied (2 virilizing, 1 without virilism in mother or child) androgen production appears to be a constant feature. The major steroid measured in the virilizing luteoma reported by O'Malley and co-workers[4] was testosterone. The non-virilizing luteoma previously studied by us contained 4-androstene-3,17-dione as its major 4-ene-3-keto-steroid[1]. The present luteoma contained 4-androstene-3, 17-dione as its major 4-ene-3-keto-steroid but DHA was present in highest concentration in the tumor. Our results, based on the studies of the virilizing and non-virilizing luteomas, suggest that luteomas of pregnancy probably do not differ significantly in their ability to produce androgenic steroids. The availability of ultramicro techniques enabled us to identify additional androgenic steroids in

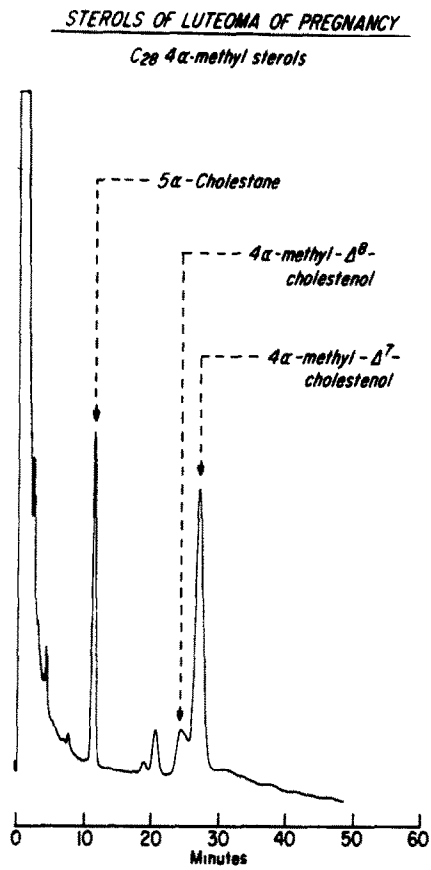

Fig. 10. Chromatographic tracing of C_{28} sterol zone from luteoma of pregnancy. Retention times of authentic compounds are shown by labelled arrow.

this virilizing luteoma that could not be detected by conventional methods in the initial study of the typical non-virilizing luteoma of pregnancy.

Androgen production seems to be characteristic of the luteoma of pregnancy whether or not there is evidence of virilization. At the present time it is difficult to say why more patients with luteomas of pregnancy do not become virilized or have virilized infants. The *typical* patient has no evidence of virilization. The production of large amounts of a potent androgen-like testosterone might account for the differing clinical pictures. Placental aromatization of secreted androgens should, however, be an efficient mechanism for protection from the virilizing effect of the secreted androgen [14]. The biological effect of the secreted androgen might not be clinically evident unless there were an abnormality in placental aromatization or secretion of androgens by the luteoma in amounts which exceed the 'clearance' capacity of the placenta. This concept is supported by the report of defects in placental metabolism of androgens in association with maternal hirsutism and fetal malformations [15]. The rarity of masculinizing syndromes during pregnancy attests to the usual efficiency of the placental mechanism [16].

Recently Dominguez and Huseby [20] described a transplantable luteoma of

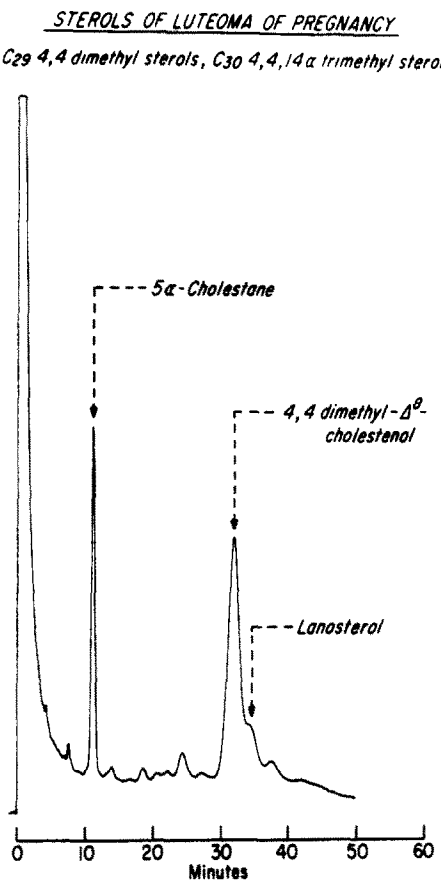

Fig. 11. Chromatographic tracing of $C_{30} + C_{29}$ sterol zone from luteoma of pregnancy on 3% OV-1. Retention times of authentic compounds are shown by labelled arrow.

the BALB/c mouse. Their studies indicated the secretion of androgens and corticoids as evidenced by stimulation of the seminal vesical-coagulating gland complex and atrophy of the adrenals in castrated male mice. After incubation of homogenates of luteoma tissue with labelled pregnenolone and progesterone, 4-androstene-3,17-dione, testosterone, and deoxycorticosterone were identified as radioactive products. The androgen, 4-androstene-3,17-dione, was the major radioactive steroid identified. It appears that the mouse luteoma and the human luteoma resemble each other in their ability to form androgens. Corticoids were not sought in the human luteoma specimen. Adrenocortical suppression by corticoids from the luteoma might explain the episode of suspected adrenal insufficiency in the female infant observed at 10 days of age.

Histological studies suggest that luteomas of pregnancy originate from stimulated theca-lutein or stroma-lutein cells. The steroidal products of the luteomas of pregnancy that have been studied do not quantitatively resemble those formed by the intact follicle or corpus luteum[17, 18]. The formation of androgens would suggest that the luteoma is derived from the stromal compartment of the ovary since androgens are the major steroids produced by normal

Table 3. Approximate sterol content of virilizing luteoma of pregnancy

Sterol	Content (μg/g)*
Cholesterol	1228
7-Cholestenol	20·1
4α-Methyl-7-cholestenol	9·3
4,4 Dimethyl-8-cholestenol	5·1
Lanosterol	1·1
β-Sitosterol	1·1
Campesterol	0·8
Stigmasterol	0·2

*Approximate values are determined from peak height of individual sterols using cholesterol as a standard and are corrected for recovery of [^{3}H] cholesterol tracer added after countercurrent distribution (Fig. 1). When [^{3}H] cholesterol was added to tumor homogenate before extraction with diethyl ether, the cholesterol content, as measured by gas–liquid chromatography, was 4·65 mg/g of tissue after correction for all methodologic losses.

ovarian stromal tissue[19]. It appears that the steroid data and the morphologic studies are in good agreement and indicate that the luteoma of pregnancy is derived from the ovarian stromal compartment.

The tentative identifications of the many sterols found in this luteoma of pregnancy are significant for several reasons. In our initial study of the non-virilizing luteoma[1], [1-^{14}C] acetate incorporation into steroid could not be demonstrated although normal ovarian stromal tissue from pregnant women incorporates large amounts of [1-^{14}C] acetate into androgenic steroids[19]. Among the possible explanations, dilution of the ^{14}C label by large amounts of endogenous sterols seems to be the most likely reason that radioactive steroid hormone synthesis could not be demonstrated in the initial *in vitro* study. Unfortunately, fresh tissue was not available from the current patient for *in vitro* study of [1-^{14}C] acetate incorporation into sterols and steroid hormones.

In addition to cholesterol, lanosterol, 4,4-dimethyl-8-cholestenol, 4α-methyl-7-cholestenol, and 7-cholestenol were tentatively identified in this luteoma of pregnancy. This is the first tentative identification of these sterols in human tissue, although 7-stenols have been measured spectrophotometrically in several non-endocrine human tissues[21]. These compounds have been identified in rat skin[22] and several of them have been isolated from a transplantable preputial gland tumor of the mouse[23]. The instability of 4α-methyl-8-cholestenol makes even tentative identification of this sterol difficult and final judgment will have to await more definitive studies.

Little is known about specific sterol metabolism, other than cholesterol, and steroid hormone biosynthesis in gonadal tissues. Gaylor and Tsai[24] showed that lanosterol may serve as a steroid hormone precursor, presumably through cholesterol, in rat testis homogenates. Hellig and Savard[25] studied sterol biosynthesis in the bovine corpus luteum and concluded that the pathway from acetate to

cholesterol was qualitatively similar but quantitatively different from that in liver tissue. Our identifications have no direct bearing on the actual pathway of cholesterol biosynthesis but are in accord with the pathway described in the preputial gland tumor study of Kandutsch and Russel[23]. The identification of lanosterol, 4,4-dimethyl-8-cholestenol, 4α-methyl-7-cholestenol, 7-cholestenol, and cholesterol from this luteoma of pregnancy suggests the operation of a metabolic pathway which includes these sterols in the sequence listed. Possible alternative pathways for cholesterol biosynthesis have been reviewed by Bloch [26] and Frantz and Schroepfer[27].

Campesterol, stigmasterol, and β-sitosterol have been tentatively identified in this luteoma. These 24 methyl and ethyl C_{28} and C_{29} sterols are usually thought to be of plant origin. Evidence based on tracer studies suggests that β-sitosterol can be absorbed from the human gastrointestinal tract[28]. We have tentatively identified these sterols in human breast cancer tissue and 2 different transplantable rat testis tumor tissues. Incubation studies with [1-^{14}C] acetate and testis tumor tissue have not shown these sterols to be synthesized *in vitro* in a transplantable rat testis tumor although they are present within the tissue (unpublished observations). It appears that these particular sterols are probably dietary in origin and are found wherever large amounts of lipids are stored provided they are available in the diet. This interpretation is difficult to document in this particular study and can only be considered as tentative at this point. It is of interest, however, that [^{3}H]β-sitosterol has been shown to be capable of serving as a precursor for cortisol synthesis in the guinea pig[29] and suggests that these sterols might serve as precursors of steroid hormones if present in gonadal tissues.

Formalin fixation of the luteoma was inadvertent but did not greatly interfere in the analysis. Sterols have been identified in formalin fixed tissues in the past [21]. We have not found any report of steroid hormone analyses of formalin fixed endocrine tissues. We would not recommend formalin fixation but it does appear that certain sex steroids are not harmed by exposure to 10% formalin. Progesterone and 4-androstene-3,17-dione were quantitatively recovered after incubation with formalin which suggests that the quantitative aspects of the steroid hormone analysis of this luteoma are meaningful. The similarity of our results to those done on unfixed human luteoma tissue[1, 4] and mouse luteoma tissue[20] suggests that formalin fixed tissue might be utilized for analyses of sex steroids providing relatively specific chromatographic techniques are employed.

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