Cholestanol metabolism in patients with cerebrotendinous xanthomatosis: absorption, turnover, and tissue deposition

Ashim K. Bhattacharyya,* Don S. Lin,† and William E. Connor[1,†]

Departments of Pathology and Physiology,* Louisiana State University Health Sciences Center, New Orleans, LA; and Department of Medicine,† Oregon Health and Science University, Portland, OR

Abstract **To study the metabolism of cholestanol in patients with cerebrotendinous xanthomatosis (CTX), we measured the cholestanol absorption, the cholesterol and cholestanol turnover, and the tissue content of sterols in two patients. Cholestanol absorption was ~5.0%. The rapid exchangeable pool of cholestanol was 233 mg, and the total exchangeable pool was 752 mg. The production rate of cholestanol in pool A was 39 mg/day. [4-^{14}C]cholestanol was detected in the xanthomas, but neither [4-^{14}C]cholestanol nor [4-^{14}C]cholesterol was detected in peripheral nerves biopsied at 49 and 97 days after [4-^{14}C]cholesterol given intravenously. Of the 18 tissues analyzed at biopsy and autopsy, the cholestanol content varied from 0.09 mg/g in psoas muscle to 76 mg/g in a cerebellar xanthoma. With the assumption that the cholestanol-to-cholesterol ratio is 1.0, the relative cholestanol-to-cholesterol ratio varied from 1.0 in plasma and liver to 30.0 in the cerebellar xanthoma; cholestanol was especially high in nerve tissue. Our data indicate that CTX patients absorb cholestanol from the diet. They have a higher than normal cholestanol production rate. Cholestanol was derived from cholesterol. In CTX patients, the blood-brain barrier was intact to the passage of [4-^{14}C]cholesterol and [4-^{14}C]cholestanol. The deposition of large amounts of cholestanol (up to 30% of total sterols in cerebellum) in nerve tissues must have an important role in the neurological symptoms in CTX patients. In view of the intact blood-brain barrier, several other explanations for the large amounts of cholestanol in the brain were postulated.—** Bhattacharyya, A. K., D. S. Lin, and W. E. Connor. **Cholestanol metabolism in patients with cerebrotendinous xanthomatosis: absorption, turnover, and tissue deposition.** ***J. Lipid Res.*** **2007.** 48: **185–192.**

Supplementary key words neurological disorders • cholesterol and cholestanol turnover • blood brain barrier • brain sterols • brain cholestanol • chenodeoxycholic acid • sterol 27-hydroxylase • 27-hydoxy cholesterol • 7α-hydroxy-4 cholesten-3-one

Cerebrotendinous xanthomatosis (CTX), first reported by van Bogaert, Scherer, and Epstein (1) in 1937, is a rare autosomal recessive neurologic disease characterized by xanthomas of tendons, lungs, and the brain despite normal or low plasma cholesterol concentrations. Other major clinical manifestations include diarrhea, cataracts, premature atherosclerosis and myocardial infarction, progressive cerebellar ataxia, dementia, spinal cord paresis, and below-normal intelligence. Greatly increased concentrations of cholestanol, the 5α-dihydro derivative of cholesterol, in plasma and in practically all tissues, notably xanthoma, nerve tissues, and brain, have been reported (2–4).

Small amounts of cholestanol normally accompany cholesterol in virtually every mammalian tissue. The normal biosynthesis and metabolism of cholestanol have been well reviewed by Bjorkhem and Skrede (4). The major metabolic defect in the CTX syndrome is the impaired synthesis of chenodeoxycholic acid from cholesterol. There is a deficiency of the mitochondrial sterol 27-hydroxylase enzyme, which catalyzes the initial steps in the oxidation of the side chain of the cholesterol structure in the conversion of cholesterol to bile acids (3–6). The CTX syndrome has been traced to a mutation of the sterol 27 hydroxylase (CYP27A1) gene on chromosome 2q33-qter (7). Several other mutations of the sterol 27-hydroxylase gene have now been reported (7–11). It has been suggested that the accumulation of cholestanol in the tissues is secondary to this mutation of the CYP27A1 gene (3).

Salen, Shefer, and Berginer (3) proposed that large accumulations of cholesterol and cholestanol in tissues result from an increased synthesis of cholestanol and cholesterol. Besides increased biosynthesis of cholestanol, increased intestinal absorption of dietary cholestanol may also contribute to the plasma levels, so we studied the intestinal absorption of cholestanol in two patients with CTX. We also measured the turnovers of plasma cholestanol and

Manuscript received 7 March 2006 and in revised form 20 April 2006 and in re-revised form 22 June 2006 and in re-re-revised form 2 August 2006 and in re-re-re-revised form 12 September 2006 and in re-re-re-re-revised form 29 September 2006.

Published, JLR Papers in Press, October 1, 2006.
DOI 10.1194/jlr.M600113-JLR200

[1] To whom correspondence should be addressed.
e-mail: connorw@ohsu.edu

cholesterol and determined the equilibration of isotopic cholestanol and cholesterol between plasma, xanthoma, and peripheral nerve tissue. In addition, we also analyzed 14 autopsy samples from one patient to assess the cholestanol and cholesterol contents and their relative ratios in various tissues. In particular, we suggest some new hypotheses for the profound accumulation of cholestanol in nerve tissue, which may result from synthesis in nerve tissue.

MATERIALS AND METHODS

Patients

Two brothers, aged 26 and 30 years and weighing 78 and 68 kg, respectively, displayed all of the clinical manifestations of the CTX syndrome: large tendon xanthoma (Achilles, patellar, and extensor tendons of the hands), cataracts, ataxia, and below-normal intelligence. Their plasma cholestanol concentrations were 3.5 and 3.6 mg/dl, respectively, and plasma cholesterol levels were 195 and 181 mg/dl, respectively. The plasma cholestanol concentrations of their mother, eldest brother and his two children, and a maternal uncle were normal (0.25–0.45 mg/dl).

Both patients were studied at the Clinical Research Center of the University of Iowa Hospital in the 1970s. The experimental protocols were explained to the patients, their mother, and their court-appointed protector, and informed consent was obtained from all persons concerned according to the policies of the Committee on Investigation involving Human Beings of the University of Iowa College of Medicine. The Committee approved the study protocol. At that time, their treatment was a low-cholestanol diet; the value of chenodeoxycholic acid in the CTX syndrome had not yet been demonstrated.

Cholestanol absorption

The intestinal absorption of cholestanol was measured in both patients by feeding a single dose of [4-^{14}C]cholestanol in a formula breakfast that provided ~800 calories and contained ~50 g of fat, consisting of 19% monounsaturated, 16% polyunsaturated, and 18% saturated fatty acids. The carbohydrate and protein contents of the meal were 63 and 25 g, respectively. The cholesterol content was ~100 mg. The isotope (~3 μCi) along with ~2.5 μCi of [22,23-^{3}H]β-sitosterol was dissolved in 5 g of peanut oil with 150 mg of crystalline β-sitosterol and 0.5 mg of crystalline cholesterol added as carriers and then mixed with egg yolk. An aliquot of the formula was analyzed for sterol mass and radioactivities to obtain the exact amounts fed (12). Feces were collected daily for the next 7 days and stored in plastic bags at −20°C. The 7 day feces pool was homogenized with water (1:1), and an aliquot was analyzed for radioactivity in the neutral sterol fraction (12). The absorption of cholestanol was calculated as the difference between the amount fed and the amount excreted in the neutral sterol fraction of the feces after correcting on the basis of sitosterol radioactivity recovery and was expressed as percentage of intake (13).

Cholesterol and cholestanol turnover

For the cholesterol turnover studies, both patients were given intravenously a single dose of ~50 μCi of [4-^{14}C]cholesterol (New England Nuclear Corp., Boston, MA) dissolved in 5 ml of ethanol and suspended in 500 ml of 0.9% NaCl solution as described previously (14). A plasma cholestanol turnover study was carried out in both patients ~2 years later. Approximately 50 μCi of [4-^{14}C]cholestanol [New England Nuclear; purified by $AgNO_3$-TLC, with>99% radiopurity as checked by $AgNO_3$-TLC and gas liquid chromatography (GLC)] was given intravenously as a single dose as described above.

Venous blood samples were obtained every morning in the fasting state for the first 5 days and then twice weekly for the next 12 weeks. Plasma was separated by centrifugation at 4°C and stored at −20°C for determination of plasma cholestanol- and cholesterol-specific radioactivities as described below.

Plasma cholestanol- and cholesterol-specific radioactivities were plotted semilogarithmically against time. For both patients, the plasma cholesterol-specific radioactivity decay curve could be resolved precisely into the sum of two exponentials. The various parameters of cholesterol turnover were calculated on the basis of the kinetic analysis of the two-pool model (15). However, the semilogarithmic plots of the plasma cholestanol-specific radioactivity were widely scattered in both patients. In patient 1, the plasma cholestanol-specific radioactivity decay curve could be resolved, although not very precisely, into the sum of two exponentials; therefore, approximate values for cholestanol turnover were calculated. In patient 2, the plasma cholestanol-specific radioactivity decay curve could not be resolved into the sum of two exponentials. Hence, no attempt was made to calculate the plasma cholestanol turnover in this patient.

Biopsies of tendon xanthomas, sural nerve, liver, skin, and adipose tissue were obtained using routine hospital procedures. Duodenal bile samples were obtained by duodenal intubation using $MgSO_4$ solution to enhance the bile flow. All procedures were carried out while both patients were inpatients at the Clinical Research Center.

Determination of sterol concentrations in plasma, erythrocytes, and tissues

One milliliter of plasma or serum was saponified with alcoholic KOH and extracted with hexane according to the procedure of Abell et al. (16). The erythrocytes were washed repeatedly with 0.9% NaCl solution, and the lipids were extracted with chloroform-isopropanol (7:11, v/v) according to the method of Rose and Oklander (17) and made up to 50 ml with the solvent. All tissue specimens were cut into small pieces and washed repeatedly with 0.9% NaCl solution to remove blood. The pieces were blotted on a filter paper, weighed, and dried in a hot vacuum oven at 80°C to a constant weight. The dried tissue was repeatedly extracted by boiling with chloroform-methanol (2:1, v/v); the lipid extract was made up to a suitable volume (usually 50 ml) with the solvent. An aliquot was then saponified and extracted with hexane as described above. The hexane extract was evaporated to dryness under N_2, redissolved in a small volume of hexane, and subjected to $AgNO_3$-TLC using a 0.25 mm thick silica gel H-coated glass plate (Applied Science Laboratories, Inc., State College, PA). The plate was pre-run in chloroform, air-dried, and impregnated with $AgNO_3$ by allowing a 10% aqueous solution of $AgNO_3$ to migrate to the top. The plate was then air-dried and activated at 120°C for 1 h. The hexane extract was applied on the plate as a narrow band using the TLC streaker (Applied Science Laboratories) along with reference cholestanol and cholesterol. The plate was developed at 4°C in chloroform-acetone (97:3, v/v), and the solvent was allowed to ascend to the top of the plate. The sterol bands were visualized under ultraviolet light after spraying lightly with a saturated aqueous solution of Rhodamine 6G (Applied Science Laboratories), scraped, eluted with ethyl ether, and subjected to GLC as the trifluoroacetate derivative, with 5α-cholestane as the internal standard (18).

GLC was carried out on a dual-column gas chromatograph equipped with a hydrogen flame ionization detector and an automatic digital integrator (Hewlett-Packard Co., Avondale, PA).

The column was a coiled glass of 183 cm length and 2 mm internal diameter packed with 3% QF-1 on 80–100 mesh Gas-chrome Q (Supelco, Inc., Bellefonte, PA). The temperatures of the column, detector, and flash heater were 210, 240, and 260°C, respectively. Nitrogen was the carrier gas at a flow rate of 24 ml/min, and the inlet pressure was 50 p.s.i. The sensitivity of our GLC method for the detection of sterols was in the 10–20 ng range.

Free and esterified sterols in the tissue extracts were separated by TLC using a silica gel G plate. The plate was developed in petroleum ether-diethyl ether-glacial acetic acid (80:20:1, v/v). The free and ester sterol bands were scraped, eluted with diethyl ether, and subjected to $AgNO_3$-TLC as described above to separate cholestanol from cholesterol. The individual sterols were quantitated by GLC as described above.

Determination of cholestanol and cholesterol radioactivities

Cholestanol and cholesterol radioactivities in the plasma and tissues were determined on aliquots of the same eluate obtained after separation of cholestanol from cholesterol by $AgNO_3$-TLC. Ten milliliters of scintillation fluid [4 g of 2,5,5-diphenyloxazole and 0.1 g of l,4-bis(2-5-phenyloxazolyl) benzene per liter of toluene] was used in each counting vial. A liquid scintillation spectrometer equipped with external standardization (Packard Instrument Co., Inc., Downers Grove, IL) was used for radioactivity counting.

RESULTS

Plasma lipid concentrations

The plasma levels of cholestanol, cholesterol, and triglyceride as well as erythrocyte concentrations of cholestanol and cholesterol in the two patients are shown in **Table 1**. Both patients had more than eight times higher concentrations of plasma cholestanol than for the normal subjects. Cholestanol was also found in the membranes of erythrocytes. The ratios of cholestanol to cholesterol in the plasma and erythrocytes were similar, showing ready equilibration. Plasma cholesterol levels were <200 mg/dl in both patients. Plasma triglyceride levels were normal.

Cholestanol absorption

The results presented in **Table 2** show that the intestinal absorption of cholestanol in the two patients measured by feeding a single dose of radioactive cholestanol was 5.5% and 3.4% of the dose fed. In six normal human subjects, the mean intestinal absorption of cholestanol was 3.3 ± 2.7% (SD) of a single oral dose (Table 2).

TABLE 1. Plasma and erythrocyte lipid concentrations in two CTX patients

Variable	Cholestanol	Cholesterol	Triglycerides
Plasma			
Patient 1	3.5 ± 0.06	195 ± 13	169 ± 13
Patient 2	3.6 ± 0.5	181 ± 29	129 ± 13
Normal subjects (n = 13)	0.4 ± 0.2	197 ± 28	150 ± 22
Erythrocytes			
Patient 1	1.9 ± 0.6	98 ± 6	
Patient 2	2.0 ± 0.6	99 ± 6	

CTX, cerebrotendinous xanthomatosis. Plasma values are mean mg/100 ml ± SD. Erythrocyte values are mean mg/100 ml packed cells ± SD.

TABLE 2. Percentage intestinal absorption of cholestanol in two patients with CTX after a single oral dose of [4-^{14}C]cholestanol

Subject	Percent
Patient 1	5.5
Patient 2	3.4
Normal subjects (n = 6)	3.3 ± 2.7

For normal subjects, the values are mean percent ± SD.

Plasma cholesterol and cholestanol turnover

The plasma cholesterol turnover calculated from the plasma cholesterol-specific radioactivity decay curve according to the two-pool model (15) showed that the half-lives of the first exponential were 4.5 and 4 days and the half-lives of the second exponential were 51 and 46 days in patients 1 and 2, respectively (**Table 3**). In the two patients, the sizes of rapidly exchangeable pool A (M_A) were 26 and 21 g and those of total exchangeable pools ($M_A + M_B$) were 84 and 70 g, respectively. The production rate of cholesterol in pool A (PR_A) in patient 1 was 2.57 g/day, and that in patient 2 was 2.16 g/day (Table 3).

Plasma cholestanol turnover could only be calculated according to the two-pool model for patient 1. For patient 2, the plasma cholestanol radioactivity decay curve could not be determined for the required length of time because the patient left the hospital for family and personal reasons; thus, not enough blood samples could be obtained. The results of the plasma cholestanol turnover, according to the two-pool model (15), in patient 1 are shown in Table 3. The rapidly exchangeable pool (M_A) was 233 mg,

TABLE 3. Plasma cholesterol and cholestanol turnover in two CTX patients

	Patient 1		Patient 2
Variable	Cholesterol	Cholestanol	Cholesterol
Half-life of the first exponential, days	4.5	3	3
Half-life of the second exponential, days	51	31	46
Pool size A (M_A), g	25.9	0.233	20.9
Pool size B (M_B),[a] g	58.2	0.419	49.3
Total exchangeable pool ($M_A + M_B$), g	84.1	0.752	70.2
Production rate of cholesterol in pool A, g/day	2.57	0.039	2.16
Rate constants			
K_{AA} per day	−0.1465	−0.2707	−0.1631
K_{AB} per day	0.0475	0.0698	0.0597
K_A per day	0.0990	0.2009	0.1033
K_{BB} per day	−0.0211	−0.0239	−0.0253
K_{BA} per day	0.0211	0.0239	0.0253

K_{AA}, rate constant for total removal from pool A; K_{AB}, rate constant for transfer from pool A to pool B; K_A, rate constant for excretion from pool A and the whole system; K_{BB}, rate constant for total removal from pool B; K_{BA}, rate constant for transfer from pool B to pool A.

[a] Minimum estimate assuming no synthesis of cholesterol or cholestanol in pool B (i.e., $K_B = 0$) (6).

TABLE 4. Cholestanol and cholesterol content of tissues biopsied from the two CTX patients

Variable	Cholestanol			Cholesterol			Relative Cholestanol-to-Cholesterol Ratio[a]
	Total	Free	Ester	Total	Free	Ester	
Patient 1							
Plasma	3.5			195			1.0
Xanthoma							
Left tendo-Achilles	25.9 (9.8)	13.8	12.1	239.3 (90.2)	110.7	128.6	5.5
Right tendo-Achilles	20.2 (11.2)	2.9	17.3	160.4 (88.8)	24.9	135.5	6.5
Left patellar	5.1 (11.7)	1.1	4.0	38.0 (88.3)	2.7	35.3	6.5
Skin	0.1 (5.7)			1.2 (94.3)			4.0
Adipose tissue	0.2 (11.8)			1.5 (88.2)			6.5
Nerve (sural)							
Left heel	1.5 (20.8)			5.6 (79.2)			13.5
Right heel	1.7 (20.3)	0.7	1.0	6.8 (79.7)	3.2	3.6	12.5
Patient 2							
Plasma	3.6			181			1.0
Skin	0.1 (7.0)			1.3 (93.0)			4.0
Adipose tissue	0.2 (18.2)			0.9 (81.8)			11.0

Values are mg/g dry weight of tissue. The numbers in parentheses are percentage of total sterols.
[a] Ratio in plasma was designated as 1.00.

and the total exchangeable pool was 752 mg. The PR_A of cholestanol was ~39 mg/day (Table 3).

Tissue sterols, radioactivity, and the blood-brain barrier

Cholestanol was found in large amounts in tendon xanthoma, peripheral nerve, skin, and adipose tissue of patient 1 (**Table 4**). It constituted between 6% and 12% of total sterols in the tendon xanthomas, skin, and adipose tissue. In the sural nerve, cholestanol constituted ~20% of total sterols. In patient 2, in skin and adipose tissue, cholestanol constituted 7% and 18% of total sterols, respectively. The relative cholestanol-cholesterol ratios varied from 1.0 in plasma to 13.5 in sural nerve in patient 1. It varied from 1.0 in plasma to 11.0 in adipose tissue in patient 2.

Between 47% and 86% of total cholestanol in tendon xanthomas was found as esterified cholestanol. Similarly, in the sural nerve, 59% of total cholestanol was in the esterified form. Esterified cholesterol ranged between 54% and 93% in the tendon xanthomas and was 53% in the sural nerve.

The free and ester cholesterol-specific radioactivities of the left tendo-Achilles xanthoma biopsied 49 days after intravenous administration of [4-^{14}C]cholesterol were 240 and 184 dpm/mg cholesterol, and those for xanthoma cholestanol were 228 and 140 dpm/mg for free and ester cholestanol, respectively (**Table 5**). The plasma free and ester cholesterol-specific radioactivities on the day of biopsy were 247 and 251 dpm/mg cholesterol. Similarly, free and ester cholesterol-specific activities of the right tendo-Achilles xanthoma biopsied 87 days after injection of [^{14}C]cholesterol were 252 and 149 dpm/mg cholesterol, respectively, whereas plasma cholesterol-specific activities were 152 and 151 dpm/mg and those for cholestanol were 166 and 111 dpm/mg. Furthermore, free and esterified cholesterol-specific activities in the left patellar xanthoma obtained 415 days after intravenous [^{14}C]cholesterol were 165 and 58 dpm/mg, and those for cholestanol were 105 and 34 dpm/mg. In the plasma, no radioactivity was detected at 415 days.

In contrast, no radioactivity was detected in the peripheral nerves biopsied 49 and 87 days after intravenous [^{14}C]cholesterol (Table 5). The most direct way to demonstrate that the blood-brain barrier was functionally intact in the CTX patient is to ask whether [4-^{14}C]cholesterol injected intravenously and found in other tissues of the body is also found in the tissues of the nervous system. The answer from two nerve biopsies was that no radioactive cholesterol or cholestanol was found in the nerve tissue after injection of isotopic cholesterol. The specific radioactivity (dpm/mg) of both cholesterol and cholestanol was easily measurable in plasma and tendon xanthomas at

TABLE 5. Plasma and xanthoma free and esterified sterol/stanol-specific radioactivity in patient 1 with CTX

Days[a]	Plasma		Xanthoma					Sural Nerve	
	Cholesterol			Cholesterol		Cholestanol			
	Free	Ester	Tendo-Achilles	Free	Ester	Free	Ester	Cholesterol	Cholestanol
49	247	251	Left	240	184	228	140	0	0
87	152	151	Right	252	149	166	111	0	0
415			Left patellar	165	58	105	34	—	—

Values are dpm/mg.
[a] After intravenous dose of [4-^{14}C]cholesterol.

TABLE 6. Isotopic equilibration between plasma, liver, and bile cholestanol after an intravenous dose of [4-^{14}C]cholestanol

Patient	Days after Intravenous Isotope	Cholestanol-Specific Activities			Percent of Equilibration		
		Plasma	Liver[a]	Bile[b]	Liver/Plasma	Bile/Liver	Bile/Plasma
		dpm/mg					
1	2	352,381	—	263,077			75
	7	91,892	225,077	50,071	245	22	54
	49	4,890	—	3,747			77
2	3	167,513		139,403			83
	8	27,136	62,176	29,555	229	47	109
	72	1,562		1,437			92

[a] Liver cholesterol was 0.013 and 0.119 mg/g dry weight for patients 1 and 2, respectively.
[b] Bile cholesterol was 0.90, 1.11, and 0.63 mg/100 ml for patient 1 at 2, 7, and 49 days and 0.20, 0.19, and 0.17 mg/100 ml for patient 2 at 3, 8, and 72 days.

similar time points. This is evidence for the intact blood-brain barrier for cholestanol and cholesterol.

Isotopic equilibration between liver, bile, and plasma cholestanol after intravenous [4-^{14}C]cholestanol

The data presented in **Table 6** show that liver cholestanol-specific activity was more than double the plasma cholestanol-specific activity in both patients at 7–8 days after an intravenous dose of [4-^{14}C]cholestanol. Biliary cholestanol-specific activity equilibrated to only 22% of that in liver in patient 1 and to 47% in patient 2.

Sterol composition of autopsy samples from patient 1

During the course of our study, patient 1 died from an accidental fall at home. Tissues were obtained for cholesterol and cholestanol analysis. The plasma and 14 different tissues were analyzed. The plasma cholesterol and cholestanol concentrations were 118 and 3.2 mg/dl, respectively (**Table 7**). Tissue cholestanol level varied from 0.08 mg/g in periadrenal fat to 76.16 mg/g in cerebellar xanthoma. The cholestanol-to-cholesterol ratio varied greatly among the tissues. Most notably, high ratios were found in nerve tissues. Plant sterols were not found in the brain tissue.

Calculation of relative cholestanol-to-cholesterol ratios

To compare the relationship of these two sterols (cholesterol and cholestanol) and, in turn, shed some light on the metabolism of these two sterols in different tissue compartments, we designated the cholestanol-to-cholesterol ratio as 1.0 in the plasma. Other tissues had ratios relative to plasma and varied greatly, up to 30-fold in the cerebellar xanthoma. The relative cholestanol-to-cholesterol ratios of all tissues (total of 18 tissues from both autopsy and biopsies) are presented in **Fig. 1**. All tissues (except liver) had higher ratios than plasma. Liver and plasma had the same ratio. The ratio of pericardial fat was 3. That of adrenal cortex was 5. The brain tissue ratio varied between 9 and 14. The cerebellar xanthoma had the highest ratio, 30. No plant sterols were found in the tissues, including nerve tissue. The same relative ratio between plasma and liver indicated rapid equilibration between these compartments. The higher ratio in nonnerve tissues can be attributable to two possibilities: *1*) selective retention of cholestanol in the tissue and less uptake of cholesterol from the blood; or *2*) increased conversion of cholesterol to cholestanol. For nerve tissue, if the blood-brain barrier is intact, there are two possibilities for the high concentrations of cholestanol: *1*) increased syntheses of cholestanol from cholesterol; or *2*) the conversion of an intermediate product to cholestanol after the intermediate product enters the nerve tissue, as will be discussed below.

DISCUSSION

The principal findings of this investigation are as follows: *1*) the blood-brain barrier was intact to the passage of both cholesterol and cholestanol; *2*) there was an especially high content of cholestanol in the nerve tissue, which led to an especially high cholestanol-to-cholesterol ratio (the implication of these findings is that brain synthesis accounts for the large amount of cholestanol in the brain); *3*) the whole body synthesis of cholestanol was also

TABLE 7. Cholesterol and cholestanol composition in plasma and tissue of CTX patient 1

Specimen	Sterol Composition		Relative Cholestanol-to-Cholesterol Ratio[a]
	Cholesterol	Cholestanol	
Plasma (mg/dl)	118.00	3.20	1.00
Tissues (mg/g)			
Liver	11.59	0.38	1.00
Myocardium	3.46	0.13	1.33
Iliac atheroma	19.66	1.18	2.00
Pericardial fat	2.06	0.13	2.00
Kidney	10.82	0.78	2.33
Psoas muscle	0.73	0.09	4.00
Periadrenal fat	0.74	0.08	3.67
Adrenal medulla	31.00	3.71	4.00
Adrenal cortex	55.21	8.52	5.00
Cerebrum, gray	45.29	12.57	9.33
Cerebrum, white	65.48	23.33	12.00
Cerebellum, gray	55.69	21.98	13.33
Cerebellum, white	85.33	36.05	14.00
Cerebellar xanthoma	85.15	76.19	30.00

[a] Ratio in plasma was designated as 1.00.

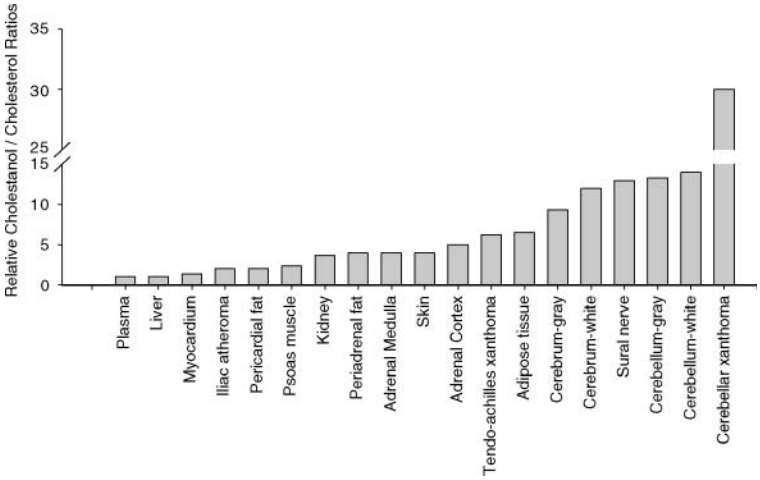

Fig. 1. Relative cholestanol-to-cholesterol ratios of tissues in cerebrotendinous xanthomatosis patients relative to plasma, in which the ratio was assumed to be 1.0.

increased; and *4*) there was cholestanol absorption from the diet. Each of these topics will be discussed sequentially.

The peripheral nerve biopsies obtained at 49 and 87 days after an intravenous dose of [4-^{14}C]cholesterol did not show any radioactivity in either the cholesterol or the cholestanol fraction of the nerve. This finding suggested that the brain-nerve barrier for these sterols was intact in these patients. This barrier would prevent the transport of sterols from the blood into nerve tissue. The blood-nerve barrier is similar to the blood-brain barrier (19). The blood-brain barrier, therefore, is intact in these patients as well. Our finding of a high cholestanol-to-cholesterol ratio in the nerve tissue, compared with liver and other tissues, is consistent with this interpretation. Furthermore, with a disrupted blood-brain barrier, plant sterols, such as sitosterol, should be found in milligram quantities in the brain. We found no plant sterols. Three other analyses of brain tissue from CTX patients, by Menkes, Schimschock, and Swanson (20), Salen (21), and Philippart and van Bogaert (22), did not allude to plant sterols in their GLC analyses. These data further attest to the intactness of the blood-brain barrier in the CTX syndrome. However, Salen and colleagues (23) have postulated that bile acid alcohols in CTX alter the blood-brain barrier and allow circulating apolipoprotein B-100 to enter the central nervous system. The reason for this discrepancy is not clear.

The two CTX brothers whom we studied had already suffered severe neurological damage. We surmise that this disease is progressive with time. The older the patient, the greater the deposition of cholestanol in the nerve tissue and the greater the ratio of cholestanol to cholesterol in the brain. This progression is also seen in the cataract formation in the lens, which does not become manifested until late childhood. CTX children are probably normal at birth but may develop intractable diarrhea. This "cholestanol progression" of the disease makes therapy with chenodeoxycholic acid even more important (6), and early diagnosis by genetic screening at birth would be important to implement. However, still to be understood is how therapy with chenodeoxycholic acid will stop cholestanol production in the brain. The decline of cholestanol levels in cerebrospinal fluid (23) suggests that chenodeoxycholic acid crosses the blood-brain barrier and, as a product, then inhibits cholestanol production in the brain, liver, and other tissues by feedback inhibition.

Norlin and colleagues (24) have postulated a number of pathways in the CTX syndrome that can lead to increased amounts of cholestanol in the tissues. All tissues of the body express the CYP27A1 gene, so the metabolism of oxysterols may be important in extrahepatic tissues as well as in the liver. An attractive mechanism is that in CTX, the accumulation of cholestanol could be caused by a reduced removal of the sterol from macrophages because of a deficiency in CYP27A1 in the macrophages (6). Menkes, Schimschock, and Swanson (20), the discoverers of cholestanol storage in this disease, had, in fact, postulated a defect in the transport of cholesterol across the cell membrane in CTX syndrome, with the result that excess cholesterol may be converted to cholestanol, which is stored either as the free or the esterified form. This hypothesis has, as one of its attractive premises, that there is a marked limitation of capacity for the removal of cholestanol from cells. The data of Gardner-Medwin et al. (25) and Derby et al. (26) on the in vivo labeling of cerebral and cerebellar sterols in one CTX patient provide some support to the idea of a deficiency in the mechanism for the removal of cholestanol from tissues. Bjorkhem (27) showed net flux of 7-hydroxy-3-oxo-4 cholestenoic acid out of the human central nervous system. Because this oxysterol is a metabolic product of 27-hydroxycholesterol, it is conceivable that a reduction in output of this oxysterol could lead to cholestanol overaccumulation in the brain. We suggest that HDL, because of its well-known role in the reverse cholesterol transport process, may be involved. In CTX patients, HDL cholesterol and apolipoprotein A composition have been reported to be abnormally low (28).

However, the most attractive explanation for a large amount of cholestanol in the tissues of CTX patients is an exaggerated synthesis of cholestanol as a result of the C27-hydroxylase deficiency (6). The synthesis of cholestanol has been depicted in elegant pathways by Norlin and

colleagues (24). Perhaps the simplest pathway is that the 7α-hydroxy-4-cholesten-3-one formed from cholesterol in the tissues is the normal precursor for chenodeoxycholic acid. In the absence of CYP27A1, chenodeoxycholic acid is not formed, and the secondary pathway to cholestanol from 7α-hydroxy-4-cholesten-3-one results. This possible alternative pathway for the accumulation of cholestanol in the brain of CTX patients could result from the uptake of this bile acid precursor (e.g., 7α-hydroxy-4-cholesten-3-one) from the blood by the brain. This compound is known to be converted into cholestanol in peripheral tissues (29). But how does the explanation for the hepatic synthesis of cholestanol apply to the nervous system, where until now bile acid synthesis has not been demonstrated? Recently, Mano and colleagues (30) showed that chenodeoxycholic acid is synthesized from 3β-hydroxy-5-cholenoic acid by rat brain enzyme systems, and Nariyasu et al. (31) detected bile acids in the rat brain. This cholenoic acid may be synthesized from cholesterol via 24-hydroxy cholesterol as an intermediate (30). Large amounts of 24-hydroxy cholesterol exist in rat brain microsomal fractions (32). Although there is no evidence that chenodeoxycholic acid can be synthesized from cholesterol in the brain, we speculate that there may be a pathway from 24-hydroxy cholesterol to bile acid in the brain. The mutation of the CYP27A1 gene (identified in the brain) may conceivably lead to disturbances in the synthesis of chenodeoxycholic acid, so that small, daily amounts of cholestanol are produced in the brain over many years. This explanation would help account for the large amount of cholestanol in the central and peripheral nervous systems.

One other defect in cholestanol metabolism in the CTX syndrome was reported to be the increased production of cholestanol in the body. Salen and Grundy (33) showed that cholestanol turnover in two CTX patients and in five human subjects (one normal and four hyperlipidemic) conformed to the two-pool kinetics described by Goodman and Noble (15). They reported that PR_A values in two CTX patients were 48 and 57 mg/day (average, 52.5 mg/day), much greater than those in the control subjects (mean, 11.8 ± 6.0 mg/day; range, 6–18 mg/day). The production rate of cholestanol in our patient was ~39 mg/day, lower than the average value reported by Salen and Grundy (33) in their two patients; yet, our value is ~3.5 times higher than the mean value of 11.8 mg/day reported for five normal human subjects by those authors. Thus, we concur with the conclusion of Salen and Grundy (33) that in the CTX syndrome, the biosynthesis of cholestanol for the whole body is increased.

In our two CTX patients, the intestinal absorption of cholestanol was found to be 5.5% and 3.4% after a single oral test meal containing [4-^{14}C]cholestanol (Table 2). In six normal human subjects, the intestinal absorption of cholestanol was 3.3 ± 2.7% of the dose. In the1930s, using balance methods, Burger and Wintersteel (34) and Dam (35) could not demonstrate cholestanol absorption in normal humans. To the best of our knowledge, no other studies on cholestanol absorption in CTX have been reported. Thus, the capacity to absorb cholestanol from the diet by CTX patients has implications for the dietary therapy of this disease, because cholestanol is present in certain foods, such as eggs and high-fat dairy products.

The great accumulation of cholestanol in the cerebrum, cerebellum, and peripheral nerves suggests that cholestanol in all probability plays a key role in the neurological dysfunction of CTX patients. Structurally, cholestanol is a saturated sterol compared with the unsaturated cholesterol, and it may have different electrical conductivity than cholesterol. Replacing large amounts of cholesterol with cholestanol (up to 30%) in the nerve tissue conceivably would have significant effects upon function. Philippart and van Bogaert (22) suggested that a part of the cholesterol normally present in myelin as the integral part of the cell membrane structure is replaced by cholestanol, thereby altering the cellular membrane structure and producing the neurological dysfunction observed in CTX. Stahl, Sumi, and Swanson (36) have demonstrated the presence of free cholestanol in myelin. The change in myelin lipid composition has also been observed in the frontal lobe of the cerebrum. This change in myelin composition may account for the severe changes in mentation that are so typical in CTX.

In conclusion, although the underlying defect(s) producing the clinical manifestations in the CTX syndrome, particularly the neurological manifestations, has yet to be established, the presence of high concentrations of cholestanol in relation to cholesterol in the brain may have important pathological consequences in CTX patients. Several hypotheses for the increased cholestanol concentration in the brain of these patients have been postulated based upon the presence of CYP27A1 and the synthesis of chenodeoxycholic acid by a brain enzyme system. In the absence of the CYP27A1 gene, the pathway to chenodeoxycholic acid from cholesterol is blocked, with diversion then to the synthesis of cholestanol. This hypothesis fits the data of an intact blood-brain barrier for cholestanol and cholesterol and the high cholestanol-to-cholesterol ratio in the brain compared with the plasma and other nonnerve tissue. Cholestanol may be synthesized in the brain from cholesterol or from a circulating precursor to chenodeoxycholic acid synthesis that enters the brain. The removal of cholestanol from the brain could also be impaired and result in its accumulation in nerve tissue.

This study was supported by research Grants HE-l4230 and HL-08974 from the National Heart, Lung, and Blood Institute and from the General Clinical Research Centers Program (Grants MOI-FR-59 and 5 M01 RR-000334) of the Division of Research Resources, National Institutes of Health (Bethesda, MD). The expert technical assistance of Mary J. Garst, Susan Weickert, and Adrianne Hakes is gratefully acknowledged. The authors are grateful to Dr. William H. Elliot (Department of Biochemistry, St. Louis University School of Medicine) for kindly providing a pure sample of allocholic acid (3α,7α,l2α-trihydroxy-5-cholan-24-oic acid). The authors thank Dr. Anuradha Pappu of our department for helping in the discussion of bile acids and cholestanol and Carolyn Reid for the careful prepa-

ration of many revisions of the manuscript. Finally, the authors are grateful for the participation of the two brothers with CTX in our studies and to their mother, who encouraged them. Some of these studies were carried out when the investigators were affiliated with the General Clinical Research Center and the Department of Medicine, University of Iowa College of Medicine (Iowa City, IA).

REFERENCES

1. van Bogaert, L., H. J. Scherer, and E. Epstein. 1937. Une Forme Cerebrale de la Cholesterinose Generalisee. Masson et Cie, Paris.
2. Bhattacharyya, A. K., and W. E. Connor. 1978. Familial diseases with storage of sterols other than cholesterol. Cerebrotendinous xanthomatosis and β-sitosterolemia and xanthomatosis. *In* The Metabolic Basis of Inherited Disease. 4th edition. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill, New York. 656-669.
3. Salen, G., S. Shefer, and V. M. Berginer. 1983. Familial diseases with storage of sterols other than cholesterol: cerebrotendinous xanthomatosis and sitosterolemia with xanthomatosis. *In* The Metabolic Basis of Inherited Disease. 5th edition. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, editors. McGraw-Hill, New York. 713-730.
4. Bjorkhem, I., and S. Skrede. 1989. Familial diseases with storage of sterols other than cholesterol: cerebrotendinous xanthomatosis and phytosterolemia. *In* The Metabolic Basis of Inherited Disease. 6th edition. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 1283-1302.
5. Skrede, S., I. Bjorkhem, E. A. Kvittingen, M. S. Buchmann, S. O. Lio, C. East, and S. Grundy. 1986. Demonstration of 26-hydroxylation of C27-steroids in human skin fibroblasts and a deficiency of its activity in cerebrotendinous xanthomatosis. *J. Clin. Invest.* **78:** 729–735.
6. Bjorkhem, I., K. Muri-Boberg, and E. Leitersdorf. 2000. Inborn errors of bile acid biosynthesis and storage of sterols other than cholesterol. *In* The Metabolic Basis of Inherited Disease. 8th Edition. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 2961-2988.
7. Cali, J. J., C-L. Hsieh, U. Francke, and D. W. Russell. 1991. Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylase underlie cerebrotendinous xanthomatosis. *J. Biol. Chem.* **266:** 7779–7783.
8. Leitersdorf, E., A. Reshef, V. Meiner, R. Levitzki, S. P. Schwartz, E. J. Dann, N. Berkman, J. J. Cali, L. Klapholz, and V. M. Berginer. 1993. Frameshift and splice-junction mutations in the sterol 27-hydroxylase gene cause cerebrotendinous xanthomatosis in Jews of Moroccan origin. *J. Clin. Invest.* **91:** 2488–2496.
9. Nakashima, N., Y. Sakai, H. Sakai, T. Yanase, M. Haji, F. Umeda, S. Koga, T. Hoshita, and H. Nawata. 1994. A point mutation in the bile acid biosynthetic enzyme sterol 27-hydroxylase in a family with cerebrotendinous xanthomatosis. *J. Lipid Res.* **35:** 663–668.
10. Kim, K-S., S. Kubota, M. Kuroyama, J. Fujiyama, I. Bjorkhem, G. Eggertsen, and Y. Seyama. 1994. Identification of new mutations in sterol 27-hydroxylase gene in Japanese patients with cerebrotendinous xanthomatosis. *J. Lipid Res.* **35:** 1031–1039.
11. Lee, M. H., S. Hazard, J. D. Carpten, S. Yi, J. Cohen, G. T. Gerhardt, G. Salen, and S. B. Patel. 2001. Fine-mapping, mutation analyses and structural mapping of cerebrotendinous xanthomatosis in U.S. pedigrees. *J. Lipid Res.* **42:** 159–169.
12. Miettinen, T. A., E. H. Ahrens, Jr., and S. M. Grundy. 1965. Quantitative isolation and gas-liquid chromatographic analysis of total dietary and fecal neutral steroids. *J. Lipid Res.* **6:** 411–424.
13. Connor, W. E., and D. S. Lin. 1974. The intestinal absorption of dietary cholesterol by hypercholesterolemic (type II) and normocholesterolemic humans. *J. Clin. Invest.* **53:** 1062–1077.
14. Bhattacharyya, A. K., W. E. Connor, and A. A. Spector. 1976. Abnormalities of cholesterol turnover in hypercholesterolemic (type II) patients. *J. Lab. Clin. Med.* **88:** 202–214.
15. Goodman, D. S., and R. P. Noble. 1968. Turnover of plasma cholesterol in man. *J. Clin. Invest.* **47:** 231–241.
16. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. 1952. A simplified method for the estimation of total cholesterol in serum and determination of its specificity. *J. Biol. Chem.* **195:** 357–366.
17. Rose, H. G., and M. Oklander. 1965. Improved procedure for the extraction of lipids from human erythrocytes. *J. Lipid Res.* **6:** 428–431.
18. Bhattacharyya, A. K., W. E. Connor, F. A. Mousoif, and A. E. Flatt. 1976. Turnover of xanthoma cholesterol in hyperlipoproteinemia patients. *J. Lab. Clin. Med.* **87:** 503–518.
19. Neuwelt, E. A., editor. 1989. Implications of the Blood Brain Barrier and Its Manipulation. Vol. 1. Basic Science Aspects. Plenum Press, New York.
20. Menkes, J. H., J. R. Schimschock, and P. D. Swanson. 1968. Cerebrotendinous xanthomatosis: the storage of cholestanol within the nervous system. *Arch. Neurol.* **19:** 47–53.
21. Salen, G. 1971. Cholestanol deposition in cerebrotendinous xanthomatosis. *Ann. Intern. Med.* **76:** 843–851.
22. Philippart, M., and M. L. van Bogeart. 1969. Cholestanotosis (cerebrotendinous xanthomatosis): a follow-up study on the original family. *Arch. Neurol.* **21:** 603–610.
23. Salen, G., V. Berginer, V. Shore, I. Horak, E. Horak, G. S. Tint, and S. Shefer. 1987. Increased concentrations of cholestanol and apolipoprotein B in the cerebrospinal fluid of patients with cerebrotendinous xanthomatosis. Effect of chenodeoxycholic acid. *N. Engl. J. Med.* **316:** 1233–1238.
24. Norlin, M., S. von Bahr, I. Bjorkehm, and K. Wikvall. 2003. On the substrate specificity of human CYP27A1: implications for bile acid and cholestanol formation. *J. Lipid Res.* **44:** 1515–1522.
25. Gardner-Medwin, D., Y. Kishimoto, B. M. Derby, and H. W. Moser. 1971. Cerebrotendinous xanthomatosis: in vivo labeling of cerebral sterols and sterol esters. *Trans. Am. Neurol. Assoc.* **96:** 241–244.
26. Derby, B. M., S. Pogacar, C. Muckenhausen, H. W. Moser, and E. P. Richardson. 1970. Cerebrotendinous xanthomatosis: a clinicopathological, biochemical, and metabolic study (Abstract). *J. Neuropathol. Exp. Neurol.* **29:** 139.
27. Bjorkhem, I. 1992. Mechanism of degradation of the steroid side chain in the formation of bile acids. *J. Lipid Res.* **33:** 455–471.
28. Shore, V., G. Salen, F. W. Cheng, T. Forte, S. Shefer, G. S. Tint, and F. Lindgren. 1981. Abnormal high density lipoproteins in cerebrotendinous xanthomatosis. *J. Clin. Invest.* **68:** 1295–1304.
29. Chiang, J. Y. L. 2002. Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr. Rev.* **23:** 443–463.
30. Mano, N., Y. Sato, M. Nagata, T. Goto, and J. Goto. 2004. Biosynthesis of 3β-hydroxy-5-cholenoic acid into chenodeoxycholic acid by rat brain enzyme systems. *J. Lipid Res.* **45:** 1741–1748.
31. Nariyasu, M., T. Goto, M. Uchida, K. Nishimura, M. Ando, N. Kobayashi, and J. Goto. 2004. Presence of protein-bound unconjugated bile acids in the cytoplasmic fraction of rat brain. *J. Lipid Res.* **45:** 295–300.
32. Zhang, J., Y. Akwa, M. El-Etr, E. E. Baulieu, and J. Sjovall. 1997. Metabolism of 27-, 25- and 24-hydroxycholesterol in rat glial cells and neurons. *Biochem. J.* **322:** 175–184.
33. Salen, G., and S. M. Grundy. 1973. The metabolism of cholestanol, cholesterol and bile acids in cerebrotendinous xanthomatosis. *J. Clin. Invest.* **52:** 2822–2835.
34. Bürger, M., and W. Wintersteel. 1931. Das Schicksal peroral verabreichten Cholesterins und Koprosterins im menschlichen Darm Zeitschrift fur physiologische Chemie. *Z. Physiol. Chem.* **202:** 237–245.
35. Dam, H. 1934. The formation of coprosterol in the intestine. I. Possible role of dihydroxycholesterol, and a method of determining dihydroxycholesterol in presence of coprosterol. *Biochem. J.* **28:** 815–819.
36. Stahl, W. L., S. M. Sumi, and P. D. Swanson. 1971. Subcellular distribution of cerebral cholestanol in cerebrotendinous xanthomatosis. *J. Neurochem.* **18:** 403–413.