JCI The Journal of Clinical Investigation

A novel pathway for biosynthesis of cholestanol with 7 alpha-hydroxylated C27-steroids as intermediates, and its importance for the accumulation of cholestanol in cerebrotendinous xanthomatosis.

S Skrede, … , G Hopen, O Fausa

J Clin Invest. 1985;75(2):448-455. https://doi.org/10.1172/JCI111719.

Research Article

A mixture of 7 alpha-3H- and 4-14C-labeled cholesterol was administered intravenously to rats. Cholestanol with 20-30% lower ratio between 3H and 14C than in cholesterol could be isolated from different organs. In a healthy human control, cholestanol isolated from feces had a 3H/14C ratio which was 28% lower than in administered cholesterol. Cholesterol and coprostanol reisolated in these experiments had the same ratio between 3H and 14C as in the precursor. A previously unknown pathway for formation of cholestanol, involving 7 alpha-hydroxylated intermediates, may explain these results. Under normal conditions, this pathway is responsible for at most 30% of the cholestanol synthesized from cholesterol. Intravenous administration of the 7 alpha-3H- and 4-14C-labeled cholesterol to a patient with cerebrotendinous xanthomatosis (CTX) resulted in formation of cholestanol which had 70-75% lower 3H/14C ratio. It is concluded that the novel pathway involving 7 alpha-hydroxylated intermediates is accelerated in patients with CTX. This acceleration may contribute essentially to the accumulation of cholestanol, which is a predominant feature of this disease. 7 alpha-Hydroxycholesterol and 7 alpha-hydroxy-4-cholesten-3-one might be intermediates in the novel pathway to cholestanol. After intravenous administration of 7 beta-3H-labeled 7 alpha-hydroxycholesterol in a patient with CTX, significant amounts of 3H were incorporated into plasma and fecal cholestanol. Only small amounts of 7 alpha-hydroxycholesterol and 7 alpha-hydroxy-4-cholesten-3-one are excreted into the intestine, and we […]

A Novel Pathway for Biosynthesis of Cholestanol with 7α-Hydroxylated C_{27}-Steroids as Intermediates, and Its Importance for the Accumulation of Cholestanol in Cerebrotendinous Xanthomatosis

Sverre Skrede, Ingemar Björkhem, Marie S. Buchmann, Gunnar Hopen, and Olav Fausa

Institute of Clinical Biochemistry and Medical Department A, Rikshospitalet, University of Oslo, Norway; Department of Clinical Chemistry and Research Center at Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden; and Medical Department B, Haukeland Hospital, University of Bergen, Bergen, Norway

Abstract

A mixture of 7α-^{3}H- and 4-^{14}C-labeled cholesterol was administered intravenously to rats. Cholestanol with 20–30% lower ratio between ^{3}H and ^{14}C than in cholesterol could be isolated from different organs. In a healthy human control, cholestanol isolated from feces had a ^{3}H/^{14}C ratio which was 28% lower than in administered cholesterol. Cholesterol and coprostanol reisolated in these experiments had the same ratio between ^{3}H and ^{14}C as in the precursor. A previously unknown pathway for formation of cholestanol, involving 7α-hydroxylated intermediates, may explain these results. Under normal conditions, this pathway is responsible for at most 30% of the cholestanol synthesized from cholesterol.

Intravenous administration of the 7α-^{3}H- and 4-^{14}C-labeled cholesterol to a patient with cerebrotendinous xanthomatosis (CTX) resulted in formation of cholestanol which had 70–75% lower ^{3}H/^{14}C ratio. It is concluded that the novel pathway involving 7α-hydroxylated intermediates is accelerated in patients with CTX. This acceleration may contribute essentially to the accumulation of cholestanol, which is a predominant feature of this disease.

7α-Hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one might be intermediates in the novel pathway to cholestanol. After intravenous administration of 7β-^{3}H-labeled 7α-hydroxycholesterol in a patient with CTX, significant amounts of ^{3}H were incorporated into plasma and fecal cholestanol. Only small amounts of 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one are excreted into the intestine, and we therefore conclude that the 7α-dehydroxylation step mainly occurs in the liver. In CTX, the synthesis of cholestanol may be accelerated because the concentrations of 7α-hydroxylated bile acid intermediates in the liver are increased. A possible mechanism for the conversion of a minor fraction of 7α-hydroxycholesterol into cholestanol is suggested.

Introduction

Cerebrotendinous xanthomatosis (CTX)[1] is a rare inherited disease. The most important symptoms are caused by increased biosynthesis of cholestanol and cholesterol, with depositions in brain, cerebellum, and tendons (2).

In CTX, the capacity to convert cholesterol to bile acids is impaired (3, 4). The incomplete oxidation of the cholesterol side chain leads to the accumulation of abnormal tetra- and penta-hydroxylated bile alcohols (3, 4). Feedback control of the cholesterol 7α-hydroxylase is decreased, and its activity is therefore increased several fold (5). By studies in vitro (6) and in vivo (7), we have recently found evidence for the concept that the primary enzymic defect in CTX is a deficiency of a mitochondrial steroid 26-hydroxylase. Salen and coworkers (8) have previously concluded that a microsomal 24β-hydroxylase active on 3α,7α,12α,25-tetrol is deficient in CTX.

It is difficult to understand the relation between the deficient cleavage of the cholesterol side-chain and the increased biosynthesis of 5α-cholestan-3β-ol (cholestanol) in CTX. Cholestanol is derived from cholesterol, but apparently not from cholesterol precursors (9). It has previously been concluded that cholestanol and bile acids have no common intermediates in their biosynthesis (10). The biosynthesis of cholestanol from cholesterol is generally believed to proceed via 4-cholesten-3-one (11), but the capacity of this pathway is low and it has so far only been demonstrated in rat liver tissue (12). Recently, we showed that intermediates of bile acid biosynthesis are accumulated in liver tissue in CTX (13). Increased amounts of, e.g., 7α-hydroxy-4-cholesten-3-one are excreted in the bile (14). 7α-hydroxylated intermediates may be dehydroxylated in the gut, analogous to the formation of secondary bile acids, and 4-cholesten-3-one may thus be formed (14). However, the quantitative importance of a 7α-hydroxylation/dehydroxylation route for the formation of cholestanol in normal individuals is not known.

The present study was performed to evaluate the possibility that in man cholestanol may be formed from early 7α-hydroxylated bile acid intermediates, and to elucidate the role of this route in CTX.

Methods

Materials

Clinical. Two sisters (A.F., born 27 July 1941, and I.J., born 6 May 1935) with CTX were studied. Detailed records of clinical findings and laboratory data have been published previously (6, 7, 15). A.F. was only slightly mentally retarded when studied in June 1983, but had rather extensive xanthomatosis. Laboratory data (normal reference ranges are indicated in parentheses): serum total cholesterol, 10.2 mmol/L (3.5–7.5); nonesterified cholesterol, 23% of total cholesterol (18–33%); high density lipoprotein (HDL)-cholesterol, 2.2 mmol/L (0.9–1.9); triglycerides, 1.6 mmol/L (0.6–1.4); ASAT, 23 U/L (13–38); ALAT, 41 U/L (9–29); and alkaline phosphatases, 248 U/L (55–195). A needle liver biopsy specimen showed only slight steatosis by light microscopy.

When studied in November 1983, I.J. was more disabled than during hospitalization in 1981 (7), with mental retardation and neurological dysfunction. Laboratory data: serum total cholesterol, 6.8

Address correspondence to Dr. Skrede, Institute of Clinical Biochemistry.

Received for publication 5 January 1984 and in revised form 15 October 1984.

1. *Abbreviations used in this paper:* CTX, cerebrotendinous xanthomatosis; cholestanol, 5α-cholestan-3β-ol; coprostanol, 5β-cholestan-3βol; 7α-hydroxycholesterol, 5-cholesten-3β,7α-diol; epicoprostanol, 5β-cholestan-3α-ol; GC/MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography.

J. Clin. Invest.

0021-9738/85/02/0448/08 $1.00
Volume 75, February 1985, 448–455

mmol/L; nonesterified cholesterol, 24% of total cholesterol; HDL-cholesterol, 0.6 mmol/L; triglycerides, 4.2 mmol/L; ASAT, 11 U/L; ALAT, 14 U/L; and alkaline phosphatases, 396 U/L. A needle liver biopsy specimen showed steatosis by light microscopy.

The ethical aspects of the present study were approved by the ethical board of the Norwegian Research Council for Science and the Humanities, Division for Medical Research. Informed consent was obtained from the patients and their family.

Three subjects without CTX served as controls for the analyses of faecal sterols (S.S., male, born 8 October 1932, L.A., female, born 25 September 1925, and P.L.I., male, born 18 December 1950). The last two subjects have been described previously (7). Samples from them were stored at −20°C for 2 yr before analysis, whereas unstored samples from S.S. were used. From both CTX patients, fresh samples and samples stored for 2 yr were analyzed.

Labeled steroids. 4-^{14}C-cholesterol, with a specific radioactivity of 58.4 mCi/mmol, was obtained from The Radiochemical Centre (Amersham, Great Britain).

7α-^{3}H-cholesterol, with a specific radioactivity of ~18.7 mCi/mmol, was prepared as described previously (16). The radiochemical purity of 7α-^{3}H-cholesterol was investigated by addition of 4-^{14}C-cholesterol, dilution with unlabeled cholesterol, and conversion into the acetate of 7-oxocholesterol by the method described by Fieser (17). The material obtained was purified by thin-layer chromatography (TLC), using benzene-ethylacetate 4:1 (vol/vol) as solvent, and was crystallized twice from methanol-water and acetone-water. The same preparation of ^{3}H-labeled cholesterol (A) was used in all experiments, except in the experiment with the healthy subject S.S. The recrystallized material had lost >99% of the ^{3}H (with both preparation A and B). Thus, >99% of the ^{3}H label is present in the 7α and/or 7β position. The mixture of 7α-^{3}H- and 4-^{14}C-cholesterol was further incubated with liver microsomes from a rat with a biliary fistula (18). The isolated and purified product, 5-cholesten-3β,7α-diol (7α-hydroxycholesterol), had lost 81% of the ^{3}H with preparation A and 92% with preparation B. Since this hydroxylation is known to occur with a stereospecific replacement of hydrogen (19), it may be concluded that ~81% of the label was present in the 7α position and ~19% in the 7β position in preparation A, whereas the corresponding fractions for preparation B were 92% (7α-^{3}H) and 8% (7β-^{3}H). Cholic acid was isolated from rabbits treated with the same mixture of 4-^{14}C- and 7α-^{3}H-cholesterol of preparation A and had lost ~84% of the ^{3}H label (see below). The slightly lower retention of ^{3}H in this case may be due to oxidoreduction at C-7 during the enterohepatic circulation of cholic acid. The cholic acid isolated from the bile of A.F. collected 7 d after the administration of 4-^{14}C- and 7α-^{3}H-cholesterol also had a ^{3}H/^{14}C ratio corresponding to removal of all 7α-^{3}H but retention of all 7β-^{3}H present in precursor cholesterol. 4-^{14}C-7α-hydroxy-4-cholesten-3-one (14) and 7β-^{3}H-7α-hydroxycholesterol (20) were synthesized as described previously. All compounds were purified by high performance liquid chromatography (HPLC) (see below) immediately before preparation of the mixture to be injected. To exclude the possibility of any cholestanol contamination of the labeled cholesterol preparation used, aliquots of 4-^{14}C- and 7α-^{3}H-cholesterol were added to unlabeled cholestanol and cholesterol and carried through the epoxidation procedures and analyzed by HPLC as described below (Fig. 1). All radioactivity had R_t as cholesterol before epoxidation, and as cholesterol epoxides after epoxidation. The cholestanol fraction had unchanged R_t (41 min) and was completely devoid of radioactivity (^{3}H or ^{14}C) both before and after epoxidation.

Chemicals. 5β-cholestan-3α-ol (epicoprostanol) was purchased from Steraloids, Inc., Wilton, NH. All other chemicals and solvents used in the present study were standard commercial high purity materials.

Procedures

Administration of labeled steroids and collection of samples. The mixture of 4-^{14}C-cholesterol (22.3 μCi) and 7α-^{3}H-cholesterol (60 μCi) given to A.F. was dissolved in 12 ml of ethanol/water containing 1% Tween 80 and was passed through a 0.22-μm filter Millex (Millipore Corp., Bedford, MA). These procedures were performed at the Norwegian Institute of Energy Technology, Kjeller, Norway. The 7β-^{3}H-7α-hydroxycholesterol (32.4 μCi) and 4-^{14}C-7α-hydroxy-4-cholesten-3-one (0.84 μCi) given to I.J. were dissolved in 0.5 ml of ethanol together with 1% Tween 80 in 4.5 ml of water and sterile-filtered as above. The solutions were slowly injected intravenously. In CTX patient A.F., blood samples were drawn after 15 min (contralateral arm) and after 16, 88, and 160 h. In both CTX patients, urine and feces were collected quantitatively for 8 d after the injection of the labeled precursor. In A.F., bile was collected by duodenal incubation 160 h after the administration of the labeled cholesterol, as described previously (7). Duodenal contents were collected on ice in 10-min periods. The sampling period was 60 min after the intravenous injection of cholecystokinin (1 clinical unit/kg body weight). The total volume obtained during this period was 89 ml (in the 10- to 20-min period the volume was 32 ml). Control subject S.S. received the mixture of 4-^{14}C-cholesterol (19.5 μCi) and 7α-^{3}H-cholesterol (60.2 μCi) in olive oil orally to ensure sufficient radioactivity in coprostanol for calculation of the ^{3}H/^{14}C ratio. Feces was collected quantitatively for 8 d.

Isolation and analyses of sterols and bile acids. Cholesterol, cholestanol, and 5β-cholestan-3β-ol (coprostanol) were isolated from feces, bile, and blood plasma by the following procedures: the samples were weighed, and hydrolysis and lipid extractions were performed as described previously (14) by refluxing at 80°C for 1 h with KOH/ethanol (5:100 wt/vol), followed by extraction twice with *n*-hexane. The hexane extracts of feces were initially subjected to TLC on plates coated with silica gel H using benzene/ethyl acetate (5:1, vol/vol) as solvent. The combined sterol zone (R_f 0.26–0.44) was scraped off, epicoprostanol was added as internal standard, and eluted on a glass sinter funnel with chloroform/methanol (2:1, vol/vol). Epicoprostanol was added directly to hexane extracts of plasma and bile. The hexane extracts were blown to dryness, and the sterols were separated by HPLC. A Supelcosil-LC18 column (250 × 4.6 mm, 5 μm particle size, obtained from Supelco, Inc., Bellefonte, PA) was used with methanol/water (95:5, vol/vol) as the mobile phase and a flow rate of 1 ml/min. The chromatograph was equipped with a constant flow pump (ConstaMetric III, Laboratory Data Control, Milton Roy Co., St. Petersburg, FL) and a differential refractometer (R-401, Waters Associates, Milford, MA). The retention times (minutes) were as follows: cholesterol-5,6α-epoxide 18; cholesta-4,6-dien-3-one 27; epicoprostanol 27; 4-cholesten-3-one 31; cholesterol 36; coprostanol 36; cholestanol 42; campesterol 42; 24-methyl-5β-cholestan-3β-ol 42; and 24-ethyl-5β-cholestan-3β-ol 48.

To obtain cholestanol and coprostanol fractions free from unsaturated sterols, an additional purification by HPLC was performed after quantitative conversion of cholesterol and phytosterols to epoxides. Aliquots of the hexane extract (blown to dryness with N_2) were incubated with 10 or 20 mg of *m*-chloroperbenzoic acid in 1 ml of chloroform at 45°C for 30 min. Identification (and in some cases also quantitation) of cholestanol, coprostanol, 24-methyl-5β-cholestan-3β-ol, and 24-ethyl-5β-cholestan-3β-ol was performed by gas chromatography/mass spectrometry (GC/MS) as described previously (14). 7α-hydroxy-4-cholesten-3-one was quantitated by isotope dilution mass spectrometry as described previously (14).

Hydrolysis of bile acids. The water phase of the bile remaining after the alkaline hydrolysis and the extraction with hexane was adjusted to pH 8 with HCl (5 mol/L), incubated at 80°C for 10 min, and centrifuged. Bile acids were isolated by means of SepPak cartridges, essentially as described by Setchell (21). The bile acid conjugates were hydrolyzed by choloylglycine hydrolase essentially as described by Ross et al. (22), except that incubations were performed at 37°C overnight. Extraction of bile acids with ethyl-acetate was performed as described by Ross et al. (22). The bile acids were separated by HPLC as described for sterols (see above) except that the mobile phase was methanol/water (70:30, vol/vol). The water was adjusted to pH 3.0 with phosphoric acid. The retention times (minutes) were: cholic acid 18; chenodeoxycholic acid 36; deoxycholic acid 44½; and lithocholic acid 90.

Measurement of radioactivity. A Packard Tri-Carb liquid scintillation spectrometer, model 3385 (Packard Instrument Co., Inc., Downers Grove, IL) was used. Simultaneous estimation of ^{3}H and ^{14}C activity was usually performed in 1-ml fractions (methanol/water 95:5, vol/

vol) collected from the outlet of the liquid chromatograph. Sometimes radioactivity was assayed in fractions blown to dryness (which were compared with dry standards). Packard Insta Gel II (10 ml) (Packard Instrument Co., Inc.) was used as scintillant. The counting efficiency under these conditions was 31% for ^3H and 55% for ^{14}C. From the activities recorded in the ^3H-channel, 46% of the ^{14}C activity had to be subtracted, whereas the influence of ^3H in the ^{14}C channel was insignificant (<0.04%). Corrections for ^3H cholesterol label in positions other than 7α were performed by calculation from analysis of cholic acid isolated from the bile of A.F. and from gallbladder bile from two rabbits (23). Assuming that all 7α-^3H had been removed during the introduction of the 7α-hydroxyl group (24), residual ^3H activity in cholic acid (16%) was reckoned to be localized in other positions.

The corrected $^3H/^{14}C$ ratios (cpm ^3H/cpm ^{14}C) were always compared with those of the ingested cholesterol. $^3H/^{14}C$ ratios in cholestanol and cholesterol were estimated after separation by rechromatography by means of HPLC.

Results

We have previously shown that cholestanol may be formed from 7α-hydroxy-4-cholesten-3-one by intestinal 7α-dehydroxylation (14), but we concluded that this route probably was of minor quantitative importance. Table I shows results of studies evaluating the relative contribution of 7α-hydroxylated intermediates in the biosynthesis of cholestanol, by estimating the loss of 7α-^3H from the precursor 7α-^3H cholesterol. Rats and a normal human subject were studied. Table I shows that cholesterol was recovered from different rat organs and from rat and human feces with the same $^3H/^{14}C$ ratio as in ingested cholesterol. This ratio was the same in coprostanol as in its precursor cholesterol. In cholestanol, however, the $^3H/^{14}C$ ratio was 20–30% lower than in cholesterol, both in the rat and in the normal human subject. (Cholestanol was purified and identified as described below.) The extent of the decrease of the $^3H/^{14}C$ ratio suggests that at most ~¼ of cholestanol is normally formed by a pathway involving 7α-hydroxylated intermediates.

Table I. $^3H/^{14}C$ Ratios of Cholesterol and Cholestanol Isolated from Rats and a Healthy Human Subject Given 7α-^3H and 4-^{14}C-Cholesterol Orally

Organ or material	Animal or subject	$^3H/^{14}C$ ratio		
		Cholesterol	Cholestanol	Coprostanol
Small intestinal and cecal wall	Rat A	1.21	0.91	—
	Rat B	1.40	1.06	—
Liver	Rat B	1.38	1.09	—
Feces	Rat B	—	—	1.38
	Human subject (S.S.)	1.74*	1.25	1.74

The total doses given to two rats in the chow during 10 d were as follows: 4-^{14}C-cholesterol: rat A, 3.2 μCi; rat B, 15.3 μCi; 7α-^3H-cholesterol: rat A, 6.9 μCi; rat B, 38.0 μCi. Normal human subject S.S. received a single oral dose of 4-^{14}C-cholesterol (19.5 μCi) and 7α-^3H-cholesterol (60.2 μCi) (refer also to Methods). The $^3H/^{14}C$ ratios (cpm/cpm) in ingested cholesterol were rat A, 1.21; rat B, 1.40; and S.S., 1.74.

* Measured in cholesterol epoxide separated from coprostanol by HPLC.

Table II. $^3H/^{14}C$ Ratios and Specific Activities of Cholesterol and Cholestanol Isolated from a Patient with CTX (A.F.) Given 4-^{14}C- and 7α-^3H-Labeled Cholesterol

Source	$^3H/^{14}C$ ratio		Specific activity of ^{14}C (*dpm/mg*)	
	Cholesterol	Cholestanol	Cholesterol	Cholestanol
Plasma	1.54	0.44 (0.010)	650	290
Bile	1.54	0.35 (0.008)	478	510
Feces				
19 June 1983	—	0.48 (0.008)	—	420
20 June 1983	—	0.45 (0.013)	—	460

The mixture of ^3H- and ^{14}C-labeled cholesterol (with a $^3H/^{14}C$ ratio of 1.54) was given intravenously to A.F. on 13 June 1983) (refer to Methods). Time (days) for sampling after precursor administration: plasma, 8; bile, 7; feces, 6–7. Amount of cholestanol (milligram) isolated: plasma, 0.7; bile, 1.5; feces, 0.8. Arithmetical means for 15–30 repeated countings/calculations are shown (in parentheses, SEM). (The amounts of cholestanol in bile and feces are shown in Table IV.)

Table II shows a similar study in a patient with CTX. In this case, labeled cholesterol was administered intravenously. In purified cholesterol fractions reisolated from bile and plasma, the $^3H/^{14}C$ ratio was the same as in the injected precursor. Cholestanol isolated from plasma, bile, and feces had a much lower $^3H/^{14}C$ ratio than cholesterol. In all three materials, aliquots of the cholestanol fraction purified by the HPLC method shown by Fig. 1 and containing the cited radioactivity were analyzed by GC/MS (14), both underivatized and after trimethylsilyl-derivatization. Only one peak appeared on GC, and the MS-spectra were typical of cholestanol and its TMS-derivative, respectively. (Underivatized: *m/e* 388 (M), 373 (M-15), 355, 234, 233, 215. TMS-derivative: 460, 445, 403, 370, 355, 306, 230, 215.) The $^3H/^{14}C$ ratio in cholestanol differed strikingly from those in the normal control person, since for patient A.F., 7α-^3H was removed in as much as three-quarter of the cholestanol. The amounts of coprostanol in the feces of A.F. were too small to enable calculation of the $^3H/^{14}C$ ratio (refer to Table IV). Table II also shows that 7 d after the administration of precursor to CTX subject A.F., the specific activity of ^{14}C was slightly higher in biliary cholestanol than in biliary cholesterol. In cholic acid isolated from this bile sample we found that all 7α-^3H had been removed by 7α-hydroxylation of cholesterol. Only 0.3% of the ^{14}C in the precursor dose was present in the collected bile, whereas 6.8% was excreted in the urine during the first 8 d. (The excretion of bile alcohol glucuronides in the urine of our patients with CTX was on the average 300–400 mg/d.)[2]

The results shown in Table II indicate that in CTX a major part of the cholestanol is formed via 7α-hydroxylated intermediates. In the normal state, on the other hand (Table I), such a pathway can at the most only account for 20–30%

2. Sjövall, J., G. Karlaganis, K. D. R. Setchell, A. Lawson, and S. Skrede, unpublished observations.

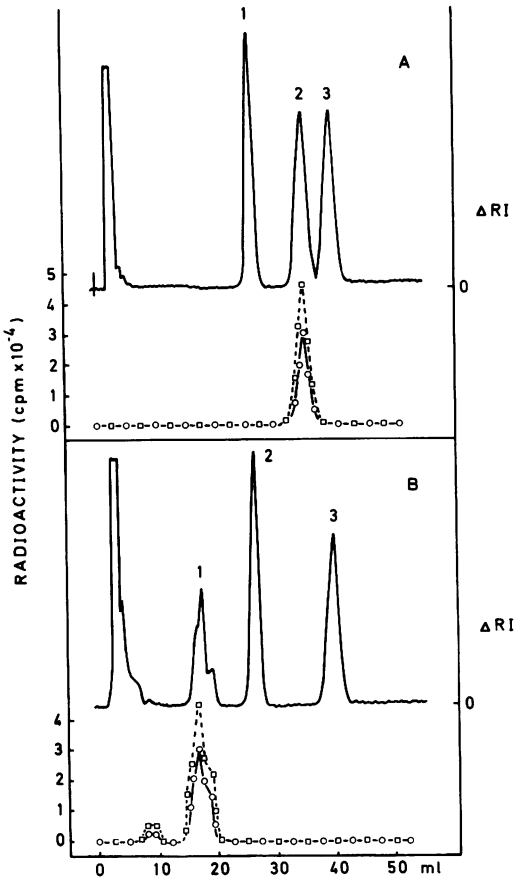

Figure 1. Characterization of the HPLC system and the precursor 7α-^{3}H- and 4-^{14}C-cholesterol used in the present study. (*A*) To an aliquot of the purified 7α-^{3}H- and 4-^{14}C-cholesterol administered to patient A.F. with CTX (refer to Table II) was added 0.3 mg unlabeled epicoprostanol (1), cholesterol (2), and cholestanol (3). The separation is shown as ΔRI (upper curve). The lower curve shows that all ^{3}H (□ - - - □) and ^{14}C (○ - - - ○) was present in cholesterol (no activity migrated as cholestanol). (*B*) Chromatogram of a mixture similar to that shown in *A*, after carrying it through the epoxidation procedure described in Methods. The cholesterol was quantitatively converted to cholesterol epoxides (1), and contained all ^{3}H and ^{14}C. Traces of radioactivity were not present in cholestanol (3). Internal standard (as in *A*): epicoprostanol (2).

of the cholestanol formed. Potential precursors of cholestanol in this novel pathway would be 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one. Table III shows that after intravenous administration of 7β-^{3}H-7α-hydroxycholesterol and 4-^{14}C-7α-hydroxy-4-cholesten-3-one to patient I.J. with CTX, ^{3}H label was incorporated into cholestanol. The cholestanol fraction of plasma was purified by epoxidation, followed by HPLC. The methods described in connection with Table II showed that the fraction did not contain contaminating sterols. Fecal cholestanol was isolated by HPLC after epoxidation of a combined sterol fraction obtained by TLC of a lipid extract of feces. By GC/MS, traces of 24-methyl-5β-cholestan-3β-ol were also found in this fraction. Since plant sterols cannot be synthesized in the mammalian organism, ^{3}H could only be present in cholestanol. The specific activity of ^{3}H in cholestanol was slightly higher in plasma than in feces, indicating that cholestanol formed via bile acid intermediates is not preferentially excreted in the bile. Detectable amounts of ^{14}C were

Table III. Incorporation of ^{3}H into Cholestanol After Intravenous Administration of 7β-^{3}H-7α-Hydroxycholesterol and 4-^{14}C-7α-Hydroxy-4-cholesten-3-one in a Patient with CTX (I.J.)

Source	Date of sampling	Specific activity of ^{3}H in cholestanol	Total ^{3}H label present in: Cholestanol	Total ^{3}H label present in: Water soluble metabolites
		dpm/mg	*dpm × 10⁻³/24 h*	*dpm × 10⁻³/24 h*
Plasma	10 Nov. 1983	1,014	—‡	—
Feces	10–11 Nov. 1983	851	20.5	3,450
	11–12 Nov. 1983	852	17.8	2,364
	12–13 Nov. 1983	772	11.7	692
	13–14 Nov. 1983	660	13.7	1,923
Urine	10–11 Nov. 1983	—	—	1,300
	11–12 Nov. 1983	—	—	1,200
	12–13 Nov. 1983	—	—	755
	13–14 Nov. 1983	—	—	900

A mixture of 7β-^{3}H-7α-hydroxycholesterol (32.4 μCi) and 4-^{14}C-7α-hydroxy-4-cholesten-3-one (0.84 μCi)* was given intravenously to I.J. on 7 November 1983.

* 0.03% of this dose might have been detected in the cholestanol fractions isolated from feces.

‡ If the plasma volume is assumed to be 3 liters, total ^{3}H activity in plasma cholestanol would be ~82 × 10^3 dpm.

not found in cholestanol, but it should be noted that only a small dose of ^{14}C-7α-hydroxy-4-cholesten-3-one was given (refer to Discussion).

It is shown by Table III that the ^{3}H present in fecal cholestanol corresponded to ~1% (0.6–1.7%) of the total ^{3}H in feces. The urinary excretion of ^{3}H was ~50% of the fecal excretion. In the period 10–11/11-83, I.J. excreted 735 mg of bile alcohols in the urine.[2] Assuming that this fraction contained all ^{3}H-activity (1.3 × 10^6 dpm), the specific activity of urinary bile alcohols would be ~1,770 dpm/mg. At the same time, the specific activity of plasma cholestanol was 1,014 dpm/mg and of fecal cholestanol 851 dpm/mg. Differences in the size and turnover of the pools of cholestanol and bile alcohols and their different precursors do not allow calculations of the relative contribution of the 7α-dehydroxylation route (Fig. 2 *B*) to the total biosynthesis of cholestanol. It is evident also from these data, however, that the activity of this pathway is significant.

Table IV shows that the fecal excretion of cholestanol in CTX patient I.J. was on the average ~20 mg/24 h. She was more severely affected than her younger sister, A.F., who excreted twice as much cholestanol in the feces. Also, the fecal excretion of cholesterol was much higher in A.F. than in I.J. There was no constant relation between the excretion of cholestanol and coprostanol in feces. The ratio between cholestanol and cholesterol was ~1:15 in the bile of A.F., and about the same in feces of both sisters (fecal cholesterol and coprostanol were regarded as one fraction). A.F. and the two control subjects L.Aa and P.I. were "low intestinal converters" (25) of cholesterol and excreted only traces of coprostanol. I.J. and the third control (S.S.) were "high converters" and excreted mainly coprostanol.

The concentration of plasma cholestanol was increased

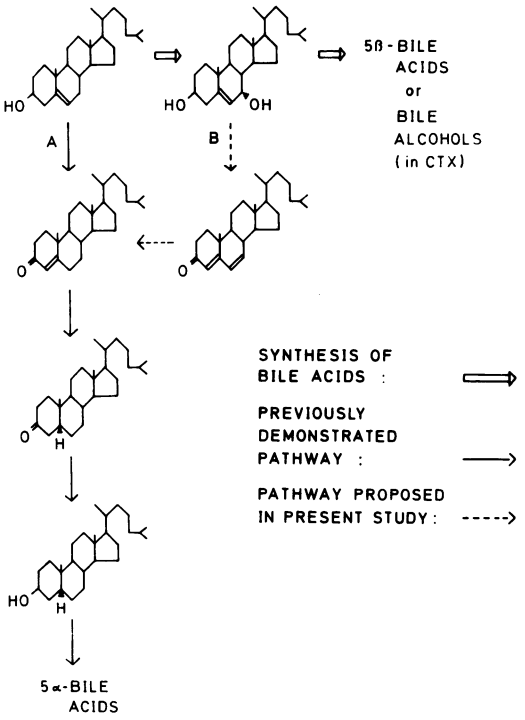

Figure 2. Mechanisms for the biosynthesis of cholestanol. Routes A and B are both localized in the liver. The initial reaction of route B also occurs in the intestine (14).

above normal by a factor of ~10 in both our patients with CTX. In A.F., bile was collected, and its relative concentration of cholestanol was 6.5% of total sterols. This is abnormally high, as previously found in patients with CTX (10). In the bile of A.F., the concentration of 7α-hydroxy-4-cholesten-3-one was 4 μg/ml, which was only ~6% of the concentration of cholestanol.

Discussion

Pathways for the biosynthesis of cholestanol in the normal state. It is well established that cholesterol may serve as precursor of cholestanol in man (9, 11). Cholestanol is readily formed from 4-cholesten-3-one (26), which in all probability is an intermediate in the route from cholesterol (11, 12). This precursor is converted into cholestanol in liver tissue by a Δ^4-3-oxo-steroid 5α-reductase (27) and a 3β-hydroxysteroid dehydrogenase (28) (route A in Fig. 2). The mechanism for formation of 4-cholesten-3-one in mammalian tissues is not clear, however, and this step is evidently rate limiting. A very slow NAD^+-dependent conversion of cholesterol to 4-cholesten-3-one has been demonstrated in rat liver microsomes (12), but not convincingly in human tissues.

In a previous study (14) we discussed the possibility that 7α-hydroxylated bile acid intermediates might be precursors of 4-cholesten-3-one and cholestanol. It was shown that 7α-hydroxy-4-cholesten-3-one, which is a key intermediate in bile acid biosynthesis, could be converted into cholestanol after oral administration to rabbits. The conversion occurred via 7α-dehydroxylation and intermediate formation of cholesta-4,6-dien-3-one and 4-cholesten-3-one. We concluded from radiotracer experiments and quantitation of 7α-hydroxy-4-cholesten-3-one in human bile that formation of cholestanol with intestinal 7α-hydroxy-4-cholesten-3-one as an intermediate only can represent a minor pathway (14).

Table IV. Amounts of Sterols in Plasma, Bile, and Feces from Two Patients with CTX and in Control Subjects

Source	Subject	Date of sampling	Cholesterol		Coprostanol, total amount (*mg*)	Cholestanol	
				Total amount (*mg*)			Total amount (*mg*)
			mmol/L			*mmol/L*	
CTX							
Plasma	A.F.	21 June 1983	10.2	—	—	0.11*	—
	I.J.	10 Nov. 1983	6.8	—	—	0.07*	—
Bile	A.F.	20 June 1983	2.8	83‡	—	0.20	5.8‡
Feces§	A.F.	25 Feb. 1981		907	Trace		43.5
		17 June 1983		871	Trace		49.8
		19 June 1983		913	Trace		52.1
		20 June 1983		—	Trace		45.4
	I.J.	21 May 1981		Trace	282		29.0
		22 May 1981		34	113		17.4
		11 Nov. 1983		151	671		26.7
		12 Nov. 1983		—428‖	—		20.9
		13 Nov. 1983		—295‖	—		15.1
		14 Nov. 1983		—496‖	—		20.8
Controls							
Feces§	L.Aa.	12 June 1981		272	20		2.5
	P.I.	23 Sept. 1981		698	Trace		4.5
	S.S.	24 Nov. 1983		57	428		5.8

—, not examined. * Level in normal controls, 0.001–0.019 mmol/L (15). ‡ In 89 ml collected from duodenum after cholecystokinin. § mg/24 h. ‖ Cholesterol plus coprostanol.

The above experiments did not exclude that other 7α-hydroxylated bile acid intermediates might be precursors of cholestanol, or that 7α-dehydroxylation might occur in other organs than the intestine. In the present work we used a more general approach by studying the loss of ^3H from 7α-^3H-labeled cholesterol during its conversion into cholestanol in rats and in one human control subject. There was a significant (20–30%) loss of tritium during the conversion both in the rats and in the human subject.

The simplest explanation of this finding is that there is a pathway to cholestanol involving 7α-hydroxylated intermediates which is responsible for 20–30% of the biosynthesis of cholestanol (route B in Fig. 2). Although less likely, the possibility cannot be completely excluded that the reduced $^3H/^{14}C$ ratio to some extent may be caused by an isotope effect in the conversion of 7α-^3H-cholesterol into 7α-^3H-4-cholesten-3-one via the previously known, direct pathway (12). Attempts to study this possibility in vitro have failed owing to the very low conversion (12). The results of the experiments with the control subject, however, do not favor the possibility of such an isotope effect. Thus the coprostanol formed from the mixture of 7α-^3H- and 4-^{14}C-labeled cholesterol had the same $^3H/^{14}C$ ratio as the precursor. Since the biosynthesis of coprostanol also involves 4-cholesten-3-one as an intermediate (29), an isotope effect in the conversion of cholesterol into 4-cholesten-3-one should have been reflected by a lowered $^3H/^{14}C$ ratio in coprostanol.

Acceleration of the novel pathway for formation of cholestanol from bile acid intermediates in patients with CTX. In the patient with CTX who received 7α-^3H- and 4-^{14}C-labeled cholesterol, the ratios between ^3H and ^{14}C in cholestanol isolated from plasma, bile, and feces were reduced as compared with corresponding ratios in a healthy control. Our findings indicate that the novel pathway discussed above (Fig. 2 *B*) accounts for ~75% of the total biosynthesis of cholestanol in this patient. The remaining 25% may be formed by the direct route which would not lead to removal of 7α-^3H. The novel pathway for biosynthesis of cholestanol is thus considerably accelerated in this patient as compared with the normal state. Our evidence for the presence of the novel pathway for biosynthesis of cholestanol in the normal state might not be accepted because of the possibility of an isotope effect in the conversion of 7α-^3H cholesterol into cholestanol in vivo. It is still evident from the additional decrease of the $^3H/^{14}C$ ratio in CTX, however, that at least 50% of all the cholestanol synthesized in this patient must have been formed by the pathway involving 7α-hydroxylated intermediates. If there is an isotope effect, it must be the same in the patient with CTX as in the normal subject. Our results thus provide complete evidence that the novel pathway is responsible for between 50 and 75% of the total synthesis of cholestanol in the patient with CTX.

Direct evidence that 7α-hydroxycholesterol is a precursor in the novel pathway to cholestanol. A possible mechanism for conversion. In the present study we have shown that ^3H from 7β-^3H-labeled 7α-hydroxycholesterol was incorporated to a significant extent into plasma and fecal cholestanol in one of our CTX patients. It is thus established that 7α-hydroxycholesterol may serve as an intermediate in a novel route for the biosynthesis of cholestanol. However, we found little or no incorporation of ^{14}C from ^{14}C labeled 7α-hydroxy-4-cholesten-3-one. The latter finding is in accordance with previous investigations (7, 10, 30). Less ^{14}C labeled precursor than desirable was available for our previous study (7) and the present work. (Informations on the amounts of labeled 7α-hydroxy-4-cholesten-3-one used in Salen's study were not given [10, 30].) It is also evident that a labeled precursor administered intravenously may be metabolized in a manner different from when it is formed within the cell. We therefore believe that our study does not exclude the possibility that 7α-hydroxy-4-cholesten-3-one is a cholestanol precursor of biological significance. We have previously shown that 7α-hydroxy-4-cholesten-3-one may be dehydroxylated in the gut and thereby serve as a cholestanol precursor. The concentrations of 7α-hydroxy-4-cholesten-3-one (14) and 7α-hydroxycholesterol (31) in the bile from control subjects and from patients with CTX, however, were very low. In view of this it seems more likely that the dehydroxylation step occurs in the liver than in the intestine. A quite large fraction of cholestanol newly synthesized from 7β-^3H-7α-hydroxycholesterol was found in the plasma of our patient. This finding may also point to the liver as the site of synthesis of plasma cholestanol, which is probably incorporated into nascent lipoproteins. Studies are in progress to investigate whether administration of cholesta-4,6-dien-3-one may lead to its incorporation into lipoproteins and conversion to cholestanol in organs in which cholestanol deposits are found in CTX (e.g., brain).

The detailed mechanism for conversion of 7α-hydroxycholesterol into cholestanol has not been investigated in the present work. Since a 7α-dehydroxylation is required, cholesta-4,6-dien-3-one would be a likely intermediate. Cholesta-4,6-dien-3-one has previously been shown to be an efficient precursor of 4-cholesten-3-one, and therefore also of cholestanol (14, 32). In view of the great amounts of 7α-hydroxycholesterol formed in the liver, conversion of only a low percentage into cholesta-4,6-dien-3-one would be required to explain the biosynthesis of cholestanol (refer below). In our laboratories, we have recently shown that 7α-hydroxycholesterol is partly converted to cholesta-4,6-dien-3-one via 7α-hydroxy-4-cholesten-3-one (Skrede, S., M. Buchmann, and I. Björkhem, unpublished observations) by rat liver microsomes in the presence of NAD (results not shown).

The possible mechanisms for formation of 4-cholesten-3-one from 7α-hydroxycholesterol and the different metabolic interrelations are summarized in Fig. 2.

The basic metabolic defect in CTX and its relation to cholestanol biosynthesis. We have previously presented evidence from in vitro (6) and in vivo (7) studies for the concept that the metabolic defect in CTX is a deficiency of a mitochondrial C_{27}-steroid 26-hydroxylase, which belongs to the physiologically most important pathway for the side chain degradation (6, 7). Our conclusions are thus at variance with those of Salen and coworkers (8), who interpreted their results as due to a deficiency of a microsomal 24β-hydroxylase active on 5β-cholestane-3α,7α,12α,25-tetrol, which was apparently more slowly converted to cholic acid than normal (reduced to ¼). We think that their findings can be explained by an increased endogenous pool of 3α,7α,12α,25-tetrol, which is present in CTX (6). Our results also indicate that side-chain degradation initiated by 25-hydroxylation is normally a minor pathway (6,

7). In CTX, however, this route probably substitutes for normal 26-hydroxylation, but the capacity is too low for normal biosynthesis of bile acids. Accordingly, abnormal 25-hydroxylated tetrols and pentols accumulate and are excreted in bile (4) and urine[2] (33), whereas 26-hydroxylated bile alcohols are not found[2] (4, 33).

Because of reduced feedback control, the activity of the 7α-hydroxylase is increased (5). Bile acid intermediates, such as 7α-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one, are accumulated in the liver (7). The rate of 7α-dehydroxylation may be increased because of increased substrate concentrations. Increased flow through the 7α-hydroxylase combined with the deficient side-chain degradation leads to enormously increased formation of bile alcohols (4, 34). In our patients, the output of bile alcohols in feces and urine sometimes exceeded 1,000–1,500 mg/d.[2] The present study shows that the fecal excretion of cholestanol was 20–50 mg/24 h, i.e., 1–3% of the sum of 7α-hydroxylated cholesterol metabolites excreted.[2] This fraction is of the same order of magnitude as the relative amount of ^{3}H found in cholestanol when compared with the total amount of ^{3}H-labeled metabolites excreted after injection of 7β-^{3}H-7α-hydroxycholesterol in one of our patients (I.J.). It can further be roughly calculated from the decrease of the ^{3}H/^{14}C ratio during conversion of cholesterol to cholestanol in the other patient (A.F.) that the novel pathway accounts for a maximum of 70–75% of all cholestanol formed, i.e., 30–40 mg/d in this CTX patient. The increased activity of the novel pathway and the deficiency of the 26-hydroxylase—which would be expected to affect also the metabolism of cholestanol—are probably the most important causes of the accumulation of cholestanol in CTX. Another expression of the quantitative importance of the pathway described in the present study is that treatment with cholestyramine, which increases 7α-hydroxylation of cholesterol, strongly increases the output of cholestanol in CTX (34). Our observations probably also explain why treatment with chenodeoxycholic acid, which decreases 7α-hydroxylation of cholesterol, leads to decreased biosynthesis of cholestanol in patients with CTX (34).

Acknowledgments

The skillful technical assistance of Anne Marie Lund and Manfred Held is gratefully acknowledged.

This work was supported by The Norwegian Research Council for Science and the Humanities (project 13.10.99-009) and the Swedish Medical Research Council (project 03X-3141).

References

1. van Bogaert, L., H. J. Scherer, and E. Epstein. 1937. Une forme cérébrale de cholestérinose généralisée. Masson et Cie, Paris.

2. Menkes, J. H., J. R. Schimschock, and P. D. Swanson. 1968. Cerebrotendinous xanthomatosis. The storage of cholestanol within the nervous system. *Arch. Neurol. (Chic.).* 19:47–53.

3. Salen, G. 1971. Cholestanol deposition in cerebrotendinous xanthomatosis. A possible mechanism. *Ann. Intern. Med.* 75:843–851.

4. Setoguchi, T., G. Salen, G. S. Tint, and E. H. Mosbach. 1974. A biochemical abnormality in cerebrotendinous xanthomatosis. *J. Clin. Invest.* 53:1393–1401.

5. Nicolau, G., S. Shefer, G. Salen, and E. H. Mosbach. 1974. Determination of hepatic cholesterol 7α-hydroxylase activity in man. *J. Lipid Res.* 15:146–151.

6. Oftebro, H., I. Björkhem, S. Skrede, A. Schreiner, and J. I. Pedersen. 1980. Cerebrotendinous xanthomatosis: a defect in mitochondrial 26-hydroxylation required for normal biosynthesis of cholic acid. *J. Clin. Invest.* 65:1418–1430.

7. Björkhem, I., O. Fausa, G. Hopen, H. Oftebro, J. I. Pedersen, and S. Skrede. 1983. Role of the 26-hydroxylase in the biosynthesis of bile acids in the normal state and in cerebrotendinous xanthomatosis. *J. Clin. Invest.* 71:142–148.

8. Salen, G., S. Shefer, F. W. Cheng, B. Dayal, A. K. Bath, and G. S. Tint. 1979. Cholic acid biosynthesis. The enzymatic defect in cerebrotendinous xanthomatosis. *J. Clin. Invest.* 63:38–44.

9. Salen, G., and A. Polito. 1972. Biosynthesis of 5α-Cholestan-3β-ol in cerebrotendinous xanthomatosis. *J. Clin. Invest.* 51:134–140.

10. Salen, G., and E. H. Mosbach. 1976. The metabolism of sterols and bile acids in cerebrotendinous xanthomatosis. *In* The Bile Acids, Vol. 3. P. P. Nair and D. Kritchevsky, editors. Plenum Press, New York. 115–153.

11. Rosenfeld, R. S., B. Zumoff, and L. Hellman. 1967. Conversion of cholesterol injected into man to cholestanol via a 3-ketonic intermediate. *J. Lipid Res.* 8:16–23.

12. Björkhem, I., and K.-E. Karlmar. 1974. Biosynthesis of cholestanol: conversion of cholesterol into 4-cholesten-3-one by rat liver microsomes. *Biochim. Biophys. Acta.* 337:129–131.

13. Björkhem, I., H. Oftebro, S. Skrede, and J. I. Pedersen. 1981. Assay of intermediates in bile acid biosynthesis using isotope dilution-mass spectrometry: hepatic levels in the normal state and in cerebrotendinous xanthomatosis. *J. Lipid Res.* 22:191–200.

14. Skrede, S., and I. Björkhem. 1982. Biosynthesis of cholestanol from intestinal 7α-hydroxy-4-cholesten-3-one. *J. Biol. Chem.* 257: 8363–8367.

15. Schreiner, A., G. Hopen, and S. Skrede. 1975. Cerebrotendinous xanthomatosis (cholestanolosis). Investigations on two sisters and their family. *Acta Neurol. Scand.* 51:405–416.

16. Corey, E. J., and G. A. Gregoriou. 1959. Stereospecific syntheses of the 7-deuterio- and 7-tritiocholesterols. The mechanism of enzyme-catalyzed hydroxylation at a saturated carbon atom. *J. Am. Chem. Soc.* 81:3127–3133.

17. Fieser, L. F. 1953. Cholesterol and companions. II. Exhaustive dichromate oxidation. *J. Am. Chem. Soc.* 75:4386–4394.

18. Björkhem, I., and H. Danielsson. 1975. 7α-hydroxylation of exogenous and endogenous cholesterol in rat-liver microsomes. *Eur. J. Biochem.* 53:63–70.

19. Bergström, S., S. Lindstedt, B. Samuelsson, E. J. Corey, and G. A. Gregoriou. 1958. The stereochemistry of 7α-hydroxylation in the biosynthesis of cholic acid from cholesterol. *J. Am. Chem. Soc.* 80:2337–2338.

20. Danielsson, H., and K. Einarsson. 1966. On the conversion of cholesterol to 7α,12α-dihydroxycholest-4-en-3-one. *J. Biol. Chem.* 241: 1449–1454.

21. Setchell, K. D. R., and J. Worthington. 1982. A rapid method for the quantitative extraction of bile acids and their conjugates from serum using commercially available reverse-phase octadecylsilane bonded silica cartridges. *Clin. Chim. Acta.* 125:135–144.

22. Ross, P. E., C. R. Pennington, and I. A. D. Bouchier. 1977. Gas-liquid chromatographic assay of serum bile acids. *Anal. Biochem.* 80:458–465.

23. Skrede, S., and I. Björkhem. 1982. Intestinal 7α-hydroxy-4-cholesten-3-one may contribute to the accumulation of cholestanol in cerebrotendinous xanthomatosis. *Falk-Symp. 35, VI. Int. Congr. Liver Dis.*, Basel. 173. (Abstr.)

24. Samuelsson, B. 1960. Bile acid and steroids. 96. On the mechanism of the biological formation of deoxycholic acid from cholic acid. *J. Biol. Chem.* 235:361–366.

25. Wilkins, T. D., and A. S. Hackman. 1974. Two patterns of neutral steroid conversion in the feces of normal North Americans. *Cancer Res.* 34:2250–2254.

26. Anker, H. S., and K. Bloch. 1949. On the metabolism of $\Delta^{4,5}$-cholestenone. *J. Biol. Chem.* 178:971–976.

27. Shefer, S., S. Hauser, and E. H. Mosbach. 1966. Studies on the biosynthesis of 5α-cholestan-3β-ol. I. Cholestenone 5α-reductase of rat liver. *J. Biol. Chem.* 241:946–952.

28. Shefer, S., S. Hauser, and E. H. Mosbach. 1966. Biosynthesis of cholestanol: 5α-cholestan-3-one reductase of rat liver. *J. Lipid Res.* 7:763–771.

29. Björkhem, I., and J.-Å. Gustafsson. 1971. Mechanism of microbial transformation of cholesterol into coprostanol. *Eur. J. Biochem.* 21:428–432.

30. Salen, G. 1974. The metabolism of cholest-4-en-3-one and 7α-hydroxycholest-4-en-3-one: transformation into bile acids and cholestanol in man. *Clin. Res.* 22:367A.

31. Björkhem, I., M. S. Buchmann, and S. Skrede. 1983. Isolation of 5α-cholestane-3β,7α-diol from bile of patients with cerebrotendinous xanthomatosis. *Biochim. Biophys. Acta.* 753:220–226.

32. Kandutsch, A. A. 1963. Metabolism of cholesta-4,7-dien-3-one and cholesta-4,6-dien-3-one by mouse liver microsomes. *J. Lipid Res.* 4:179–187.

33. Wollhers, B. G., M. Volmer, J. van der Molen, B. J. Koopman, A. E. J. de Jager, and R. J. Waterreus. 1983. Diagnosis of cerebrotendinous xanthomatosis (CTX) and effect of chenodeoxycholic acid therapy by analysis of urine using capillary gas chromatography. *Clin. Chim. Acta.* 131:53–65.

34. Salen, G., and S. M. Grundy. 1973. The metabolism of cholestanol, cholesterol, and bile acids in cerebrotendinous xanthomatosis. *J. Clin. Invest.* 52:2822–2835.