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-Δ^4 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-hydroxylated metabolites were 0.21, 0.12 and 0.02 nmol/24 h per 10^6 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-cholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid and 7α-hydroxy-3-oxo-4-cholestenoic acid. In addition to the two hydroxycholesterols, other 3β-hydroxy-Δ^5 steroids, dehydroepiandrosterone, pregnenolone, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid underwent 7α-hydroxylation. Competitive experiments did not distinguish between the presence of one or several 7α-hydroxylases. In astrocyte incubations, 27-hydroxycholesterol also underwent 25-hydroxylation, and 12% of its metabolites carried a 25-hydroxy group. 25-Hydroxylation of added 24-hydroxycholesterol was also observed in the astrocyte incubations, as was the formation of 7α,25-dihydroxy-4-cholesten-3-one, 25-hydroxycholesterol and 7α,25-dihydroxycholesterol from endogenous precursor(s). Our study indicates that side-chain oxygenated cholesterol can undergo metabolic transformations that may be of importance for cholesterol homoeostasis in the brain.

INTRODUCTION

The conversion of cholesterol into bile acids in the liver can start either in the endoplasmic reticulum with 7α-hydroxylation or in the mitochondria with 27-hydroxylation, the latter pathway leading preferentially to chenodeoxycholic acid [1–3]. 7α-Hydroxylation of 27-hydroxycholesterol is catalysed by 27-hydroxycholesterol 7α-hydroxylase(s), an enzyme different from cholesterol 7α-hydroxylase [4–8]. Cholesterol 7α-hydroxylase is confined to the liver [1] whereas cholesterol 27-hydroxylase is present in both the liver and many extrahepatic tissues [9–12]. Recently, we reported on the presence of 27-hydroxycholesterol 7α-hydroxylase(s) in human diploid fibroblasts [13] and rat brain microsomes [14]. This reaction may be important for metabolic removal of cholesterol from certain cells [15]. We and others showed that 25-hydroxycholesterol was equally as good a substrate as 27-hydroxycholesterol for the 7α-hydroxylation in both liver and fibroblasts [13,16]. A recent paper described the 7α-hydroxylation of 25-hydroxycholesterol in the rat ovary [17].

27-Hydroxycholesterol has been found in the brain where 24-hydroxycholesterol is the major hydroxycholesterol [18]. The possibility that bile acids are formed in the brain has been suggested [19,20]. Our finding that 7α,27-dihydroxycholesterol is formed from 27-hydroxycholesterol by rat brain microsomes supported this suggestion [14]. Since the cellular distribution of the reaction is unknown, we have investigated the occurrence of 7α-hydroxylase(s) active on 24-, 25- and 27-hydroxycholesterol in cultures of different cell types from the nervous system. Since pregnenolone (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 octadecylsilane (ODS)-bonded silica (preparative C18) from Waters Associates (Milford, MA, U.S.A.). Triethylaminohydroxypropyl 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-^{3}H]Hydroxycholesterol (3186 GBq/mmol) was from NEN Research Products (Dreieich, Germany) and [1,2,6,7-^{3}H]DHEA (3182 GBq/mmol) and [7-^{3}H(n)]pregnenolone (925 GBq/mmol) were from NEN (les Ulis, France). 25-Hydroxycholesterol and 3β-hydroxy-5-cholenoic acid were from Steraloids (Wilton, NH, U.S.A.), and pregnenolone and 5α-androstane-3β,17β-diol from Sigma. DHEA, testosterone and oestradiol were gifts from Roussel-Uclaf (Romainville, France). 27-Hydroxycholesterol was prepared from kryptogenin, kindly

Abbreviations used: DHEA, dehydroepiandrosterone; GC/MS, gas–liquid chromatography–mass spectrometry; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HPBCD, hydroxypropyl-β-cyclodextrin; RI, retention index; TEAP-LH-20, triethylaminohydroxypropyl Sephadex LH-20; TMS, trimethylsilyl; MEM, minimal essential medium; DMEM, Dulbecco's modified Eagle's medium; ODS, octadecylsilane.

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supplied by Dr. L. Tökes (Syntex Research, Palo Alto, CA, U.S.A.) [26]. 25- and 27-Hydroxycholesterol were used as starting materials for synthesis of 25-[3α-^{3}H]hydroxycholesterol (259 GBq/mmol), 27-[3α-^{3}H]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-hydroxy-5-cholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid and 7α-hydroxy-3-oxo-4-cholestenoic acid [26]. 24-Hydroxycholesterol was prepared from 24-oxocholesterol (Searle, Chicago, IL, U.S.A.) by reduction with $NaBH_4$ 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(L-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 $NaHCO_3$, 5 mM Hepes, 5 units/ml penicillin and 5 μg/ml streptomycin] supplemented with 10% hormone mixture (25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 20 nM progesterone and 30 nM $NaSeO_3$) 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(L-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(L-lysine)-coated flasks. Finally, after 3 weeks of culture, cells were treated with trypsin, seeded on poly(L-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 with or without 25 nmol of unlabelled hydroxycholesterol, in 10 ml of serum-free MEM or DMEM. The solutions of hydroxycholesterol in 45% HPBCD 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 0.9×10^6/dish; Schwann cells 1.6×10^6/dish; neurons 3.5×10^6/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 N_2 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., Hicksville, NJ, U.S.A.), and redissolved 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 Na_2HPO_4/KH_2PO_4, 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 mg 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 HCO_3^- 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 (Gilson, Villiers Le Bel, France), a μ-Bondapak C_{18} 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.

RESULTS

Metabolism of 27-hydroxycholesterol and 25-hydroxycholesterol in cultures of astrocytes, Schwann cells and neurons

To simplify reading of the following text, experiments are referred to by the numbers given in Table 1 and compounds by underlined arabic numerals in italics as given in Figure 3.

27-[3α-^{3}H]Hydroxycholesterol (*3*; 0.28 nmol/10 ml of medium) was incubated for 24 h with astrocytes (0.9×10^6 cells per dish), in the absence (expt. A1) and presence (A2) of unlabelled 27-hydroxycholesterol (25 nmol) or unlabelled 25-hydroxycholesterol (25 nmol) (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 tritium 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-cholestenoic acid (*12*), 3β,7α-dihydroxy-5-cholestenoic acid (*13*) and 7α-hydroxy-3-oxo-4-cholestenoic acid (*14*).

HPLC of the neutral fractions showed unchanged tritiated substrate in fractions 28 and 29, accounting for about 19% of the total tritium in the absence of unlabelled hydroxycholesterol (A1), 77% in the presence of unlabelled 27-hydroxycholesterol (A2) and 57% in the presence of unlabelled 25-hydroxycholesterol (A3). Several metabolites more polar than the substrate were observed. HPLC fraction 10 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,27-dihydroxy-4-cholesten-3-one (*8*) [13]. Fractions 11 and 12 contained 10.4, 9.5 and 14.3% of the total tritium in incubations A1, A2 and A3 respectively. GC/MS analysis showed this to represent 7α,27-dihydroxycholesterol (*7*). In the incubation containing unlabelled 25-hydroxycholesterol (A3), unlabelled 7α,25-dihydroxy-4-cholesten-3-one (*6*) was formed and was found in fraction 9. 7α,25-Dihydroxycholesterol (*5*) was identified in fractions 10 and 11.

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 trimethylsilanols 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-ditrimethylsiloxy 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-cholestene-3β,7α,25,27-tetrol in patients with 3β-hydroxy-Δ^5-C_{27}-steroid 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 27-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,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-Δ^4 metabolites calculated from the release of ^{3}H from the 3α-^{3}H-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

Table 1 Distribution of ^{3}H in different fractions and metabolites

^{3}H-labelled hydroxycholesterols were incubated with astrocytes, neurons and Schwann cells for 24 h in 10 ml of medium in the absence or presence of unlabelled substrates. C is cholestane, and the superscript indicates the position of the double bond and greek letters the configuration of the hydroxy groups. The values for ^{3}H distribution are based on the radioactivities in the respective TEAP-LH-20 and HPLC fractions (see the text) and are the means from duplicate incubations. The differences between single values were usually less than 10%. The value for C^4-7α,25-diol-3-one or C^4-7α,27-diol-3-one is based on the radioactivity released in water-soluble form when the substrates were labelled in the 3α position.

			^{3}H distribution (% of total in medium)						
	Substrate added			Neutral metabolites					
Expt. no.	^{3}H-labelled (2 μCi)	Unlabelled (25 nmol)	Unchanged substrate	C^5-3β,7α,25-triol (*5*) or C^5-3β,7α,27-triol (*7*)	C^4-7α,25-diol-3-one (*6*) or C^4-7α,27-diol-3-one (*8*)	C^5-3β,25,27-triol (*9*)	C^5-3β,7α,25,27-tetrol (*10*)	Unidentified	Acids (*12*–*14*)
Astrocytes									
A1	[3α-^{3}H]C^5-3β,27-diol	–	19.3	10.4	47.1	2.6	9.7	< 1.0	3.7
A2	[3α-^{3}H]C^5-3β,27-diol	C^5-3β,27-diol	76.5	9.5	9.4	2.4	1.6	< 1.0	3.1
A3	[3α-^{3}H]C^5-3β,27-diol	C^5-3β,25-diol	57.1	14.3	9.4	7.9	2.8	< 1.0	3.5
A4	[3α-^{3}H]C^5-3β,25-diol	–	13.2	38.6	45.2	< 1.0	< 1.0	< 1.0	1.1
A5	[3α-^{3}H]C^5-3β,25-diol	C^5-3β,25-diol	64.2	26.5	9.3	< 1.0	< 1.0	< 1.0	< 1.0
A6	[3α-^{3}H]C^5-3β,25-diol	C^5-3β,27-diol	69.0	22.9	5.8	< 1.0	< 1.0	< 1.0	< 1.0
A7	[26,27-^{3}H]C^5-3β,25-diol	–	5.4	6.6	74.0	< 1.0	< 1.0	3.1	1.2
A8	[26,27-^{3}H]C^5-3β,25-diol	C^5-3β,25-diol	58.2	13.5	18.2	< 1.0	< 1.0	1.0	< 1.0
Schwann cells									
S1	[3α-^{3}H]C^5-3β,27-diol	–	20.6	7.7	61.9	< 1.0	1.2	< 1.0	3.9
S2	[3α-^{3}H]C^5-3β,27-diol	C^5-3β,27-diol	69.0	6.7	14.9	< 1.0	< 1.0	< 1.0	4.4
S3	[3α-^{3}H]C^5-3β,27-diol	C^5-3β,25-diol	71.8	6.1	15.1	< 1.0	< 1.0	< 1.0	4.0
S4	[3α-^{3}H]C^5-3β,25-diol	–	5.5	16.2	71.9	< 1.0	1.3	< 1.0	< 1.0
S5	[3α-^{3}H]C^5-3β,25-diol	C^5-3β,25-diol	58.6	19.0	17.1	< 1.0	< 1.0	< 1.0	< 1.0
S6	[3α-^{3}H]C^5-3β,25-diol	C^5-3β,27-diol	60.7	26.0	14.3	< 1.0	< 1.0	< 1.0	< 1.0
S7	[26,27-^{3}H]C^5-3β,25-diol	–	5.4	3.6	59.0	< 1.0	< 1.0	4.6	< 1.0
S8	[26,27-^{3}H]C^5-3β,25-diol	C^5-3β,25-diol	47.6	6.2	20.4	< 1.0	< 1.0	1.7	< 1.0
Neurons									
N1	[3α-^{3}H]C^5-3β,27-diol	–	78.4	12.5	9.1	< 1.0	< 1.0	< 1.0	3.2
N2	[3α-^{3}H]C^5-3β,27-diol	C^5-3β,27-diol	97.3	2.7	2.6	< 1.0	< 1.0	< 1.0	2.2
N3	[3α-^{3}H]C^5-3β,27-diol	C^5-3β,25-diol	96.9	3.1	2.0	< 1.0	< 1.0	< 1.0	3.0
N4	[3α-^{3}H]C^5-3β,25-diol	–	53.0	37.4	9.6	< 1.0	< 1.0	< 1.0	< 1.0
N5	[3α-^{3}H]C^5-3β,25-diol	C^5-3β,25-diol	93.8	6.2	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
N6	[3α-^{3}H]C^5-3β,25-diol	C^5-3β,27-diol	94.2	5.8	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
N7	[26,27-^{3}H]C^5-3β,25-diol	–	36.6	33.1	30.2	< 1.0	< 1.0	< 1.0	< 1.0
N8	[26,27-^{3}H]C^5-3β,25-diol	C^5-3β,25-diol	93.4	4.7	1.9	< 1.0	< 1.0	< 1.0	< 1.0

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-cholesten-3-one (*8*) was formed and found in fraction 10. 7α,27-Dihydroxycholesterol (*7*) was identified in fractions 11 and 12.

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

The metabolism of side-chain-labelled 25-[26,27-^{3}H]hydroxycholesterol (0.022 nmol/10 ml of medium) gave an HPLC profile of ^{3}H different from that obtained after incubation of ring-labelled 25-[3α-^{3}H]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 ^{3}H-labelled 25- and 27-hydroxycholesterol, the extracts of cells contained between 5 and 7% of the total amount of ^{3}H 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 ^{3}H 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

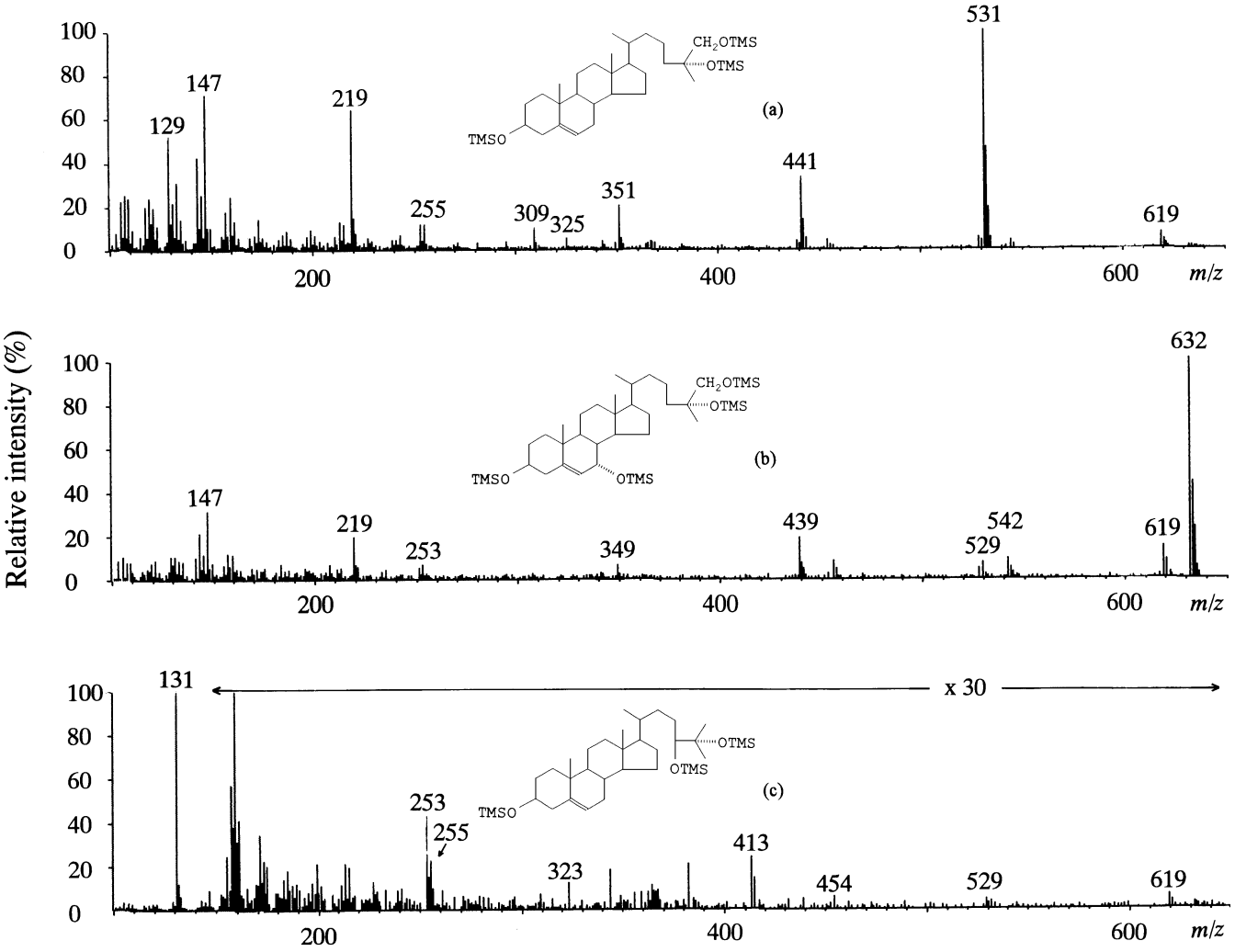

Figure 1 Mass spectra of the TMS derivatives of the 25-hydroxylated metabolites in rat brain astrocytes

(**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 1.6×10^6 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-^{3}H]hydroxycholesterol (*3*; S1), only 1.2% of added ^{3}H was found in fraction 7 (7α,25,27-trihydroxycholesterol, *10*), and almost no ^{3}H was found in fraction 13 (25,27-dihydroxycholesterol, *9*). As was the case in the astrocyte incubations, 13.4 and 12.2% of the radioactivity from 25-[26,27-^{3}H]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 ^{3}H 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 ^{3}H in the cell extracts).

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

Neurons (about 3.5×10^6 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-cholestenoic 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 ^{3}H 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 ^{3}H 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 (24*R*/*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 C_{27} 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 (*15*). 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-Δ^4 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 (*7*) and 7α,27-dihydroxy-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-cholestenoic and 3β-hydroxy-5-cholenoic 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-cholestenoic 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-hydroxy-5-cholestenoic acid. The 3β-hydroxy-5-cholenoic 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-hydroxylations in rat brain microsomes

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

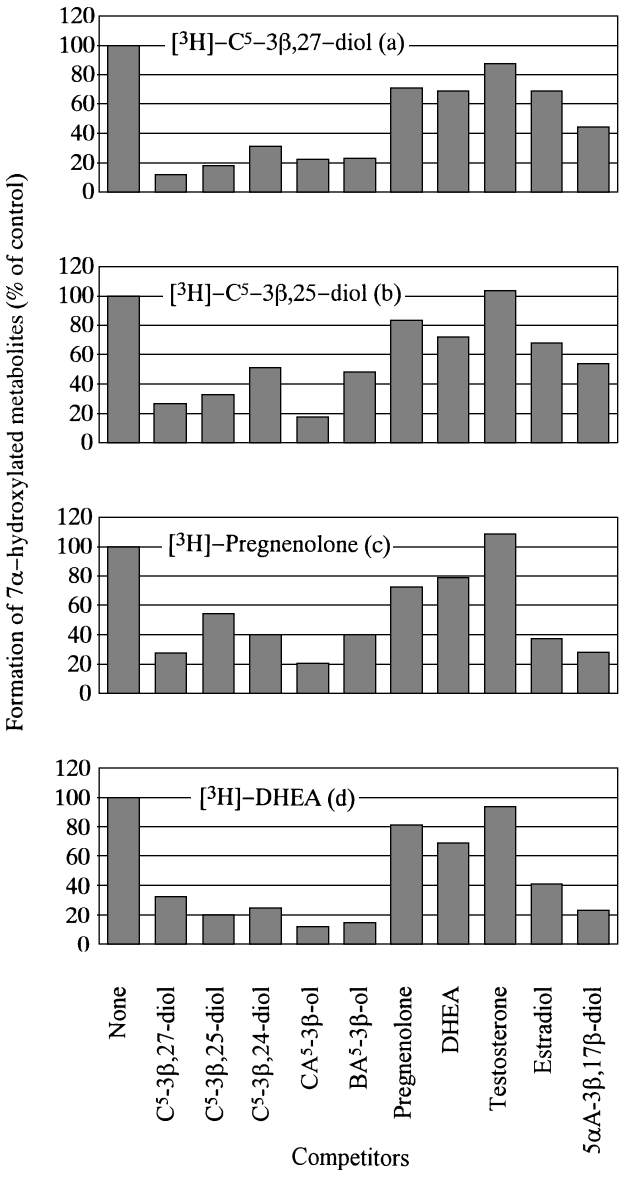

Figure 2 Formation of 7α-hydroxylated metabolites in rat brain microsomes in the presence of competitors

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 [^{3}H]-C^5-3β,27-diol (**a**), 35.3% for [^{3}H]-C^5-3β,25-diol (**b**), 12.0% for [^{3}H]pregnenolone (**c**) and 18.0% for [^{3}H]DHEA (**d**). For abbreviations see Table 1. CA, cholestanoic acid; BA, cholanoic acid; A, androstane; Estradiol, oestradiol.

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-cholestenoic acid, 3β-hydroxy-5-cholenoic 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.

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-Δ^4 steroids. A small fraction of the 27-hydroxycholesterol and its 7α-hydroxylated metabolites was also oxidized to 3β-hydroxy-5-cholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid and 7α-hydroxy-3-oxo-4-cholestenoic 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-Δ^5 and 7α-hydroxy-3-oxo-Δ^4 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-Δ^5-C_{27}-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-Δ^5 and 7α-hydroxy-3-oxo-Δ^4 steroid was always higher at the higher substrate concentration. The oxidation of the 3β-hydroxy-Δ^5 to the 3-oxo-Δ^4 steroids was subject to an isotope effect as seen by a comparison of the conversions of the 3α-^{3}H and 26,27-^{3}H 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-Δ^5-C_{27}-steroid dehydrogenase in

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

Cultures of astrocytes, Schwann cells and neurons were incubated with 27-[3α-^{3}H]hydroxycholesterol or 25-[3α-^{3}H]hydroxycholesterol in 10 ml of medium at 37 °C for 24 h. Radioactive steroids were extracted from the incubation media and analysed by HPLC (see the Experimental section).

Substrate	Amount of substrate (nmol)	Production of 7α-hydroxylated metabolites (nmol/24 h per 10^6 cells)		
		Astrocytes	Schwann cells	Neurons
27-Hydroxycholesterol	0.28	0.209	0.124	0.017
	25	5.694	3.375	0.379
25-Hydroxycholesterol	0.022	0.021	0.009	0.004
	0.28	0.256	0.156	0.038
	25	9.889	5.641	0.429

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 C_{27}, C_{21} and C_{19} 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-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-cholestenoic acid (*14*) 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-cholestenoic 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-hydroxy-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 (*4*) was not 7α-hydroxylated, by either the nervous cells or brain microsomes. This was not due to a lack of uptake of the sterol 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 radiolabelled 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-hydroxylation 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 D_3 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 homoeostasis [52,53]. However, the tissue distribution of 25-hydroxycholesterol has not been well studied. Our results suggest that 25-hydroxycholesterol (*2*), 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-Δ^5-C_{27}-steroid 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-Δ^5-C_{27}-steroid 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 (*3*) undergoes 7α-

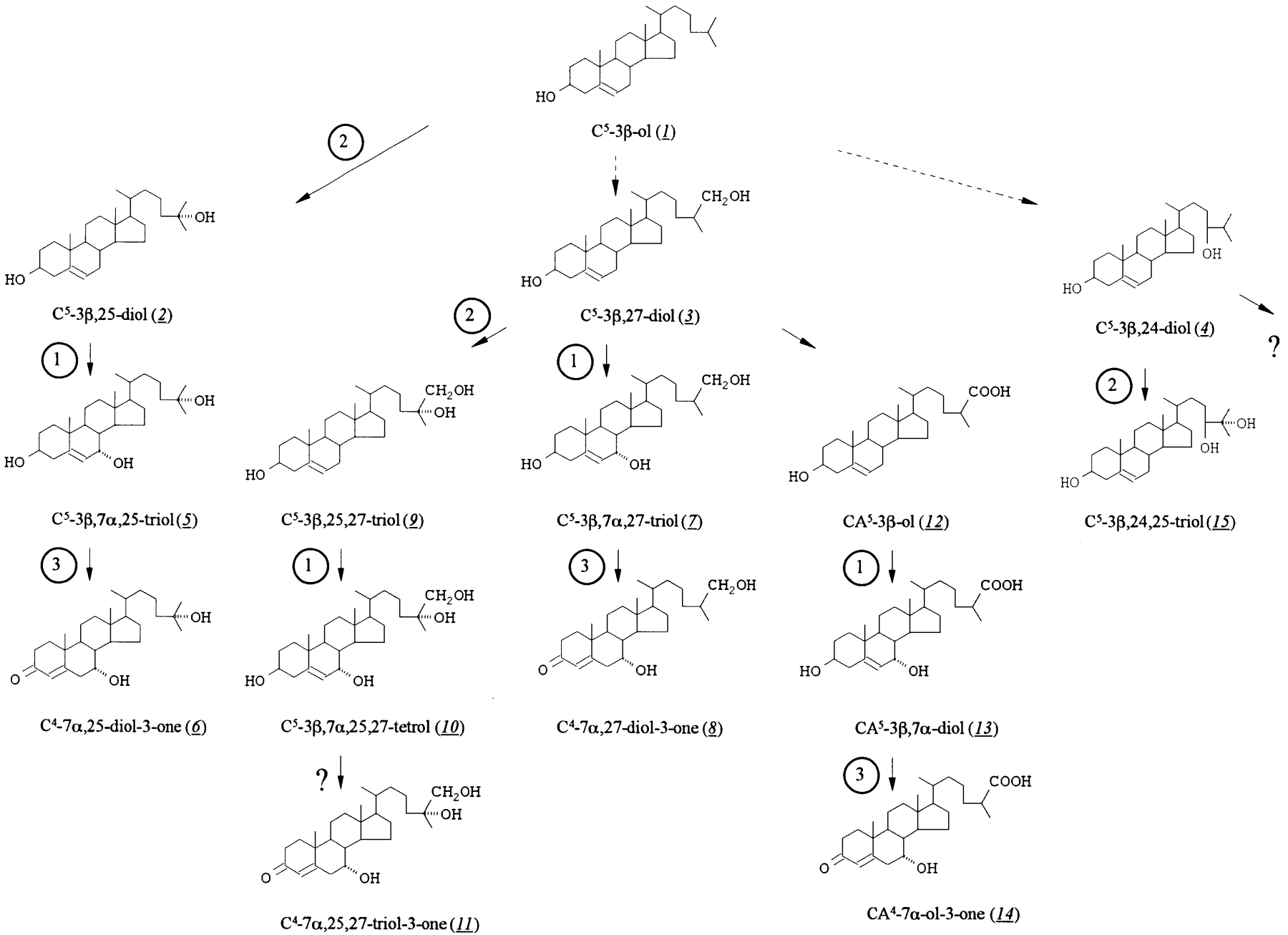

Figure 3 Suggested metabolic pathways of cholesterol in astrocytes

For abbreviations of the steroid structures see Table 1. Reactions marked by an encircled number involve: 1, 7α-hydroxylation; 2, 25-hydroxylation; 3, oxidation by 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase. Broken arrows indicate reactions previously demonstrated with brain tissue.

hydroxylation with subsequent oxidation to a 7α-hydroxy-3-oxo-Δ^4 steroid (*8*). Part of 27-hydroxycholesterol may be 25-hydroxylated (*9*) or converted into the C_{27} acid (*12*) before 7α-hydroxylation and oxidation by 3β-hydroxy-Δ^5-C_{27} steroid dehydrogenase. 25-Hydroxycholesterol (*2*) is converted into 7α,25-dihydroxy-4-cholesten-3-one (*6*) through the intermediate, 7α,25-dihydroxycholesterol (*5*). The 24-hydroxycholesterol (*4*) is not 7α-hydroxylated and is therefore not oxidized by the 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase [13] to a 3-oxo-Δ^4 metabolite. So far 25-hydroxylation of 24-hydroxycholesterol is the only reaction found in astrocytes and Schwann cells. Whether the metabolites with a 3-oxo-Δ^4 structure can be further converted into saturated steroids and secreted from cells is not clear. 3-Oxo-Δ^4-steroid 5α-reductase is present in the brain [54], but so far we have not detected saturated C_{27} or C_{24} steroids or bile acids in the incubations with cells from the nervous tissue.

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REFERENCES

1 Björkhem, I. (1985) in Sterols and bile acids (Danielsson, H. and Sjövall, J., eds.), pp. 231–278, Elsevier Science Publishers, Amsterdam
2 Axelson, M. and Sjövall, J. (1990) J. Steroid Biochem. **36**, 631–640
3 Princen, M. G., Meijer, P., Wolthers, B. G., Vonk, R. J. and Kuipers, F. (1991) Biochem. J. **275**, 501–505
4 Axelson, M., Shoda, J., Sjövall, J., Toll, A. and Wikvall, K. (1992) J. Biol. Chem. **267**, 1701–1704
5 Toll, A., Shoda, J., Axelson, M., Sjövall, J. and Wikvall, K. (1992) FEBS Lett. **296**, 73–76
6 Shoda, J., Toll, A., Axelson, M., Pieper, F., Wikvall, K. and Sjövall, J. (1993) Hepatology **17**, 395–403
7 Björkhem, I., Nyberg, B. and Einarsson, K. (1992) Biochim. Biophys. Acta **1128**, 73–76
8 Martin, K. O., Budai, K. and Javitt, N. B. (1993) J. Lipid Res. **34**, 581–588
9 Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H. and Russell, D. W. (1989) J. Biol. Chem. **264**, 8222–8229
10 Skrede, S., Björkhem, I., Kvittingen, E. A., Buchmann, M. S., Lie, S. O., East, C. and Grundy, S. (1986) J. Clin. Invest. **78**, 729–735
11 Pedersen, J. I., Oftebro, H. and Björkhem, I. (1989) Biochem. Int. **18**, 615–622
12 Cali, J. I. and Russell, D. W. (1991) J. Biol. Chem. **266**, 7774–7778
13 Zhang, J., Larsson, O. and Sjövall, J. (1995) Biochim. Biophys. Acta **1256**, 353–359
14 Zhang, J., Akwa, Y., Baulieu, E. E. and Sjövall, J. (1995) C. R. Acad. Sci. Paris **318**, 345–359
15 Lund, E., Andersson, O., Zhang, J., Babiker, A., Ahlborg, G., Diczfalusy, U., Einarsson, K., Sjövall, J. and Björkhem, I. (1996) Arterioscler. Thromb. Vasc. Biol. **16**, 208–212
16 Toll, A., Wikvall, K., Sudjan-Sugiaman, E., Kondo, K. H. and Björkhem, I. (1994) Eur. J. Biochem. **224**, 309–316
17 Payne, D. W., Shackleton, C., Toms, H., Ben-Shlomo, I., Kol, S., deMoura, M., Strauss, J. F. and Adashi, E. Y. (1995) J. Biol. Chem. **270**, 18888–18896
18 Smith, L. L., Ray, D. R., Moody, J. A., Wells, J. D. and Lier, J. E. (1972) J. Neurochem. **19**, 899–904
19 Nicholas, H. J. (1976) in The Bile Acids, (Nair, P. P. and Kritchevsky, D., eds.), vol. 3, pp. 1–15, Plenum Press, New York
20 Nagata, K., Takakura, K., Asano, T., Seyama, Y., Hirofa, H., Shigematsu, N., Shima, I., Kasama, T. and Shimizu, T. (1992) Biochim. Biophys. Acta **1126**, 229–236
21 Akwa, Y., Morfin, R. F., Robel, P. and Baulieu, E. E. (1992) Biochem. J. **288**, 959–964
22 Johansson, G. (1971) Eur. J. Biochem. **21**, 68–69
23 Sulcova, J. and Starka, L. (1972) Experientia **28**, 1361–1362
24 Khalil, M. W., Strutt, B., Vachon, D. and Killinger, D. W. (1994) J. Steroid Biochem. Mol. Biol. **48**, 545–552
25 Axelson, M., Sahlberg, B. L. and Sjövall, J. (1981) J. Chromatogr. Biomed. Appl. **224**, 355–370
26 Shoda, J., Axelson, M. and Sjövall, J. (1993) Steroids **58**, 119–125
27 El-Etr, M., Cordier, J., Glowinski, J. and Prémont, J. (1989) J. Neurosci. **9**, 1473–1480
28 Akwa, Y., Sananès, N., Gouézou, M., Robel, P., Baulieu, E. E. and Le Goascogne, C. (1993) J. Cell Biol. **121**, 135–143
29 Akwa, Y., Schumacher, M., Jung-Testas, I. and Baulieu, E. E. (1993) C. R. Acad. Sci. Paris **316**, 410–414
30 Schumacher, M., Jung-Testas, I., Robel, P. and Baulieu, E. E. (1993) Glia **8**, 232–240
31 Groyer, A. and Robel, P. (1980) Anal. Biochem. **106**, 262–268
32 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
33 Axelson, M., Mörk, B. and Sjövall, J. (1988) J. Lipid Res. **29**, 629–641
34 Ichimiya, H., Egestad, B., Nazer, H., Baginski, E. S., Clayton, P. T. and Sjövall, J. (1991) J. Lipid Res. **32**, 829–841
35 Tint, C. S., Dayal, B., Batta, A. K., Shefer, S., Cheng, F. W., Salen, C. G. and Mosbach, E. H. (1978) J. Lipid Res. **19**, 956–966
36 Dahlbäck, H. (1989) Dissertation. University of Uppsala
37 Smith, A. G., Gilbert, J. D., Harland, W. A. and Brooks, C. J. W. (1974) Biochem. J. **139**, 793–795
38 Leighton, J. K., Dueland, S., Straka, M. S., Trawick, J. and Davis, R. A. (1991) Mol. Cell. Biol. **11**, 2049–2056
39 Dueland, S., Trawick, J. D., Nenseter, M. S., MacPhee, A. A. and Davis, R. A. (1992) J. Biol. Chem. **267**, 22695–22698
40 Axelson, M., Larsson, O., Zhang, J., Shoda, J. and Sjövall, J. (1995) J. Lipid Res. **36**, 290–298
41 Lin, Y. Y. and Smith, L. L. (1974) Biochim. Biophys. Acta **348**, 189–196
42 Lund, E., Björkhem, I., Furster, C. and Wikvall, K. (1993) Biochim. Biophys. Acta **1166**, 177–182
43 Lund, E., Breuer, O. and Björkhem, I. (1993) J. Biol. Chem. **267**, 25092–25097
44 Dhar, A. K., Teng, J. I. and Smith, L. L. (1973) J. Neurochem. **21**, 51–60
45 Saucier, S. E., Kandutsch, A. A., Clark, D. S. and Spencer, T. A. (1993) Biochim. Biophys. Acta **1166**, 115–123
46 Smith, L. (1987) Chem. Phys. Lipids **44**, 87–125
47 Björkhem, I. and Gustafsson, J. (1974) J. Biol. Chem. **249**, 2528–2535
48 Björkhem, I. (1992) J. Lipid Res. **33**, 455–471
49 Okuda, K. I., Usui, E. and Ohyama, Y. (1995) J. Lipid Res. **36**, 1641–1652
50 Dahlbäck, H. (1988) Biochem. Biophys. Res. Commun. **157**, 26–30
51 Björkhem, I., Holmberg, I., Oftebro, H. and Pedersen, J. I. (1980) J. Biol. Chem. **255**, 5244–5249
52 Kandutsch, K. A., Chen, H. W. and Heiniger, H. J. (1978) Science **201**, 498–501
53 Smith, L. L. and Johanson, B. H. (1989) Free Radic. Biol. Med. **7**, 285–332
54 Celotti, F., Melcangi, R. C., Negri-Cesi, P., Ballabio, M. and Martini, L. (1987) J. Steroid Biochem. **26**, 125–129

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