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SIDE-CHAIN CLEAVAGE OF 4-CHOLESTEN-3-ONE, 5-CHOLESTEN-3α-OL, β-SITOSTEROL, AND RELATED STEROIDS IN ENDOCRINE TISSUES FROM RAT AND MAN

LEIF ARINGER, PETER ENEROTH and LENNART NORDSTRÖM*
Hormone Laboratory, Department of Obstetrics and Gynecology, Karolinska Sjukhuset, S-104 01 Stockholm 60, Sweden

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SUMMARY

Fourteen 4-^{14}C-labeled C_{27}- and C_{29}-steroids were incubated with mitochondria prepared from rat adrenals, ovaries, testes and placenta, fortified with NADPH. Of the eleven C_{27}-steroids six were available also with 26-^{14}C-label. The main purpose was to study which of the substrates tested could be converted to C_{21}- and/or C_{19}-steroids. When experiments with a 4-^{14}C- and a 26-^{14}C-labeled steroid were run in parallel, the metabolites not labeled in 26-^{14}C-experiments but in 4-^{14}C-experiments were considered as steroids containing a side-chain shorter than that of the C_{27}-substrate. Identifications were also based on chromatography data (TLC and GLC combined with radioactivity detection).

In the rat, cholesterol and β-sitosterol were converted into C_{21}- and C_{19}-steroids in mitochondria from all tissues tested. 5-Cholesten-3α-ol was converted into the 3α-epimer of pregnenolone in the adrenal and ovarium 8500 *g* sediment. 4-^{14}C-Labeled 4-cholesten-3-one was converted in the testes to labeled androstenedione, without the presence of labeled C_{21}-steroids. Using the conditions employed in studies with testis mitochondria, adrenal 8500 *g* sediments were able to metabolize 4-cholesten-3-one into progesterone and 17α-hydroxyprogesterone. Other C_{27}- and C_{29}-steroids tested yielded polar metabolites to different degrees. None of those metabolites was identified as a C_{21}- or C_{19}-steroid.

Pregnenolone and progesterone were shown to be formed from cholesterol and β-sitosterol in mitochondria prepared from human adrenals, testes and term placentae.

INTRODUCTION

The enzyme(s) responsible for the cleavage of the cholesterol side-chain in adrenal glands have been shown also to utilize steroids with different side-chain structures as substrate. Thus Werbin *et al.*[1] demonstrated that in the guinea pig, β-[^{3}H]-sitosterol yields tritiated urinary cortisol. In studies on the reaction sequence in the cleavage of the side-chain by acetone powders of rat adrenal mitochondria Burstein and Gut[2] demonstrated that β-[4-^{14}C]-sitosterol was converted to pregnenolone. Although information regarding the adrenal enzyme substrate specificity in terms of side-chain structure is well established [1–13] there are less data available from other endocrine tissues. However, Kuksis and Subbiah[14] have presented results which indicate that rat testes are able to utilize β-sitosterol for steroid-hormone formation.

The structural requirements in terms of the steroid nucleus have been less extensively studied in all steroid-hormone producing organs [6, 15]. In an attempt to explore the substrate specificity of cholesterol metabolizing enzymes we have synthesized a number of labeled C_{27}-steroids closely related to cholesterol. These steroids have previously been tested in reactions related to bile acid biosynthesis [16–18]. The aim of the present investigation was to explore whether or not steroid-hormone producing organs in the rat would discriminate between C_{27}-steroids with a structure different from that of cholesterol in the A- and B-rings of the steroid nucleus. In addition β-sitosterol and other C_{29}-steroids have been used to see if the steroid side-chain requirements differed amongst different endocrine tissues.

MATERIALS

Solvents. All solvents and reagents were analytical grade and purchased from E. Merck A.G., Darmstadt, West Germany, unless otherwise stated. They were used without further purification.

Substrates. [4-^{14}C]-Cholesterol (55–61 mCi/mmol), [26-^{14}C]-cholesterol (55–58 mCi/mmol) and β-[^{14}C]-

*To whom correspondence should be sent.

Abbreviations: Systematic names of the steroids referred to in the text by trivial names are as follows: cholesterol, 5-cholesten-3β-ol; epicholesterol, 5-cholesten-3α-ol; cholestanol, 5α-cholestan-3β-ol; β-sitosterol, 24α-ethyl-5-cholesten-3β-ol; and epipregnenolone, 3α-hydroxy-5-pregnen-20-one. PAD refers to pathological and anatomical diagnosis. Other abbreviations: LC, liquid chromatography; TCV, total column volume; TLC, thin-layer chromatography; TLC-RD, thin-layer chromatography combined with radioactivity detection; GLC, gas–liquid chromatography; GLC-RD, gas–liquid chromatography combined with radioactivity detection; GLC-MS, gas–liquid chromatography–mass spectrometry; TMS ether, trimethylsilyl ether; t_R is the retention time relative to that of 5α-cholestane.

sitosterol (58 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England. All other substrates containing 27 carbon atoms were prepared from [4-^{14}C]-cholesterol or [26-^{14}C]-cholesterol, respectively, as described previously for the 4-^{14}C-labeled substrates [16]. 4-^{14}C-Labeled 24α-ethyl-5α-cholestan-3β-ol and 24α-ethyl-4-cholesten-3-one were prepared from β-[4-^{14}C]-sitosterol as described in reference [16]. All substrates were purified and the purity was checked as described previously [16].

Reference compounds. Reference C_{19}- and C_{21}-steroids were purchased from Sigma Chemical Co., St. Louis, U.S.A. and purity was checked in TLC system 1 (Table 1, footnote §) and by GLC. 20α-Hydroxy- and 20α,22R-dihydroxycholesterol were gifts from Prof. J.-Å. Gustafsson, Dept. of Chemistry, Karolinska Institute, S-104 01 Stockholm 60, Sweden.

Enzymes and coenzymes. 3α-Hydroxysteroid-dehydrogenase, dissolved in 50% glycerol (114 IU/μl) was a gift from Prof. H. Carstensen, Institute of Biology, Dept. of Physiology, The University of Umeå, S-104 87 Umeå, Sweden. The steroid substrate, in 100 μl of methanol, was mixed with 25 μl of enzyme solution and 2.8 ml of redistilled water containing 100 μmoles of $Na_4P_2O_7$ and 0.5 μmol of NAD. The mixture was incubated for 2 h at 25°C and worked up as described for the tissue 8500 *g* sediments, see below.

NADPH (type I) and NAD (grade III) were purchased from Sigma Chemical Co. and were used without further purification.

Chromatography techniques. The liquid chromatography (LC) system used are described in Table 1, footnote‡; Table 4, footnote ‡, and in the text. TLC was performed with Merck's precoated 20 × 20 cm Silica gel 60 plates in the systems described in Table 1 (footnote §) and in the text. Separated compounds were detected by iodine vapor or by radioautography (see below). Compounds were extracted from the gel as described previously [19]. For GLC a Pye gas chromatograph (model 104) with a hydrogen flame ionization detector was used. The columns (1.3 m × 4 mm, QF-1; or 2 m × 4 mm, SP-2100) contained Supelcoport (80–100 mesh, QF-1; 100–120 mesh, SP-2100) coated either with 3% QF-1 or 3% SP-2100 (Supelco Inc. Bellefonte Pa., U.S.A.). The temperatures were: flash heater, 270°C and column oven 240°C (QF-1) or 255°C (SP-2100). Carrier gas was N_2 with a gas flow rate of 480 ml/cm^2/min. Unless otherwise stated, the t_R-values given are from analysis on the QF-1 column. All hydroxysteroids were analyzed as their TMS ethers, prepared according to the procedure of Makita and Wells[20].

Measurement of radioactivity. A Packard Model 2009 liquid scintillation spectrometer was used. Aliquots for radioactivity determination were transferred to glass scintillation vials. The organic solvent was evaporated under a stream of nitrogen and 10 ml of a scintillation fluid (Insta Gel, Packard Instrum.) was added. At least 2000 counts above the background were counted. Counting efficiency for ^{14}C was 61%. ^{14}C-Labeled spots on thin-layer chromatograms were localized by exposing the plate to a sheet of Agfa Gevaert Osray M3 film for 1–14 days. GLC-RD was accomplished with a Packard Instrument, Model 893. At least 4000 d.p.m. were injected.

EXPERIMENTAL PROCEDURES

Animals. Sprague–Dawley rats (Anticimex, Stockholm) 200 g, were kept for 3–5 days preceding the experiments. They were fed a standard food fortified with 0.1% (w/w) DL-α-tocopherol acetate [19] and water *ad libitum*. For each experiment, pools of organs from 4–6 animals were used. They were killed by a blow to the head at 8 a.m.

Experiments with mitochondria from rat adrenals. To 100 mg of wet adrenal tissue from male animals were added 10 ml of a 0.25 M sucrose solution, pH 7.4. The mixture was homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle (clearance 0.15 mm). The mitochondria (8500 *g* sediments) were prepared as described by Björkhem and Karlmar [21]. All steps were carried out at 4°C. Protein in each incubation flask as determined according to the method of Lowry *et al.*[22] was 1.0 mg. For each incubation, 1.0 ml of the homogenate and 2.0 ml of a 0.1 M Tris–Cl-buffer, pH 7.4 containing 0.36 mmol of KCl were used. The mitochondria were incubated for 10 min. at 37°C in the presence of 2.4 μmoles of NADPH. One-10 μg of 4-^{14}C- or 26-^{14}C-labeled substrates dissolved in 100 μl of acetone were added to the homogenate under a stream of nitrogen and with concomitant vibration of the mixture (Supermixer, Labline Instruments Inc. Ill. U.S.A.) as previously reported [23].

Experiments with mitochondria from rat ovaries. Human chorionic gonadotropin (600 IE of Gonadex, LEO, Sweden, dissolved in 0.5 ml of saline) was injected intraperitoneally once a day for 2 days. One additional injection was carried out 1 h prior to the killing. The ovaries were excised and homogenized in a Potter Elvehjem homogenizer, as described for the adrenals. To 100 mg of wet ovary tissue, 10 ml of the sucrose solution were added. The mitochondria were prepared and incubated as described for the adrenal mitochondria. The amount of protein per flask was 0.4 mg.

Experiments with mitochondria from rat testes. Gonadex was administered as described under experiments with ovaries. The testes were pooled and the mitochondria (6500 *g* sediments) were prepared as described by Forchielli[24]. The procedure involved washing of the mitochondria. The mitochondria isolated from 1.5 g of wet testis tissue was suspended in 3 ml of a 0.20 M sucrose solution containing 0.02 M Tris–Cl buffer, pH 7.4, 80 μmol of $MgCl_2$ and 250 μmoles of KCl. The suspension was incubated for 60 min at 35°C in the presence of 4.8 μmoles of NADPH. The amount of protein in each incubation

Table 1. Yields of C_{27}- and C_{29}-steroid metabolites after incubations with rat adrenal 8500 *g* sediments and NADPH*

Substrate†	Yields of metabolites (% of added dose) after LC purification‡			Mean distribution of 4-^{14}C-labeled metabolites (% of added dose) after TLC purification§		
	4-^{14}C-steroid	26-^{14}C-steroid	*n*	Zone 1 (R_F 0.00–0.05)	Zone 2 (R_F 0.06–0.35)	Zone 3 (R_F 0.36–0.70)
4-Cholesten-3α-ol	1.7 (0.6–3.7)	0.9 (0.3–1.0)	3	0.9 (0.4)	0.2 (0.1)	0.2 (0.2)
4-Cholesten-3β-ol	2.4 (1.8–3.0)	1.3 (1.0–2.0)	3	1.6 (0.5)	0.4 (0.2)	0.4 (0.4)
5-Cholesten-3α-ol	1.9 (0.7–3.0)	1.0 (0.5–2.0)	3	0.3 (0.0)	0.4 (0.0)	1.2 (0.9)
5-Cholesten-3β-ol	8.7 (3.3–18.0)	1.5 (0.8–2.3)	16	1.4 (0.7)	1.9 (1.4)	5.4 (5.0)
24α-Ethyl-5-cholesten-3β-ol	7.6 (5.3–9.8)		3	0.7	1.0	5.3
5α-Cholestan-3α-ol	0.1 (0.0–0.2)		2	0.1	0.0	0.0
5α-Cholestan-3β-ol	1.2 (0.2–2.9)	0.3 (0.1–0.8)	3	0.4 (0.1)	0.3 (0.2)	0.3 (0.2)
5β-Cholestan-3α-ol	0.5 (0.2–0.8)		3	0.1	0.2	0.2
5β-Cholestan-3β-ol	0.5 (0.2–1.0)		3	0.2	0.2	0.1
4-Cholesten-3-one	15.0 (1.6–24.0)	14.6 (2.0–20.0)	3	10.1 (0.1)	3.1 (0.1)	0.6 (0.2)
5α-Cholestan-3-one	9.8 (5.9–13.8)		2	3.3	5.1	0.2
5β-Cholestan-3-one	12.6 (12.0–13.0)		3	2.3	7.9	1.4

* Incubations (37°C, 10 min) with 5 μg of a ^{14}C-labeled substrate were carried out in a 3 ml suspension of a rat adrenal 8500 *g* sediment, isolated from 10 mg of wet adrenal weight and fortified with 2.4 μmoles of NADPH. Each incubation flask contained 1 mg of protein.

† Experiments with 4-^{14}C- and 26-^{14}C-labeled steroids were run in parallel. For synthesis, see Materials.

‡ Liquid chromatography (LC), *system 1*: Lipidex-5000 (Packard instruments) in methanol–water–dichloroethane (95:5:25, by vol.). Column dimensions: 0.75 cm^2 × 13 cm. Flow rate: 0.5 ml/cm^2/min at 24°C. The metabolites were collected between 40 and 150% TCV. Non-metabolized substrate was collected between 200 and 400% TCV. Metabolites remaining on the column were eluted with one column volume of diethyl ether–acetone (1:1, v/v). Ranges in yields are given within parenthesis. The recovery of radioactivity after the initial extraction and LC was for all substrates at least 90%. The recovery of unchanged substrate after TLC purification varied between substrates. With 3-hydroxysteroids the recoveries ranged between 67 and 85%; for 3-oxosteroids lower values were obtained i.e. 40–58%. Some losses of radioactivity were caused by the LC fractionation procedure i.e. between 0 and 40% TCV and between 150 and 200% TCV but did not differ much between the different substrates. The radioactivity remaining on the LC column amounted to 1–4% in all instances.

§ Figures within parenthesis denote the difference between yields (in % of added substrate) of 4-^{14}C- and 26-^{14}C-metabolites. TLC on precoated Silica gel 60 plates (20 × 20 cm, Merck) in solvent *system 1*: chloroform–ethyl acetate (6:1, v/v). R_F-values for cortisol, 5α,6α-oxidocholestan-3β-ol and progesterone were 0.01, 0.18 and 0.60, respectively.

flask was 15 mg. Labeled steroids were added dissolved in 100 μl of propyleneglycol.

Experiments with mitochondria from rat placenta. Placentae from animals, pregnant for 17–19 days, were excised and homogenized in a solution composed of a 0.25 M sucrose solution and 0.1 M potassium phosphate buffer in the proportion (4:1, v/v), pH 7.35, with an all glass homogenizer [24]. The mitochondria were prepared as described for testes [24] and were then homogenized in 0.1 M phosphate buffer (pH 7.0), so that mitochondria isolated from 300 mg of wet placenta tissue were suspended in 3 ml of buffer. The mitochondria were incubated for 30 min at 37°C in the presence of 4.8 μmol of NADPH. The amount of protein per flask was 1.0 mg. Labeled steroids were added to the homogenate dissolved in acetone as described above.

Experiments with mitochondria prepared from endocrine tissues from man. Part of tissues, taken for PAD, from patients suffering from a Conn tumor (adrenals), tumor uteri (ovaries), testicular feminization (testes) and after normal term deliveries (placenta), were homogenized and the mitochondria were prepared as described for the respective rat organ. Only some of the substrates used for the experiments with rat tissues were studied, see Table 2, footnote ‖.

Extraction and work-up procedures. All incubations were terminated and extracted with chloroform–methanol (2:1, v/v) as described previously [19]. The combined chloroform phases were taken to dryness and then purified by LC in system 1 (adrenals, ovaries, and placenta; see Table 1, footnote ‡) or in LC system 2 (testes; see Table 4, footnote ‡). The metabolites were collected as described in the footnotes to Table 1 and 4 and were further purified and analyzed by TLC and GLC-RD. The identification of C_{19}- and C_{21}-steroids was accomplished by comparison with chromatography data of reference compounds.

RESULTS

Metabolism of C_{27}- and C_{29}-steroids in rat adrenal mitochondria

The extent of metabolism for each steroid substrate tested is summarized in Table 1. From data on the 4-^{14}C-labeled substrates it is clear that the highest yields of metabolites were obtained with cholesterol, β-sitosterol and the 3-oxo-steroids. 5α-Cholestan-3α-ol and the isomeric 5β-cholestan-3-ols were converted to a very limited degree. Cholesterol was the only steroid which afforded 4-^{14}C-labeled metabolites much in excess over 26-^{14}-labeled metabolites. But higher yields of 4-^{14}C-metabolites than of 26-^{14}C-labeled metabolites were also noted for the other substrates tested.

When 4-^{14}C- and 26-^{14}C-labeled cholesterol had been used as substrates, side by side analysis on TLC (Fig. 1) of metabolite fractions from LC revealed that most of the difference in yields was confined to a region containing comparatively nonpolar steroids (zone 3, see Table 1). Three metabolites with R_F-values equal to those of pregnenolone, androstenedione, and progesterone, respectively, were obtained. GLC-RD analysis of the extracted TLC-zones verified the identity of 4-^{14}C-labeled pregnenolone (t_R = 1.74), androstenedione (t_R = 6.30) and progesterone (t_R = 8.09). The net yields of these metabolites are shown in Table 2. No evidence for the formation of 17α-hydroxyprogesterone or testosterone were obtained. Other metabolites that were labeled in 4-^{14}C-experiments but not in experiments with 26-^{14}C-labeled steroids (see Fig. 1) were obtained in yields too low to permit further identification. The metabolite pattern obtained in experiments with β-[4-^{14}C]-sitosterol, resembled that obtained in experiments with [4-^{14}C]-cholesterol. GLC-RD analysis verified the identity of pregnenolone and progesterone as β-sitosterol metabolites. The extent of their formation is shown in Table 2.

Two 4-^{14}C-labeled metabolites (R_F = 0.26 and 0.50) that were not seen in experiments with the 26-^{14}C-labeled sterol were obtained in experiments with 5-cholesten-3α-ol (see Fig. 1). TLC-RD revealed that the one with R_F = 0.26 migrated like 17α-hydroxyprogesterone. The epicholesterol metabolite with an R_F = 0.50 (about 10 μg, from three experiments) was subjected to oxidation with 3α-hydroxysteroid dehydrogenase. Subsequent work up and analysis by TLC-RD in system 1 (Table 1, footnote §) revealed that the product had shifted to a TLC position equal to that of progesterone. Subsequent GLC-RD afforded a labeled peak at t_R = 8.09, confirming that the original compound was 3α-hydroxy-5-pregnen-20-one. The yields are presented in Table 2.

Incubations with labeled 4-cholesten-3α-ol or 4-cholesten-3β-ol yielded 4-^{14}C-labeled metabolites mainly in the lowest TLC region (zone 1, see Table 1), where the most polar metabolites appeared. TLC zones 2 and 3 (see Table 1) from each of these experiments did not contain any distinct spots. The metabolites extracted from TLC zone 1 (experiments with 4-cholesten-3β-ol) were subjected to LC system 2 (Table 4, footnote ‡). Ten % of the amount of ^{14}C-activity applied to the column was eluted before 150% TCV, indicating the presence of steroids with no or a short side-chain (steroids containing a C_{27} side-chain are selectively retarded in LC system 2; Aringer L. and Nordström L., unpublished results). No further attempts were made to identify these metabolites.

TLC-RD analysis (system 1, Table 1) of metabolite fractions after LC purification of extracts from incubations with 4-^{14}C- and 26-^{14}C-labeled 5α-cholestan-3β-ol, did not reveal any specific localization of 4-^{14}C-radioactivity in zone 2 or 3. Similar negative results were obtained in experiments with the other 5α- and 5β-saturated C_{27}-steroids.

The radioactivity, recovered in zone 1 from incubations with 4-^{14}C- and 26-^{14}C-labeled 4-cholesten-3-

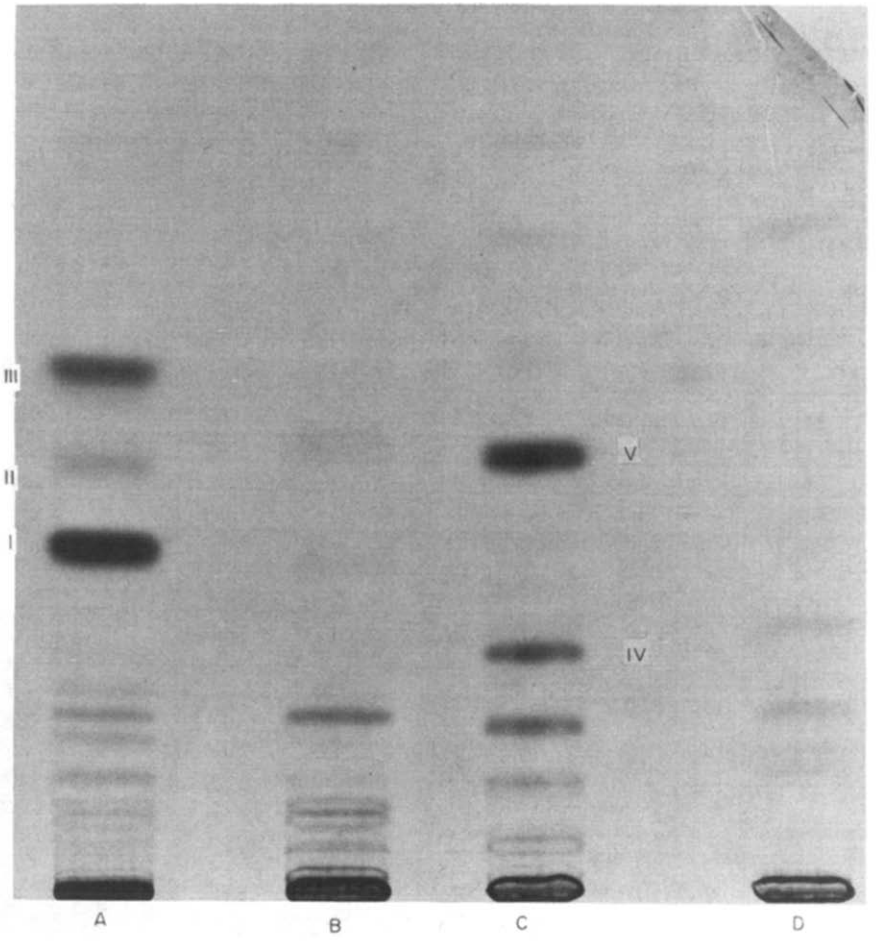

Fig. 1. Radioautography after TLC purification (system 1, see Table 1, footnote §) of metabolite fractions from liquid chromatography of extracts from incubations with a rat adrenal 8500 *g* sediment fraction, fortified with NADPH, and [4-^{14}C]-cholesterol (A), [26-^{14}C]-cholesterol (B), 5-[4-^{14}C]-cholesten-3α-ol (C), or 5-[26-^{14}C]-cholesten-3α-ol (D). Compounds I-V denote metabolites that contain 4-^{14}C, but are not labeled in incubations with the respective 26-^{14}C-steroid. Compounds **I–IV** migrated as the following reference compounds: pregnenolone (**I**, $R_F = 0.41$); androstenedione (**II**, $R_F = 0.48$); progesterone (**III**, $R_F = 0.60$); 17α-hydroxyprogesterone (**IV**, $R_F = 0.26$). Compound **V** was identified as 3α-hydroxy-5-pregnen-20-one ($R_F = 0.50$). Subsequent GLC combined with radioactivity detection verified the tentative identifications of compounds **I–III**.

one and with 4-^{14}C-labeled 5α-cholestan-3-one and 5β-cholestan-3-one, was found not to be associated with polar C_{21}- or C_{19}-steroids. The radioactivity in zone 2 and 3 from incubations with labeled 4-cholesten-3-one was not found in a distinct spot on TLC-RD analysis. The comparably high yields of metabolites recovered in zone 2 and 3 from incubations with labeled 5α-cholestan-3-one and 5β-cholestan-3-one were not due to the formation of C_{21}- or C_{19}-steroids. Thus, long retention times on SP-2100 columns (t_R between 3.50 and 6.00) were noted on GLC-RD analysis, which indicated that the metabolites were C_{27}-steroids. The nature of those compounds was not further studied.

Metabolism of C_{27}- and C_{29}-steroids in rat ovary mitochondria. The yields of 4-^{14}C- and 26-^{14}C-labeled metabolites are presented in Table 3. Cholesterol was the only steroid which gave rise to 4-^{14}C-labeled metabolites much in excess over 26-^{14}C-labeled metabolites.

The metabolite patterns from incubations with 4-^{14}C- and 26-^{14}C-labeled cholesterol and β-[4-^{14}C]-sitosterol were similar to those seen in incubations with cholesterol and adrenal mitochondria (Fig. 1). Thus, 4-^{14}C-labeled cholesterol and β-sitosterol yielded, after TLC purification (system 1, see Table 1), metabolites with R_F-values equal to those of pregnenolone ($R_F = 0.41$, major metabolite formed) and progesterone ($R_F = 0.60$). The suggested identities were subsequently confirmed by GLC-RD. The yields of these compounds are shown in Table 2. The formation of [4-^{14}C]-androstenedione could not be shown.

Metabolism of 5-cholesten-3α-ol (4-^{14}C- or 26-^{14}C-labeled) yielded a product (4-^{14}C-labeled) with a TLC-mobility equal to that of 3α-hydroxy-5-pregnen-20-one (cf. adrenals). Subsequent enzymatic oxi-

Table 2. Conversion of 5-cholesten-3β-ol, 24α-ethyl-5-cholesten-3β-ol, 5-cholesten-3α-ol, and 4-cholesten-3-one into pregnenolone, progesterone and androstenedione in NADPH supplemented mitochondria, prepared from different tissues from rat and man

Mitochondria prepared from	Products formed* Pregnenolone from $3\beta\Delta5C_{27}$	Pregnenolone from $3\beta\Delta5C_{29}$	$3\alpha\Delta5C_{27}$†	Androstenedione from $3\beta\Delta5C_{27}$	Androstenedione from $3\beta\Delta5C_{29}$	$3CO\Delta4C_{27}$	Progesterone from $3\beta\Delta5C_{27}$	Progesterone from $3\beta\Delta5C_{29}$	$3CO\Delta4C_{27}$
Rat									
Adrenals	4.1 (0.2–6.0)	3.5 (2.0–5.0)	0.9 (0.5–2.0)	0.1 (0.0–0.1)	0.0	0.0	0.6 (0.3–1.4)	0.7 (0.1–1.0)	0.0
Adrenals‡	3.6 (2.7–4.4)	1.1	—	0.1 (0.1–0.2)	0.1	0.0	0.9 (0.8–0.9)	0.3	0.3 [0.4§] (0.1–0.5)
Ovaries	2.1 (1.3–3.4)	1.3 (1.1–1.4)	0.2 (0.2)	0.0	0.0	0.0	0.3 (0.0–0.8)	0.3 (0.0–0.5)	0.0
Testes	0.2 (0.0–0.7)	0.1 (0.1–0.2)	0.0	0.3 (0.1–0.8)	0.2 (0.2)	1.1 (0.0–2.9)	0.1 [0.7§] (0.0–0.1)	0.1 [0.4§] (0.1–0.2)	0.0
Placenta	0.1 (0.1–0.2)	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Man‖									
Adrenals	0.1	0.1	—	0.0	0.0	—	0.0	0.0	—
Testes	0.0	0.0	—	0.0	0.0	—	0.4	0.4	—
Placenta	0.1 (0.1–0.2)	0.1 (0.0–0.1)	0.0 (0.0–0.1)	0.0	0.0	0.0	0.1 (0.0–0.1)	0.0	0.0

* The net yields of products formed are given in % of added dose. The yields from incubations with 26-^{14}C-labeled substrates were subtracted from those obtained from incubations with 4-^{14}C-labeled steroids. Figures within parenthesis denote the range in net yields. The figures are results from 1–7 experiments. The abbreviations used denote 5-cholesten-3β-ol ($3\beta\Delta5C_{27}$), 24α-ethyl-5-cholesten-3β-ol ($3\beta\Delta5C_{29}$), 5-cholesten-3α-ol ($3\alpha\Delta5C_{27}$), and 4-cholesten-3-one ($3CO\Delta4C_{27}$).

† The product formed from this steroid was identified as 3α-hydroxy-5-pregnen-20-one.

‡ These mitochondria were prepared and incubated as described for mitochondria from the testes.

§ This figure denotes the sum of the yields of progesterone and 17α-hydroxyprogesterone.

‖ The tissues were available due to a Conn tumor (adrenals), tumor uteri (ovaries), a testicular feminization, and after normal termed deliveries, respectively. Except for the substrates listed in the table the following labeled compounds were incubated: 5-Cholesten-3β-ol, 5α-cholestan-3β-ol, 4-cholesten-3-one and 24α-ethyl-5-cholesten-3β-ol (ovaries); 4-cholesten-3α-ol, 4-cholesten-3β-ol, 5α-cholestan-3β-ol, 5β-cholestan-3α-ol, 5β-cholestan-3-one and 24α-ethyl-5α-cholestan-3β-ol (placenta). 4-^{14}C- and 26-^{14}C-labeled substrates were used as in the analogous experiments with rat tissues (see Tables 1, 3, 4 and 5).

Table 3. Yields of C_{27}- and C_{29}-steroid metabolites after incubations with rat ovary 8500 *g* sediments and NADPH*

Substrate†	Yields of metabolites (% of added dose) after LC purification‡			Mean distribution of 4-^{14}C-labeled metabolites (% of added dose) after TLC purification§		
	4-^{14}C-steroid	26-^{14}C-steroid	*n*	Zone 1 (R_F 0.00–0.05)	Zone 2 (R_F 0.06–0.35)	Zone 3 (R_F 0.36–0.70)
4-Cholesten-3α-ol	1.3 (0.8–1.8)	1.2 (1.2–1.3)	2	0.7 (0.0)	0.3 (0.1)	0.2 (0.0)
4-Cholesten-3β-ol	1.4 (1.2–1.6)	1.3 (1.1–1.5)	2	0.7 (0.0)	0.4 (0.0)	0.3 (0.0)
5-Cholesten-3α-ol	1.6 (1.4–1.8)	1.3 (1.2–1.4)	2	0.5 (0.0)	0.6 (0.0)	0.5 (0.3)
5-Cholesten-3β-ol	4.3 (2.8–6.6)	1.7 (0.6–2.8)	6	0.9 (0.1)	1.0 (0.1)	2.4 (2.4)
24α-Ethyl-5-cholesten-3β-ol	3.7 (2.3–5.7)		3	0.8	1.3	1.6
5α-Cholestan-3β-ol	0.4 (0.3–0.5)	0.2 (0.1–0.3)	2	0.2 (0.1)	0.1 (0.1)	0.1 (0.0)
5β-Cholestan-3α-ol	0.9 (0.8–0.9)		2	0.2	0.3	0.2
5β-Cholestan-3β-ol	0.8 (0.6–1.1)		2	0.3	0.2	0.2
4-Cholesten-3-one	6.9 (5.3–8.4)	4.9 (3.2–6.0)	3	3.2 (1.0)	0.9 (0.6)	0.4 (0.2)
5α-Cholestan-3-one	18.5 (17.0–20.0)		2	9.0	8.0	0.4
5β-Cholestan-3-one	17.0 (16.0–18.0)		2	5.2	10.6	0.7

* Incubations (37°C, 10 min) with 5 μg of a ^{14}C-labeled substrate were carried out in a 3 ml suspension of a rat ovary 8500 *g* sediment, isolated from 10 mg of wet ovary weight, fortified with 2.4 μmol of NADPH. Each incubation flask contained 0.4 mg of protein.

† Experiments with 4-^{14}C- and 26-^{14}C-labeled steroids were run in parallel. For synthesis, see Materials.

‡ For the fractionation procedure, see Table 1 footnote ‡. The recovery of radioactivity after the initial extraction and LC was for all substrates at least 90%. The recovery of unchanged substrate after TLC purification were quite similar to those reported in Table 1 footnote‡.

§ See Table 1.

Table 4. Yields of C_{27}- and C_{29}-steroid metabolites after incubations with rat testis 6500 *g* sediments and NADPH*

Substrate†	Yields of metabolites (% of added dose) after LC purification‡			Mean distribution of 4-^{14}C-labeled metabolites (% of added dose) after TLC purification§		
	4-^{14}C-steroid	26-^{14}C-steroid	*n*	Zone 1 (R_F 0.00–0.05)	Zone 2 (R_F 0.06–0.35)	Zone 3 (R_F 0.36–0.70)
4-Cholesten-3α-ol	0.4 (0.3–0.5)	0.4 (0.4)	2	0.2 (0.0)	0.2 (0.1)	0.0 (0.0)
4-Cholesten-3β-ol	0.2 (0.1–0.3)	0.2 (0.1–0.2)	2	0.1 (0.0)	0.0 (0.0)	0.1 (0.0)
5-Cholesten-3α-ol	0.3 (0.3)	0.3 (0.3)	2	0.2 (0.0)	0.1 (0.0)	0.0 (0.0)
5-Cholesten-3β-ol	1.2 (0.6–1.7)	0.4 (0.1–1.4)	8	0.2 (0.0)	0.4 (0.2)	0.6 (0.6)
24α-Ethyl-5-cholesten-3β-ol	0.9 (0.7–1.2)		4	0.1	0.3	0.3
5α-Cholestan-3β-ol	0.1 (0.0–0.1)	0.1 (0.0–0.1)	2	0.1 (0.0)	0.0 (0.0)	0.0 (0.0)
4-Cholesten-3-one	1.6 (0.4–1.8)	0.4 (0.2–0.7)	4	0.3 (0.1)	0.1 (0.1)	1.2 (1.2)
24α-Ethyl-4-cholesten-3-one	1.6		1	1.3	0.2	0.1
5α-Cholestan-3-one	14.2 (11.0–17.4)		2	11.6	1.9	0.0
5β-Cholestan-3-one	13.7 (12.0–15.4)		2	11.0	2.0	0.7

* Incubations (35°C, 60 min) with 5 μg of a ^{14}C-labeled substrate were carried out in a 3 ml suspension of a rat testis 6500 *g* sediment, isolated from 1.5 *g* of wet testis weight, fortified with 4.8 μmol of NADPH. Each incubation flask contained 15 mg of protein.

† Experiments with 4-^{14}C- and 26-^{14}C-labeled steroids, were run in parallel. For synthesis, see Materials.

‡ Liquid chromatography (LC), *system 2*: Lipidex-1000 (Packard instruments) in methanol–water–butanol–chloroform (70:30:7:3, by vol.). Column dimensions, 0.75 cm^2 × 13 cm. Flow rate. 0.5 ml/cm^2/min at 24°C. The metabolites were collected between 70 and 300% TCV. Non-metabolized substrate was collected after 300% TCV by elution with one column volume of acetone. Ranges in yields are given within parentheses. The recovery of radioactivity after initial extraction and LC was for all substrates at least 90%. The recovery of unchanged substrate after TLC purification varied much between substrates. With 3-hydroxysteroids the recoveries ranged between 77 and 81%; for 3-oxosteroids lower values were obtained i.e. 9–79%. The amount of radioactivity eluted before 70% TCV never exceeded 0.1% of the added substrate.

§ See Table 1.

dation and TLC-RD (see above) afforded labeled progesterone confirming the identification of the ovarian metabolite.

The other C_{27}-steroids tested with two isotopes gave slightly higher yields of 4-^{14}C- than of 26-^{14}C-labeled metabolites (see Table 3). The differences were less than those noted for experiments with adrenal mitochondria (see Table 1), and TLC-analysis of the metabolites failed to show any spot specifically 4-^{14}C-labeled. Also in the ovarian mitochondria both the [4-^{14}C]- and [26-^{14}C]-3-oxo-steroids yielded polar labeled metabolites. One difference from the results obtained with adrenal mitochondria was noted in that 4-cholesten-3-one was less extensively converted to polar metabolites than the saturated 3-oxo-C_{27}-steroids.

Metabolism of C_{27}- and C_{29}-steroids in rat testis mitochondria

The extent of metabolism for each substrate tested is summarized in Table 4. Except for 5α-cholestan-3-one and 5β-cholestan-3-one, low yields of 4-^{14}C-labeled metabolites were obtained from C_{27}- and C_{29}-steroids. When yields from incubations with 26-^{14}C-labeled steroids were subtracted from the respective 4-^{14}C-figure, net conversions into metabolites in zone 2 and 3 were recorded for cholesterol, and 4-cholesten-3-one.

TLC-RD (Fig. 2) revealed at least five distinct zones from incubations with 4-^{14}C-labeled cholesterol and β-sitosterol, which migrated like reference compounds. Metabolite **I** migrated as 17α-hydroxyproges-

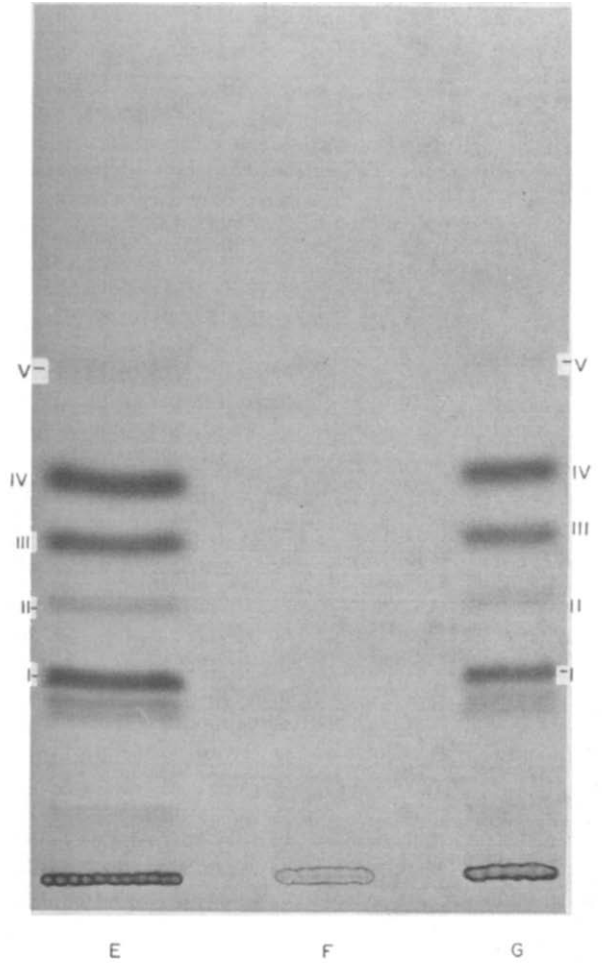

Fig. 2. Radioautography after TLC purification (system 1, see Table 1, footnote §) of metabolite fractions from liquid chromatography of extracts from incubations with a rat testis 6500 *g* sediment fraction, fortified with NADPH, and [4-^{14}C]-cholesterol (E), [26-^{14}C]-cholesterol (F), or β-[4-^{14}C]-sitosterol (G). Compounds **I–V** denote metabolites that contain 4-^{14}C, but are not labeled in incubations with the respective 26-^{14}C-steroid. Compounds **I–V** migrated as the following reference compounds: 17α-hydroxyprogesterone and testosterone (**I**, R_F = 0.26); dehydroepiandrosterone (**II**, R_F = 0.36); pregnenolone (**III**, R_F = 0.41); androstenedione (**IV**, R_F = 0.48); and progesterone (**V**, R_F = 0.60). Subsequent GLC combined with radioactivity detection verified the tentative identifications made for compounds **I** and **III–V**.

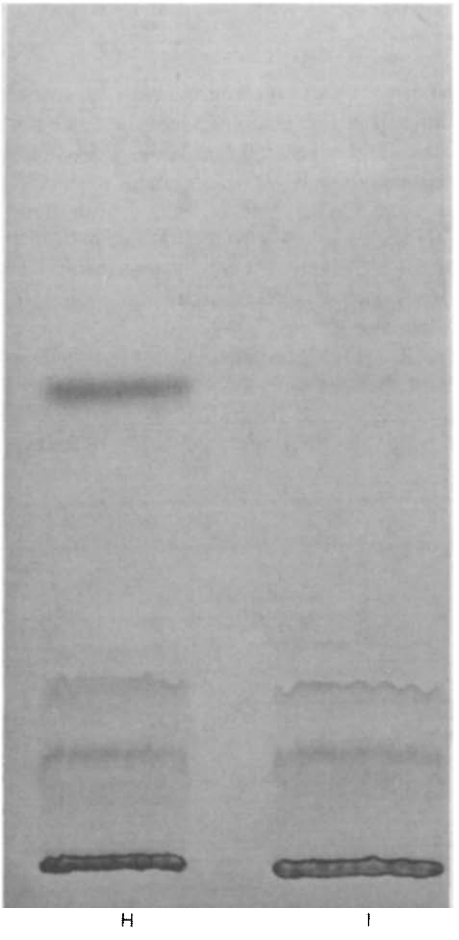

Fig. 3. Radioautography after TLC-purification (system 1, see Table 1, footnote §) of metabolite fractions from liquid chromatography of extracts from incubations with a rat testis 6500 *g* sediment fraction, fortified with NADPH, and 4-[4-^{14}C]-cholesten-3-one (H) and 4-[26-^{14}C]-cholesten-3-one (**I**). Compound **I** denotes a metabolite that contained 4-^{14}C, but was not seen in incubations with the 26-^{14}C-steroid. Compound **I** migrated as androstenedione (R_F = 0.48). Subsequent GLC combined with radioactivity detection verified this tentative identification.

terone and testosterone, metabolite **II** as dehydroepiandrosterone, metabolite **III** as pregnenolone, metabolite **IV** as androstenedione, and metabolite **V** as progesterone. GLC-RD verified the tentative identifications of compounds **I** and **III**–**V**. Thus, when metabolites **III**–**V** were injected (after preparation of TMS ethers [20]) peaks were recorded at t_R = 1.74 (metabolite **III**), t_R = 6.30 (metabolite **IV**), and t_R = 8.09 (metabolite **V**), which were the same as found for the authentic reference compounds. However, when authentic 17α-hydroxyprogesterone was analyzed by GLC-RD (after preparation of the TMS ether derivative according to the standard procedure) it was decomposed. Therefore trimethylsilylation of metabolite(s) **I** was carried out with a trimethylbromosilane containing reagent which affords the dienol TMS ether derivatives of 3-oxo-4-ene-steroids and 17-hydroxy-20-oxo-steroids [25]. Subsequent GLC-RD analysis of metabolite(s) **I** yielded radioactive peaks at the same t_R-values as authentic 17α-hydroxyprogesterone and testosterone i.e. at t_R = 1.57 (persilylated 17α-hydroxyprogesterone), t_R = 2.26 (17α-hydroxyprogesterone with the 20-oxo-group not reacted) and at t_R = 0.75 (persilylated testosterone).

In experiments with 4-cholesten-3-one (4-^{14}C- as well as 26-^{14}C-labeled, Fig. 3) a compound with the TLC- and GLC-mobility of androstenedione was obtained (metabolite **I**). Attempts to shown the presence on TLC of any other C_{21}- or C_{19}-steroid carrying 4-^{14}C from incubations with labeled 4-cholesten-3-one, failed.

No other C_{21}- or C_{29}-steroid could be identified from other C_{27}- or C_{29}-steroids tested.

Metabolism of C_{27}- and C_{29}-steroids in rat placenta mitochondria

Although conversion with most substrates tested was comparatively high (Table 5) the conversion to steroid-hormones was low as can be seen in Table 2. The formation of [4-^{14}C]-pregnenolone from incubations with 4-^{14}C-labeled cholesterol and β-sitosterol could be demonstrated by TLC-RD and GLC-RD (radioactive peak at t_R = 1.74). No compounds that had lost the 26-^{14}C-label were detected after incubations with the other substrates that were available both as 4-^{14}C- and 26-^{14}C-labeled steroids. The labeled metabolites with an R_F-value between 0.06 and 0.35 in TLC system 1 (zone 2, see Table 5) from all substrates were subjected to GLC-RD and a number of peaks with t_R-values higher than 2.00 (SP-2100 column) were recorded, indicating the presence of steroids with an intact C_{27}- or C_{29}-side-chain.

The conversion of 4-cholesten-3-one into progesterone in rat adrenal 8500 g sediments

In view of the finding that 4-cholesten-3-one could be transformed by testis mitochondria to androstenedione, the conditions in preparing and incubating mitochondria from testes, were adopted for adrenal mitochondria. The resulting conversions of cholesterol and β-sitosterol to C_{21}- and C_{19}-steroids were not much changed (see Fig. 1 and 4, and Table 2) compared to those obtained when the standard adrenal preparation was used. The differences between the yields of labeled metabolites from 4-^{14}C- and 26-^{14}C-labeled cholesterol (see Fig. 4) were: in zone 2 = 0.9% and in zone 3 = 4.7% of added dose. When 4-cholesten-3-one (4-^{14}C- or 26-^{14}C-labeled) was used as substrate, the conversion pattern changed markedly from that obtained under standard conditions. Thus 4-cholesten-3-one was found to afford two metabolites labeled in experiments with [4-^{14}C]- but not with [26-^{14}C]-4-cholesten-3-one. Metabolite **IV** (Fig. 4) was found to be labeled 17α-hydroxyprogesterone, according to TLC-RD (R_F = 0.26) and GLC-RD (t_R = 1.57 and 2.26; trimethylbromosilane catalyzed reaction, see above) and metabolite **V**

Table 5. Yields of C_{27}- and C_{29}-steroid metabolites after incubation with rat placenta 6500 *g* sediments and NADPH*

Substrate†	Yields of metabolites (% of added dose) after LC purification‡			Mean distribution of 4-^{14}C-labeled metabolites (% of added dose) after TLC purification§		
	4-^{14}C-steroid	26-^{14}C-steroid	*n*	Zone 1 (R_F 0.00–0.05)	Zone 2 (R_F 0.06–0.35)	Zone 3 (R_F 0.36–0.70)
4-Cholesten-3α-ol	4.9	5.2	1	1.9 (0.3)	2.1 (0.0)	0.7 (0.0)
4-Cholesten-3β-ol	6.7 (4.6–8.9)	5.3 (5.1–5.5)	2	1.8 (0.5)	4.1 (0.6)	0.5 (0.0)
5-Cholesten-3α-ol	12.1	13.4	1	5.5 (0.0)	6.0 (0.0)	0.3 (0.0)
5-Cholesten-3β-ol	5.7 (1.7–10.4)	5.1 (1.1–11.5)	3	1.6 (0.4)	3.2 (0.0)	0.4 (0.2)
24α-Ethyl-5-cholesten-3β-ol	0.8		1	0.2	0.1	0.2
5α-Cholestan-3β-ol	2.5 (1.6–3.4)	2.5 (2.4–2.6)	2	0.6 (0.0)	1.3 (0.0)	0.5 (0.0)
24α-Ethyl-5α-cholestan-3β-ol	0.9		1	0.2	0.5	0.2
5β-Cholestan-3α-ol	2.4		1	0.0	0.1	0.1
5β-Cholestan-3β-ol	2.1		1	0.3	0.8	1.0
4-Cholesten-3-one	7.6 (1.8–16.4)	8.4 (3.3–13.5)	3	3.6 (0.3)	2.2 (0.0)	1.0 (0.1)
24α-Ethyl-4-cholesten-3-one	13.3		1	7.1	3.9	0.7
5α-Cholestan-3-one	8.9		1	5.1	2.4	0.9
5β-Cholestan-3-one	5.5		1	0.3	0.3	2.7

* Incubations (37°C, 30 min) with 5 μg of a ^{14}C-labeled substrate were carried out in a 3 ml suspension of a rat term placenta 6500 *g* sediment, isolated from 300 mg of wet placenta weight, fortified with 4.8 μmol of NADPH. Each incubation flask contained 1.0 mg of protein.

† Experiments with 4-^{14}C- and 26-^{14}C-labeled steroids were run in parallel. For synthesis, see Materials.

‡ The recovery of radioactivity after the initial extraction and LC was for all substrates at least 85%. The recovery of unchanged substrate after TLC purification varied between substrates. With 3-hydroxysteroids the recoveries ranged between 67 and 85%; for 3-oxosteroids lower values were obtained i.e. 40–58%. Some losses of radioactivity were caused by the LC fractionation procedure i.e. between 0 and 40% TCV and between 150 and 200% TCV but did not differ much between the different substrates. The radioactivity remaining on the LC column amounted to 1–4% in all instances. Also see Table 1 footnote‡.

§ See Table 1.

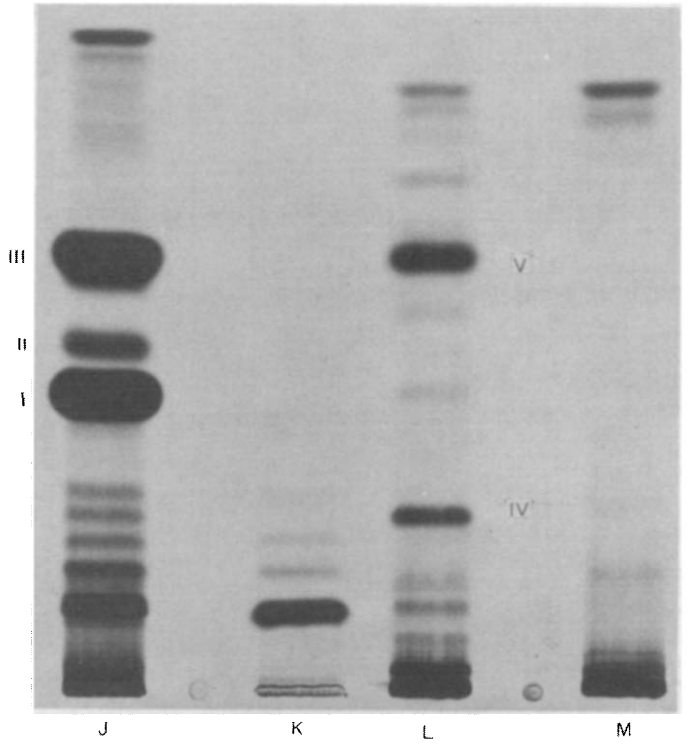

Fig. 4. Radioautography after TLC purification (system 1, see Table 1, footnote §) of metabolite fractions from liquid chromatography of extracts from incubations with a rat adrenal 8500 *g* sediment fraction (incubations under conditions employed in studies with testis mitochondria, see text) fortified with NADPH, and [4-^{14}C]-cholesterol (J), [26-^{14}C]-cholesterol (K), 4-[4-^{14}C]-cholesten-3-one (L), or 4-[26-^{14}C]-cholesten-3-one (M). Compounds **I**–**V** denote metabolites that contain 4-^{14}C, but are not labeled in incubations with the respective 26-^{14}C-steroid. Compounds **I**–**V** migrated as the following reference compounds pregnenolone (**I**, R_F = 0.41); androstenedione (**II**, R_F = 0.48); progesterone (**III** and **V**, R_F = 0.60); 17α-hydroxyprogesterone (**IV**, R_F = 0.26). Subsequent GLC combined with radioactivity detection verified the tentative identifications made for compounds **I**–**V**.

labeled progesterone (R_F = 0.60; t_R = 8.09). No evidence for the formation of testosterone was obtained. The differences between the yields of labeled metabolites in the three different TLC-zones (cf. Table 1) from experiments with 4-^{14}C- and 26-^{14}C-labeled 4-cholesten-3-one were: R_F = 0.00–0.05: 1.08%; R_F = 0.06–0.35: 2.2% and R_F = 0.36–0.70: 1.8%. The net yields of identified steroid metabolites are given in Table 2.

Metabolism of C_{27}- and C_{29}-steroids in mitochondria prepared from human adrenals, ovaries, testes and placenta

The conversion into polar products seen after LC purification was comparatively low for all substrates tested (see Table 2, footnote ‖). For instance [4-^{14}C]-cholesterol was converted to 2.4%, 0.3%, 1.4% and 1.8% of the added dose into metabolites, in mitochondria from adrenals, ovaries, testes and placenta, respectively. The net transformations, after LC and TLC purification into identified steroids are listed in Table 2. TLC-analysis of an extract from a placenta incubation with 4-^{14}C- and 26-^{14}C-labeled cholesterol can be seen in Fig. 5. No other steroid hormones but 4-^{14}C-labeled pregnenolone and progesterone could be shown to be formed in this incubation.

DISCUSSION

When common C_{21}- and C_{19}-steroids are formed from a labeled C_{27}-steroid precursor other than cholesterol, the products mix with the endogenous cholesterol metabolites, and GLC-MS analysis of the compound eluted at the t_R-value where a labeled compound was observed, naturally affords a mass spectrum of the endogenous compound. Mass spectrometry does not provide definite evidence that the exogenous C_{27}-steroid gave rise to the endogenous metabolite nor does it contradict it. When a common cholesterol metabolite is not formed from an exogenous C_{27}-steroid, the low conversion rate makes an identification of the atypical steroid metabolite by GLC-MS very difficult. It was therefore realized that when low conversions were to be expected another approach had to be tried. Hence, to be certain that a labeled metabolite did not carry the C_{27}-steroid side-chain, the amount of ^{14}C in a labeled metabolite

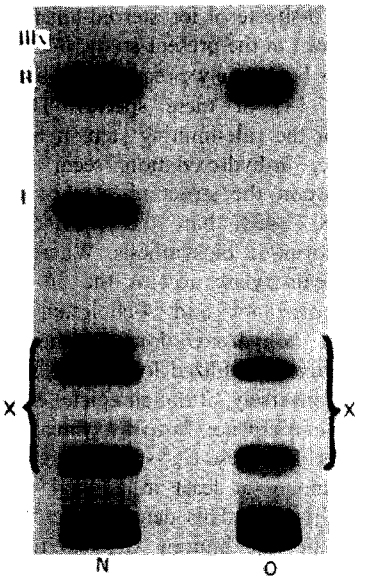

Fig. 5. Radioautography after TLC purification (system 1, see Table 1, footnote §) of metabolite fractions from liquid chromatography of extracts from incubations with a human placenta 6500 *g* sediment fraction, fortified with NADPH, and [4-^{14}C]-cholesterol (N) or [26-^{14}C]-cholesterol (O). Compounds **I** and **III** were metabolites that contained 4-^{14}C, but were not seen in incubations with 26-^{14}C cholesterol. Compounds **I–III** migrated as the following reference compounds: pregnenolone (**I**, $R_F = 0.41$) cholesterol (**II**, $R_F = 0.55$); and progesterone (**III**, $R_F = 0.60$). Subsequent GLC combined with radioactivity detection verified the tentative identifications made. 4-^{14}C- and 26-^{14}C-Labeled metabolites X, denote auto-oxidation products (7α-hydroxy-, 7β-hydroxy-, 7-oxo and 5,6-oxido-derivatives) of cholesterol.

spot upon TLC-RD analysis was compared in parallel experiments with 4-^{14}C- and 26-^{14}C-labeled steroid substrates. Additional evidence that the side-chain had been split off was obtained by reversed phase chromatography on Lipidex-1000, which retains steroids with a C_{27}-side-chain to a remarkable degree even if the compound has been polyhydroxylated in the steroid nucleus (Aringer L. and Nordström L., unpublished results). In addition the retention times upon GLC-RD analysis clearly could distinguish between a mono- or dioxygenated C_{27}-steroid and a di- or trioxygenated C_{21}- or C_{19}-steroid.

Using the above mentioned analytical approach it is clear that of the substrates tested only cholesterol, epicholesterol, 4-cholesten-3-one, and β-sitosterol appeared to be converted to identifiable steroid-hormones by endocrine tissues. In mitochondria from rat organs all substrates tested were metabolized to a comparably high extent by these tissues to a number of unidentified compounds with an intact steroid side-chain. This was particularly striking with the 3-oxo-C_{27}-steroids. It may be that 20,22-epoxy metabolites [26] or side-chain hydroperoxides [13, 27, 28] were formed.

In the experiments with rat adrenal mitochondria but not with e.g. ovaries the metabolite yields from the 4-^{14}C-labeled substrates were consistently larger than those obtained from the corresponding 26-^{14}C-labeled substrates. The specifically 4-^{14}C-labeled metabolites from 4-cholesten-3α-ol and 4-cholesten-3β-ol appeared in the LC and the TLC fractions containing very polar steroids, but the radioactivity could not be recovered in distinct spots on TLC. The possibility exists that these results mean that the substrates mentioned do yield C_{21}- and C_{19}-steroids in a sequence which bypasses at least dioxygenated C_{21}- and C_{19}-intermediates. However, the loss of carbon atom 26 does not prove that the side-chain is split off between C-20 and C-22. 26-Nor and for instance 25,26-bisnor compounds may arise. Our results do not permit a distinction between the different possibilities.

Availability of cholesterol to the mitochondrial cholesterol side-chain cleaving enzyme(s) is a major factor in steroidgenesis, as stressed most recently by Mason *et al.*[29]. Since a cholesterol carrier protein seems to be involved in the steroid-hormone production in adrenals at least [30], this carrier may determine cholesterol availability. Our results demonstrating that epicholesterol can be converted to epipregnenolone in rat adrenal and ovarian mitochondria may indicate that when the carrier has been washed out in the preparation of mitochondria, part of the substrate specificity of the steroid side-chain cleaving system is removed. A similar explanation can be offered for the adrenal conversion of 4-cholesten-3-one to progesterone. This metabolism only took place when adrenal mitochondria were prepared and incubated in the same fashion as rat testis mitochondria, emphasizing the importance of the composition of the buffer used in the *in vitro* studies. The conversion of 4-cholesten-3-one to progesterone in rat adrenals in very low yields has previously been reported by Lommer *et al.*[31] who used quartered rat adrenals incubated for 3 h. Similar results were obtained by Raggatt and Whitehouse[6] who used bovine adrenal cortex preparations and Kobayashi and Ichii[7] who worked with hog adrenocortical mitochondria. A pathway for adrenal C_{21}-steroid biosynthesis from non-cholesterol precursors has recently been suggested by Diedrichsen *et al.*[32]. It involves a 21-hydroxylation of desmosterol (see also ref. [3, 33]) followed by side-chain cleavage to yield 21-hydroxypregnenolone. Burstein *et al.*[12] have previously demonstrated the conversion of 20(S)-20,21-dihydroxycholesterol to 21-hydroxypregnenolone by bovine adrenocortical preparations. The possibility exists that the noncholesterol steroid-hormone precursors studied in our investigation at least in part were metabolized through a 21-hydroxy pathway but we have no data to support this.

Incubation of rat testis mitochondria with cholesterol afforded C_{21}-steroids (i.e. pregnenolone, progesterone and 17α-hydroxyprogesterone) as well as testosterone and androstenedione. When the testis mitochondria were incubated with 4-cholesten-3-one

only androstenedione was obtained and no C_{21}-steroids could be detected. These findings add evidence to the controversy as to whether or not there exists a direct pathway from C_{27}- to C_{19}-steroids in endocrine tissues. Thus Jungman[34, 35] presented data to show that calf testes and rat adrenals ovaries and testes *in vitro* were capable of removing the entire C_{27}-steroid side-chain from cholesterol in a direct pathway to C_{19}- steroids. The results could not be confirmed by the experiments conducted by Burstein *et al.*[36] or Hochberg *et al.*[37]. Our finding may indicate that such a direct C_{27}- to C_{19}-steroid pathway may exist in rat testes. Our data are not in disagreement with those of Burstein *et al.* or Hochberg *et al.* and do not confirm the findings of Jungman since our substrate was 4-cholesten-3-one not cholesterol. A possible pathway for a C_{27}- to C_{19}-steroid conversion has been indicated by Shimitzu *et al.*[38, 39], who demonstrated that 5-cholestene-3β,17α,20α-triol is a steroid-hormone precursor in human adrenals *in vitro* and by Burstein *et al.*[40] in guinea pig adrenal preparations. The intermediary formation of 17α,20α-dihydroxy-4-cholesten-3-one [4] might explain the conversion of 4-cholesten-3-one to androstenedione in rat testis mitochondria in the present investigation. In this connection it should be mentioned that we have investigated whether or not cholesterol is converted to 4-cholesten-3-one in the mitochondria prepared from rat endocrine tissue, but this conversion could not be demonstrated. However, the possibility remains that small amounts of 4-cholesten-3-one or 5-cholesten-3-one [41] formed from cholesterol is directly used for steroid-hormone biosynthesis.

From the results of incubations with 8500 *g* sediment of endocrine tissues in man, it is concluded that apart from cholesterol, β-sitosterol could be used as a substrate in the formation of C_{21}-steroids. Although the results indicate that the endocrine tissues from man do not discriminate between sterols with a side-chain different from that of cholesterol, it must be remembered that the tissues, taken for PAD after surgery, were part of organs considered not to be normal. However, term placentae from normal rats and women afforded similar results *in vitro* with β-sitosterol as a steroid-hormone precursor, indicating perhaps that the results obtained *in vitro* in rat endocrine organs may be valid also for human endocrine tissue.

The results of the present investigation confirm that the steroid substrate specificity in steroid-hormone biosynthesis is not dependent upon the side-chain structure of the precursor sterol. Thus, as indicated by the results of Werbin *et al.*[1] in the guinea pig, Subbiah and Kuksis[14], with rat testes, Burstein and Gut[2] with acetone powders of bovine adrenal mitochondria, and indirect results obtained by Miettinen[42] with rat adrenals, β-sitosterol may be converted to steroid-hormones although at a slower conversion rate than that of cholesterol. Whereas the endocrine systems in the rat and man may utilize the plant sterol β-sitosterol for steroid-hormone biosynthesis as shown in the present study, β-sitosterol does not seem to be a likely precursor in bile acid biosynthesis [17, 18] in these species. The substrate specificity of the rate-limiting step in bile acid biosynthesis i.e. 7α-hydroxylation, seem to be more dependent upon the structure of the steroid side-chain [16, 18, 23, 43] than the enzyme(s) involved in steroid-hormone biosynthesis. Whereas the C_{27}-steroid 7α-hydroxylase accepts the 3β-hydroxylated steroids—cholestanol and 4-cholesten-3β-ol—as a substrate in 7α-hydroxylation [16] these substrates are apparently not utilized for steroid-hormone biosynthesis. Conversely, although epicholesterol and 4-cholesten-3-one are not 7α-hydroxylated by rat liver preparations [16], these C_{27}-steroids are converted to steroid hormones at least in adrenal, ovarian and testis mitochondria. This demonstrates the different natures of the rate limiting steps in the two most important pathways in animal metabolism of cholesterol.

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