Biosynthesis of cholesterol, lanosterol, and Δ^7-cholestenol, but not cholestanol, in cultured fibroblasts from normal individuals and patients with cerebrotendinous xanthomatosis

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Abstract The cholesterol and cholestanol biosynthetic pathways and the control of cholesterolgenesis were investigated in skin fibroblasts, from patients with cerebrotendinous xanthomatosis (CTX) and from normal subjects, grown in a lipoprotein deficient (d < 1.25 g/ml) medium. [^{3}H]Acetate was added to the culture medium and its incorporation into sterols was assayed by both argentation and reversed-phase thin-layer chromatography (TLC). The labeling patterns were similar in both CTX and control cells with ^{3}H being found, in order of increasing activity, in lanosterol, Δ^7-cholestenol, and cholesterol. No ^{3}H-labeled material at all, however, could be detected in the TLC mobility region corresponding to cholestanol. The ratio of cholestanol to cholesterol in the low density lipoprotein (LDL) subfraction from the plasma of individuals with CTX ranged from 1.4 to 5.3%, which is equal to or slightly greater than the ratio in whole plasma. Approximately 65–70% of the total plasma and LDL cholestanol and cholesterol were esterified. Since CTX-LDL added to incubates of normal cells and normal LDL added to CTX fibroblasts suppressed HMG-CoA reductase activity and stimulated cholesterol esterification equally, and since ^{125}I-labeled control LDL was degraded with normal kinetics from the surface of CTX fibroblasts, both CTX-LDL and CTX fibroblast LDL membrane receptors appear to be biologically normal. These results suggest that *1*) cholesterol is synthesized in cultured CTX and control fibroblasts via Δ^7-cholestenol, a C-24,25 saturated intermediate; *2*) cholestanol is not synthesized in the skin of CTX patients but is transported there from the liver via the plasma LDL; and *3*) CTX is not a disease associated with a defect of peripheral tissue LDL receptors.—**Tint, G. S., and G. Salen.** Biosynthesis of cholesterol, lanosterol, and Δ^7-cholestenol, but not cholestanol, in cultured fibroblasts from normal individuals and patients with cerebrotendinous xanthomatosis. *J. Lipid Res.* 1982. **23:** 597–603.

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In man and in the rat, cholesterol (cholest-5-en-3β-ol) may be synthesized from its obligate precursor lanosterol (4,4',14-trimethyl-5α-cholest-8,24-diene-3β-ol) by two different routes (1, 2). We have previously studied (3–5) one of these pathways by the in vivo stereospecific labeling of a patient with the rare genetic sterol storage disease, cerebrotendinous xanthomatosis (CTX). In this subject, the initial step in the formation of cholesterol was shown to be the hepatic reduction of the C24-25 double bond of lanosterol to form 24,25-dihydrolanosterol (4,4',14-trimethyl-5α-cholest-8-en-3β-ol). This latter compound was demethylated and isomerized, in a number of steps, to yield Δ^7-cholestenol (lathosterol, 5α-cholest-7-en-3β-ol) and then cholesterol.

The alternate pathway involves intermediates which retain the Δ^{24} double bond and follows the sequence, lanosterol, zymosterol (5α-cholest-8,24-diene-3β-ol), desmosterol (cholest-5,24-diene-3β-ol), cholesterol (1, 2). The evidence for this, however, rests primarily on the observation that desmosterol was found to accumulate in the plasma and tissues of man and of the rat when the potent Δ^{24} reductase inhibitor triparanol (1-[p-(2-diethylaminoethoxy)phenyl]-1-(p-tolyl)-2-(p-chlorophenyl)ethanol) was administered (6–8). It has been suggested (1) that this may be evidence, only, that hepatic enzymes are active enough to transform the sterol nucleus even though the side chain is *not* reduced.

Individuals with CTX exhibit a rate of cholesterol synthesis that is 50% greater than normal (9) and accumulate lanosterol, 24,25-dihydrolanosterol, and Δ^7-cholestenol in their blood, bile, feces, and peripheral tissue (3, 5, 9, 10).

We felt, therefore, that if fibroblasts obtained from these patients were to be grown under conditions of

Abbreviations: CTX, cerebrotendinous xanthomatosis; LDL, low density lipoprotein; HMG-CoA, hydroxy methylglutaryl coenzyme A; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; RP, reversed phase.

maximal cholesterol synthesis, a similar accumulation of these or other cholesterol precursors might occur.

Cholestanol (5α-cholestan-3β-ol) is a cholesterol *product* (3–5) which also accumulates in the bile, feces, plasma, and peripheral tissues of subjects with CTX (3, 9, 10). These patients synthesize cholestanol at a rate that is three to four times normal (9), but it is not known whether biosynthesis can take place in the peripheral tissue or whether this compound must be carried there from the liver. Since fibroblasts grown in a lipoprotein-deficient medium will synthesize radio-labeled cholesterol from labeled precursors (11) and cholestanol is synthesized in vivo from cholesterol, the absence of [^{3}H]cholestanol in skin cells incubated with [^{3}H]acetate would demonstrate that fibroblasts cannot synthesize this sterol.

Finally, although CTX has never been associated with a defect in fibroblast membrane low-density lipoprotein (LDL) receptors, as in familial hypercholesterolemia (11–14), this has never been tested.

MATERIALS AND METHODS

Clinical

Skin biopsies were taken from two CTX patients (J.C. and L.R.) hospitalized at the VA Medical Center, East Orange, NJ; plasma samples were obtained from J.C. and two other CTX subjects (W.H. and B.D.). Clinical descriptions of these and other CTX patients have been reported previously (9, 10). Control fibroblasts (No. 76 and No. 234) and data on normal cells were supplied by Drs. J. L. Goldstein and M.S. Brown, Department of Molecular Genetics, The University of Texas Health Science Center at Dallas, Texas.

Cell cultures

A total of 2×10^6 cells from each normal line and from each CTX biopsy were incubated in Eagle's minimum essential medium to which were added 1% (v/v) non-essential amino acids and 10% fetal calf lipoprotein-deficient serum ($d < 1.25$ g/ml) (12). On day 7, 160 μCi of [^{3}H]acetate was added and 24 hr later the cells were harvested. A second group of 2×10^6 cells was incubated identically except that the radioactive acetate was omitted. Lipids were obtained from the incubates by extraction with chloroform–methanol 2:1.

The experiments with cultured cells were carried out using previously published methods (12–14). Measurements of HMG-CoA reductase activity in normal and cultured CTX fibroblasts and its suppression by 0.5 to 10 μg/ml of normal LDL protein were carried out, together with measurements of the suppression of HMG-CoA reductase activity in normal fibroblasts by 5 and 21 μg/ml of both normal and CTX LDL (12).

Degradation of bound ^{125}I-labeled LDL from CTX fibroblasts (13) and LDL-stimulated esterification of cholesterol (14) were also determined. In this latter experiment, either 25 μg/ml of normal LDL was added to the CTX fibroblast incubation mixture or 21 μg/ml of CTX or normal LDL was added to normal cells, and the rate of incorporation of [^{14}C]oleate into cholesteryl [^{14}C]oleate was determined.

All of these cell culture studies were carried out by Drs. J. L. Goldstein and M. S. Brown.

Argentation thin-layer chromatography ($AgNO_3$–TLC)

The sterols extracted from the incubates were applied to argentation TLC plates (4, 15). The cholesterol band ($R_f = 0.34$) and the band above cholesterol ($R_f \geq 0.40$), which would contain cholestanol and the cholesterol precursors, were eluted with ethyl ether. Recovery of 7 to 55 μg of added cholesterol and cholestanol was 60–80% while recovery of ^{3}H-labeled material from the plated cell extracts (calculated by counting an aliquot of the sterol extract before and after plating) ranged from 90–110%.

Reversed-phase thin-layer chromatography (RP–TLC)

An aliquot of the eluted upper band (cholestanol and cholesterol precursors) from each $AgNO_3$–TLC plate was applied, as a 3-cm wide band, to a reversed-phase TLC plate. These were made by developing a Kieselguhr plate (20 cm × 20 cm × 0.25 mm, Analtech Inc., Newark, DE) in a 5% solution of paraffin oil in petroleum ether (5, 16). Since the mass of the sterols in the cells was too small to be visualized under ultraviolet illumination after spraying with Rhodamine 6G, 10–15 μg of cholestanol, Δ^7-cholestenol, lanosterol, and 24,25-dihydrolanosterol were added to the aliquot before plating.

Recoveries of ^{3}H-labeled material from the plated cell extracts ranged from 70–108%. Mass recoveries were not determined since the chromatographic oil-removal step (5, 16) needed before mass measurements by GLC, which can result in the loss of sterol, was found to be unnecessary for liquid scintillation counting.

Gas–liquid chromatography (GLC)

The sterol content of the extracts of the unlabeled CTX and normal cells was determined by GLC following $AgNO_3$–TLC. The eluates were converted to the trimethylsilyl (TMSi) ether derivatives and were chromatographed on a silanized 180 cm × 4 mm ID glass

column packed with 3% QF-1 on Gas Chrom Q 80/100 mesh (Applied Science Laboratories, State College, PA) at 240°C with a nitrogen flow of 40 ml/min. All mass measurements used 5α-cholestane as an internal standard (17).

Radioassay (zonal scraping)

In order to determine the ^{3}H radioactivity distribution, the developed TLC plates were divided into 3-mm-high zones starting at the origin and ending at the top of the plate. Each zone was then scraped and drawn by vacuum into a Pasteur pipet plugged at the opposite end with glass wool. The silica gel was eluted with the scintillation cocktail (toluene plus 42 ml/liter of Liquifluor (New England Nuclear, Boston, MA)) through the glass wool plug directly into counting vials. The small amounts of rhodamine 6G, and, in the case of the RP plates, paraffin oil dissolved in the cocktail had no detectable quenching effect.

Lipoprotein isolation

Lipoprotein fractions were isolated by preparative ultracentrifugation (18) by Dr. Virginia Shore, Biomedical Sciences Division, Lawrence Livermore Laboratory, University of California, Livermore, CA.

Determination of free and esterified cholesterol and cholestanol

Two ml of plasma and the LDL ($1.1019 < d < 1.063$ g/ml) fraction from 2–3 ml of the plasma from the three CTX subjects were extracted exhaustively with chloroform–methanol 2:1. After evaporation and concentration, the residue from plasma of one of the subjects (B.D.) was applied to silica gel 0 plates (Analtech Inc., Newark, DE) and developed in petroleum ether–ethyl ether–acetic acid 85:15:0.5 (9). The free sterol and steryl ester bands were eluted with ethyl ether and the latter was hydrolyzed in N NaOH ethanol for 1 hr at 70°C (4, 5). The chloroform–methanol extracts of the plasma from the other two subjects (J.C. and W.H.) were hydrolyzed without separating the esterified sterols. Cholesterol and cholestanol were then purified by $AgNO_3$–TLC and the concentration was measured by GLC.

RESULTS

Sterol content

The extracts of the unlabeled cell incubates each contained 40–100 μg of cholesterol and GLC peaks having the same retention times as Δ^7-cholestenol (0.6–0.9 μg) and lanosterol (0.1–0.3 μg) were detected. The areas under the Δ^7-cholestenol and lanosterol GLC peaks were approximately 1% and 0.4%, respectively, of the area of the cholesterol peak. Further identification of these two compounds was not possible due to the low masses.

Distribution of ^{3}H radioactivity on the TLC plates

The ^{3}H radioactivity distribution on a RP–TLC plate from one of the control cells (cell line No. 76) is illustrated in **Fig. 1a,** and from one of the CTX patients (L.R.) is shown in Fig. 1b. The plots from the other normal and the other CTX cell culture were virtually identical to these.

Since cholesterol and cholestanol can overlap on a reversed phase TLC plate, an aliquot of the region above cholesterol extracted from a $AgNO_3$–TLC plate was rechromatographed by $AgNO_3$–TLC (**Fig. 2**). The absence of ^{3}H radioactivity in the cholesterol mobility region demonstrated that the upper $AgNO_3$–TLC band was cholesterol-free. Cholestanol ($R_f = 0.46$) would have chromatographed between cholesterol ($R_f = 0.34$) and Δ^7-cholestenol ($R_f = 0.52$).

The large radioactivity peak in the center of both Figs. 1a and 1b (RP–TLC, $R_f = 0.44$) and Fig. 2 ($AgNO_3$–TLC, $R_f = 0.52$) corresponds exactly to a compound with the mobility of Δ^7-cholestenol. An average of 46% of the ^{3}H radioactivity recovered from the region of the argentation TLC plates above cholesterol was included under this peak. A smaller ^{3}H radioactivity peak was also detected in both systems (Figs. 1a, 1b, and 2) that had the same mobility as lanosterol.

No ^{3}H radioactivity could be detected, however, in either TLC system (Figs. 1a, 1b and 2) that corresponded to cholestanol.

Table 1 summarizes the total ^{3}H-label extracted from the four cell cultures and the percentage of the total incorporated ^{3}H radioactivity that was recovered as ^{3}H-labeled cholesterol, Δ^7-cholestenol, and lanosterol. An average of 23% of the ^{3}H-label extracted from the incubates was recovered in the $AgNO_3$–TLC cholesterol band while approximately 3% of the total was found in the RP–TLC Δ^7-cholestenol band and 0.2% in the lanosterol band.

HMG-CoA reductase activity

The HMG-CoA reductase activity of CTX fibroblasts (subject J.C.) grown in lipoprotein deficient serum was 145 pmol/min per mg protein while the activity of normal fibroblasts grown under identical conditions was 159 pmol/min per mg protein. The addition of 1, 5, and 10 μg/ml of LDL protein from a control subject reduced the HMG-CoA reductase activity in the CTX fibroblasts to 141, 47, and 16 pmol/min per mg protein, respectively, and the HMG-CoA reductase activity in the control cells to 114, 25, and 16 pmol/min per mg protein.

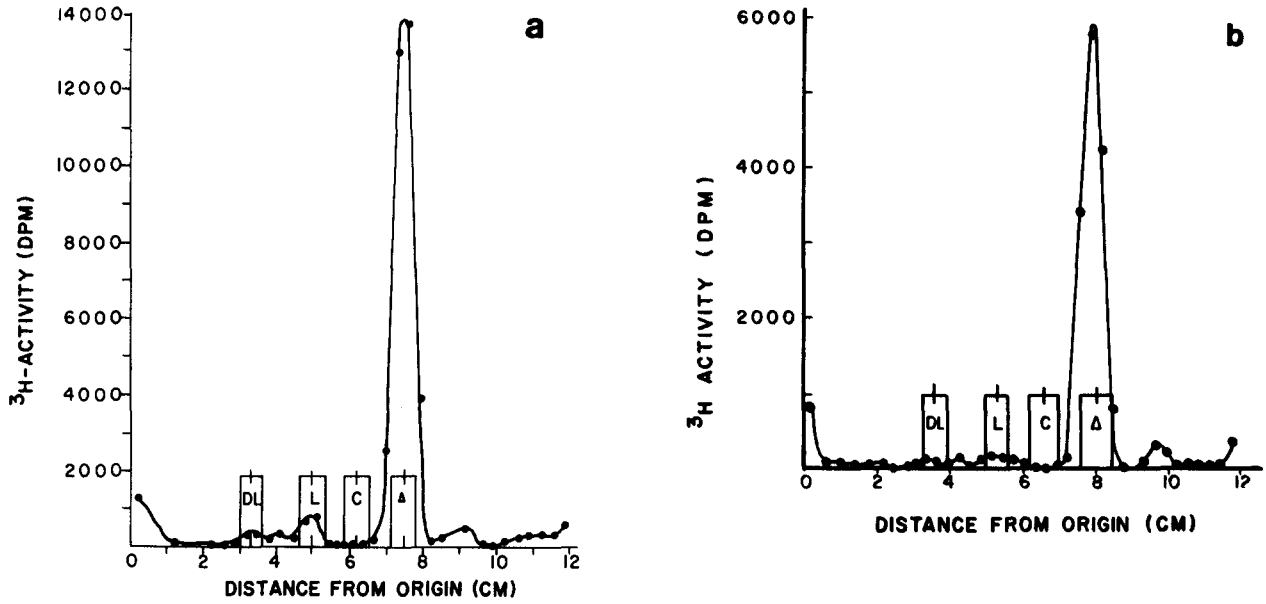

Fig. 1. ^{3}H Radioactivity (dpm) distribution on reversed-phase TLC plates obtained by zonal scraping. The lipid extract from fibroblasts grown in a lipoprotein-deficient medium with added [^{3}H]acetate was first chromatographed by argentation–TLC and the mobility region above cholesterol ($0.4 \leq R_f \leq 1.0$) was eluted with ethyl ether and then replated as a 3-cm-wide band on a reversed-phase plate and developed in acetone–water 80:20. Ten to fifteen μg of 24,25-dihydrolanosterol (DL), lanosterol (L), cholestanol (C), and Δ^7-cholestenol (Δ) was added. The width of the blocks denotes the visual limits of the sterol bands after spraying with rhodamine 6G and viewing under ultraviolet illumination. The small vertical lines on the top of each block represent the center of each band and the heights of the blocks are arbitrary. a, Control fibroblasts (#76); b, CTX fibroblasts (subject L.R.).

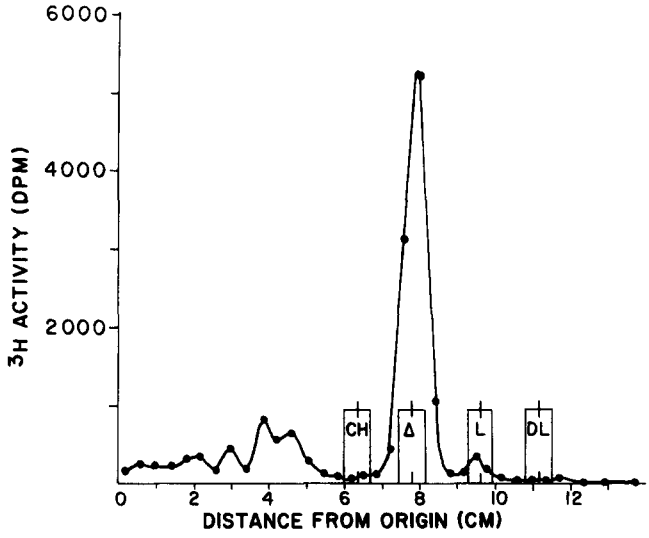

Fig. 2. ^{3}H Radioactivity (dpm) distribution on an argentation TLC plate obtained by zonal scraping. The lipid extract from one of the control fibroblasts cultures (#76) was chromatographed by argentation TLC and the mobility region above cholesterol ($0.4 < R_f < 1.0$) was eluted and replated on another argentation TLC plate developed in chloroform–acetone 97:3 at 4°C. Ten to fifteen μg of cholesterol (CH), Δ^7-cholestenol (Δ), lanosterol (L), and dihydrolanosterol (DL) was added. The width of the blocks denotes the visual limits of the sterol bands after spraying with rhodamine 6G and viewing under ultraviolet illumination. The small vertical lines on top of each block represent the center of the band and the heights of the blocks are arbitrary. The mobility of cholestanol is about two-thirds of the way between cholesterol and Δ^7-cholestenol.

In a separate experiment, the HMG-CoA reductase of normal fibroblasts incubated in a lipoprotein-deficient medium was 210 pmol/min per mg protein. The addition of 5 μg and 21 μg/ml of normal LDL protein reduced this value to 63 and 13 pmol/min per mg protein, respectively, while adding 5 μg/ml and 21 μg/ml of LDL from CTX subject J.C. reduced the activity to 36 and 31 pmol/min per mg protein.

Stimulation of cholesterol esterification by LDL

When normal cells were incubated in a lipoprotein-deficient medium, the rate of incorporation of [^{14}C]oleate into cholesteryl [^{14}C]oleate was 36 pmol/hr per mg protein. After the addition of 21 μg/ml of either normal or CTX LDL protein the rate increased to 1100 (30-fold stimulation). Similarly, CTX fibroblasts experienced a 25-fold increase of oleate incorporation when 25 μg/ml of normal LDL was added.

Degradation of ^{125}I-labeled LDL

At 37°C, ^{125}I-labeled LDL (control) incorporated into CTX fibroblasts (J.C.) was degraded normally (13) with V_{max} = 1 μg/hr per mg protein and K_m = 10 μg/ml.

Sterol content of LDL

Table 2 lists the cholesterol and cholestanol concentration in whole plasma and in the LDL subfractions

TABLE 1. ^3H Label recovered by argentation ($AgNO_3$–TLC) and reversed-phase (RP–TLC) thin-layer chromatography of extracted skin fibroblasts from control and CTX subjects grown in lipoprotein-deficient fetal calf serum plus [^3H]acetate

Cells	Total ^3H Label Extracted from Cells[a]	^3H recovered (Percentage Compared to Total ^3H Label Extracted)		
		Cholesterol: $AgNO_3$–TLC Band	Δ^7-Cholestenol: RP–TLC Band	Lanosterol: RP–TLC Band
	dpm	%[b]	%[c]	%[d]
76 (Normal)	67×10^6	28	3.9	0.3
234 (Normal)	77×10^6	31	1.9	0.1
J.C. (CTX)	63×10^6	17	4.1	0.2
L.R. (CTX)	57×10^6	18	3.3	0.1

[a] Measured from an aliquot of the cells extracted with chloroform–methanol 2:1.
[b] Silica gel H:10% $AgNO_3$; developed two times in chloroform–acetone 97:3 at 4°C.
[c] Kieselguhr–paraffin oil; developed in acetone–water 80:20. Δ^7-Cholestenol band (Δ), Figs. 1a and 1b.
[d] Lanosterol band (L), Figs. 1a and 1b.

of five subjects. In every case the percentage of cholestanol (relative to cholesterol) in the LDL fraction is equal to or greater than the relative percentage of cholestanol in the whole plasma.

The ratios of free and esterified cholesterol and cholestanol in the LDL fraction of subject B.D. were found to be similar, with about 35% of each sterol being free and 65% being esterified (Table 2).

Data from reference 9 are also included in Table 2 and demonstrate that about 70% of total plasma cholestanol is also esterified.

DISCUSSION

Fibroblasts from both CTX and control subjects grown in a cholesterol-deficient medium containing [^3H]acetate and analyzed by RP–TLC (Figs. 1a and 1b) and by $AgNO_3$–TLC (Fig. 2) exhibited a ^3H radioactivity peak (band Δ) that had a mobility equal to that of Δ^7-cholestenol, and a ^3H peak (band L) with the same mobility as lanosterol. Although direct identification of these compounds was not possible due to the low mass of each sterol (<1 μg), the TLC separations were carried out in two very dissimilar systems in which the mobilities, as well as the elution order, of all of the sterols were considerably different. In the direct phase ($AgNO_3$–TLC) system, mobility varies inversely with polarity, while in the reversed-phase system, this order is reversed. It is possible, however, that there are other sterols with mobilities identical to those of Δ^7-cholestenol and lanosterol in both systems, so that these identifications must remain tentative.

Of the total label recovered from the cell cultures, about 23% was found in the cholesterol band on the $AgNO_3$–TLC plates, 3% appeared in the RP–TLC Δ^7-cholestenol band and 0.2% in the RP–TLC lanosterol band. GLC measurements of the extracts from unlabeled normal and CTX fibroblast cultures demonstrated a peak having the same retention time as Δ^7-cholestenol,

TABLE 2. Cholesterol and cholestanol concentration and the percent esterified in the plasma and LDL fraction (1.019 < d < 1.063 g/ml) of CTX subjects

Subject	Plasma				LDL			
	Cholesterol		Cholestanol		Cholesterol		Cholestanol	
	mg/ml	% Ester	%[b]	% Ester	mg/ml	% Ester	%[b]	% Ester
E.D.E.[a]	1.45	71	1.2	68	1.06		1.4	
E.D.S.[a]	1.51		1.6		1.13		1.7	
J.C.	1.03	70[a]	4.5	72[a]	0.58		4.5	
V.R.	1.61		2.8		1.03		3.0	
B.D.	1.54		1.4		0.80	65	5.3	67

[a] All measurements on E.D.E. and E.D.S. and the % cholesterol and cholestanol ester in plasma of J.C. from reference 9.
[b] Percentage compared to cholesterol in plasma or LDL.

with an area equal to about 1% of the cholesterol peak, and another GLC peak having the same retention time as lanosterol having an area of about 0.4% of that of the cholesterol peak.

We have shown by stereospecific labeling studies in a CTX patient (3, 5) that cholesterol biosynthesis is compatible with a sequence of Δ^{24}-saturated intermediates following lanosterol, i.e., 24,25-dihydrolanosterol and Δ^{7}-cholestenol. The finding of the Δ^{7}-cholestenol activity peak in all of the fibroblast cultures serves to confirm the existence of this pathway in control subjects as well as in CTX patients. Therefore, if normal human fibroblasts and hepatocytes synthesize cholesterol in the same way, early reduction of the lanosterol Δ^{24}-double bond is a common pathway for cholesterolgenesis in all humans. The intermediates in the pathway seem to be detectable, however, only under the conditions of greatly increased synthesis found in CTX patients (9) and in fibroblasts incubated in a cholesterol-deficient medium (12).

We could not detect any ^{3}H radioactivity in the cholestanol TLC mobility region from the extracted incubates of ^{3}H-labeled control and CTX fibroblasts when they were chromatographed on argentation and reversed-phase plates (Figs. 1a, 1b, 2). Therefore, although normal individuals produce an average of 12 mg/day and CTX patients 50 mg/day of cholestanol (9), this synthesis cannot occur in the fibroblasts. Cholestanol, however, is available for deposition in peripheral tissue of individuals with CTX since cholestanol and cholestanyl ester are found, in quantity, in the LDL subfraction of all of these subjects (Table 2). The LDL from affected individuals appears to be biologically normal in all respects since it suppresses HMG-CoA reductase activity and stimulates cholesterol esterification in control fibroblasts to the same extent as does control LDL. Similarly, CTX fibroblast membrane LDL-receptors are completely normal since control LDL suppresses HMG-CoA reductase activity, stimulates cholesterol esterification, and is degraded from the membrane surface just as in normal fibroblasts. Thus, the cholestanol found in the peripheral tissue of CTX subjects is not synthesized in situ but is carried there and deposited by the same mechanism by which cholesterol enters these tissues: via the plasma LDL fraction (19). Large amounts of cholestanol (and cholesterol) accumulate in the peripheral tissue of these individuals because hepatic synthesis of these sterols is significantly elevated (9), transport of the esters by LDL and transfer to tissue membranes proceeds normally but their removal will likely be inhibited by the severe defect in the plasma high density lipoproteins which we have recently demonstrated (18).

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