Demonstration of 26-Hydroxylation of C_{27}-Steroids in Human Skin Fibroblasts, and a Deficiency of this Activity in Cerebrotendinous Xanthomatosis

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Abstract

26-Hydroxylation of 5β-cholestane-3α,7α,12α-triol and other C_{27}-steroids was demonstrated in cultured skin fibroblasts from healthy individuals. Activities in skin fibroblasts were ~5–10% of those previously found in human liver homogenates, and were inhibited by CO. The apparent K_m was lowest for 5β-cholestane-3α,7α,12α-triol (1.3 μmol/liter) and highest for 5-cholestene-3β,7α-diol (12 μmol/liter). The rate of 26-hydroxylation was highest with 7α-hydroxy-4-cholesten-3-one. These characteristics are similar to those of hepatic mitochondrial C_{27}-steroid 26-hydroxylase.

In skin fibroblasts from three patients with cerebrotendinous xanthomatosis (CTX), 26-hydroxylation of C_{27}-steroids proceeded at a rate of only 0.2–2.5% of healthy controls. No accumulation of endogenous 5β-cholestane-3α,7α,12α-triol could be demonstrated in these cells, and the lowered formation of radioactive, 26-hydroxylated products could not be explained by dilution of the labeled exogenous substrate.

The present results add strong evidence to the concept that the primary metabolic defect in CTX is a deficiency of C_{27}-steroid 26-hydroxylase.

Introduction

Cerebrotendinous xanthomatosis (CTX)[1] is a rare disorder of cholesterol metabolism, inherited by an autosomal recessive pattern (1). The most striking symptoms in CTX are tendinous xanthomas, accumulation of sterols in the central nervous system with dementia and cerebellar ataxia, premature coronary heart disease, and gallstones (1). Excess cholestanol (5α-cholestan-3β-ol) is present in brain (2), xanthomas, plasma, and bile. The concentration of bile acids in the bile is subnormal, and high levels of abnormal bile alcohols are found (3, 4). This is due to a deficient degradation of the cholesterol side chain, as shown by Salen et al. (3, 4).

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1. *Abbreviations used in this paper:* CTX, cerebrotendinous xanthomatosis; HPLC, high pressure liquid chromatography; m/e, mass to charge ratio; MEM, minimal essential medium; R_t, retention time; TMS, trimethylsilyl.

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On the basis of our previous studies in vitro (5) and in vivo (6), we have proposed that the underlying defect is a deficiency of the mitochondrial C_{27}-steroid 26-hydroxylase. Our findings are also consistent with the concept that the "26-hydroxylation pathway" is the major pathway for bile acid synthesis (7). However, a different metabolic defect has been postulated by Salen et al. These workers suggest that the specific enzymatic defect in CTX is at the microsomal 24β-hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol (8, 9). This would be compatible with their proposal that the "25-hydroxylation pathway" may be, physiologically, the most important route for the formation of bile acids (10). Recently, Salen's group has also found evidence for depressed 26-hydroxylation in CTX (11), and they conclude that the primary enzymatic defect in this disease remains to be established (11).

In the present study, we show the presence of C_{27}-steroid 26-hydroxylase in cultured skin fibroblasts from healthy human controls. In skin fibroblasts from three patients with CTX, 26-hydroxylation of C_{27}-steroids was almost completely deficient.

Methods

Materials

Clinical. We studied two Norwegian sisters with CTX (A.F., born 27 July 1941, and I.J., born 6 May 1935). Detailed records of clinical findings and laboratory data have been published previously (5, 6, 12, 13). Patient H.E. is a 58-yr-old black man with a history of tendon xanthomas that began in his teens. Over his lifetime, the xanthomas gradually enlarged and progressed to involve numerous extensor tendons on his hands, bilateral tibial tuberosities, and bilateral Achilles tendons. He was otherwise in good health until 1979, when he developed exertional angina pectoris. Cardiac catheterization revealed severe atherosclerotic disease of two coronary arteries, with complete occlusion of the distal left anterior descending artery. In 1981, he underwent cholecystectomy after an episode of pancreatitis associated wtih cholelithiasis and biliary duct dilatation. In 1984, a lesion was excised from one large, firm xanthoma in the distribution noted above; the patient also had bilateral corneal arcus, but no xanthelasmas. A left femoral bruit was present with mildly diminished peripheral pulses bilaterally. Neurological examination disclosed decreased distal reflexes consistent with a mild peripheral neuropathy. In all other respects, however, neurological examination, including intellectual function, was normal.

All laboratory values were normal including tests for hepatic, thyroid, and renal function, and the patient's fasting cholesterol was 181 mg/dl and triglycerides were 148 mg/dl. The patient's plasma cholestanol level, analyzed on an SP-1000 gas-liquid chromatographic column (Supelco, Inc., Bellefonte, PA) (14), was 2.24 mg/dl (normal, <1 mg/dl (1)). Sitosterol could not be detected. In duodenal bile, neutral sterols were composed of 89.8% cholesterol and 10.2% cholestanol; normally, the percentage of cholestanol is <0.2%. Distribution of bile acids in the bile sample was 22.7% taurocholic acid, 54.7% glycocholic acid, 13.0% glycochenodeoxychoic acid, and 9.6% glycodeoxycholic acid.

Several biochemical parameters (i.e., elevated plasma and biliary cholestanol, relatively low plasma cholesterol in the presence of tendon xanthomas, and a low percentage of chenodeoxycholic acid in bile acids) are consistent with the diagnosis of CTX in H.E. The diagnosis is further supported clinically by the presence of severe tendon xanthomatosis and premature coronary artery disease. The patient did not have progressive deterioration of central or peripheral nervous system function, but this finding is not invariably present in CTX (1).

Labeled steroids. 5β-[2,2,3,4-$^2H_4$]-7β-^{3}H-cholestane-3α,7α,12α-triol was synthesized as described previously (5). 7β-^{3}H-5β-Cholestane-3α,7α,12α-triol (9.9 mCi/mmol), 7β-^{3}H-5β-cholestane-3α,7α-diol (6.7 mCi/mmol), 7β-^{3}H-5-cholestene-3β-7α-diol (158 mCi/mmol) and 4-^{14}C-7α-hydroxy-4-cholesten-3-one (1.3 mCi/mmol) were synthesized as described previously (5, 6, 13). All these compounds were purified by high performance liquid chromatography (HPLC) (see below) before use.

Unlabeled steroids. 5β-cholestane-3α,7α,12α,26-tetrol, 5β-cholestane-3α,7α,26-triol, 7α,26-dihydroxy-4-cholesten-3-one and 5-cholestene-3β,7α,26-triol were synthesized chemically or biosynthetically as described previously (5, 6, 15, 16).

Other reagents. NADPH, glucose-6-phosphate, ATP, and malic acid were purchased from Sigma Chemical Co., St. Louis, Mo. Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was obtained from Calbiochem-Behring Corp., La Jolla, CA, glucose-6-phosphate dehydrogenase was from Boehringer, Mannheim, Federal Republic of Germany and 5β-cholestane-3α-ol (epicoprostanol) from Steraloids, Inc., Wilton, NH. Minimum essential medium (MEM, modified) and fetal calf serum were products of Flow Laboratories, Irvine, Scotland. All other chemicals and solvents used in the present study were standard commercial high purity materials.

Procedures

Fibroblast cultures and preparation of cell suspensions. Fibroblasts were derived from skin. Skin biopsies were obtained from patient H.E. and two American controls (A.A. and J.J.) simultaneously. Control A.A. is a 58-yr-old Latin American man with a history of moderate hypercholesterolemia and hypertriglyceridemia. Control J.J. is a 50-yr-old white man with a history of myocardial infarction and moderate hypercholesterolemia. The biopsies were handled in an identical fashion and mailed to Norway for analysis blinded to the Scandinavian investigators. Fibroblasts were grown from the biopsies and the studies completed under blinded conditions. The other six controls were adults without any known disease, and the fibroblast cultures were started in Norway.

The cells were grown and maintained as monolayers in tissue culture flasks (75 cm^2) (Costar; Cambridge, MA) in MEM supplemented with 17% fetal calf serum. Penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin (2 U/ml) and L-glutamine (1.5 μmol/ml) were added. The cells were harvested with trypsin after 5–10 passages, washed three times in isotonic salt solution, and stored as a pellet at −70°C for a short period before assay. The cells were resuspended in sucrose (0.25 mol/liter) and frozen and thawed three times. By light microscopy, all cells in these suspensions were found to be disrupted. Incubations were performed as described below, and cells from one culture flask were used for each assay of 26-hydroxylation. Protein was determined by a Lowry method.

Assay of C_{27}-steroid 26-hydroxylase was performed in fibroblasts, essentially according to the method of Atsuta and Okuda (17) and as previously described (5). When not otherwise stated, incubations were performed at 37°C for 2 h in air, as follows: to albumin (final concentration 12 μmol/liter), the substrate was added dissolved in acetone (which was evaporated under a stream of nitrogen) followed by the additions of (mmol/liter): sucrose, 10; Hepes buffer (pH 7.4), 33; ATP, 5; potassium malate, 5; glucose-6-phosphate, 1; glucose-6-phosphate-dehydrogenase, 0.5 U; NADPH, 1.2; and $MgCl_2$, 15. The reaction was started by the addition of the frozen and thawn fibroblast suspension corresponding to a final protein concentration of 1.6–3.3 mg/ml. Termination of the incubation was achieved by adding 0.1 ml of HCl (1 mol/liter). Extractions were performed twice with ethyl acetate, and the solvent was evaporated with N_2. The extent of the conversion was calculated from the sum of radioactivity in the product zone. The experiments were designed so that with normal fibroblasts, product label corresponding to ~15.000 dpm was usually obtained. In some experiments, the extent of 26-hydroxylation was detected by mass fragmentography (ref. 16 and Fig. 5). All determinations were done in duplicate, and the overall coefficient of variation for the analysis was 4.9%. Carnitine palmitoyl transferase was assayed according to Norum and Bremer (18). Incubations in an atmosphere of 5% oxygen, 40% carbon monoxide, and 55% nitrogen were performed as described by Björkhem and Holmberg (19).

Chromatography and mass spectrometry. Duodenal bile from the U. S. citizen H.E. was aspirated and analyzed for sterols and bile acids by reverse-phase high pressure liquid chromatography (HPLC) by Dr. Lyman Bilhartz in Dallas, TX (20). A C-18 column with a pump and refractometer was employed. For neutral sterols, the mobile phase was 60% 2-propanol, 30% 0.6 M acetate buffer, pH 4.5. For the assay of 26-hydroxylase, aliquots of the extract of the different incubation mixtures were subjected to HPLC. A Supelcosil-LC18 column (250 × 4.6 mm, 5-μm particle size, obtained from Supelco, Inc., Bellefonte, PA) was used with 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). Two different mobile phases were used: (*a*) Methanol/water (85:15, vol/vol) for the separation of 5β-cholestane-3α,7α,12α-triol (retention time [R_t], 38 min) and 5β-cholestane-3α,7α,12α,26-tetrol (R_t, 11 min). (*b*) Methanol/water (90:10, vol/vol) for the separation of 5β-cholestane-3α,7α-diol (R_t, 33 min) and 5β-cholestane-3α,7α,26-triol (R_t, 10 min); 5-cholestene-3β,7α-diol (R_t, 22 min) and 5-cholestene-3β,7α,26-triol (R_t, 9 min); 7 α-hydroxy-4-cholesten-3-one (R_t, 19 min) and 7α,26-dihydroxy-4-cholesten-3-one (R_t, 7 min).

Combined gas chromatography. Mass spectrometry was performed with an LKB 9000 instrument (LKB Instruments, Inc., Gaithersburg, MD) equipped with a 1.5% SE30 capillary column. 5β-cholestane-3α,7α,12α,26-tetrol was identified by a complete mass spectrum (Fig. 1), whereas the identity of the other 26-hydroxylated products was established by monitoring of selected typical ions (15) using a multiple ion detector (see ref. 16). Endogenous 5β-cholestane-3α,7α,12α-triol was quantitated by isotope dilution–mass spectrometry with use of $^2H_4$-labeled 5β-cholestane-3α,7α,12α-triol and monitoring the ions at mass to charge ratio (m/e) 456 and 460 according to the same principles as described previously (5).

Quantitation of endogenous intermediates in the fibroblasts. Unincubated aliquots of skin fibroblast suspensions (0.5–1 mg of protein) from two CTX subjects (I.J. and H.E.) and two normal controls were each added to 0.1 μg of $^2H_4$-7β-^{3}H-5β-cholestane-3α,7α,12α-triol at 0°C and immediately extracted as described for cells incubated with substrates (see above). The extracts were subjected to HPLC, and the fractions containing ^{3}H were pooled. The steroids were converted to trimethylsilyl (TMS) ethers with bis (trimethylsilyl) trifluoroacetamide (BSTFA) and analyzed by isotope dilution mass spectrometry as described above.

Results

During incubations of frozen and thawed normal skin fibroblasts with 5β-cholestane-3α,7α,12α-triol (R_t, 38 min in the present HPLC system) and a NADPH-regenerating system, a polar product (R_t, 11 min) was formed. Fig. 1 shows that the mass spectrum of the isolated product (*bottom*) was identical to that of 5β-cholestane-3α,7α,12α,26-tetrol used as reference (*top*). Note that under the gas-chromatographic conditions employed, 5β-cholestane-3α,7α,12α,26-tetrol was almost completely separated from 5β-cholestane-3α,7α,12α,25-tetrol and completely separated from 5β-cholestane-3α,7α,12α,24-tetrol and 5β-cholestane-3α,7α,12α,23-tetrol (see ref. 16). The complete absence of 5β-cholestane-3α,7α,12α,25-tetrol in the isolated material was

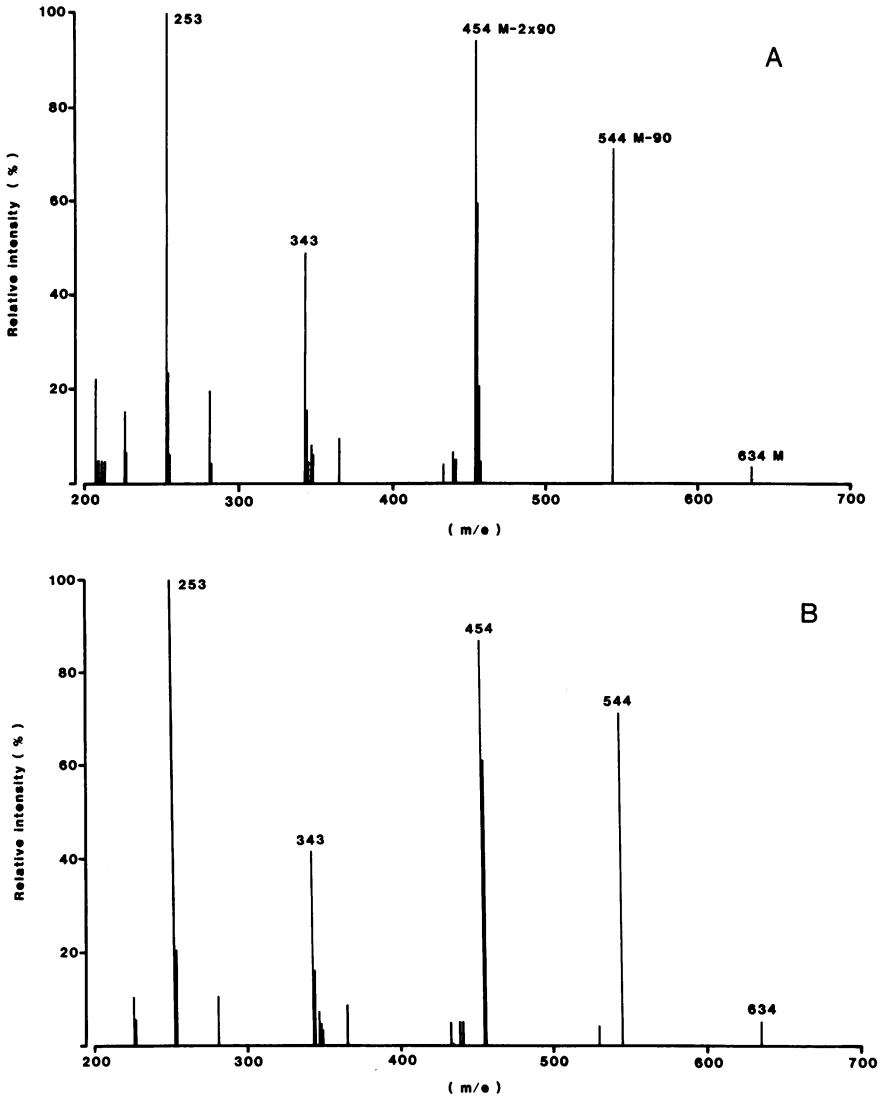

Figure 1. Mass spectrum of 5β-cholestane-3α,7α,12α,26-tetrol formed during incubation of 5β-cholestane-3α,7α,12α-triol with skin fibroblasts from a healthy human subject. Incubations were performed as described in Methods. (*A*) mass spectrum of trimethylsilylated derivative of the reference compound (5β-cholestane-3α,7α,12α,26-tetrol). (*B*) Mass spectrum of trimethylsilylated product from 5β-cholestane-3α,7α,12α-triol incubated with control fibroblasts, purified by HPLC (see Methods).

ascertained by selected ion monitoring of the characteristic ion at m/e 131 (see ref. 16). Fig. 2 shows that under the conditions chosen (see refs. 5, 15), 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol proceeded linearly to a protein concentration of ~5 mg/ml. The time-course was linear for at least 4 h (Fig. 3). Activity of 26-hydroxylase was lowered by further disruption of the cells by the addition of Triton X-100 (0.05%) or treatment with ultrasonic vibrations. 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol in skin fibroblasts from a healthy control was strongly inhibited by CO (76% inhibition) as compared with incubations in air, as might be expected for a cytochrome P-450 monooxygenase (19). The substrate specificity of the 26-

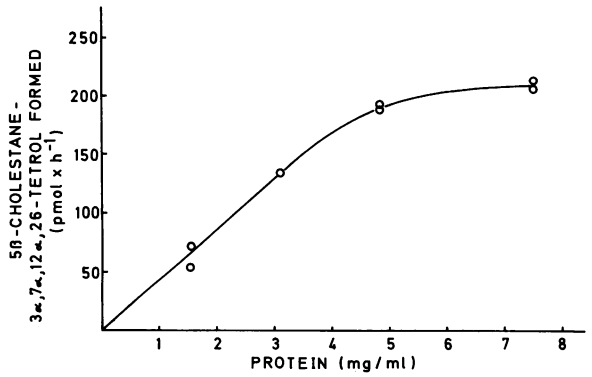

Figure 2. 26-Hydroxylation of 5β-cholestane-3α,7α,12α-triol in skin fibroblasts, related to the concentration of protein. 5β-cholestane-3α,7α,12α-triol (30 μmol/liter) was incubated at 37°C for 2 h with skin fibroblasts from healthy controls as described in Methods.

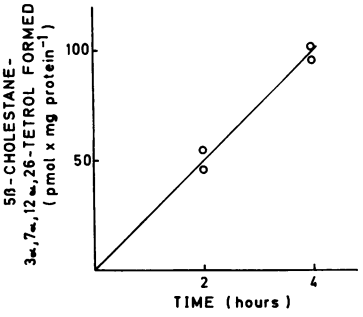

Figure 3. Time-course of the formation of 5β-cholestane-3α,7α,12α,26-tetrol in human skin fibroblasts. 5β-cholestane-3α,7α,12α-triol (17 μmol/liter) was incubated at 37°C with skin fibroblasts from healthy controls (4.8 mg of protein/ml) as described in Methods.

hydroxylase was studied by incubation also of 5β-cholestane-3α,7α-diol, 7α-hydroxy-4-cholesten-3-one and 7α-hydroxycholesterol with skin fibroblast suspensions.

The product obtained after incubation with 5β-cholestane-3α,7α-diol was purified by HPLC, converted into TMS ether, and analyzed by combined gas chromatography—mass spectrometry. Due to the small amounts available, no complete mass spectrum of the compound could be obtained. However, the identity of the compound as TMS ether of 5β-cholestane-3α,7α,26-triol was established by selected monitoring of the typical ions at m/e 546 (M-90), 531 (M-90-15), and 441 (M-2×90-15) (15). All these tracings gave a peak with a retention time identical to that obtained with authentic 5β-cholestane-3α,7α,26-triol.

The purified product obtained after incubation with 7α-hydroxy-4-cholesten-3-one was also identified by combined gas chromatography—mass spectrometry, using selected ion monitoring of the trimethylsilyl ether derivative. The typical ions at m/e 545 (M-15), m/e 470 (M-90), and m/e 380 (M-2×90) were used (15). All these tracings gave a peak with a retention time identical to that of derivative of authentic 7α,26-dihydroxy-4-cholesten-3-one isolated after incubation of 7α-hydroxy-4-cholesten-3-one with rat liver mitochondria (15).

The trimethylsilyl ether of the product isolated after incubation of 7α-hydroxycholesterol was analyzed by selected monitoring of the ions at m/e 634 (M), 544 (M-90), 529 (M-90-15), and 454 (M-2×90) (15). All the tracings gave a peak with a retention time identical to that of TMS ether of authentic 5-cholestene-3β,7α,26-triol. In addition, however, a peak with a slightly longer retention time was obtained in some tracings, indicating also that another product than 5-cholestene-3β,7α,26-triol may have been formed. No attempts were made to identify this product.

Table I lists apparent K_m and V_{max} for the different substrates. These results should be interpreted with caution, inter alia because of the restricted water solubility of the substrates. In addition, the rate of 26-hydroxylation of 7α-hydroxycholesterol may have been overestimated due to the presence of the additional compound in the product fraction. Michaelis constant (K_m) increased in the order 5β-cholestane-3α,7α,12α-triol < 7α-hydroxy-4-cholesten-3-one < 5β-cholestane-3α,7α-diol < 5-cholesten-3β,7α-diol. The highest rate of 26-hydroxylation was observed with 7α-hydroxy-4-cholesten-3-one. A Lineweaver-Burk plot of the 26-hydroxylation of this substrate is shown by Fig. 4.

Table I. Apparent K_m and V_{max} for C27-steroid 26-hydroxylase with Different Substrates Incubated with Skin Fibroblasts from Normal Human Subjects

Substrate	"V_{max}"	"K_m"
	pmol × mg protein^{-1} × h^{-1}	*μmol/liter*
5β-cholestane-3α,7α,12α-triol	79	1.3
5β-cholestane-3α,7α-diol	56	4.6
5-cholestene-3β,7α-diol	71	12.0
7α-hydroxy-4-cholesten-3-one	149	3.2

Incubations were performed as described in Methods.

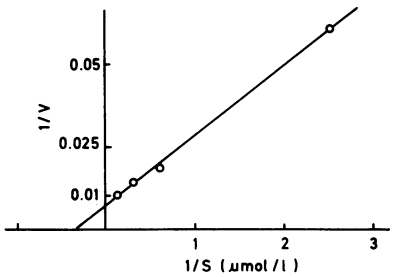

Figure 4. Lineweaver-Burk plot of C_{27} steroid 26-hydroxylase activity in skin fibroblasts with 7α-hydroxy-4-cholesten-3-one as substrate. Incubation was performed at 37°C for 2 h with skin fibroblasts from a healthy control (2.8 mg of protein/ml) in the same system as used in the experiments of Figs. 2 and 3. V, pmol mg protein^{-1} × h^{-1}. The product −7α,26-dihydroxy-4-cholesten-3-one was identified by its R_t on HPLC.

Table II shows 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol in skin fibroblasts from three patients with cerebrotendinous xanthomatosis (I.J., A.F., and H.E.). In fibroblasts from all three CTX-subjects, 26-hydroxylation was deficient, and proceeded at a rate of only 0.2–2.5% of the controls. By isotope dilution mass spectrometry, the concentration of endogenous 5β-cholestane-3α,7α,12α-triol was found to be lower than 6 ng/mg of protein in the cells from CTX-patients and healthy controls. Since this was only ~1/1,000 of added, labeled triol, the lowered formation of radioactive 5β-cholestane-3α,7α,12α-triol from the CTX-subjects could not be explained by dilution of the labeled exogenous substrate. Fibroblasts from CTX patient I.J. were tested also with 5α-cholestane-3α,7α-diol and 7α-hydroxy-4-cholesten-3-one, and the 26-hydroxylase activity was <3% of the controls also with these substrates (Table II).

The experiments shown in Table II were based upon the detection of labeled product by the described HPLC method (see Methods), which separated the products very efficiently from the substrates (e.g., for 5β-cholestane-3α,7α,12α,26-tetrol and 5β-cholestane-3α,7α,12α-triol, the R_t were 11 and 38 min, respectively). Fig. 5 shows another method for the detection of product. Cells from CTX subject A.F. and control cells were incubated with 5β-cholestane-3α,7α,12α-triol, and the TMS ether of the isolated product was detected by a mass fragmentographic recording at m/e 253 (see ref. 16). After incubation of the triol with control cells, significant amounts of 5β-cholestane-3α,7α,12α,26-tetrol were detected, whereas 26-hydroxylated product hardly could be identified with cells from CTX subject A.F.

It is possible that the cells from the CTX patients had been inactivated before or during the incubations, in contrast with all normal controls tested. Further, the 26-hydroxylase of the skin biopsy from CTX subject H.E. might theoretically have been selectively inactivated in shipment from the United States. However, skin biopsies were taken from two control subjects in Dallas at the same time the biopsy was made on H.E. The biopsies were handled in the same manner both for the CTX patient H.E. and the controls, and they were sent and studied blindly. Table II shows separate results for the American controls, whose 26-hydroxylation capacity was within the observed normal range. In a parallel assay of Δ^5-3β-hydroxysteroid dehydrogenase with 5-cholestene-3β,7α-diol as substrate, this enzymatic activity

Table II. Deficient 26-hydroxylation of C27-steroids in Skin Fibroblasts from Three Patients with CTX

Substrate	26-hydroxylated product formed		
	5β-cholestane-3α,7α,12α-triol	5β-cholestane-3α,7α-diol	7α-hydroxy-4-cholesten-3-one
	pmol × mg protein^{-1} × h^{-1}	*pmol × mg protein^{-1} × h^{-1}*	*pmol × mg protein^{-1} × h^{-1}*
CTX subjects			
I.J.	1.2	0.3	3.3
	(0.7)	(0.1–0.5)	(3.2–3.5)
A.F.	0.1	—	—
	(0.01)		
H.E.*	0.9	—	—
	(0.7)		
Healthy controls			
A.A.*	43.3	—	—
J.J.*	48.0	—	—
Other (n = 6)	48.1	64	104
	(4.3)	(51–77)	(102–106)

Skin fibroblasts were grown and prepared as described in Methods from two Norwegian sisters (I.J. and A.F., cf. ref. 12) and one U. S. male (H.E.)—all three suffering from CTX. Incubations were performed as described in Methods. With 5β-cholestane-3α-7α,12α-triol, 4–6 separate assays were performed with cells from CTX patients. With 5β-cholestane-3α,7α-diol or 7α-hydroxy-4-cholesten-3-one, analyses were done in duplicate. Mean values are shown, and SEM (triol) or range (other substrates) are in parentheses. * Skin biopsies were sent from the United States by the same shipment.

could easily be detected in normal cells from the control subjects and was not lowered in fibroblasts from any of the three CTX subjects (results not shown). Further, the inner mitochondrial membrane marker enzyme, carnitine palmitoyl transferase (18), had similar activity in the cells from all three CTX patients and the healthy human controls (Table III).

Discussion

Presence in human skin fibroblasts of enzymes acting on bile acid intermediates. In this study, we have shown C_{27}-steroid 26-hydroxylase activity in human skin fibroblasts. All cultures derived from healthy subjects that were tested had such activity, which was strongly inhibited by CO. Also, C_{27}-Δ5-3β-hydroxysteroid dehydrogenase was present in normal fibroblasts. The activity of C_{27}-steroid 26-hydroxylase in frozen and thawed fibroblasts from healthy human subjects was ~5–10% of that previously found in total homogenates of human liver (5). In the liver, the 26-hydroxylase is present in the mitochondria (5, 17, 21), where it is localized in the inner membrane. In human liver, there is little or no 26-hydroxylation in the microsomal fraction (21). In the rat, on the other hand, 26-hydroxylation

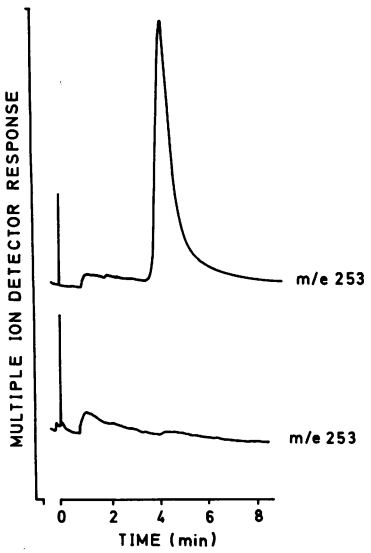

Figure 5. Mass fragmentographic recording of trimethylsilylated HPLC-purified 5β-cholestane-3α,7α,12α, 26-tetrol formed during incubation with skin fibroblasts from a healthy human control (top curve) and CTX patient A.F. (bottom curve). Simultaneous incubations of 5β-cholestane-3α,7α,12α-triol with skin fibroblasts from a healthy control (7.6 mg of protein/ml) and CTX patient A.F. (8.4 mg of protein/ml) were performed as described in Methods. 5β-cholestane-3α,7α,12α,26-tetrol formed was purified by HPLC. Equal parts of purified product formed in fibroblasts from the control and the CTX patient were trimethylsilylated and used for mass fragmentographic recordings at m/e 253.

Table III. Carnitine Palmitoyl Transferase Activity in Skin Fibroblasts from Patients with CTX and Healthy Controls

Subjects	Carnitine palmitoyl transferase
	dpm × mg protein^{-1} × h^{-1}
CTX subjects	
I.J.	116–129
H.E.*	95–104
Healthy controls	
A.A.*	99–102
J.J.*	112–111
M.N.	142–111
R.G.	94–86
L.C.	154–177

Skin fibroblasts from the same preparations as used in the experiments shown by Table II were assayed for carnitine palmitoyl transferase according to Norum and Bremer (18). The assays were performed with two different protein concentrations in the assays; both series are shown.

* Skin biopsies were sent from the United States by the same shipment.

occurs both in the mitochondrial and the microsomal fraction (21).

Our attempts to isolate different subcellular fractions of cultured human skin fibroblasts have failed so far, and we cannot be sure that we have assayed the mitochondrial 26-hydroxylase. However, it is interesting to compare the present results with known properties of the mitochondrial and microsomal hydroxylases. We found that the 26-hydroxylase of human skin fibroblasts has a low substrate specificity, as has the mitochondrial 26-hydroxylase (21). The affinities of the enzyme for the substrates tested decreased in this order: 5β-cholestane-3α,7α,12α-triol > 7α-hydroxy-4-cholesten-3-one > 5β-cholestane-3α,7α-diol, which is also the case for the mitochondrial 26-hydroxylase in the liver (21). We observed the highest rate of 26-hydroxylation with 7α-hydroxy-4-cholesten-3-one. The apparent K_m for 5β-cholestane-3α,7α,12α-triol found in the present study (1.3 μmol/liter) is closer to values previously found in liver mitochondria (4.5–7.1 μmol/liter) (5, 17) than in liver microsomes (15 μmol/liter) (5). The latter 26-hydroxylase, furthermore, has a much higher substrate specificity than the mitochondrial enzyme, and is only active to a significant extent with 5β-cholestane-3α,7α,12α-triol as substrate. Thus, the properties of the activity we studied resemble mitochondrial 26-hydroxylase more than the microsomal enzyme.

26-Hydroxylated C_{27}-steroids in serum, brain, and atheromata. The major part of the 26-hydroxylation of C_{27}-steroids probably occurs in liver mitochondria (21). 26-Hydroxycholesterol has been identified in human meconium, in serum from normal adults (22), and in brain (23). Further, 26-hydroxycholesterol has been isolated from human atheromata, where it is probably not a product of autoxidation. It has been suggested that it may be accumulated from plasma (24). The present finding of 26-hydroxylation of C_{27}-steroids in fibroblasts from healthy controls shows that such enzymatic activity may be expressed in some other organs than the liver.

It has been proposed that hydroxylated sterols may be involved in the normal regulation of cellular sterol metabolism (25), but direct evidence for this concept is lacking. 26-Hydroxycholesterol is reduced or absent in serum from patients with CTX (26). It may be speculated that 26-hydroxylation normally occurs also in some extrahepatic tissues and participates in the regulation of sterol metabolism. In CTX patients, however, extrahepatic 26-hydroxylation will probably not occur, as suggested by the present findings. At this time, it is difficult to see how an extrahepatic absence of this enzymatic activity in CTX can contribute to the accumulation of sterols in the central nervous system and to the premature atherosclerosis in this disease.

Enzymic defect in CTX. In this study we showed with cultured skin fibroblasts from three subjects with CTX, that 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol was deficient, proceeding at a rate of only 0.2–2.5% of normal controls. Deficiency of 26-hydroxylation could also be demonstrated with 5β-cholestane-3α,7α-diol, 7α-hydroxy-4-cholesten-3-one, and 5-cholestene-3β,7α-diol.

This finding adds strong evidence to our concept that the primary enzymatic defect in CTX is a deficiency of C_{27}-steroid 26-hydroxylase. Moreover, it appears that the two Norwegian sisters with CTX studied by us (5, 6, 12, 13) do not suffer from a particular genetic variant of the disaese, since the deficiency of 26-hydroxylase was also found in skin fibroblasts from an American CTX-patient.

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