Metabolism of cholesteryl palmitate by rat brain in vitro; formation of cholesterol epoxides and cholestane-3β,5α,6β-triol

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Abstract Incubation of [4-^{14}C]cholesteryl palmitate with the 12,000 *g* supernatant fraction of adult rat brain fortified with an NADPH-generating system and β-mercaptoethylamine resulted in formation (2–5%) of more polar metabolites characterized as a mixture of cholesterol-5,6-epoxides. Under extended incubation conditions, cholestane-3β,5α,6β-triol was isolated as the major end product of the incubations. Free [4-^{14}C]cholesterol incubated under similar conditions was not oxidized, whereas oxidation of [4-^{14}C]cholesteryl palmitate appeared to be dependent upon hydrolysis of the ester by the rat brain microsomal subcellular fraction. Elimination of the NADPH-generating system or the addition of EDTA to the incubation mixture inhibited epoxide formation, suggesting that the products are derived from an NADPH-dependent enzymatic lipoperoxidation mechanism. The in vitro conversion of [4-^{14}C]cholesterol-5α,6α-epoxide to cholestane-3β,5α,6β-triol was also demonstrated in rat brain subcellular fractions in the absence of added cofactors.

Supplementary key words brain cholesterol · cholesterol oxidation

Although cholesterol is the major sterol of the central nervous system, a number of minor neutral sterols have been detected in brain tissue (1, 2). Because of the well-established capacity of cholesterol to undergo autoxidation (3, 4), especially during isolation procedures, and because brain contains such a high concentration of cholesterol, it has generally been assumed that these minor sterols are artifacts produced during the purification of brain cholesterol. However, recent studies from other laboratories have suggested that trace sterols such as 7-ketocholesterol, 7α- and 7β-hydroxycholesterol, and cholestane-3β,5α,6β-triol are the direct result of microsomal NADPH-dependent enzymatic lipid peroxidation (5, 6), at least in liver.

A program has been initiated to ascertain whether or not cholesterol is converted to other products during the course of the turnover of this sterol in the central nervous system (7). That some degree of turnover does occur in brain has been established in several laboratories (8, 9). For the present, our approach to this general problem has been to determine what products can be produced in vitro by brain tissue preparations. We feel that these studies may be of more than academic interest because some minor steroids have now been found associated with certain diseases involving the brain. For example, it has been reported that a small percentage of sterols (0.39%) in normal brain tissue compared with a larger percentage (2.90%) in brain tumors is represented in part by unidentified oxygenated products of cholesterol catabolism (10). Cholestanol, normally a minor sterol accompanying cholesterol in most tissues, accumulates in the brain in the disease cerebrotendinous xanthomatosis (11, 12). In addition, some monohydroxy bile acids have been detected in the brains of guinea pigs afflicted with experimental allergic encephalomyelitis (13) and in multiple sclerosis brain tissue (14). It seemed of import, therefore, that efforts be made to determine the extent, if any, to which brain tissue can catabolize as well as synthesize cholesterol.

During the course of the present investigation, a wide variety of experiments gave negative results when attempts were made to isolate products of cholesterol metabolism when appropriate antioxidants and the *free sterol* were incubated with brain preparations. Unexpectedly, incubation of [4-^{14}C]cholesteryl palmitate, used to minimize formation of cholesterol autoxidation products (15, 16), produced cholesterol-5α,6α-epoxide (I, Fig. 1), which was hydrolyzed on longer incubation periods to cholestane-3β,5α,6β-triol (II, Fig. 1).

MATERIALS AND METHODS

Steroids and cofactors

[4-^{14}C]Cholesterol was obtained from New England Nuclear (Boston, Mass.) and had a specific activity of 1·54

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; RRT, relative retention time.

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μCi/mg. [4-^{14}C]Cholesteryl palmitate was prepared by microscale synthesis from [4-^{14}C]cholesterol, which was initially purified by preparative TLC, using palmitylchloride–pyridine according to the method of Deykin and Goodman (17). The ester was purified by repeated TLC. It was shown to be homogeneous by TLC and GLC after mild alkaline hydrolysis under nitrogen in a sealed vial containing 2 mg of unlabeled cholesteryl palmitate as a carrier, 2 mg of BHT (butylated hydroxytoluene), and 3.0 ml of 5% KOH in ethanol.

[4-^{14}C]Cholesterol-5α,6α-epoxide was synthesized from labeled [4-^{14}C]cholesterol and *m*-chloroperbenzoic acid (Eastman Kodak) employing a microscale procedure (18). The radioactive epoxide was purified by preparative TLC. Homogeneity of the pure compounds was verified using GLC combined with a radioactivity monitoring system.

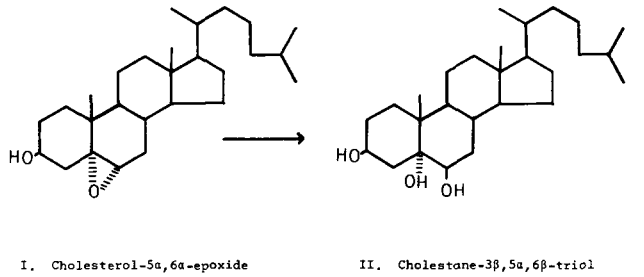
Unlabeled steroids required for isotope dilution experiments were prepared by synthesis and purified by recrystallization. Cholesterol-5α,6α-epoxide (mp 142°C) was prepared by a macroscale adaptation of the procedure described for preparation of the radioactive compound (18). Cholesterol-5β,6β-epoxide (mp 131°C) was synthesized from cholestane-3β,5α,6β-triol triacetate by the method of Davis and Petrow (19). Cholestane-3β,5α,6β-triol (mp 232°C) was synthesized by the method of Fieser and Rajagopalan (20). Triphosphopyridine nucleotide cofactors (NADP$^+$), D-glucose-6-phosphate, ethylenediaminetetraacetic acid (EDTA), and β-mercaptoethylamine were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Tissue preparations

Adult 250-g rats (Wistar-derived strain, National Laboratories, St. Louis, Mo.) of either sex were killed by decapitation under light ether anesthesia. The brains were removed immediately and freed of blood and meninges. All subsequent operations were carried out at 4°C. The brain tissue was homogenized in modified Bucher's phosphate medium (21), pH 7.4, using an all-glass homogenizer with a loose-fitting Teflon pestle. After four plunges, the 30% homogenate was centrifuged at 1000 *g* for 10 min to remove cellular debris. The 1000 *g* cell-free fraction, the crude mitochondrial fraction, the microsomal fraction, and the 105,000 *g* supernatant fraction of adult rat brain were prepared by methods previously developed in this laboratory (22). Each fraction contained 10–20 mg of protein and 1.5–2 mg of cholesterol.

Incubation conditions

Each incubation mixture contained 3.5 ml of cell-free homogenate or the subcellular fraction equivalent of one adult rat brain. The radioactive substrate dissolved in 50 μl of acetone was added with the aid of a micropipette at the start of the incubation. The cofactor solution in 1.0 ml of phosphate buffer contained an NADPH-generating system consisting of glucose-6-phosphate (60 μmoles) and NADP (6 μmoles), plus 10 mM β-mercaptoethylamine as an antioxidant. Incubations were carried out under normal atmospheric conditions at 37°C in 50-ml Erlenmeyer flasks. Heat-treated controls were prepared by heating the incubation protein mixtures on a steam bath for 20 min. Streptomycin sulfate (2 mg) and penicillin G salt (12,000 units) were included in incubations exceeding the 90-min time period.



I. Cholesterol-5α,6α-epoxide II. Cholestane-3β,5α,6β-triol

Fig. 1.

The incubation was terminated by the addition of 20 ml of methanol, and the precipitate was removed by centrifugation. The aqueous methanolic supernate was extracted with a mixture of 2 vol of chloroform and 0.1 vol of 0.1% potassium chloride solution, and the mixture was allowed to partition overnight in the cold room. The lower chloroform phase was removed and evaporated under N_2. The residue was reconstituted in ethyl acetate, and aliquots were removed for assay of the radioactive metabolites after their separation by TLC on Chromar AR 500 glass fiber sheets. 95–97% of the total radioactivity was recovered in the final incubation extracts.

Chromatographic methods

TLC was carried out using Chromar AR 500 and AR 1000 glass fiber sheets (Mallinckrodt Chemical Co., St. Louis, Mo.). Solvent systems for the separation of neutral steroids consisted of trimethylpentane–methyl ethyl ketone in the following ratios: S-1, 80:20 (v/v), and S-2, 60:40 (v/v). Reference steroids were detected as fluorescent zones after spraying the chromatograms with 8-hydroxy-1,3-pyrene trisulfonic acid sodium salt (23), 5 mg in 100 ml of methanol, and viewing under long-wave UV light.

GLC was carried out on a Barber-Colman model 5000 gas chromatograph equipped with a radioactive monitoring system (Barber-Colman model 5190). Separations of neutral steroid metabolites were made using a 2-m U-shaped column (ID 4 mm) packed with either 3% QF-1 or 3% OV-17 on Gas-Chrom Q (100–120). For isothermal separation on 3% QF-1, the column temperature was 250°C; for 3% OV-17 the column temperature was 275°C. The radioactivity monitoring system was used for detection of labeled reference sterols and unknown metabolites. The hydrogen flame detector was used for detection of unlabeled reference sterols. All relative retention times

				Front
20,200	32,300	20,200	17,900	1
690	2,500	1,510	820	
180	260	340	140	
15,700	8,160	260	24,600	2
200	180	240	120	
2,300	280	140	270	3, 4
320	50	50	60	
110	50	40	50	
129	118	100	150	5
150	140	130	180	Origin
A	B	C	D	

Fig. 2. Chromar AR 500 TLC chromatogram showing the distribution of radioactivity (dpm) recovered after 90-min incubations of [4-^{14}C]cholesteryl palmitate (1 μCi, 20 nmoles) with adult rat brain 12,000 g supernatant fraction prepared in modified Bucher's phosphate medium, pH 7.4, under the following conditions: (*A*) fortified with NADPH-generating system and 10 mM β-mercaptoethylamine, (*B*) incubation without NADPH-generating system, (*C*) control incubation with heat-treated protein and NADPH-generating system, and (*D*) incubation with NADPH-generating system and 1 mM EDTA. Reference standards are as follows: (*1*) cholesteryl palmitate, (*2*) cholesterol, (*3*) cholesterol-5α,6α-epoxide, (*4*) 7-ketocholesterol, and (*5*) cholestane-3β,5α,6β-triol. Solvent system was trimethylpentane–methyl ethyl ketone 80:20 (S-1).

1.5 hr	5 hr	18 hr	
			Front
30,500	44,000	32,200	1
1,550	2,700	1,800	
21,000	56,700	21,400	2
340	380	420	
2,500	1,700	500	3, 4
160	3,000	570	
260	510	280	
290	2,100	1,700	5
280	620	220	Origin

Fig. 3. Incubation of [4-^{14}C]cholesteryl palmitate over extended time periods. Chromar AR 500 TLC chromatogram shows the distribution of radioactivity (dpm) recovered from individual incubations of [4-^{14}C]cholesteryl palmitate (1 μCi, 20 nmoles) for various periods of time with the 12,000 g supernatant fraction of adult rat brain prepared in modified Bucher's phosphate medium, pH 7.4. Each incubation was fortified with an NADPH-generating system and 10 mM β-mercaptoethylamine. Reference sterols are as follows: (*1*) cholesteryl palmitate, (*2*) cholesterol, (*3*) 7-ketocholesterol, (*4*) cholesterol-5α,6α-epoxide, and (*5*) cholestane-3β,5α,6β-triol. The solvent system was S-1.

are referred to cholesterol = 1.00. Absolute retention times of cholesterol were: QF-1, 4.4 min; OV-17, 4.0 min.

Assay of radioactive metabolites

Radioactive metabolites, after separation on Chromar AR 500 TLC, were characterized by sectioning chromatographic zones corresponding to reference sterols and placing the cut sections directly in counting vials containing 15 ml of Omnifluor scintillation fluid (New England Nuclear). All radioactive measurements were made using an Ansitron liquid scintillation counter. Efficiency for ^{14}C was approximately 80%.

RESULTS

Incubation of [4-^{14}C]cholesteryl palmitate

Individual 90-min incubations of [4-^{14}C]cholesteryl palmitate (1 μCi, 20 nmoles) with the 12,000 g supernatant fraction of adult rat brain under various incubation conditions are shown in Fig. 2. In the presence of an NADPH-generating system and 10 mM β-mercaptoethylamine (Fig. 2, *A*), 40–50% of the ^{14}C on the chromatogram was free cholesterol and 2–5% of the ^{14}C existed as oxidized metabolites of intermediate polarity having the same mobility (on Chromar AR 500 TLC) as 7-ketocholesterol. In the absence of the NADPH-generating system (Fig. 2, *B*), both sterol ester hydrolysis and the formation of polar oxidized metabolites were inhibited. In the heat-treated control incubation (Fig. 2, *C*), about 88% of the ^{14}C was recovered unchanged as [4-^{14}C]cholesteryl palmitate. The addition of 1 mM EDTA to the incubation (Fig. 2, *D*) in place of 10 mM β-mercaptoethylamine stimulated sterol ester hydrolysis without the formation of more polar, oxidized cholesterol metabolites.

Incubation of [4-^{14}C]cholesteryl palmitate for periods of up to 5 hr in duration (Fig. 3) resulted in production of oxidized products of both intermediate polarity (1–3%) and greater polarity (1–2%). Extension of the incubation periods to 18 hr resulted in production of only cholesterol metabolites of greater polarity (1–3%) having the same mobility on Chromar AR 500 TLC as cholestane-3β,5α,6β-triol. In each case, the extension of the incubation periods had little effect on the hydrolysis of cholesteryl palmitate to free cholesterol. Under similar and extended incubation conditions with the cell-free and 12,000 g supernatant fractions of adult rat brain, free [4-^{14}C]cholesterol was recovered unchanged from the incubation mixtures. However, in the absence of either 1 mM EDTA or 10 mM β-mercaptoethylamine, some nonspecific, nonenzymatic autoxidation of [4-^{14}C]cholesterol was observed.

Identification of metabolites

The extracts from at least 12 separate incubations for each time period were combined, and the polar products resulting from cholesterol oxidation were separated by preparative TLC. These metabolites were then characterized by GLC combined with a radioactivity monitoring system. Cholestane-3β,5α,6β-triol was identified as the only major cholesterol metabolite eluted from the preparative TLC chromatogram zone of the polar metabolites from the combined extracts of the 18-hr incubations of [4-^{14}C]cholesteryl palmitate.

Examination of relative retention data for radioactive metabolites eluted from the 7-ketocholesterol region of the preparative TLC chromatogram of the combined 90-min incubation extracts of [4-^{14}C]cholesteryl palmitate indi-

cated a total absence of 7-ketocholesterol. The radioactive metabolite gave a single symmetrical peak on GLC radiochromatograms with a relative retention time on both QF-1 (RRT = 1.92) and OV-17 (RRT = 1.47) columns corresponding to cholesterol-5α,6α-epoxide (RRT 1.90, 1.47, respectively). Reexamination of the 7-ketocholesterol region (containing less than 1% of total radioactivity) recovered from the 18-hr incubation extracts indicated a 1:1 mixture of two metabolites with RRTs (QF-1 = 1.92, 3.90; OV-17 = 1.47, 2.14) corresponding to reference cholesterol-5α,6α-epoxide (RRT of QF-1, 1.92; OV-17, 1.47) and 7-ketocholesterol (QF-1, 3.90; OV-17, 2.14). Examination of the dihydroxy sterol regions from the preparative TLC extracts recovered from both 90-min and 18-hr incubations gave no indication of the presence of epimeric 7α- or 7β-hydroxycholesterols in these fractions.

Final identifications of cholesterol-5α,6α-epoxide (Table 1) and cholestane-3β,5α,6β-triol (Table 2) were made by cocrystallization to constant specific activity after dilution with the authentic sterols. Of the total radioactivity eluted from the cholesterol epoxide region of the preparative TLC chromatogram, approximately 60% cocrystallized as the α-epoxide isomer. The remaining radioactivity may possibly consist of the β-epoxide isomer which resisted cocrystallization to constant specific activity even after formation of the chlorohydrin benzoate ester derivatives. Cholesterol-5β,6β-epoxide is known to have the ability to form mixed crystalline complexes (24). The β-epoxide epimer is thought to be present because only one radioactive metabolite was detected on 3% OV-17, 3% QF-1, and 3% SE-30 GLC columns, which unfortunately failed to resolve satisfactorily a mixture of cholesterol α- and β-epoxide epimers as the free steroids and because cholestane-3β,5α,6β-triol, isolated as the only metabolite over extended incubation periods, is formed from both α- and β-epoxide precursors in vitro in rat liver preparations (5).

TABLE 1. Identification of cholesterol-5α,6α-epoxide by reverse isotope dilution

Solvent System	Recrystallization No.	Amount	Specific Activity
		mg	*dpm/mg*
	0	100	1380
Methanol–water	1	93	838
	2	77	858
Acetone–water	3	65	849
	4	51	835
Cocrystallization of chlorohydrin monobenzoate derivative			
(Calculated value)	0	66	619
Hexane–ethylacetate	5	54	573
	6	42	619
Hexane	7	22	608

Unlabeled cholesterol-5α,6α-epoxide (100 mg) was added to an aliquot, containing 1.38 × 10^5 dpm, eluted from the cholesterol epoxide region of the preparative TLC chromatogram of the combined extracts of the 90-min incubation of [4-^{14}C]cholesteryl palmitate. After four recrystallizations, the chlorohydrin monobenzoate derivative (3β-benzyl,5α-hydroxy-6β-chlorocholestane) was prepared using benzoylchloride–pyridine.

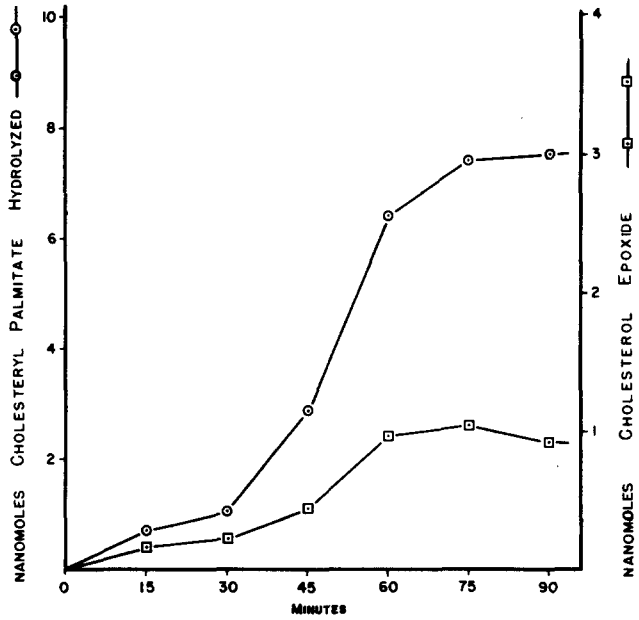


Fig. 4. In vitro incubation of [4-^{14}C]cholesteryl palmitate. Each point on the curve represents the percentage distribution of total radioactivity in the free cholesterol and cholesterol epoxide chromatogram zones recovered in the extracts from individual incubations of [4-^{14}C]cholesteryl palmitate (1 μCi, 20 nmoles) with the 12,000 *g* supernatant fraction of adult rat brain for various periods of time. Each incubation mixture (3.5 ml) contained the supernatant protein, equivalent to one adult rat brain, which was prepared in modified Bucher's phosphate medium, pH 7.4, and fortified with an NADPH-generating system and 10 mM β-mercaptoethylamine. Incubation extracts were assayed on Chromar AR 500 glass fiber sheets with solvent system S-1.

Time course and subcellular distribution

Fig. 4 shows the oxidation of [4-^{14}C]cholesteryl palmitate by the 12,000 *g* supernatant fraction followed over a period of 90 min. The formation of cholesterol epoxides parallels the hydrolysis of [4-^{14}C]cholesteryl palmitate to free cholesterol, indicating an initial slow induction period followed by a rapid increase in both hydrolysis and epoxide formation in the 30- to 60-min time period. After 90 min, sterol ester hydrolysis and epoxide formation ceased, and only the slow conversion of cholesterol epoxide to cholestane-3β,5α,6β-triol was observed. The presence of

TABLE 2. Identification of cholestane-3β,5α,6β-triol by reverse isotope dilution

Solvent System	Recrystallization No.	Amount	Specific Activity
		mg	*dpm/mg*
	0	100	1220
Methanol–water	1	89	1110
	2	74	1100
	3	55	1160
Acetone–water	4	34	1140
	5	24	1130

Unlabeled cholestane-3β,5α,6β-triol (100 mg) was added to an aliquot, containing 1.22 × 10^5 dpm, eluted from the cholestane-3β,5α,6β-triol region after preparative TLC of the combined extract of the 18-hr incubations of [4-^{14}C]cholesteryl palmitate.

TABLE 3. Subcellular localization of cholesterol-5,6-epoxidase activity in adult rat brain

Subcellular Fraction	Free Cholesterol (% hydrolysis)	Cholesterol-5,6-epoxides
12,000 *g* supernate	48.0	3.6
Mitochondrial fraction	4.7	1.0
Microsomes	39.7	4.4
105,000 *g* supernate	5.2	1.2
Microsomes + 105,000 *g* supernate	59.5	5.2

Values are percentages of radioactivity recovered in the free cholesterol and cholesterol-5,6-epoxide regions on the Chromar AR 500 TLC chromatogram. Each subcellular fraction, equivalent to one adult rat brain, was prepared in modified Bucher's medium and incubated for 90 min with [4-^{14}C]cholesteryl palmitate (1 μCi, 20 nmoles) in the presence of an NADPH-generating system containing 10 mM β-mercaptoethylamine. Values are averages of duplicate incubations. Solvent system S-1 was used for the TLC separations.

cholesterol epoxides or cholestane-3β,5α,6β-triol was not observed after an examination of the unhydrolyzed ester fraction after mild alkaline hydrolysis, which indicated that only free cholesterol had been oxidized during the course of the incubation.

The subcellular distribution of both cholesterol ester hydrolase and cholesterol-5,6-epoxidase activity is shown in Table 3. The bulk of the cholesterol ester hydrolase and cholesterol epoxidase enzyme activity was observed in the microsomal and the reconstituted microsomal and soluble fractions of adult rat brain.

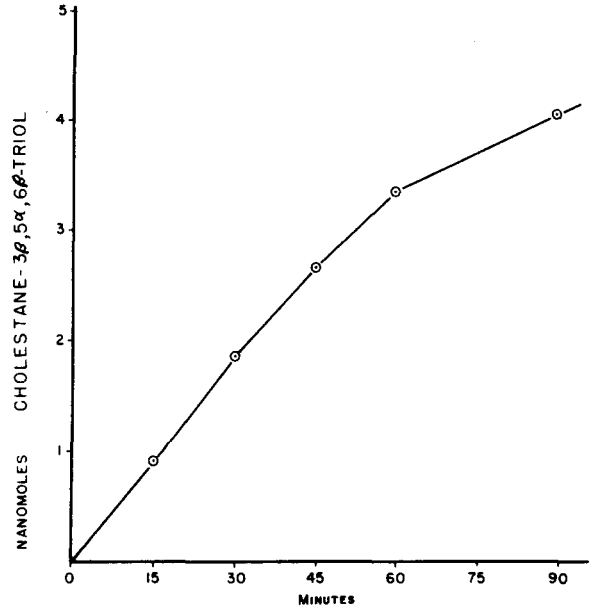


Fig. 5. In vitro incubation of [4-^{14}C]cholesterol-5α,6α-epoxide. Each point on the curve is based on the assays of the percentage distribution of total radioactivity, in the cholestane-3β,5α,6β-triol chromatogram zone, recovered from individual incubations of cholesterol-5α,6α-epoxide (1 μCi, 20 nmoles) with the 12,000 *g* supernatant fraction of adult rat brain for various periods of time. Each incubation mixture (3.5 ml) contained the supernatant protein, equivalent to one adult rat brain, which was prepared in modified Bucher's phosphate medium, pH 7.4. No cofactors were added. Incubation extracts were assayed on Chromar AR 500 glass fiber sheets with solvent system S-2.

TABLE 4. Subcellular localization of cholesterol-5α,6α-epoxide hydrolase activity in adult rat brain

Subcellular Fraction	Cholestane-3β,5α,6β-triol
12,000 *g* supernate	22.0
Microsomes	17.8
105,000 *g* supernate	7.0

Values are percentages of radioactivity recovered in the cholestane-3β,5α,6β-triol region of the Chromar AR 500 TLC chromatogram. Each subcellular fraction, equivalent to one adult rat brain, was prepared in modified Bucher's phosphate medium and incubated for 90 min with [4-^{14}C]cholesterol-5α,6α-epoxide (1 μCi, 20 nmoles). Values are averages of duplicate incubations.

Incubation of [4-^{14}C]cholesterol-5α,6α-epoxide

Fig. 5 shows the conversion of [4-^{14}C]cholesterol-5α,6α-epoxide (1 μCi, 20 nmoles) to cholestane-3β,5α,6β-triol by the cell-free preparations of adult rat brain followed over a 90-min incubation. Hydrolysis of the cholesterol α-epoxide epimer to the 5α,6β-*trans* glycol functional group of cholestane-3β,5α,6β-triol was almost linear throughout the incubation time. Hydrolysis of the epoxide occurred in the absence of cofactors and did not occur in control incubations with heat-treated cell-free protein preparations. Cholestane-3β,5α,6β-triol was identified as the only metabolite of the incubation of [4-^{14}C]cholesterol-5α,6α-epoxide by TLC, by relative retention data on GLC combined with a radioactivity monitoring system, and finally by cocrystallization to constant specific activity after dilution with authentic sterol.

Table 4 shows the distribution of cholesterol-5α,6α-epoxide hydrolase activity in the microsomal and soluble fractions of adult rat brain. The epoxide hydrolase activity was observed mainly in the microsomal subcellular fraction, although significant activity was also noted in the soluble fraction.

DISCUSSION

The present experiments indicate that the 12,000 *g* supernatant fraction of adult rat brain, fortified with an NADPH-generating system, has the capacity to oxidize [4-^{14}C]cholesteryl palmitate to a mixture of more polar, oxygenated steroids identified as cholesterol-5α,6α-epoxide and cholestane-3β,5α,6β-triol. Free [4-^{14}C]cholesterol incubated under similar conditions was not oxidized. The evidence suggests that the formation of the oxidized products may possibly be dependent on the hydrolysis of cholesteryl palmitate to free cholesterol by the microsomal fraction where subsequent oxidation could occur perhaps via a microsomal NADPH-dependent lipid peroxidation mechanism leading to the formation of cholesterol α- and possibly β-epoxides. The resulting sterol epoxides are then cleaved by a microsomal sterol epoxide hydrolase to yield cholestane-3β,5α,6β-triol as the only metabolite. Although the exact role of the sterol ester in the reaction mechanism or reaction sequence was not determined, the evidence suggests that only esterified cholesterol can reach the site

where microsomal oxidation of cholesterol may occur. These findings suggest that sterol esters may possibly play an important role in cholesterol turnover in brain especially when there are abnormal increases of esterified cholesterol in a variety of pathological demyelinating conditions (25, 26).

The in vitro formation of cholestane-3β,5α,6β-triol as a product of the oxidative degradation of cholesterol was previously demonstrated in rat liver subcellular fractions fortified with an NADPH-generating system (5, 6). These investigations demonstrated that triol formation was inhibited under conditions where NADPH-dependent microsomal lipid peroxidation was inhibited by the addition of either EDTA or β-mercaptoethylamine. In the present experiments, the addition of 10 mM β-mercaptoethylamine stimulated cholesterol epoxide formation in rat brain preparations fortified with NADPH while supressing the formation of 7-ketocholesterol and other nonspecific autoxidation products. Addition of EDTA stimulated ester hydrolysis and completely inhibited epoxide formation.

The in vitro conversion of [4-^{14}C]cholesterol-5α,6α-epoxide to cholestane-3β,5α,6β-triol by the 12,000 *g* supernatant fraction of adult rat brain was demonstrated in the present experiments. The *trans-triol* was the only product isolated from the reaction catalyzed by a steroid epoxide hydrolase, located mainly in the rat brain microsomes. The possible precursor–product relationship between cholesterol epoxides and the biosynthesis of cholestane-3β,5α,6β-triol have recently been demonstrated in rat liver subcellular fractions (5). Only a few examples of steroid epoxidases and steroid epoxide hydrolases in mammalian systems have been reported. The 11β-hydroxylating system of bovine adrenals was shown to form small amounts of 9β,11β-epoxide from 17,21-dihydroxypregna-4,9-diene-3,20-dione (27). The 16α,17α-epoxidation of unsaturated estrogens and the subsequent epoxide cleavage were demonstrated in rat liver (28). The formation of steroid epoxides is an important metabolic transformation in microorganisms (29) in which the mechanism of epoxidation is apparently closely related to the mechanism of steroid axial hydroxylations (30, 31). In general, epoxide intermediates have been shown to play an important role as microsomal metabolites of polycyclic hydrocarbons in many different mammalian tissues (32). The enzymatic oxidation of olefins to glycols in rat liver was also shown to occur through the formation of epoxide intermediates which were rapidly hydrolyzed by a microsomal epoxide hydrolase in the absence of added cofactors (33).

Only recently, the isolation and identification of cholesterol-5α,6α-epoxide and other trace sterols in human serum was reported (34). In this latter investigation, higher levels of cholesterol-α-epoxide were found in patients with varying degrees of hypercholesterolemia and atherosclerosis than in normal subjects. Cholestane-3β,5α,6β-triol was not isolated. This was apparently the first reported isolation of cholesterol-α-epoxide from a natural source. The metabolic fate of ingested cholesterol-α-epoxide in the rat was investigated (35, 36). The toxic effects of dietary cholesterol-α-epoxide were attributed to its subsequent conversion to cholestane-3,5,6-triol in the gastrointestinal tract of the rat (36).

Cholestane-3β,5α,6β-triol, a companion sterol of cholesterol, has been isolated as a trace sterol from a wide range of natural sources including ox liver, pig testicles, atherosclerotic human aorta, and human brain tissue (2, 3, 37). Cholestane-3β,5α,6β-triol was reported to constitute 0.5% of the total neutral sterols isolated from human brain tissue under conditions that precluded the autoxidation of cholesterol (38). Since the triol has been isolated and identified as a trace product of cholesterol autoxidation under a variety of conditions including photolysis, irradiation, and air oxidation (4), the biosynthetic origin of this polyhydroxy sterol from brain tissue has remained uncertain. We believe that the present data demonstrate that the formation of the triol is a true biosynthetic phenomenon, and we must accordingly take issue with Boyd's concept (5) that the production of the 5α,6β-diol is "nonenzymatic," even though, paradoxically, requiring protein and an NADPH-generating system.

The physiological significance of the in vitro demonstration of cholestane-3β,5α,6β-triol biosynthesis in rat brain in relationship to cholesterol turnover in brain tissue cannot be fully evaluated at this time. Cholestane-3β,5α,6β-triol is known to have marked hypocholesterolemic and antiatherogenic properties (39, 40). In rat liver the triol has been shown to inhibit cholesterol biosynthesis by interfering with enzymatic demethylation at C-4 (41), and it is metabolized to 3β,5α,6β-trihydroxycholanoic acid (42). Whether cholestane-3β,5α,6β-triol can exert similar inhibitory properties during brain cholesterol biosynthesis, either in vivo or in vitro, or may undergo further side-chain cleavage to form acidic steroid metabolites in brain tissue, will require further investigation.

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