Bile acid synthesis in rat liver peroxisomes: metabolism of 26-hydroxycholesterol to 3β-hydroxy-5-cholenoic acid[1]

Skaidrite K. Krisans,[2,*] Sara L. Thompson,[*] Louis A. Pena,[*] Engeline Kok,[†] and Norman B. Javitt[†]

Department of Biology and Molecular Biology Institute, San Diego State University, San Diego, CA 92182,[*] and Division of Hepatic Diseases, New York University Medical Center, 550 First Avenue, New York, NY 10016[†]

Abstract Rat liver peroxisomes have been found to oxidize 26-hydroxycholesterol, the product of cholesterol C-26 hydroxylation to 3β-hydroxy-5-cholenoic acid. Peroxisomes were purified by differential and equilibrium density centrifugation in a steep linear metrizamide gradient to greater than 95% purity. Purity of peroxisomes was determined by measurement of specific marker enzymes. The activities of cytochrome oxidase (a mitochondrial marker) and acid phosphatase (a lysosomal marker) in the purified peroxisome fractions were below the level of detection. Esterase activity indicated a 2–4% microsomal contamination. Subsequent to incubation of peroxisomes with [16,22-^{3}H]-26-hydroxycholesterol, the reaction products were extracted, methylated, acetylated, and subjected to thin-layer, high pressure liquid, and gas–liquid chromatographic analyses. 3β-Hydroxy-5-cholenoic acid was the major identifiable metabolite of 26-hydroxycholesterol. Incubations of pure microsomal fractions (>99%) with 26-hydroxycholesterol under the same conditions demonstrated that the production of 3β-hydroxy-5-cholenoic acid by peroxisomes was not attributable to microsomal contamination. This study demonstrates that peroxisomes participate in the side-chain oxidation of intermediates in bile acid synthesis. —**Krisans, S. K., S. L. Thompson, L. A. Pena, E. Kok, and N. B. Javitt.** Bile acid synthesis in rat liver peroxisomes: metabolism of 26-hydroxycholesterol to 3β-hydroxy-5-cholenoic acid. *J. Lipid Res.* 1985. **26:** 1324–1332.

Supplementary key words bile acid synthesis

Until recently, the conversion of cholesterol and its derivatives to bile acids has been assumed to involve only enzymes of the endoplasmic reticulum (ER), the soluble compartment of the cell, and the mitochondria. The two accepted pathways include the major ER-initiated pathway in which alterations in the steroid nucleus by ER and soluble enzymes are followed by the hydroxylation of the C-26 by either ER or mitochondrial hydroxylases and then the side-chain oxidation in mitochondria to produce a C-24 bile acid (2–7). The second is believed to be a minor pathway in which C-26 hydroxylation and side-chain oxidation of cholesterol by mitochondria precedes steroid hydroxylations (8–10). Enriched peroxisomal fractions have recently been shown to convert 3α,7α,12α-trihydroxy-5β-cholestanoic acid, a major intermediate in the ER-initiated pathway, to cholic acid in the rat (11, 12). We here propose that peroxisomes also have a role in the cholesterol side-chain oxidation pathway. In a previous study (13), we showed that peroxisomal fractions (70–75% pure) can oxidize cholesterol to an unidentified bile acid and propionic acid, the expected 3-carbon fragment produced in both of the above-mentioned pathways. In this study, we demonstrate the ability of highly purified peroxisomes (96–98% pure) to oxidize 26-hydroxycholesterol, the product of the 26-hydroxylation of cholesterol, to a C-24 bile acid, 3β-hydroxy-5-cholenoic acid.

26-Hydroxycholesterol was chosen as the substrate for these studies over cholesterol and its other derivatives for several reasons. First, we were interested in following up our previous study to determine whether pure peroxisomes were able to degrade cholesterol to a bile acid via a pathway in which side-chain oxidation precedes changes in the steroid nucleus. In the course of this study, using a peroxisomal-enriched fraction, metrizamide (employed in purifying peroxisomes) was found to be extremely inhibitory to the enzymatic reactions when cholesterol was used as a substrate and much less inhibitory when 26-hydroxycholesterol was employed as substrate. This indicates that the 26-hydroxylase is the enzyme inhibited by metrizamide. Second, we found that 26-hydroxycholes-

Abbreviations: ER, endoplasmic reticulum; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; GLC, gas–liquid chromatography.

[1]An abstract of these results has been published in reference 1.

[2]To whom correspondence should be addressed.

terol had a much higher conversion rate to acid-extractable material than did cholesterol, thus providing more sample for product identification studies.

Our study clearly demonstrates that side-chain oxidation of 26-hydroxycholesterol is an important function of rat liver peroxisomes.

EXPERIMENTAL PROCEDURES

Materials

[16,22-^{3}H]-26-Hydroxycholesterol was synthesized from kryptogenin (cholest-5-ene-3β, 26-diol-16,22 dione) as previously described (10). 3β-Hydroxy-5-[1,2-^{3}H]cholenoic acid was synthesized as described by Kok et al. (14). 3β-Hydroxy-5-cholestenal was prepared from 26-hydroxycholesterol by oxidation with pyridinium chlorochromate in dichloromethane. The products were separated by preparative thin-layer chromatography (TLC) and the identity of 3β-hydroxy-5-cholestenal was verified by mass spectrometry. 3β-Hydroxy-5-cholestenoic acid was a gift from Dr. Marcel Gut at the Worcester Institute. [Carboxyl-^{14}C]lithocholic acid (3α-hydroxy-5β-cholanoic acid) was purchased from New England Nuclear. The methyl ester of 3β-hydroxy-5-cholenoic acid was purchased from Steraloids. Other biochemicals were purchased from Sigma Chemical Co.

Animals

Male Sprague-Dawley rats (150–180 g), maintained on a stock laboratory diet, were used. Rats were fasted overnight before being killed by decapitation.

Preparation of rat liver peroxisomes

Rat liver homogenate was fractionated by differential centrifugation (15) to prepare a λ fraction that is enriched with peroxisomes and also contains smaller mitochondria and is similar to the L fraction of de Duve et al. (16). The λ fraction was then further fractionated by centrifugation in a steep linear metrizamide gradient (20–50%, w/w) (17). Routinely, 6 ml of the λ fraction, prepared from three to six rat livers, was loaded on top of a 27-ml linear metrizamide gradient containing a 0.5-ml 50% w/w metrizamide cushion. The gradient was spun in a Sorvall OTD 75B centrifuge using a TV 850 ultra-vertical rotor at 40,000 rpm for 60 min at 8°C. A total of 25–30 fractions was collected with a Densi-Flow II fraction collector, Buchler Instruments.

Preparation of rat liver microsomes

Rat liver homogenate was fractionated by differential centrifugation according to the method of de Duve et al. (16). The microsomal fraction was then further fractionated in a linear metrizamide gradient (20–35%, w/w). The gradient was centrifuged as described for the preparation of rat liver peroxisomes. The purity of the resulting microsomal preparation, as determined by marker enzyme analysis (see following section), was >99%.

Assay of marker enzymes

Catalase (a peroxisomal marker) and cytochrome oxidase (a mitochondrial marker) activities were measured as previously described (15, 18) except that molar absorptivity of 19 mM^{-1} cm^{-1} for cytochrome c was used (19). Esterase (a microsomal marker) activity was measured according to Beaufay et al. (20). Acid phosphatase (a lysosomal marker) activity was measured according to Bergmeyer, Gawehn, and Grassl (21). Enzyme units were expressed as μmol/min except for catalase which was expressed in the units used by Leighton et al. (15). Protein was determined by the method of Lowry et al. (22) using bovine serum albumin as a standard. Since metrizamide interferes with the Lowry reaction, the gradient samples were first precipitated by the addition of 10% TCA (trichloroacetic acid), redissolved in water, and the protein was then determined.

Incubation, extraction, and chromatographic procedures

The reaction conditions for both peroxisomal and microsomal incubations are based on those described by Hagey and Krisans (13). The final reaction mixture of 0.2 ml contained 150 mM Tris-HCl buffer (pH 8.5), 4.2 mM ATP, 0.4 mM NAD, 3.2 mM glutathione, 2.5 mM $Mg(NO_3)_2$, 3.5 mM sodium citrate, 1.4 mM isocitrate, 10 mg/ml of bovine serum albumin, 3.5 μM [16,22-^{3}H]-26-hydroxycholesterol (720 Ci/mol), and 0 to 30 μg of peroxisomal or microsomal protein diluted in 1 mM Tris-HCl (pH 7.5), containing sucrose (0.25 M) and ethanol (0.1%). The 26-hydroxycholesterol was first dissolved in 10 μl of 2.8% DMSO before addition to the reaction mixture. The samples were incubated in a shaking water bath (37°C) for 3 hr.

Unreacted 26-hydroxycholesterol was removed by the addition of 1.05 ml of 0.05 M potassium carbonate, 0.05 M boric acid buffer (pH 9.5), and a single extraction with methanol–chloroform–heptane 1.41:1.25:1.00 (v/v/v) (13). This procedure resulted in partitioning 99% of the unreacted 26-hydroxycholesterol into the lower, organic phase (as determined by partition testing) with the aqueous phase containing the product(s).

The rate of reaction was linear with protein concentration up to 30 μg in 200 ul and incubation times of up to 4 hr. Control samples in which the enzyme was added after termination of the reaction with methanol–chloroform–heptane 1.41:1.25:1.00 were routinely included. Concentrations greater than 1% of metrizamide were found to be severely inhibitory to the production of acid-extract-

able material. The metrizamide inhibition was determined using the peroxisomal-enriched fraction that was prepared in 0.25 M sucrose. The reaction mixtures were cultured for the presence of bacteria, before and after the 3-hr incubation. No growth was detected on blood agar plates.

The aqueous phases from the above incubations were removed, acidified, and extracted with diethyl ether. Less than 5% of the total radioactivity remained in the aqueous phase after the extraction. The products were methylated either with diazomethane or 2,2 dimethoxypropane (23) and separated by TLC (hexane–ethyl acetate 70:30, v/v) using silica gel H plates. Acetylation of the methyl esters was performed either with pyridine–acetic anhydride or acetic acid–acetic anhydride as described previously (24). The acetates were separated by TLC using hexane–ethyl acetate 85:15 (v/v) on silica gel G plates.

Samples for high pressure liquid chromatography (HPLC) were reduced in volume and injected on a stainless steel column (300 × 3.9 mm), packed with microPorasil (Waters Associates) rated at 3000 theoretical plates, and eluted with 7% ethyl acetate in hexane (14). Recovery of radioactivity from the HPLC column was essentially complete. For each determination, 60 fractions of 1.3 ml were collected and 0.3 ml was counted in a Beckman CPM 200 liquid scintillation counter. Gas–liquid chromatographic (GLC) analysis was performed using a 20 m × 0.32 mm I.D. fused silica capillary column containing SE-52 stationary phase, obtained from J. and W. Scientific, Davis, CA. Reverse isotope dilution studies were done in the conventional manner.

RESULTS

Characterization of purified peroxisomes

Peroxisomes were prepared by metrizamide gradient centrifugation; this type of gradient yields a better separation of organelles than does the traditional sucrose gradient. The purity of the peroxisomes was calculated by three different means based on the measurement of specific marker enzymes. As shown in **Table 1,** the relative specific activity of catalase (a peroxisomal marker) in the purified peroxisomes was 36.9. Based on the results of Leighton et al. (15) this implies 98% purity. Another means of calculating purity of organelles is to use the relative specific activities of the contaminating organelles (25). The activities of cytochrome oxidase (a mitochondrial marker) and acid phosphatase (a lysosomal marker) in the purified peroxisome fractions were below the level of detection. The only measurable contribution was from esterase (an endoplasmic reticulum marker). Since the endoplasmic reticulum constitutes about 20% of total liver protein (15, 26), we calculate a 4% contribution due to esterase (0.20 × 20% = 4.0%). If the specific activity of esterase in the purified peroxisome fraction is divided by the specific activity of esterase in the purified microsomal fraction, a 2% contamination of the peroxisomes by microsomal protein is obtained. Since the relative specific activity of catalase was 36.9, it is highly unlikely that the purified peroxisome fractions contain any significant amount of non-peroxisomal unidentified protein. Also, any solubilized enzymes that may not have been removed by the differential centrifugation steps would sediment at the low density end of the gradient, well separated from the peroxisomes after equilibrium density centrifugation. Using these criteria, all preparations of peroxisomes used in this study were between 96–98% pure.

Incubation of 26-hydroxycholesterol with rat liver peroxisomes

After incubation of rat liver peroxisomes with ^{3}H-labeled 26-hydroxycholesterol, the aqueous phase was removed, acidified, and extracted with diethyl ether. The products were methylated and separated by TLC using silica gel H plates. Two peaks were obtained as shown in **Fig. 1.** The R_f of one of the major product(s) (peak II in Fig. 1) was identical to the R_f of the methyl 3β-hydroxy-5-cholenoate standard. This co-migration of the radioactivity of peak II with 3β-hydroxy-5-cholenoic acid was evident on numerous TLC systems when analyzed as a free acid, methyl ester, or as a methyl ester acetate. The

TABLE 1. Marker enzyme composition of purified peroxisomes[a]

Fraction	Enzyme Specific Activities[b]			
	Acid Phosphatase	Catalase	Cytochrome Oxidase	Esterase
Peroxisome fraction (1)	—[c]	18.1 ± 0.23	—[c]	0.182 ± 0.006
Homogenate fraction (2)	10.0 ± 2.0	0.49 ± 0.01	0.105 ± 0.005	0.891 ± 0.239
Relative specific activity (1/2)		36.9 ± 0.34		0.200 ± 0.042

[a] All values are given as mean ± SD for three different gradients. Mean density 1.230 ± 0.004.
[b] Units/mg protein; units defined in Methods section.
[c] No measurable activity.

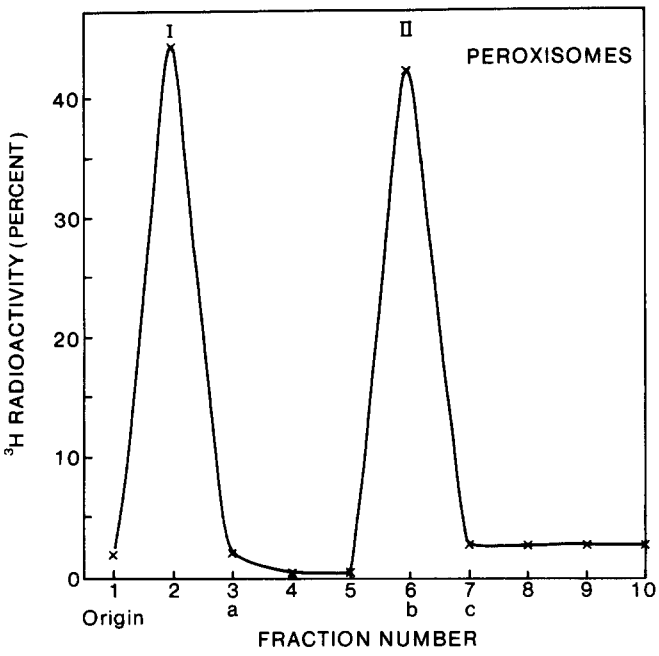

Fig. 1. Thin-layer chromatogram of the methylated product(s) from incubations of ^{3}H-labeled 26-hydroxycholesterol with purified rat liver peroxisomes as described in Experimental Procedures. The radioactivity of each of ten equal fractions was eluted and counted. The results are expressed as percent of total ^{3}H-labeled radioactivity per fraction. Reference compounds were (a) 26-hydroxycholesterol; (b) methyl 3β-hydroxy-5-cholenoate; (c) methyl 3β-hydroxy-5-cholestanoate. Solvent, hexane–ethyl acetate 70:30 (v/v).

radioactivity from peak II was eluted with methanol from the TLC plate, reduced in volume, and injected on an HPLC column as described in Methods. This HPLC system is capable of separating the methyl esters of 3β-hydroxy-5-cholenoic acid from 3α-hydroxy-5β-cholanoic acid (lithocholic acid) as well as resolving 3-keto-5β-cholanoic acid from 3β-hydroxy-5β-cholanoic acid (14). Dihydroxy and trihydroxy bile acids have very different elution volumes in this system.

Fig. 2 illustrates the results. The only tritiated compound detected on the HPLC column had the same relative elution volume to [^{14}C]lithocholate (R = 0.66) as had been determined using the authentic methyl 3β-hydroxy-5[1,2-^{3}H]cholenoate standard (14). To some samples, before the extraction step, [^{14}C]lithocholic acid had been added as an internal standard for quantification ([^{14}C]-3β-hydroxy-5-cholenoic acid is not available). Lithocholic acid serves as an excellent internal standard because throughout the extraction procedure and TLC steps it behaves in a manner identical to that of 3β-hydroxy-5-cholenoic acid. Also, as mentioned above, [^{14}C]lithocholate is clearly resolved from the putative [^{3}H]-3β-hydroxy-5-cholenoate using this HPLC system, as shown in Fig. 2.

In four different HPLC runs, taken from four different peroxisomal incubations, the ratio of ^{3}H/^{14}C activity after HPLC separation was always the same as obtained after TLC separation of the methyl esters. This indicates that all of the tritium activity in the methyl ester area after TLC separation was most likely due to the putative methyl 3β-hydroxy-5-cholenoate.

For further specificity in identification, after TLC separation, the radioactivity of peak II was eluted (including the [^{14}C]lithocholate standard), the esters were converted to the acetates with acetic acid–acetic anhydride as described in Methods, and re-chromatographed. Similarly, the putative methyl 3β-hydroxy-5-cholenoate and the methyl lithocholate standard were combined after HPLC separation, the ^{3}H/^{14}C ratios were determined, the esters were converted to acetates, and the acetates were separated by TLC. **Table 2** shows the results. In all cases the acetate ^{3}H/^{14}C ratio was similar to the methyl ester ^{3}H/^{14}C ratios, and the radioactive peaks obtained after acetylation had the same R_f as the two acetate standards. Since the acetate ^{3}H/^{14}C ratios were similar to the methyl ester ^{3}H/^{14}C ratios, this indicates that all the methyl ester area was converted to the corresponding acetate.

Final identification of peak II as methyl 3β-hydroxy-5-cholenoate was made by programmed temperature capillary column high resolution GLC. The eluate from the methyl 3β-hydroxy-5-cholenoate area after TLC separa-

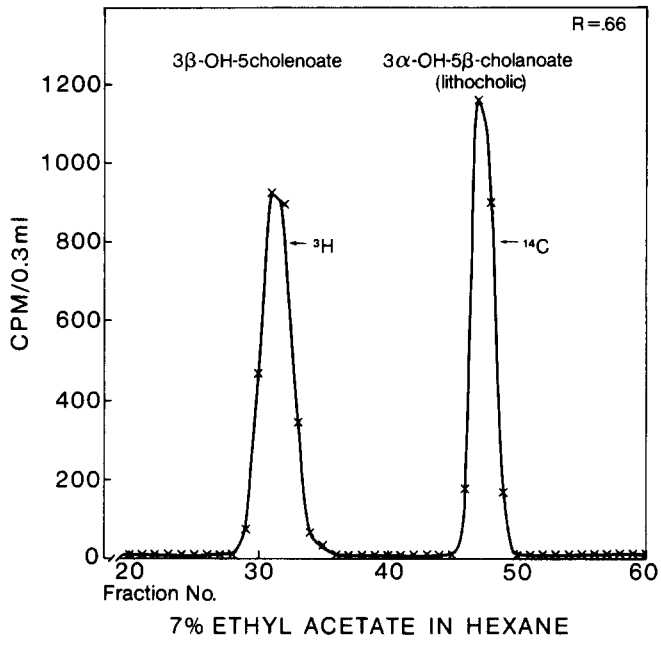

Fig. 2. High pressure liquid chromatogram of the product(s) of peak II from the thin-layer chromatogram described in Fig. 1. The bile acids were injected together as the methyl ester derivatives on a column (300 × 3.9 mm) of microPorasil using a solvent system of 7% ethyl acetate in hexane at 700 p.s.i. (flow rate, 2 ml/min). Sixty fractions of 1.3 ml were collected. The relative elution volume of the single ^{3}H-activity peak and the ^{14}C-activity peak (the methyl lithocholate internal standard) was identical to the relative elution volume of the authentic methyl 3β-hydroxy-5-cholenoate standard and the methyl 3α-hydroxy-5-cholanoate standard (methyl lithocholate).

TABLE 2. Identification of 3β-hydroxy-5-cholenoic acid as a metabolite of 26-hydroxycholesterol

Study	$^3H/^{14}C$ Ratios	
	Methyl Ester	Acetate[a]
1	0.92[b]	0.90
2	0.54[b]	0.50
3	0.58[c]	0.55
4	1.00[c]	0.97

[a] Solvent: hexane–ethyl acetate 85:15 (v/v).
[b] 3β-Hydroxy-5-cholenoate after HPLC separation.
[c] 3β-Hydroxy-5-cholenoate after TLC.

tion (including added [^{14}C]lithocholate) was pooled from a number of samples, reduced in volume, and analyzed by GLC. Two peaks were identified, one corresponding to the retention time of authentic methyl 3β-hydroxy-5-cholenoate standard, and the other peak corresponding to the retention time of authentic methyl lithocholate standard. Confirmation of the identity of methyl 3β-hydroxy-5-cholenoate was obtained by recrystallization with added nonradioactive standard. No reduction in calculated specific activity occurred. **Table 3** shows the results.

After the first partition with methanol–chloroform–heptane 1.41:1.25:1.00, before extraction, the aqueous phase from several peroxisomal incubations was initially hydrolyzed for 3 hr with 1.25 N NaOH at 1 atmos. pressure. The distribution of radioactive metabolites on TLC and HPLC was identical to that of the non-hydrolyzed samples, indicating the absence of conjugated bile acids.

A second large peak of radioactivity was consistently found near the origin (peak I, Fig. 1). To determine whether this compound was a metabolite of 3β-hydroxy-5-cholenoic acid, studies were performed whereby nonradioactive 3β-hydroxy-5-cholenoic acid was added to the standard incubation mixture containing peroxisomes. There was no difference in the relative quantities of radioactive 3β-hydroxy-5-cholenoic acid produced or in the amount of the ^{3}H-labeled unknown metabolite. This implies that the unknown compound(s) is not a metabolite of 3β-hydroxy-5-cholenoic acid.

In an attempt to identify this product(s), the area was eluted and a portion of the radioactive sample was hydrolyzed and re-extracted with acid. Part of the remaining sample was left as the methylated product(s), and a portion of the methylated product(s) was acetylated. All three samples, the acid form, the methylated form, and the acetylated form, were then analyzed by TLC and compared to a number of standards. Based on these studies, lithocholic, chenodeoxycholic, and cholic acid as well as 3β-hydroxy-5-cholestenal and 3β-hydroxy-5-cholestenoic acid were excluded as possible metabolites as their standards had different R_f values than the unknown metabolite(s).

The unknown metabolite did not extract into ether from an alkaline solution, but did after acidification, implying the presence of a carboxyl group. A shift occurred in the R_f from 0.50 to 0.71 after acetylation, implying the presence of at least one hydroxyl group, presumably the 3β-hydroxyl group. Since the R_f was much less than that of either the C-24 or C-27 monohydroxy methyl esters, we assumed that other functional groups had been added. In view of the formation of 3β-hydroxy-5-cholenoic acid, it is reasonable to propose that the unidentified compound is an intermediate with either a hydroxyl or a keto function on the side chain, as is known to occur during β-oxidation of fatty acids. No further attempt was made to identify this compound.

Incubation of 26-hydroxycholesterol with rat liver microsomes

Since the only measurable contaminant of the purified peroxisomal fraction in our study was microsomal (2–4%), we wanted to verify that the microsomes were not responsible for the formation of 3β-hydroxy-5-cholenoic acid by our peroxisomal samples. Purified microsomal fractions (99% pure) were incubated with 26-hydroxycholesterol and the products were analyzed as described for the peroxisomes. **Fig. 3** illustrates a thin-layer chromatogram of the methylated product(s) from the microsomal incubation extract. One large peak of ^{3}H-radioactivity was observed, similar to the peak I of Fig. 1. No radioactive product peak was found in the methyl 3β-hydroxy-5-cholenoate area. However, since the initial conversion of 26-hydroxycholesterol to acid-extractable radioactivity was much higher in the microsomes than in the peroxisomes (**Table 4**), the activity obtained in the methyl 3β-hydroxy-5-cholenoate area from the microsomal incubation was acetylated and compared to the peroxisomes. Table 4 summarizes the results. The calculated specific activity in the 3β-hydroxy-5-cholenoate area after acetylation of the microsomal sample was 5.0 pmol/min per mg versus 13.8 pmol/min per mg in the peroxisomal sample. Clearly, the activity in the peroxi-

TABLE 3. Identification of methyl 3β-hydroxy-5-cholenoate by reverse isotope dilution

Compound	Specific Activity
	dpm/mg
Methyl 3β-hydroxy-5-cholenoate[a]	
Crude	680
First crystallization[b]	688
Second crystallization[b]	690
Third crystallization[c]	631

[a] Methyl 3β-hydroxy-5-cholenoate (50 mg) was added to radioactivity obtained from peak II, Fig. 1.
[b] Crystallization in methanol.
[c] Crystallization in acetone.

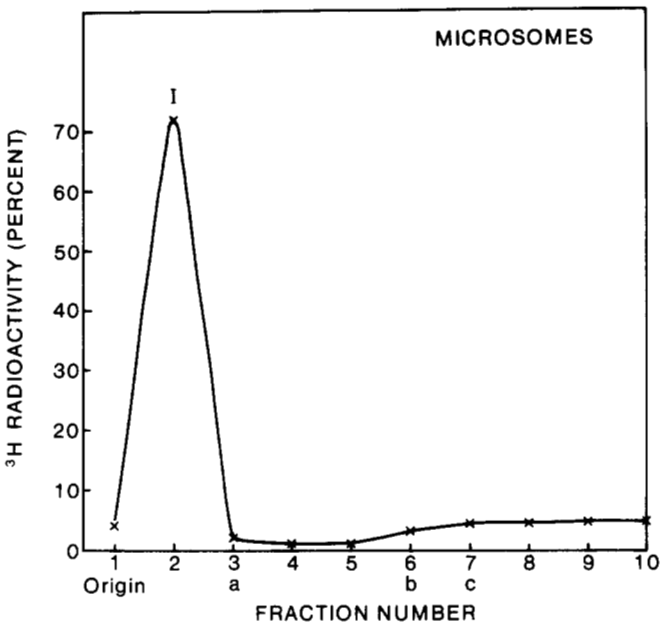

Fig. 3. Thin-layer chromatogram of the methylated product(s) from incubations of ^{3}H-labeled 26-hydroxycholesterol with purified rat liver microsomes as described in Experimental Procedures. The radioactivity of each of ten equal fractions of the chromatogram was eluted and counted. The results are expressed as percent of total ^{3}H-radioactivity per fraction. The reference compounds were (a) 26-hydroxycholesterol; (b) methyl 3β-hydroxy-5-cholenoate; (c) methyl 3β-hydroxy-5-cholestenoate. Solvent, hexane–ethyl acetate 70:30 (v/v).

somes could not be due to the small microsomal contribution. The radioactivity in the 3β-hydroxy-5-cholenoate area after acetylation of the microsomal samples was not further identified. It is doubtful that this activity represents the monohydroxy bile acid as microsomes are not known to contain enzymes capable of complete side-chain oxidation.

Cofactor requirement for the metabolism of 26-hydroxycholesterol by rat liver peroxisomes

The metabolism of 26-hydroxycholesterol by the peroxisomal fraction required the presence of NAD, Mg^{2+}, and ATP (**Table 5**). The complete system resulted in conversion of about 8% of 26-hydroxycholesterol to water-soluble, acid-extractable radioactivity, of which about 50% was identified as 3β-hydroxy-5-cholenoic acid. The 0.2% activity observed in the control, minus NAD, and minus all cofactors represents the unreacted 26-hydroxycholesterol extracted in the aqueous phase after the initial partition. The addition of 0.1 mM CoA had no effect on the reaction. Three controls, from three different gradients, were carried through the entire analysis, and the only radioactivity present was due to contamination by unreacted 26-hydroxycholesterol.

DISCUSSION

The results of this study show that the conversion of 26-hydroxycholesterol to 3β-hydroxy-5-cholenoic acid is catalyzed by rat liver peroxisomes. Although the peroxisome fraction was slightly contaminated by microsomes, studies using 99% pure microsomes exclude the possibility that 3β-hydroxy-5-cholenoic acid formation in the peroxisomal fraction is catalyzed by the contaminating microsomes. Lysosomes and mitochondria (as measured by acid phosphatase and cytochrome oxidase activity, respectively) were well separated from the peroxisomes.

A peroxisomal-mediated pathway of cholesterol oxidation may be similar to one proposed for the mitochondrion (8). A possible pathway for the peroxisomal conversion of 26-hydroxycholesterol to 3β-hydroxy-5-cholenoic acid is illustrated in **Fig. 4.** 26-Hydroxycholesterol is first converted to 3β-hydroxy-5-cholestenoic acid (III). These two steps require an alcohol and aldehyde dehydrogenase (27, 28). The C-27 acid is then activated by acyl-CoA synthetase to form 3β-hydroxy-5-cholestenoyl coenzyme A (IV). The next four conversions proceed via the β-oxidation pathway, leading to the formation of 3β-hydroxy-5-cholenoic acid (VIII) and propionic acid (IX). Rat liver peroxisomes have been shown to contain the acyl-CoA synthetase (29) and the classical β-oxidation enzymes (30, 31). However, as with the mitochondria, it is not known if the same β-oxidation enzymes are responsible for the

TABLE 4. Summary of incubations of 26-hydroxycholesterol with rat liver peroxisomes and microsomes[a]

	26-Hydroxycholesterol	Total Extracted Activity[b]	3β-OH-5-Cholenoate	
			After Methylation	After Acetylation
		pmol/min per mg		
Peroxisomes	0.5 μCi	27.3 ± 3.8 (13)[c]	12.4 ± 3.7 (10)	13.9 ± 1.2 (4)
Microsomes	0.5 μCi	235 ± 22.5 (3)	7.6 ± 1.5 (3)	5.0 ± 1.4 (2)

[a] All values are given as mean ± SD.
[b] Refers to radioactivity extracted in the aqueous phase after the initial partition.
[c] The numbers in parentheses refer to number of samples analyzed.

TABLE 5. Cofactor requirement for the metabolism of 26-hydroxycholesterol by rat liver peroxisomes

	Total Conversion[a]
	%
Complete system[b]	7.9
Minus ATP	4.6
Minus $Mg(NO_3)_2$	3.3
Minus NAD	0.2
Minus all cofactors	0.2
Control[c]	0.2

[a] Represents total extracted radioactivity in aqueous phase after the initial partition.

[b] Ten μg of peroxisomal protein was incubated with 0.5 μCi 26-hydroxycholesterol as described in Methods.

[c] Ten μg of peroxisomal protein was added after termination of reaction.

cholesterol side chain oxidation. Catalase (the major peroxisomal protein in liver) may function as an alcohol dehydrogenase (32). There is also a preliminary report of a liver peroxisomal aldehyde dehydrogenase (33). Peroxisomes from yeast cells have been shown to contain long-chain alcohol and aldehyde dehydrogenase activities (34).

The results of the co-factor requirement study are consistent with the proposed pathway. ATP and Mg^{2+} are essential for the activation step (29), and NAD is involved in the dehydrogenation steps and is critical for peroxisomal β-oxidation (30, 35). In this study the addition of 0.1 mM exogenous CoA had no effect on the reaction. Rat liver peroxisomes are known to possess their own CoA pool (36). It is possible that during the long incubation time, the CoA can become available to acyl-CoA synthetase, which is located at the cytoplasmic side of the peroxisomal membrane (37), by leakage through the membrane.

Whether peroxisomes can independently oxidize cholesterol to 3β-hydroxy-5-cholenoic acid is not clear. Previously we showed (13) that rat liver peroxisomal fractions (70–75% pure) can convert cholesterol to an unknown bile acid and propionic acid. However, because of mitochondrial contamination, the possibility exists that the 26-hydroxylase activity necessary for the conversion of cholesterol to 26-hydroxycholesterol may have resulted from the mitochondrial enzyme (8). Preliminary studies performed using cholesterol as substrate with highly purified rat liver peroxisomes are not conclusive. As mentioned before, in the incubations using cholesterol as substrate, the enzymatic reactions are greatly inhibited by metrizamide. However, initial studies performed using 5β-cholestane-3α,7α,12,-triol (believed to be the major substrate for 26-hydroxylation) with 95–98% pure rat liver peroxisomes suggests the presence of hydroxylase activity in peroxisomes (38, 39).

In previous in vivo studies, 26-hydroxycholesterol has been shown to produce lithocholic, chenodeoxycholic, and cholic acids in the rat, hamster, and human (9, 10, 40). Under the present in vitro incubation conditions with peroxisomes, these bile acids were not detected. At present, it is not clear whether rat liver peroxisomes lack the enzymes to convert 3β-hydroxy-5-cholenoic acid to lithocholic, chenodeoxycholic, and cholic acids or the incubation conditions are not favorable for these conversions.

It is possible that peroxisomes produce 3β-hydroxy-5-cholenoic acid which is then used as a substrate by soluble enzymes to produce lithocholic acid, chenodeoxycholic acid, and/or cholic acid. A fast metabolic rate for the production of these bile acids could explain the fact that little or no 3β-hdyroxy-5-cholenoic acid is detected in adults (41).

Other evidence also exists that supports the role of peroxisomes in the biosynthesis of bile acids. A recent study has shown that rat liver peroxisomal fractions of relatively low purity (65–70%) are capable of converting 3α,7α,12α-trihydroxy-5β-cholestanoic acid, a crucial intermediate in the ER-initiated pathway, to cholic acid (12). However, there are important key differences between that study and this present one. First, we have used a peroxisome preparation of much higher purity, thus definitively demonstrating bile acid synthesis in the peroxisome. Second, the conversion of 26-hydroxycholesterol to 3β-hydroxy-5-cholenoic acid involves two additional enzymatic steps (requiring an alcohol dehydrogenase and an aldehyde dehydrogenase), steps not needed for the conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid to cholic acid. And third, the substrates involved, 26-hydroxycholesterol and 3α,7α,12α-trihydroxy-5β-cholestanoic acid, are produced by two distinct pathways, the latter requiring microsomal involvement.

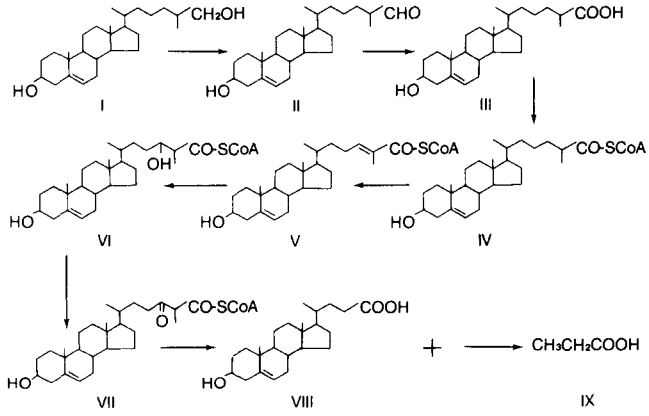

Fig. 4. Proposed peroxisomal pathway for the oxidation of 26-hydroxycholesterol to 3β-hydroxy-5-cholenoic acid. (I) 26-hydroxycholesterol; (II) 3β-hydroxy-5-cholestenal; (III) 3β-hydroxy-5-cholestenoic acid; (IV) 3β-hydroxy-5-cholestenoyl coenzyme A; (V) 3β-hydroxy-24-ene-5-cholestenoyl coenzyme A; (VI) 3β-24-dihydroxy-5-cholestenoyl coenzyme A; (VII) 3β-hydroxy-24-keto-5-cholestenoyl coenzyme A; (VIII) 3β-hydroxy-5-cholenoic acid (IX) propionic acid.

Although the peroxisome has been known to participate in lipid metabolism, a full understanding of the metabolic role of the organelle has yet to be achieved. From this and other studies it is becoming clear that bile acid synthesis may be an important function of rat liver peroxisomes. Of considerable significance are the recent observations that 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the key regulating enzyme in cholesterol biosynthesis, is found in rat liver peroxisomes (42, 43) as well as apoprotein A-I,[3] the major protein of high density lipoprotein.

This research was supported by grant AM 32852 from the National Institutes of Health. We thank Dr. Somanathan, Department of Chemistry, San Diego State University, for the synthesis of 3β-hydroxy-5-cholestenal. We thank Dr. Richard J. Laub, Department of Chemistry, San Diego State University, for performing the gas-liquid chromatography analysis.

Manuscript received 3 February 1985.

REFERENCES

1. Krisans, S. K., S. L. Thompson, L. A. Pena, E. Kok, and N. B. Javitt. 1984. Bile acid synthesis by rat liver peroxisomes. *J. Cell Biol.* **99:** 360a.
2. Berseus, O. 1965. On the stereospecificity of 26-hydroxycholesterol. *Acta Chem. Scand.* **19:** 325–328.
3. Mitropoulos, K. A., and N. B. Myant. 1965. Evidence that the oxidation of the side chain of cholesterol by liver mitochondria is stereospecific, and that the immediate product of cleavage is propionate. *Biochem. J.* **97:** 26C–28C.
4. Mendelsohn, D., and L. Mendelsohn. 1969. The catabolism of cholesterol in vitro. Formation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid from cholesterol by rat liver. *Biochem. J.* **114:** 1–3.
5. Cronholm, T., and G. Johansson. 1970. Oxidation of 5β-cholestane-3α,7α,12α-triol by rat liver microsomes. *Eur. J. Biochem.* **16:** 373–381.
6. Danielssen, H. 1973. Mechanisms of bile acid biosynthesis. *In* The Bile Acids. Vol. 2. P. P. Nair, and D. Kritchevsky, editors. Plenum Press, New York, London. 1–32.
7. Mosbach, E. H. 1977. *In* Cholesterol Metabolism and Lipolytic Enzymes. J. Polonovski, editor. Masson Publishing USA, Inc., New York. 53–68.
8. Mitropoulos, K. A., and N. B. Myant. 1967. The formation of lithocholic acid, chenodeoxycholic acid, and the α- and β-muricholic acids from cholesterol incubated with rat liver mitochondria. *Biochem. J.* **103:** 472–479.
9. Anderson, K. E., E. Kok, and N. B. Javitt. 1972. Bile acid synthesis in man: metabolism of 7α-hydroxycholesterol-^{14}C and 26-hydroxycholesterol-^{3}H. *J. Clin. Invest.* **51:** 112–117.
10. Wachtel, N., S. Emerman, and N. B. Javitt. 1968. Metabolism of cholest-5-ene-3β, 26-diol in the rat and the hamster. *J. Biol. Chem.* **243:** 5207–5212.
11. Pedersen, J. I., and J. Gustafsson. 1980. Conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid by rat liver peroxisomes. *FEBS Lett.* **121:** 345–348.
12. Kase, F., I. Björkhem, and J. I. Pedersen. 1983. Formation of cholic acid from 3α,7α,12α-trihydroxy-5β-cholestanoic acid by rat liver peroxisomes. *J. Lipid Res.* **24:** 1560–1567.
13. Hagey, L. R., and S. K. Krisans. 1982. Degradation of cholesterol to propionic acid by rat liver peroxisomes. *Biochem. Biophys. Res. Commun.* **107:** 834–841.
14. Kok, E., S. Bernstein, N. B. Javitt, M. Gut, and B. Y. Chang. 1981. Bile acid synthesis. Metabolism of 3β-hydroxy-5-cholenoic acid in the hamster. *J. Biol. Chem.* **256:** 6155–6159.
15. Leighton, F., B. Poole, H. Beaufay, P. Baudhuin, J. W. Coffey, S. Fowler, and C. de Duve. 1968. The large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR-1339. *J. Cell Biol.* **37:** 482–513.
16. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies. VI. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60:** 604–617.
17. Hajra, A., and J. Bishop. 1982. Glycerolipid biosynthesis in peroxisomes via the acyl dihydroxyacetone phosphate pathway. *Ann. NY Acad. Sci.* **386:** 170–182.
18. Lazarow, P. B., and C. de Duve. 1973. The synthesis and turnover of rat liver peroxisomes. V. Intracellular pathway of catalase synthesis. *J. Cell Biol.* **59:** 507–524.
19. Yonentani, T. 1967. Cytochrome oxidase: beef heart. *Methods Enzymol.* **10:** 332–335.
20. Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thines-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. *J. Cell Biol.* **61:** 188–200.
21. Bergmeyer, H. V., K. Gawehn, and M. Grassl. 1974. Methods of Enzymatic Analysis. 2nd English Ed. Vol. 1. H. V. Bergmeyer, editor. Verlag-Chemie, Weinheim, West Germany. 495–496.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
23. Ali, S. S., and N. B. Javitt. 1970. Quantitative estimation of bile salts in serum. *Can. J. Biochem.* **48:** 1054–1057.
24. Javitt, N. B., and S. Emerman. 1968. Effect of sodium taurolithocholate on bile flow and bile acid excretion. *J. Clin. Invest.* **47:** 1002–1014.
25. Fujiki, Y., S. Fowler, H. Shio, A. L. Hubbard, and P. B. Lazarow. 1982. Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes: comparison with endoplasmic reticulum and mitochondrial membranes. *J. Cell Biol.* **93:** 103–110.
26. Amar-Costesec, A., H. Beaufay, M. Wibo, D. Thines-Sempoux, E. Feytmans, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. *J. Cell Biol.* **61:** 201–212.
27. Okuda, K., and N. Takigawa. 1968. Separation of 5β-cholestane-3α,7α,12α,26-tetrol oxidoreductase, ethanol-NAD oxidoreductase, 5β-cholestane-3α,7α,12α-triol-26-al oxidoreductase, and acetaldehyde-NAD oxioreductase from the soluble fraction of rat liver by gel filtration. *Biochem. Biophys. Res. Commun.* **33:** 788–793.
28. Okuda, K., and N. Hoshita. 1968. Oxidation of 5β-cholestane-3α,7α,12α-triol by rat liver mitochondria. *Biochem. Biophys. Acta.* **164:** 381–388.
29. Krisans, S. K., R. M. Mortensen, and P. B. Lazarow. 1980. Acyl-CoA synthetase in rat liver peroxisomes. *J. Biol. Chem.* **255:** 9599–9607.

[3]G. A. Keller, Department of Biology, University of California at San Diego, personal communication.

30. Lazarow, P., and C. de Duve. 1976. A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. *Proc. Natl. Acad. Sci. USA.* **73:** 2043–2046.
31. Lazarow, P. 1978. Rat liver peroxisomes catalyse the β-oxidation of fatty acids. *J. Biol. Chem.* **253:** 1522–1528.
32. Masters, C., and R. Holmes. 1977. The metabolic role of peroxisomes in mammalian tissues. *Int. J. Biochem.* **8:** 549–553.
33. Smith, H. E., and R. Lindahl. 1980. Peroxisome-associated aldehyde dehydrogenase in normal rat liver. *J. Cell Biol.* **87:** 318a.
34. Tanaka, A., M. Osumi, and S. Fukui. 1982. Peroxisomes of alkane-grown yeast: fundamental and practical aspects. *Ann. NY Acad. Sci.* **386:** 183–198.
35. Mannaerts, G. P., L. J. Debeer, J. Thomas, and P. J. DeSchepper. 1979. Mitochondrial and peroxisomal fatty acid oxidation in liver homogenates and isolated hepatocytes from control and clofibrate-treated rats. *J. Biol. Chem.* **254:** 4585–4595.
36. Van Broekhoven, A., M. C. Peeters, L. J. Debeer, and G. P. Mannaerts. 1981. Subcellular distribution of coenzyme A. Evidence for a separate coenzyme A pool in peroxisomes. *Biochem. Biophys. Res. Commun.* **100:** 305–312.
37. Mannaerts, G. P., P. Van Veldhoven, A. Van Broekhoven, G. Vandebroek, and L. J. Debeer. 1982. Evidence that peroxisomal acyl-CoA synthetase is located at the cytoplasmic side of the peroxisomal membrane. *Biochem. J.* **204:** 17–23.
38. Thompson, S. L., and S. K. Krisans. 1985. Localization of 26-hydroxylase activity in rat liver peroxisomes. *Federation Proc.* **44:** 1155.
39. Thompson, S. L., and S. K. Krisans. 1985. Evidence for peroxisomal hydroxylase activity in rat liver. *Biochem. Biophys. Res. Commun.* **130:** 708–716.
40. Danielsson, H. 1961. Formation and metabolism of 26-hydroxycholesterol. *Arik. Kem.* **17:** 373–379.
41. Gibbons, G. F., K. A. Mitropoulos, and N. B. Myant. 1982. Biochemistry of Cholesterol. Elsevier, Amsterdam. 189–192.
42. Keller, G. A., M. C. Barton, D. J. Shapiro, and S. J. Singer. 1985. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase is present in peroxisomes in normal rat liver cells. *Proc. Natl. Acad. Sci. USA.* **82:** 770–774.
43. Keller, G. A., M. Pazirandeh, and S. K. Krisans. 1985. Quantitative biochemical and immunoelectron microscopic analysis of HMG-CoA reductase in rat liver peroxisomes and microsomes of normal and cholestyramine-treated animals. *Federation Proc.* **44:** 1044.