Metabolism of 27-, 25- and 24-hydroxycholesterol in rat glial cells
and neurons

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The metabolism of 27-, 25- and 24-hydroxycholesterol in cultures of rat astrocytes, Schwann cells and neurons was studied. 27- and 25-Hydroxycholesterol, but not 24-hydroxycholesterol, underwent 7α-hydroxylation with subsequent oxidation to 7α-hydroxy-3-oxo-∆5 steroids in all three cell types. When cells were incubated for 24 h with 0.28 nmol of 27-hydroxycholesterol in 10 ml of medium, the rates of conversion into 7α-hydroxy metabolites were 0.21, 0.12 and 0.02 nmol/24 h per 10⁶ cells in the media of astrocytes, Schwann cells and neurons respectively. The corresponding values for 25-hydroxycholesterol were 0.26, 0.16 and 0.04. A minor fraction of 27-hydroxycholesterol and its 7α-hydroxylated metabolites was oxidized to 3β-hydroxy-5-cholesten-3-one, 25-hydroxycholesterol and 7α-hydroxy-3-oxo-4-cholesten-3-one. In addition to the two hydroxycholesterols, other 3β-hydroxy-∆5 steroids, dehydroepiandrosterone, pregnenolone, 3β-hydroxy-5-cholesten-3-one and 3β-hydroxy-5-cholesten-3-one were detected which are also described.

α-β-Hydroxylated metabolites was oxidized to 3β-hydroxy-5-pregnen-20-one and dehydroepiandrosterone [3β-hydroxy-5-androsten-17-one (DHEA)] are also 7α-hydroxylated by microsomes from the nervous system [21] and other cell types [22–24], the substrate specificity of 7α-hydroxylation with regard to the side-chain structure was investigated. In the course of these studies other metabolic reactions were detected which are also described.

EXPERIMENTAL

Chemicals

All solvents were of analytical grade and were redistilled. Hydroxypropyl-β-cyclodextrin (HPBCD) was from Aldrich and octadecysilane (ODS)-bonded silica (preparative C18) from Waters Associates (Milford, MA, U.S.A.). Triethylamino-hydroxypropyl Sephadex LH-20 (TEAP-LH-20) was synthesized as described [25]. Other chemicals, reagents and column-packing materials were those used previously in our laboratory [6,26].

Reference compounds

25-[26,27-3H]Hydroxycholesterol (3186 GBq/mmol) was from NEN Research Products (Dreieich, Germany) and [1,2,6,7-3H]DHEA (3182 GBq/mmol) and [7-3H(n)]pregnenolone (925 GBq/mmol) were from NEN (les Ulis, France). 25-Hydroxycholesterol and 3β-hydroxy-5-cholesten-3-one were from Steraloids (Wilton, NH, U.S.A.), and pregnenolone and 5α-androstan-3β,17β-diol were from Sigma. DHEA, testosterone and oestradiol were gifts from Roussel-Uclaf (Romainville, France). 27-Hydroxycholesterol was prepared from kryptogen, kindly
supplied by Dr. L. Tókes (Syntex Research, Palo Alto, CA, U.S.A.) [26]. 25- and 27-Hydroxycholesterol were used as starting materials for synthesis of 25-[α-3H]hydroxycholesterol (259 GBq/mmol), 27-[α-3H]hydroxycholesterol (259 GBq/mmol), 7α,25-dihydroxycholesterol and 7α,25-dihydroxy-4-cholesten-3-one [13], and of 7α,27-dihydroxycholesterol, 7α,27-dihydroxy-4-cholesten-3-one, 3β,7α-dihydroxy-5-cholestenolic acid and 7α-hydroxy-3-oxo-4-cholestenolic acid [26]. 24-Hydroxycholesterol was prepared from 24-oxocholesterol (Searle, Chicago, IL, U.S.A.) by reduction with NaBH₄ in ethanol. All compounds were purified by HPLC (see below) shortly before use.

Cell culture preparation and incubation conditions

Astrocytes and neurons

Cerebral hemispheres were removed from 17-18 day Sprague-Dawley foetuses, as previously described [27,28]. Briefly, cerebral hemispheres were dissected free from meninges and mechanically dissociated. Cells were seeded in 100 mm Petri dishes pretreated with 3 μg/ml poly(t-ornithine) (Sigma) in a final volume of 10 ml of serum-free medium [consisting of minimal essential medium (MEM)/Ham’s F12 medium (1:1, v/v)] with 2 mM glutamine, 30 mM glucose, 3 mM NaHCO₃, 5 mM Hepes, 5 units/ml penicillin, 5 μg/ml streptomycin [32] supplemented with 10% hormone mixture (25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 20 nM progesterone and 30 nM NaSeO₄) for neuronal cultures. Astrocytes were grown for 3 weeks (medium change every 3 days) until they had formed a confluent monolayer, devoid of neuronal cells, fibroblasts and oligodendrocytes. Microglial cells represented less than 5% of the cells. Neurons were cultured for 6 days, without a change of medium. In these conditions, the neuronal culture was devoid of detectable glial elements.

Schwann cells

Pure cultures were established from 5-day-old Sprague-Dawley rat sciatic nerves, as previously described [29,30]. Briefly, cells were plated on to 25 cm² tissue culture flasks, coated with 20 μg/ml poly(t-lysine) (Sigma) and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated calf serum (DMEM-F), in the presence of 1 μM insulin and 5 μM forskolin. In order to eliminate contaminating fibroblasts, they were successively treated with cytosine arabinoside (10 μM) and anti-Thy-1.1 antibody and related after 1 week on 75 cm² poly(t-lysine)-coated flasks. Finally, after 3 weeks of culture, cells were treated with trypsin, seeded on poly(t-lysine)-coated Petri dishes and grown for 1 more week in 10 ml of DMEM-F.

After 6 days (neurons), 21 days (astrocytes) or 28 days (Schwann cells) of culture, cells were incubated for 24 h with 0.28 or 0.022 nmol of tritiated or without 25 nmol of unlabelled hydroxycholesterol, in 10 ml of serum-free MEM or DMEM. The solutions of hydroxycholesterol in 45% HPO occurred. The HPBCL were made as described previously [13]. Incubations were performed in duplicate. Control experiments were carried out under the same conditions with cells previously fixed in acetic acid/methanol (1:4, v/v) for 10 min (dead cells). At the end of the incubation time, media and cells were collected separately for steroid analysis. The number of cells per dish was counted (astrocytes 0.9 × 10⁶/dish; Schwann cells 1.6 × 10⁶/dish; neurons 3.5 × 10⁶/dish) and DNA was measured (11, 27 and 6 μg and of DNA respectively per dish) by the mithramycin method [31].

Isolation of microsomes and incubation conditions

Sprague-Dawley male rats (10 weeks old) of the OFA strain were purchased from Iffa-Credo (L’Arbesle, France). They were killed by decapitation, and forebrains were removed quickly, weighed and homogenized in ice-cold 0.01 M sodium phosphate buffer, pH 7.4, containing 0.8% NaCl. Microsomes were isolated as previously described [21], resuspended in 0.32 M sucrose and stored in liquid N₂ until use. Proteins were quantified as described by Bradford [32].

Tritium-labelled substrates in methanol (1 μCi, 0.14-2 nmol) were deposited into 10 ml glass tubes, dried under vacuum (Speed-Vac concentrator, Savant instrument Corp., H Hicksville, NJ, U.S.A.), and redisolved in 25 μl of acetone. For inhibition studies, the competitors (10 nmol) dissolved in methanol were added to the substrate molecules. Incubations were carried out in a total volume of 2 ml. The buffer (0.067 M NaHPO₄/KH₂PO₄, pH 7.4, containing 1 mM EDTA) was first added, the tubes were vortex-mixed and warmed at 37 °C for 5 min. NADPH was then added at final concentration of 0.5 mM. Incubations were started by the addition of microsomal suspension (0.5 μg of protein). They were carried out at 37 °C for 30 min in a shaking water bath (70 rev/min) and stopped by the addition of 3 vol of methanol. Control incubations were performed under the same conditions but the microsomes were boiled for 10 min.

Analysis of steroid metabolites

The medium (10 ml) from the incubations was separated from the cells and diluted with 10 ml of aq. 0.5 M triethylamine sulphate. The solution was passed through a bed of ODS-bonded silica (2 cm × 0.8 cm) in water at 64 °C followed by a wash with 5 ml of water [33]. A 1% aliquot was taken for counting of radioactivity in a liquid-scintillation counter (1211 Minibeta, Wallac, Sollentuna, Sweden) using Optiphase ‘HiSafe’ 2 (Wallac) as the scintillation liquid. Another aliquot was evaporated and the residue dissolved in methanol and counted for radioactivity.

Steroids were eluted from the ODS-bonded silica with 10 ml of methanol. The eluate was passed through a column (6 cm × 0.4 cm) of TEAP-LH-20 in HCO₃⁻ form packed in aq. 95% methanol, followed by a rinse with 5 ml of methanol (neutral fraction) [34]. Steroid carboxylic acids were then eluted with 4 ml of 0.15 M acetic acid in aq. 95% methanol. In some experiments a fraction of sulphated and/or glucuronidated steroids was also collected [34]. The carboxylic acid fraction was taken to dryness and methylated with freshly prepared diazomethane. The neutral fraction was evaporated and the residue was dissolved in 85% methanol. Aliquots were analysed by HPLC performed with an LKB 2150 pump (Pharmacia Biotech, Sollentuna, Sweden), a model 201 fraction collector (Gilion, Villiers Le Bel, France), a μ-Bondapak C₅ steel column (300 mm × 3.9 mm; particle size 10 μm; Waters) and a variable UV detector (LDC/Milton Roy, Riviera Beach, FL, U.S.A.). The mobile phase was 80% methanol. The effluent was collected in 1 ml fractions, and 100 μl of each fraction was taken for counting of radioactivity. The fractions were then taken to dryness. After conversion into trimethylsilyl (TMS) ethers, the compounds in each fraction were analysed by GLC and GLC-MS (GC/MS). The methylated acids from TEAP-LH-20 were also analysed by GLC and GC/MS after TMS derivatization [34]. GC/MS was performed using a VG 7070E double-focusing instrument (Micromass, Manchester, U.K.). The conditions were those described previously [34].

The cells were suspended in aq. 40% ethanol and centrifuged. The supernatant was passed through a bed of ODS-bonded silica. After a wash with 5 ml of water, the compounds adsorbed
were eluted with 5 ml of methanol and 5 ml of methanol/chloroform (1:1, v/v). The cells were extracted with 5 ml of propan-2-ol/hexane (2:3, v/v) in an ultrasonic bath for 15 min. After centrifugation, this extraction was repeated; the two extracts were combined with the eluate from the ODS-bonded silica and solvents were evaporated in vacuo. The residue was dissolved in 2 ml of hexane/dichloromethane (1:4, v/v) and purified by chromatography on a column (3 cm × 0.4 cm) of Unisil packed in hexane and washed with 5 ml of hexane/dichloromethane (1:4, v/v) before use. After application of the sample, the column was washed with 10 ml of the same solvent (ester fraction) and free steroids were then eluted with 10 ml of ethyl acetate. The ethyl acetate was evaporated and the residue was dissolved in 85% methanol and analysed by HPLC, GLC and GC/MS as described above.

The methanolic incubation mixtures of microsomes were centrifuged and diluted with water to give a 50% methanol solution. This was passed through a bed of ODS-bonded silica in water. The effluent was diluted with water to 30% methanol and passed through the same bed. This effluent was again diluted with water to 10% methanol and passed through the same bed followed by a wash with 5 ml of water. Steroids were eluted with 10 ml of methanol, separated on TEAP-LH-20 and analysed by HPLC, GLC and GC/MS as described above.

The molecular ion was at $m/z$ 619, 529, 439, and 349 resulting from loss of a side-chain volatile. The steroids were separated by ion-exchange chromatography into a neutral and an acidic fraction. Most of the trimetil was found in the neutral fraction, and 3-4% in the acidic fraction (Table 1). Three acids were identified by GC/MS of the acidic fraction from the incubation in the presence of unlabelled 27-hydroxycholesterol: 3β-hydroxy-5,7,25-trihydroxycholesterol (A2) and labelled 25-,27-dihydroxycholesterol (A3). The medium was extracted with ODS-bonded silica. Tritium was released in the water-soluble form (47.1%), in the absence of unlabelled sterols, and 9.4% in their presence. About 35-40% of this material was volatile. The steroids were separated by ion-exchange chromatography into a neutral and an acidic fraction. Most of the trimetil was found in the neutral fraction, and 3-4% in the acidic fraction (Table 1).

Two additional metabolites were found in fractions 7 and 13. Fraction 13 contained 2.6, 2.4 and 7.9% of the tritium in the respective incubations (A1–A3). Analysis of the incubation containing unlabelled 27-hydroxycholesterol (A2) showed a metabolite TMS ether with a GLC retention index (RI) of 3664. The mass spectrum (Figure 1a) showed a molecular ion at $m/z$ 634. An intense peak at $m/z$ 531 and a series of fragment ions at $m/z$ 441 and 351 resulting from loss of a side-chain fragment of mass 103 and losses of one and two trimethylsilanol suggested the presence of two hydroxy groups in the side chain. An ABCD-ring ion at $m/z$ 255 showed that no group was added on the sterol nucleus, and an intense ion at $m/z$ 219 indicated a 25,27-dimethylsiloxyl structure [35]. This metabolite is therefore identified as 25,27-dihydroxycholesterol (9).

Fraction 7 contained 9.7, 1.6 and 2.8% of the total radioactivity in the respective incubations (A1–A3). The TMS ether had an RI of 3633 and the mass spectrum (Figure 1b) was in several respects analogous to that of 25,27-dihydroxycholesterol TMS ether above. The RI was identical with that of the TMS ether of a bile alcohol identified as 5-cholene-3β,7α,25,27-tetrol in patients with 3β-hydroxysteroid dehydrogenase deficiency [34], and the mass spectra were essentially the same. The molecular ion was at $m/z$ 722, and the peak at $m/z$ 632 ($M^-90$) was intense. There were a series of fragment ions at $m/z$ 619, 529, 439, and 349 resulting from loss of a side-chain fragment of mass 103 and successive losses of one to three trimethylsilanols. The peak at $m/z$ 219 representing a side-chain fragment ion composed of carbons 25–27 was clearly seen. Thus the mass spectrum and the RI identify the metabolite as 7α,25,27-trihydroxycholesterol (10). A search was made for the possible 25-hydroxylation of 25-hydroxycholesterol and for the 7α,25-dihydroxylated metabolite. However, the two metabolites with a 25,27-dihydroxy structure appearing in fractions 7 and 13 were only found after incubation with 27-hydroxycholesterol but not after incubation of 25-hydroxycholesterol, indicating a specific 25-hydroxylation. It may be noted that unlabelled 27-hydroxycholesterol decreased the $^3$H incorporation into total 25-hydroxylated products whereas unlabelled 25-hydroxycholesterol only decreased the incorporation into the 7α,25-dihydroxylated product (Table 1, A2 and A3). This indicates that 25-hydroxycholesterol does not inhibit the 25-hydroxylation of 27-hydroxycholesterol whereas it competes for the 7α-hydroxylation. The subsequent metabolism of 7α,25,27-trihydroxycholesterol was not studied. A possible formation of 7α,25,27-trihydroxy-4-cholesten-3-one (11) would result in loss of $^3$H to the water phase and this potential metabolite will be included in the value for 7α,25,27-dihydroxy-4-cholesten-3-one (8, Table 1).

The metabolism of 25-hydroxycholesterol (2) in cultures of astrocytes was studied using sterol radiolabelled in either the A-ring or the side chain. This permitted a validation by HPLC of the oxidation to 3-oxo-Δ4 metabolites calculated from the release of $^3$H from the 3α-Δ4-labelled sterol. The incubations of 25-[3α- $^3$H]hydroxycholesterol (0.28 nmol/10 ml of medium) were made in the absence (A4) or presence (A5) of unlabelled 25-hydroxycholesterol (25 nmol) and, in the presence of unlabelled 27-hydroxycholesterol (25 nmol; A6; Table 1). The release of $^3$H in water-soluble form was 45.2% in incubations in the absence of unlabelled hydroxycholesterol (A4), 9.3% in the presence of unlabelled 25-hydroxycholesterol (A5) and 5.8% in the presence of 27-hydroxycholesterol (A6). The acidic fraction from TEAP-LH-20 did not contain significant amounts of tritium. HPLC of the neutral fractions showed unchanged tritiated substrate (2) in fractions 24 and 25, accounting for about 13, 64 and 69% of the total tritium in the three incubations (A4–A6). Fraction 9 contained a metabolite that had lost $^3$H and had a UV
absorption maximum at 239 nm. GC/MS of the TMS ether showed it to be 7α,25-dihydroxy-4-cholesten-3-one (6). Fractions 10 and 11 contained 38.6, 26.5 and 22.9 % of the total tritium in the respective incubations (A4–A6). GC/MS analysis showed this to represent 7α,25-dihydroxycholesterol (5). In the incubation containing unlabelled 27-hydroxycholesterol (A6), 7α,27-dihydroxy-4-cholesterol-3-one (8) was formed and found in fraction 10. 7α,27-Dihydroxycholesterol (2) was identified in fractions 11 and 12.

Fractions 7 and 13 from the incubations with radio labelled 27-hydroxycholesterol did not contain significant radioactivity in contrast with the incubation of radio labelled 27-hydroxycholesterol. Thus 27-hydroxylation of 25-hydroxycholesterol could not be detected.

The metabolism of side-chain-labelled 25-[26,27-3H]hydroxycholesterol (0.022 nmol/10 ml of medium) gave an HPLC profile of 3H different from that obtained after incubation of ring labelled 25-[3α-3H]hydroxycholesterol (A7, A8 compared with A4, A5). This is because one of the metabolites, 7α,25-dihydroxy-4-cholesten-3-one, did not lose tritium. This compound and 7α,25-dihydroxycholesterol appeared in HPLC fractions 9 and 10 + 11, respectively. The formation of labelled 7α,25-dihydroxy-4-cholesten-3-one (6) and 7α,25-dihydroxycholesterol (5) represented 74.0 and 6.6 %, respectively. When unlabelled 25-hydroxycholesterol was present (A8), the corresponding values were 18.2 and 13.5 %.

In both the absence and presence of unlabelled 25-hydroxycholesterol, radioactivity was released in water-soluble form (9.8 and 8.6 % respectively; A7 and A8). No metabolite resulting from side-chain cleavage or hydroxylation of 25-hydroxycholesterol at C-26 or C-27 was found. Pregnenolone, 7α-hydroxypregnenolone, 5-pregnene-3,20-diol and progesterone were less than 1 pmol.

After incubations with 3H-labelled 25- and 27-hydroxycholesterol, the extracts of cells contained between 5 and 7 % of the total amount of 3H added. HPLC analyses indicated that about 22–34 % of this material consisted of 7α-hydroxylated metabolites in the incubations without unlabelled carrier, and 7–14 % in the incubations with added unlabelled compounds. The steroid ester fraction contained negligible amounts of tritium (less than 1.5 % of the total 3H in the cell extracts).

Control incubations were carried out with dead cells. None of the above metabolites of 27- and 25-hydroxycholesterols were found in these incubations. Furthermore possible autoxidation
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Figure 1  Mass spectra of the TMS derivatives of the 25-hydroxylated metabolites in rat brain astrocytes

(a) 25,27-Dihydroxycholesterol; (b) 7α,25,27-trihydroxycholesterol; (c) 24,25-dihydroxycholesterol.

products, such as 7β,27- and 7β,25-dihydroxycholesterols and their 7-oxo derivatives, were not found in any incubation mixture.

The same protocol as for astrocytes was used to study the metabolism in Schwann cells. Each dish contained about 1.6 × 10⁶ cells. The results are shown in Table 1 (S1–S8). The incubation with 27-hydroxycholesterol (3; S1–S3) yielded 7α,27-dihydroxycholesterol (7), 7α,27-dihydroxy-4-cholesten-3-one (8), 3β-hydroxy-5-cholestenoic acid (12) and the two 7α-hydroxylated acids (13, 14) which were all identified as described above. The incubation of 25-hydroxycholesterol (2; S4–S8) yielded 7α,25-dihydroxycholesterol (5) and 7α,25-dihydroxy-4-cholesten-3-one (6). The capacity for 7α-hydroxylation was similar in incubations with Schwann cells and astrocytes whereas 25-hydroxylation was much lower in Schwann cells. In the incubation of tracer 27-[3α-3H]hydroxycholesterol (3; S1), only 1.2% of added ³H was found in fraction 7 (7α,25,27-trihydroxycholesterol, 10), and almost no ³H was found in fraction 13 (25,27-dihydroxycholesterol, 9). As was the case in the astrocyte incubations, 13 and 12.2% of the radioactivity from 25-[6β,27-³H]hydroxycholesterol was released in water-soluble form in the absence (S7) and presence (S8) of 25-hydroxycholesterol respectively. No corresponding unlabelled metabolites could be found.

The extracts of cells contained between 3 and 6% of the total amount of ³H added. HPLC analyses indicated that about 32–37% of this material consisted of 7α-hydroxylated metabolites in the incubations without unlabelled carrier, and 6–11% in the incubations with added unlabelled compounds. The steroid ester fraction contained negligible amounts of tritium (less than 1.5% of the total ³H in the cell extracts).

Control incubations with dead cells were carried out and none of the above metabolites was found.

Neurons (about 3.5 × 10⁶ cells per dish) also expressed 7α-hydroxylation towards 25- and 27-hydroxycholesterol but the conversion rates were lower than with astrocytes and Schwann cells (Table 1, N1–N8). Radioactive metabolites were observed in the HPLC analyses of the incubations with tracer amounts but their formation was markedly depressed by the addition of unlabelled 25- and 27-hydroxycholesterol (Table 1, N1, N4, N7 compared with N2, N3, N5, N6, N8). The 7α-hydroxylated metabolites of 27- and 25-hydroxycholesterol described above
were identified by GC/MS. 25-hydroxylation of 27-hydroxycholesterol and endogenous cholesterol (see below) was not detected. Only 3β-hydroxy-5-cholestenolic acid (12) was identified by GC/MS of the acidic fraction from the incubation with unlabelled 27-hydroxycholesterol.

The extracts of cells contained between 8 and 13% of the total amount of 4H added. HPLC analyses indicated that about 13–20% of this material consisted of 7α-hydroxylated metabolites in the incubations without unlabelled carrier, and 4–5% in the incubations with added unlabelled compounds. The steroid ester fraction contained negligible amounts of tritium (less than 1.5% of the total 4H in the cell extracts).

None of the above metabolites was found in control incubations with dead cells.

Metabolism of 24-hydroxycholesterol in astrocytes, Schwann cells and neurons

Radioactively labelled 24-hydroxycholesterol was not available and the cultures of astrocytes, Schwann cells and neurons were incubated in 10 ml of medium to which 25 nmol of (24R,S)-24-

hydroxycholesterol (4) was added. The medium was extracted with ODS-bonded silica, potential metabolites were separated by HPLC and all fractions were analysed by GC/MS. Only one metabolite was found, the TMS ether of which had a RI of 3515. The mass spectrum (Figure 1c) showed an intense ion at m/z 131 and other ions had a very low intensity. This is a typical spectrum of the TMS ether of a C27 sterol with a 24,25-dihydroxy structure in which fragmentation between carbons 24 and 25 is pronounced [35]. The magnified spectrum showed a molecular ion at m/z 634 and a fragment ion at m/z 413 resulting from losses of 131 and 90 mass units. These results (and the polarity on HPLC) suggest that the metabolite is a 24,25-dihydroxycholesterol (45). As in the case of other metabolites formed by 25-hydroxylation (see above), it was formed in the incubations with astrocytes and it was not formed in the dead cell controls. The amount formed in 24 h was 0.055 nmol. Less than 0.01 nmol of this metabolite was formed by Schwann cells, and it was not detectable in incubations with neurons.

Particular efforts were made in order to find the possible metabolites 7α,24-dihydroxycholesterol or 7α,24-dihydroxy-4-cholesten-3-one. Monitoring the HPLC effluent with the detector set at 239 nm did not give any peak of a 3-oxo-Δ4 structure in the expected retention time range. Two-thirds of the total material in each fraction were analysed by GC/MS, and no spectrum of 7α,24-dihydroxycholesterol TMS ether was detected. Under the conditions used, GC/MS gave spectra of a steroid at levels of a few pmol. In directed searches for peaks in selected ion current chromatograms, the detection limit was considerably lower. Therefore the formation of 7α,24-dihydroxycholesterol, if any, was less than 0.03% of the added 25 nmol of 24-hydroxycholesterol. However, the recoveries of added 24-hydroxycholesterol from the incubations with living cells were about 15% lower than from the respective dead cells. It is likely that some unknown metabolite(s) was formed. The possibility of the formation of a conjugated 24-hydroxycholesterol, e.g. sulphated and/or glucuronidated, was checked by electrospray ionization MS, but no peaks of such products were found in the conjugate fractions from the ion-exchanger where they would be eluted.

Determination of endogenous 7α,25-dihydroxy-4-cholesten-3-one in the incubation medium of astrocyte cultures

The observation of 25-hydroxylation in astrocyte cultures made it interesting to investigate the possible endogenous formation of 25-hydroxycholesterol and its 7α-hydroxylated metabolites during the incubation. In three incubations with astrocytes in the absence of added 25-hydroxycholesterol, about 0.09–0.15 nmol of 7α,25-dihydroxy-4-cholesten-3-one (6) was found in the incubation medium. This compound was also identified in cell extracts. 25-Hydroxycholesterol (2) and 7α,25-dihydroxycholesterol (5) were also found by GC/MS analysis of the media, but the amounts were lower than 0.01 nmol. 27-Hydroxycholesterol (3), 7α,27-dihydroxycholesterol (2) and 7α,27-

hydroxy-4-cholesten-3-one (8) were not detectable. None of the above oxysterols was found in the media of the dead cell controls.

If cholesterol 7α-hydroxylase were present in the cells, its products 7α-hydroxycholesterol and/or 7α-hydroxy-4-cholesten-3-one might be formed in detectable amounts. Traces (below 1 pmol) of 7α- and 7β-hydroxycholesterols and 7-oxocholesterol were identified when appropriate HPLC fractions from the incubations with tracer amounts of substrates were analysed by GC/MS and chromatograms of ions specific for these compounds were reconstructed. However, these compounds were also present at similar levels in the media from the dead cell controls. Thus the formation of 7α-hydroxycholesterol, if any, is negligible.

Metabolism of 27-, 25- and 24-hydroxycholesterol and of 3β-

hydroxy-5-cholestenolic and 3β-hydroxy-5-choleenoic acids in rat brain microsomes

The substrate and metabolites in the incubation medium were extracted and separated on TEAP-LH-20 and by HPLC of the neutral fraction. The radioactivity in each HPLC fraction was counted and the compounds were determined by both GLC and GC/MS. Various concentrations were tested and control incubations were performed with boiled microsomes. In contrast with the conversions by the cell cultures, only one reaction, i.e. 7α-hydroxylation, was observed during the incubation with microsomes (under the conditions used). All substrates except 24-hydroxycholesterol were found to be 7α-hydroxylated, and 7α,27-dihydroxycholesterol, 7α,25-dihydroxycholesterol and 3β,7α-dihydroxy-5-cholestenolic acid were identified by GC/MS as products of the respective precursors. The rates of 7α-

hydroxylation of the three substrates at 0.5, 1.0, 5.0 and 10.0 μM were respectively 16.8, 22.8, 48.9 and 79 pmol/min per mg of protein for 25-hydroxycholesterol, 8.7, 8.7, 20.7 and 22.8 pmol/ min per mg of protein for 27-hydroxycholesterol and 11.4, 23.4, 24.1 and 16.1 pmol/min per mg of protein for 3β,7α-hydroxy-5-cholestenolic acid. The 3β-hydroxy-5-cholestenolic acid was studied at 2.5 and 25 μM concentrations and found to be 7α-hydroxylated at a rate similar to that for 27-hydroxycholesterol.

Presence of 24-hydroxycholesterol in brain microsomes

Both microsomes and boiled microsomes were incubated for 30 min and the incubation mixtures were analysed. Appropriate HPLC fractions were taken to dryness followed by TMS derivatization, 24-Hydroxycholesterol (4) TMS ether was identified by GC/MS and quantified by GLC. The amount of 24-

hydroxycholesterol in two incubations with microsomes and two with boiled microsomes were between 0.49 and 0.66 nmol/mg of protein. The differences between fresh and boiled microsomes were not significant, suggesting that most of the 24-hydroxycholesterol was formed before the incubations.

Substrate specificity of 7α-hydroxylation in rat brain microsomes

Radiolabelled 27- and 25-hydroxycholesterol, pregnenolone and DHEA were incubated in the absence and presence of potentially
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DISCUSSION

This study shows that foetal rat astrocytes, newborn rat Schwann cells and foetal rat neurons express a 7α-hydroxylase that is active towards 25- and 27-hydroxycholesterol. As found in previous studies of fibroblasts [13], both steroids underwent 7α-hydroxylation with subsequent oxidation to 7α-hydroxy-3-oxo-Δ5 steroids. A small fraction of the 27-hydroxycholesterol and its 7α-hydroxylated metabolites was also oxidized to 3β-hydroxy-5-cholestenolic acid, 3β,7α-dihydroxy-5-cholestenolic acid and 7α-hydroxy-3-oxo-4-cholestenolic acid. The rates and capacities for 7α-hydroxylation were much higher in astrocytes and Schwann cells than in neurons (Table 2). This difference probably reflects differences in amounts or activities of the enzymes involved. It is unlikely to be due to differences in uptake of the substrates since the percentage of labelled substrates and products in the cellular fraction was similar in all cell cultures.

The ratios between the amounts of 3β,7α-dihydroxy-Δ5 and 7α-hydroxy-3-oxo-Δ5 compounds formed differed between incubations with 25- and 27-hydroxycholesterol and between the three cell lines. This indicates that the ratio between the activities of 7α-hydroxylase and 3β-hydroxy-Δ5-Δ5,7α,9α,11α,17α,20β,23α-steroid dehydrogenase was different in the three cell types and that the relative activities of the two enzymes towards 25-hydroxylated and 27-hydroxylated substrates were different. The endogenous 24-hydroxycholesterol present in the brain microsomes and the formation of 25-hydroxylated metabolites from endogenous precursor(s) may also affect the hydroxylation of added substrates. In all experiments with 25- and 27-hydroxycholesterol there seemed to be a rate limitation in the oxidation by the dehydrogenase since the ratio between 3β,7α-dihydroxy-Δ5 and 7α-hydroxy-3-oxo-Δ5 steroid was always higher at the higher substrate concentration. The oxidation of the 3β-hydroxy-Δ5 to the 3-oxo-Δ5 steroids was subject to an isotope effect as seen by a comparison of the conversions of the 3α,7α-H and 26,27-3H labelled 25-hydroxycholesterols at the same substrate levels (Table 1, A5 compared with A8 and S5 compared with S8).

Previous studies have shown that 27-hydroxycholesterol 7α-hydroxylase(s) and 3β-hydroxy-Δ5-Δ5,7α,9α,11α,17α,20β,23α-steroid dehydrogenase in competitive substrates at a concentration of 5 μM. 7α-Hydroxylated products were quantified from the radioactivity in the appropriate HPLC fractions (Figure 2). In the absence of competitor, about 23, 35, 12 and 18% of the radioactive steroids were converted into their respective 7α-hydroxylated products. This percentage decreased to half or less in the presence of 27-, 25- or 24-hydroxycholesterol, 3β-hydroxy-5-cholestenolic acid, 3β-hydroxy-5-cholestanolic acid or 5α-androstane-3β,17β-diol (all at 5 μM). Pregnenolone and DHEA only had a slight effect on the 7α-hydroxylation of 25- and 27-hydroxycholesterols. Surprisingly, this was also the case with their own 7α-hydroxylation. Testosterone was without effect on the 7α-hydroxylation of any of the substrates.

Table 2 Conversion rates of 27- and 25-hydroxycholesterol into 7α-hydroxylated metabolites

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount of substrate (nmol)</th>
<th>Production of 7α-hydroxylated metabolites (nmol/24 h per 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Astrocytes</td>
<td>Schwann cells</td>
</tr>
<tr>
<td>27-Hydroxycholesterol</td>
<td>0.28</td>
<td>0.209</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>5.684</td>
</tr>
<tr>
<td>25-Hydroxycholesterol</td>
<td>0.022</td>
<td>0.021</td>
</tr>
<tr>
<td>25</td>
<td>28</td>
<td>0.256</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>9.889</td>
</tr>
</tbody>
</table>

Figure 2 Formation of 7α-hydroxylated metabolites in rat brain microsomes in the presence of competitors

The radiolabelled substrates (hydroxycholesterols, 0.14 μM, pregnenolone and DHEA, 1 μM) and competitors (5 μM) were incubated with 0.5 mg of microsomal proteins for 30 min. The conversions of the added radioactive compounds in the absence of competitor were 23.1% for [3H]-C5-3β,27-diol (a), 35.3% for [3H]-C5-3β,25-diol (b), 12.0% for [3H]-pregnenolone (c) and 18.0% for [3H]-DHEA (d). For abbreviations see Table 1. CA, cholestanoic acid; BA, chelanoic acid; A, androstanone; Estradiol, oestradiol.
the liver may be located in mitochondria or microsomes and that there are species differences in these localizations [4–6]. In the present study 7α-hydroxylase activity was found in the microsomal fraction but the presence of additional activity in mitochondria cannot be excluded. pregnenolone and DHEA have previously been shown to be 7α-hydroxylated by the microsomal fraction [21]. It is not known whether there is one or several 7α-hydroxylases for the C21, C24, and C19 substrates. The 7α-hydroxylations of the four substrates studied were affected in a similar way by all the competitors added. It can be noted that 24-hydroxycholesterol (this paper) and oestradiol [21], which are not 7α-hydroxylated in rat brain, are strong inhibitors of the reactions with all four substrates. Thus it appears that factors other than the substrates influence the results. Furthermore the possible influence of endogenous 24- and 25-hydroxycholesterol in the microsomal preparation cannot be evaluated. Isolation of the enzyme(s) involved in a pure form will be required to answer the question about the number of enzymes and their substrate specificities.

27-Hydroxycholesterol 7α-hydroxylase(s) appears to be widely distributed in extrahepatic tissue and cells, including human fibroblasts [13], rat nervous cells (this paper; [14]), mouse thymus (J. Zhang, Y. T. Xue, M. Jondal and J. Sjövall, unpublished work) and rat ovary [17]. Ongoing studies show that the 7α-hydroxylation is also present in some human tumour cell lines but absent from virus-transformed human fibroblasts (J. Zhang, A. Dricu and J. Sjövall, unpublished work). Cholesterol 7α-hydroxylase, an enzyme different from 27-hydroxycholesterol 7α-hydroxylase [4–8], is not expressed in those tissues and cells.

The biological function of the extrahepatic 7α-hydroxylation is not clear. The presence of 7α-hydroxy-3-oxo-4-cholesten-3-one (28) in blood and its net uptake by the human liver indicates that this acid is formed in extrahepatic cells in vivo [15,33]. Thus the reaction may be important for the removal of cholesterol and 27-hydroxycholesterol from certain cell types [15]. Since cholesterol 27-hydroxylase is present in the brain [11,36,37], our results suggest that 7α-hydroxy-3-oxo-4-cholestenic acid, which accumulates in subdural haematomas [20], may be formed locally. 7α-Hydroxylation has also been proposed to be important for the inactivation of 25-hydroxycholesterol as a regulator of 3-oxo-3-methylglutaryl-CoA (HMG-CoA) reductase activity and the low-density lipoprotein receptor [38,39]. However, our previous results are not in agreement with this concept [13,40].

24-Hydroxycholesterol (9) was not 7α-hydroxylated, by either the nervous cells or brain microsomes. This was not due to a lack of uptake of the steroid since metabolism did occur but to other metabolites. 24-Hydroxycholesterol can be formed from cholesterol by rat brain microsomal preparations [41] and by pig and mouse liver mitochondria [42,43]. The highest levels of 24-hydroxycholesterol in any organ are found in the brain [18]. Our finding of 0.49–0.66 nmol of 24-hydroxycholesterol/mg of microsomal protein confirms an early report of a microsomal location in bovine brain [44]. Most or all of this was preformed and the slow conversion of cholesterol into 24-hydroxycholesterol that is known to occur [44] could not be detected with certainty in the 30 min of incubation. The retention of 24-hydroxycholesterol in the brain microsomes might be due to the absence of the 7α-hydroxylation that occurs with 25- and 27-hydroxycholesterols also at nanomolar levels.

The metabolic pathways for 24-hydroxycholesterol are not clear. Two unidentified metabolites were observed in the livers of mice given radioactively labelled 24-hydroxycholesterol [45]. In our search for metabolites, compounds were separated into neutral, acidic and conjugated fractions by ion-exchange chromatography. Only 24,25-dihydroxycholesterol (15) was detected as a metabolite in the neutral fractions from the incubations with astrocytes and Schwann cells. Analyses of acidic and conjugated fractions failed to detect other metabolites by MS. However, the recoveries of added 24-hydroxycholesterol from incubations with living cells were about 15%, lower than those from the incubations with dead cells. Thus it is possible that metabolites of 24-hydroxycholesterol escaped detection. Further studies with the combined use of radio-labelled 24-hydroxycholesterol and MS will be required to elucidate the metabolism of 24-hydroxycholesterol.

An interesting finding in this study is the expression of 25-hydroxylase in astrocytes. 25-Hydroxylation of cholesterol may be due to autoxidation [46] or enzyme reactions [42,47,48]. The comparison between incubations with living and dead cells makes the formation by autoxidation unlikely. 25-Hydroxylation was low or undetectable in incubations with Schwann cells and neurons. Thus the reaction in astrocytes is likely to be catalysed by a 25-hydroxylase. 25-Hydroxylases may be microsomal or mitochondrial. It is not clear whether the mitochondrial 25-hydroxylase and cholesterol 27-hydroxylase are the same or different enzymes [48,49]. COS cells transfected with cytochrome P-450-27 (CYP27) cDNA express both sterol 27- and vitamin D3 25-hydroxylase activity [36], suggesting that the activities are derived from the same gene. Experiments with antibodies towards the sterol 27-hydroxylase [50] and some kinetic experiments with crude mitochondrial fractions [51] suggest, however, that different enzymes may be involved. The extent of 25-hydroxylation of cholesterol catalysed by enzyme(s) in the liver has always been much less than that of a parallel 27-hydroxylation [42,47]. Our results show that in astrocytes 24- and 27-hydroxycholesterol were both 25-hydroxylated whereas products of 27-hydroxylation of 24- and 25-hydroxycholesterol were not detectable. Furthermore there was an accumulation of 25-hydroxylated metabolites formed from endogenous precursors. These results suggest that the 25-hydroxylation in astrocytes is catalysed by an enzyme different from cholesterol 27-hydroxylase. The selective formation of 25-hydroxylated cholesterol metabolites in astrocytes in preference to the 27-hydroxylated ones suggests a potential function in these cells. 25-Hydroxycholesterol is a highly potent suppressor of HMG-CoA reductase activity and has been suggested as a regulator of cholesterol homeostasis [52,53]. However, the tissue distribution of 25-hydroxycholesterol has not been well studied. Our results suggest that 25-hydroxycholesterol (9), when formed, is rapidly converted into 7α,25-dihydroxy-4-cholesten-3-one (6) by the combined actions of 27-hydroxycholesterol 7α-hydroxylase and 3β-hydroxy-A7-C17,sterol dehydrogenase.

The presence and concentration of 7α,25-dihydroxy-4-cholesten-3-one (6) in different cells and tissue are not known since the formation and structure of this metabolite was only recently recognized [13]. A previous study showed that when 3β-hydroxy-A7-C17,sterol dehydrogenase was deficient, many intermediates with a 25-hydroxy group were formed, including 7α,25,27-trihydroxycholesterol (10), now found as a metabolite in astrocytes, whereas 25,27-dihydroxycholesterol (9) was not found [34]. Under normal conditions, none of these compounds have been detected. This may be an indication that 25-hydroxylated compounds have a shorter half-life than 27-hydroxylated compounds. From this point of view, 25-hydroxylated intermediates are more likely to be involved in a regulation of biological functions.

On the basis of the present and previous results, the pathways of cholesterol metabolism in astrocytes may be summarized as in Figure 3. The metabolism may start with a 27-, 25- or 24-hydroxylation. 27-Hydroxycholesterol (9) undergoes 7α-
Metabolism of hydroxycholesterols in rat glial cells and neurons

Figure 3 Suggested metabolic pathways of cholesterol in astrocytes

For abbreviations of the steroid structures see Table 1. Reactions marked by an encircled number involve: 1, \(\alpha\)-hydroxylation; 2, 25-hydroxylation; 3, oxidation by \(\beta\)-hydroxy-\(\Delta_5\)-steroid dehydrogenase/isomerase. Broken arrows indicate reactions previously demonstrated with brain tissue.
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