Sterols in blood of normal and Smith-Lemli-Opitz subjects

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**Abstract Smith-Lemli-Opitz syndrome (SLOS) is a heredi-
tary disorder in which a defective gene encoding 7-dehydro-
cholesterol reductase causes the accumulation of nonchol-
lesterol sterols, such as 7- and 8-dehydrocholesterol. Using
rigorous analytical methods in conjunction with a large
collection of authentic standards, we unequivocally identi-
fied numerous noncholesterol sterols in 6 normal and 17
SLOS blood samples. Plasma or erythrocytes were saponi-
fied under oxygen-free conditions, followed by multiple
chromatographic separations. Individual sterols were iden-
tified and quantitated by high performance liquid chroma-
tography (HPLC), Ag$^+$-HPLC, gas chromatography (GC),
GC-mass spectrometry, and nuclear magnetic resonance.
As a percentage of total sterol content, the major C_{27} sterols
observed in the SLOS blood samples were cholesterol
(12–98%), 7-dehydrocholesterol (0.4–44%), 8-dehydrocholes-
terol (0.5–22%), and cholesta-5,7,9(11)-trien-3β-ol (0.02–
5%), whereas the normal blood samples contained <0.03%
each of the three noncholesterol sterols. SLOS and normal
blood contained similar amounts of lathosterol (0.05–
0.6%) and cholestanol (0.1–0.4%) and ~0.003–0.1% each
of the Δ^8, $\Delta^{8(14)}$, $\Delta^{5,8(14)}$, $\Delta^{5,24}$, $\Delta^{6,8}$, $\Delta^{6,8(14)}$, and $\Delta^{7,24}$ ste-
rols. The results are consistent with the hypothesis that
the $\Delta^{8(14)}$ sterol is an intermediate of cholesterol synthesis
and indicate the existence of undescribed aberrant path-
ways that may explain the formation of the $\Delta^{5,7,9(11)}$ sterol.
19-Norcholesta-5,7,9-trien-3β-ol was absent in both SLOS
and normal blood, although it was routinely observed as a
GC artifact in fractions containing 8-dehydrocholesterol.
The overall findings advance the understanding of SLOS
and provide a methodological model for studying other
metabolic disorders of cholesterol synthesis.**—Ruan, B.,
W. K. Wilson, J. Pang, N. Gerst, F. D. Pinkerton, J. Tsai, R. I.
Kelley, F. G. Whitby, D. M. Milewicz, J. Garbern, and G. J.
Schroepfer, Jr. **Sterols in blood of normal and Smith-Lemli-
Opitz subjects.** *J. Lipid Res.* 2001. **42: 799–812.**

Supplementary key words SLOS • noncholesterol sterols • cholesta-
5,8-dien-3β-ol • Ag$^+$-HPLC • NMR • GC-MS

Smith-Lemli-Opitz syndrome (SLOS; MIM 270400) is
an autosomal recessive disorder caused by various combi-
nations of more than 60 known mutations in the gene
encoding 7-dehydrocholesterol reductase (DHCR7; EC
1.3.1.21) (1–7). Diminished DHCR7 activity (8) leads to
a deficiency of cholesterol, accompanied by the accumu-
lation of 7-dehydrocholesterol (8–11), other sterols (8–
21), and their steroid metabolites (13, 22–24). Signs of
SLOS include mental retardation, stunted growth, be-
havioral problems, and structural anomalies affecting
the brain, face, digits, heart, kidneys, genitals, adrenals,
and lungs (1–6). Early developmental abnormalities that
are primarily responsible for the physical signs of SLOS
are attributed to aberrant embryonic patterning, which
involves covalent binding of cholesterol to the morpho-
gen sonic hedgehog with concomitant autoproteolysis,
followed by signal transduction by incompletely defined
mechanisms (see 1–5 and references therein). The se-
verity of SLOS is related to genotype, cholesterol levels,
and levels of noncholesterol sterols, but correlations
with severity are confounded by additional factors (1, 7,
12, 14). The abnormal sterol profile of the SLOS neo-
nate evolves during childhood to produce higher (but
still usually depressed) cholesterol levels, a process that
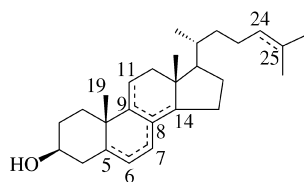is facilitated somewhat by cholesterol feeding (1–3).

Sterols reported previously in SLOS

Major sterols	Other sterols	
Δ^5	Δ^7	$\Delta^{5,7,9(11)}$
$\Delta^{5,7}$	Δ^8	$\Delta^{6,8}$
$\Delta^{5,8}$	$\Delta^{8(14)}$	$\Delta^{6,8(14)}$
19-nor-$\Delta^{5,7,9}$	$\Delta^{5,24}$	$\Delta^{5,7,24}$

Fig. 1. Unsaturated sterols reported previously in blood and tissues of SLOS subjects. Sterols are designated according to the position of their double bonds as indicated in the generalized chemical structure. Except for the $\Delta^{5,7}$ and $\Delta^{5,8}$ sterols, reported assignments of structure were based primarily on GC, GC-MS, or LC-MS of a complex mixture of sterols.

However, the relationship between sterol levels and clinical symptoms of SLOS is quite complex.

The major noncholesterol sterols in SLOS are the $\Delta^{5,7}$ and $\Delta^{5,8}$ dienes,[3] which have been found in blood, other fluids, tissues, and feces (1–5, 8–21). As shown in **Fig. 1**, many additional sterols have been reported to be present in SLOS (8–11, 13–21) or animal models (25, 26): 19-norcholesta-5,7,9-trien-3β-ol (9, 18–21, 25, 26), 24,25-dihydrolanosterol (15), and $\Delta^{5,7,9(11)}$ (19), Δ^7 (8, 15–17, 26), Δ^8 (26), $\Delta^{5,24}$ (8), $\Delta^{6,8}$ (1, 16, 17), $\Delta^{6,8(14)}$ (11), $\Delta^{8(14)}$ (16), and $\Delta^{5,7,24}$ (11, 16) sterols. Most of these substances were characterized as minor components of complex sterol mixtures by gas chromatography-mass spectrometry (GC-MS) or GC, although some analyses were done by high performance liquid chromatography (HPLC) (21), HPLC-MS (19), or thin-layer chromatography (TLC) followed by GC-MS (8, 13) and other characterization (13). Except for fecal sterols (13), identifications were generally based on comigration with an authentic standard on a single chromatographic phase, sometimes in conjunction with mass spectrometry. Although facile, these chromatographic and spectral methods alone are poorly suited to the comprehensive analysis of complex sterol mixtures.

Detailed investigations of the GC, GC-MS, and HPLC of sterols indicate serious limitations in their use for the separation and identification of unsaturated C_{27} sterols (27–29). Data from a large collection of authentic standards have shown that many pairs of sterols, including acetate and trimethylsilyl (TMS) derivatives, coelute on a variety of stationary phases commonly used in GC (27) and HPLC (28). Moreover, a number of sterol isomers relevant to SLOS cannot be distinguished by mass spectronmetry under standard electron-impact conditions (27). Because sterol mixtures of any complexity are likely to contain comigrating components on GC, HPLC, or TLC, such analyses, even in conjunction with mass spectrometry, can provide only tentative identification and quantitation of the sterols present. A further complication is the lability of several sterols to GC conditions (27). For example, the $\Delta^{5,8}$ sterol partially decomposes under the thermal stress of GC injection to form the 19-nor-$\Delta^{5,7,9}$ sterol as an artifact (30).

In addition to the chromatographic and spectral methods commonly used to analyze sterol mixtures in biological samples, we have applied more powerful techniques to separate and identify sterols in SLOS. Silver ion (Ag$^+$)-HPLC provides essentially baseline separation of a large number of C_{27} sterol acetates relevant to SLOS (28, 29), and the underivatized free sterols are also well resolved (28). [1]H nuclear magnetic resonance (NMR) is a remarkably effective method for detecting and identifying trace-level sterols, and the highly characteristic upfield methyl signals are well dispersed at 500 MHz (31). Supported by a large collection of authentic standards, we have now combined these nondestructive methods with GC, GC-MS, and HPLC to establish effective strategies for the comprehensive analysis of aberrant sterols accumulating in SLOS. Herein is described the application of practical analytical methods to the quantitation and identification of sterols in 17 SLOS and 6 normal blood samples. Comparisons between normal and SLOS samples, together with studies of the aberrant metabolic pathways of cholesterol biosynthesis (32), provide insight into the origin and fate of sterol intermediates.

MATERIALS AND METHODS

Materials

The preparation and purity of 5α-cholestan-3β-ol, its unsaturated analogs with double bonds at the Δ^4, Δ^5, Δ^6, Δ^7, Δ^8, $\Delta^{8(14)}$, Δ^{14}, $\Delta^{4,6}$, $\Delta^{5,7}$, $\Delta^{5,8}$, $\Delta^{5,8(14)}$, $\Delta^{5,24}$, $\Delta^{6,8}$, $\Delta^{6,8(14)}$, $\Delta^{7,9(11)}$, $\Delta^{7,14}$, $\Delta^{7,24}$, $\Delta^{8,14}$, $\Delta^{8,24}$, $\Delta^{5,7,9(11)}$, and $\Delta^{6,8,14}$ positions, and the corresponding acetate derivatives have been described previously (31). 25,26,26,26,27,27,27-Heptafluorocholesterol (F$_7$-cholesterol) (33) and 7α-(1′,2′-dicarbomethoxyhydrazo)cholesta-5,8-dien-3β-ol 3-acetate (31) were prepared as described previously. The following items were obtained commercially: silica gel (230-400 mesh) and Omnisolve-grade solvents (EM Science, Gibbstown, NJ); 2,5-diphenyloxazole (J. T. Baker, Phillipsburg, NJ); Scintiverse (Fisher Scientific, Fair Lawn, NJ); Hyflo Super Cel (Johns-Manville, Lompoc, CA); neutral alumina (activity I; ICN Biomedicals, Costa Mesa, CA); silicic acid (100 mesh; Mallinckrodt, Chesterfield, MO); 5α-cholestane (Aldrich, Milwaukee, WI); butylated hydroxytoluene (BHT, 2,6-di-*tert*-butyl-4-methylphenol; Sigma, St. Louis, MO); and [3H]acetic anhydride and [4-14C]cholesterol (Amersham, Arlington Heights, IL). [4-14C]cholesterol (55 μCi/mmol) was purified prior to use on a silicic acid-Super Cel column (500 mm × 10 mm i.d.; elution with toluene) to give material of 99.5% purity as judged by radio-TLC analysis on silica gel.

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[3] C$_{27}$ 3β-hydroxysterols are all of 5α configuration (or Δ^4 or Δ^5) and are designated by their unsaturation as illustrated by the following examples: Δ^0, 5α-cholestan-3β-ol (cholestanol); Δ^5, cholest-5-en-3β-ol (cholesterol); Δ^7, 5α-cholest-7-en-3β-ol (lathosterol); $\Delta^{5,7}$, cholesta-5,7-dien-3β-ol (7-dehydrocholesterol); $\Delta^{5,8}$, cholesta-5,8-dien-3β-ol (8-dehydrocholesterol); $\Delta^{6,8}$, 5α-cholesta-6,8-dien-3β-ol (isodehydrocholesterol); $\Delta^{5,7,9(11)}$, cholesta-5,7,9(11)-trien-3β-ol; 19-nor-$\Delta^{5,7,9}$, 19-norcholesta-5,7,9-trien-3β-ol.

Chromatographic and spectral methods

HPLC was carried out with acetone-hexane at 1 ml/min (or 3 ml/min for semipreparative columns) with Waters (Milford, MA) 510 or 515 pumps, a Rheodyne (Cotati, CA) 7125 injector, and ultraviolet (UV) detection at 210 nm. Normal-phase HPLC was performed on a 5 μm Adsorbosphere silica column (250 × 4.6 mm or 300 × 10 mm; Alltech, Deerfield, IL), and Ag⁺-HPLC was done on 5 μm Nucleosil SA cation-exchange columns (250 × 10 mm or 300 × 10 mm; 90-Å pore size; Alltech) prepared as described previously (29). Radio-Ag⁺-HPLC was done on similar Ag⁺-HPLC columns (300 × 3.2 mm or 300 × 4.6 mm). These six columns are designated, respectively, as CL-1, CL-2, CL-3, CL-4, CL-5, and CL-6. The following solvent systems were used: acetone-hexane 1:10 (SS-1) for the analysis of free sterols, acetone-hexane 4:96 (SS-2) or acetone-hexane 3:97 (SS-3) for diene steryl acetates, and acetone-hexane 1:99 (SS-4) for monoene steryl acetates. The elution of tritium-labeled sterols was monitored with a β-RAM flow detector (IN/US, Tampa, FL) using a 500 μl cell and added liquid scintillator or by taking aliquots for analysis by scintillation spectrometry. Medium pressure liquid chromatography (MPLC) was done on glass columns dry packed with alumina-AgNO₃ (34). Silicic acid-Super Cel chromatography was carried out as described (35). TLC was performed on aluminum-backed, silica gel 60 plates (EM Science). NMR spectra were acquired as described previously (31) at 25°C on a Bruker (Billerica, MA) AMX500 spectrometer and referenced to (CH₃)₄Si (¹H) or CDCl₃ (δ 77.0; ¹³C). NMR samples were dilute solutions (<10 mM for ¹H, <50 mM for ¹³C) in CDCl₃, which had been freshly filtered through basic alumina.

Capillary GC was carried out on a Shimadzu (Kyoto, Japan) GC-9A instrument with a 30-m DB-5 column (0.25-mm i.d., 0.1 μm film thickness; J&W Scientific, Folsom, CA) at 250°C with nitrogen carrier gas at 1.1 kg/cm². Additional GC analyses were done on a Perkin-Elmer (Norwalk, CT) Sigma 2000 chromatograph with split injection, using a 60-m DB-5 column (0.25-mm i.d., 0.1 μm film thickness) at 250°C with nitrogen carrier gas at 1.3 kg/cm². GC-MS was done on a Hewlett-Packard (Palo Alto, CA) HP-5890A GC unit containing a 60-m DB-5ms column (0.25-mm i.d., 0.1 μm film thickness, 250°C, helium carrier gas at 1.4 kg/cm²) interfaced to a ZAB-HF mass spectrometer containing an electron-impact ion source (70 eV, 200°C). Mass spectral data are presented as m/z (relative abundance). Steryl acetates were analyzed directly by GC after addition of F₇-cholesterol acetate and/or 5α-cholestane. Samples of free sterols with corresponding internal standards were converted to TMS derivatives by treatment with a 1:1 mixture (200 μl) of N,O-bis(trimethylsilyl)trifluoroacetamide and pyridine at 50°C for 2 h.

Individual sterols were quantitated by GC and GC-MS against known amounts of internal standards (5α-cholestane; F₇-cholesterol acetate or TMS derivative), after correction for differences in detector response and the increased mass arising from derivatization. The relative responses (based on mass injected) of the acetates of F₇-cholesterol, Δ⁵, Δ⁷, Δ⁵,⁷, and Δ⁵,⁸ sterols, and of 5α-cholestane to flame ionization detectors were 1.0, 1.7, 1.7, 1.7, 1.6, and 1.8, respectively. These sterols showed linear detector responses with 30-m and 60-m DB-5ms columns over the range of 3 to 150 ng, although quantitation of the broad Δ⁵,⁷ TMS or acetate peak was sometimes imprecise. Relative amounts of sterols in mixtures were also measured by ¹H NMR, mainly by comparison of the relative intensities of the upfield methyl signals (δ 0.5–1.1) after slight Gaussian apodization (line broadening, −0.8; Gaussian broadening, 0.08; 9,000–16,000 complex points). Acquisition times for trace-level analyses were typically 1.5 s, with an ~70° pulse and a 10-ms relaxation delay. T_1 relaxation times for the H-18 and H-19 signals of representative sterols in nondegassed CDCl₃ were both ~0.7 s.

Chemical synthesis and characterization of 19-norcholesta-5,7,9-trien-3β-ol[4]

7α-(1′,2′-Dicarbomethoxyhydrazo)cholesta-5,8-dien-3β-ol 3-acetate (200 mg) was pyrolyzed as described previously (36, 37) by heating under argon for 10 min at 220–235°C, followed by vacuum pyrolysis (0.2 torr argon) for 30 min. The reaction product, containing 60% starting material, was dissolved in hexane (10 ml) and purified on silica gel (SS-4, CL-2, 6-ml fraction volumes). Evaporation of fractions 6–8 gave a mixture of mainly the nortriene and Δ⁵,⁷⁽¹¹⁾ acetates, which were separated in 30-mg batches by semipreparative Ag⁺-HPLC (SS-3, CL-4). Besides dienes and trienes (t_R 16 min) and the Δ⁵,⁷,⁹⁽¹¹⁾ acetate (t_R 37 min; 50 mg), intermediate fractions (t_R 25 min) contained 19-norcholesta-5,7,9-trien-3β-ol 3-acetate (9 mg): single component by GC (t_R 2.06 relative to F₇-cholesterol acetate) and GC-MS (t_R 1.98 relative to cholesterol acetate); MS, m/z 350 (100, M–AcOH), 335 (2), 237 (13), 210 (22), 195 (51); single component by Ag⁺-HPLC (t_R 12.4 min, SS-3, CL-6); UV [ethanol; λ_max (ε)], 220 nm (6500), 269 nm (300), 277 nm (300) [literature UV (36), 225 nm (10000), 231 nm (5500), 269 nm (1700), 278 nm (1400)]; no color response after treatment with the Liebermann-Burchard reagent (1 ml added to 279 μg of sterol, observed for 48 h); ¹H NMR, δ 2.67 (H-1α), 2.70 (H-1β), 2.098 (H-2α), 1.925 (H-2β), 5.143 (dddd, 9.2, 7.8, 5.1, 3.1 Hz, H-3α), 3.082 (dd, 16.2, 5.0 Hz, H-4α), 2.865 (dd, 16.1, 7.8 Hz, H-4β), 6.879 (d, 7.9 Hz, H-6), 6.854 (d, 7.9 Hz, H-7), 2.67 (H-11α), 2.65 (H-11β), 1.659 (H-12α), 2.253 (ddd, 13.0, 6.7, 2.7 Hz, H-12β), 2.66 (H-14), 2.08 (H-15α), 1.50 (H-15β), 2.08 (H-16α), 1.47 (H-16β), 1.327 (H-17α), 0.594 (s, H-18), 1.463 (H-20), 0.997 (d, 6.6 Hz, H-21), 1.41 (H-22R), 1.061 (H-22S), 1.37 (H-23R), 1.18 (H-23S), 1.12 (H-24R), 1.16 (H-24S), 1.537 (H-25), 0.877 (d, 6.6 Hz, H-26), 0.882 (d, 6.6 Hz, H-27), 2.058 (s, OAc); ¹³C NMR, δ 24.13, 28.01, 69.81, 35.00, 130.62, 126.53, 123.87, 138.02, 134.13, 132.95, 25.05, 36.89, 41.78, 51.68, 24.17, 28.86, 55.16, 11.08, 36.17, 18.78, 36.11, 23.84, 39.48, 28.01, 22.56, 22.83, 21.47, 170.84.

The 19-nortriene acetate (4.3 mg) was treated with 15% ethanolic KOH (1 ml) at 70°C for 2 h, followed by aqueous workup. Purification on HPLC (SS-4, CL-2, 20-ml fraction volumes) and evaporation of fractions 10–19 gave the nortriene as a white solid (4 mg): melting point 118–118.5°C; single component on TLC (R_f 0.4; acetone-hexane 2:8) and Ag⁺-HPLC (t_R 16.5 min, SS-1, CL-5); high resolution MS, calcd. for C₂₆H₄₀O 368.3079, found, 368.3074; UV [ethanol; λ_max (ε)], 220 nm (6,200), 269 nm (300), 277 nm (300); single component as the TMS derivative by GC-MS (t_R 44.3 min); m/z 440 (12, M⁺), 350 (100, M-TMSOH), 237 (22), 210 (25), 195 (34), 182 (32), 141 (12), essentially identical to the GC-MS of the decomposition product of the Δ⁵,⁸ TMS derivative (t_R 44.2 min); ¹H NMR, δ 2.64 (H-1α), 2.74 (H-1β), 2.104 (H-2α), 1.823 (H-2β), 4.116 (H-3α), 3.062 (H-4α), 2.768 (H-4β), 6.897 (d, 7.8 Hz, H-6), 6.852 (d, 7.8 Hz, H-7), 2.66 (H-11α), 2.64 (H-11β), 1.650 (H-12α), 2.251 (H-12β), 2.65 (H-14), 2.07 (H-15α), 1.504 (H-15β), 2.07 (H-16α), 1.47 (H-16β), 1.321 (H-17α), 0.588 (s, H-18), 1.462 (H-20), 0.995 (d, 6.5 Hz, H-21), 1.40 (H-22R), 1.058 (H-22S), 1.38 (H-23R), 1.18 (H-23S), 1.12 (H-24R), 1.16 (H-24S), 1.536 (H-25), 0.876 (d, 6.6 Hz, H-26), 0.881 (d, 6.6 Hz, H-27); ¹³C NMR, δ 24.66, 31.66, 67.13, 38.95, 131.11, 126.76, 123.87, 138.01, 134.17, 133.05, 25.11, 36.92, 41.77, 51.71, 24.18, 28.85, 55.17, 11.06, 36.16, 18.78, 36.12, 23.83, 39.48, 28.02, 22.56, 22.82. Ring A coupling constants (1α-2α, 6.5; 1α-2β, 9.5; 1β-2α, 5.1; 1β-2β, 6.1; 2α-3α, 3.2; 2β-3α, 9.5; 3α-4α, 4.8; 3α-4β, 8.2 Hz) indicated a 2:1 ratio of conformers with the 3β-hydroxyl group equatorial or axial.

[4] Ruan et al. have reported HPLC data (28) for the nortriene and a brief account of its synthesis (30).

Blood samples

Samples of erythrocytes and plasma (about 20 ml each) were obtained from a 28-year-old SLOS subject before (A) and after (B) 1 month of a high cholesterol diet (target of 1,050 mg of cholesterol per day from meat, eggs, and dairy products). Additional plasma samples of about 1 ml represented 4 other SLOS subjects (C, E–G) and an SLOS control mixture (D). These samples were shipped on dry ice and stored at −80°C prior to analysis. Normal blood samples (40 ml; nonfasting) were obtained from healthy young American adults, a 34-year-old Caucasian female (P), a 22-year-old Asian male (Q), and a 22-year-old Caucasian male (R), and processed immediately. More limited analyses were carried out on a 0.5-ml whole blood sample from a 1-year-old girl severely affected by SLOS (H) and 0.5-ml plasma samples from 7 mildly affected SLOS subjects (I–O) and 3 normal subjects (S–U). All blood samples were obtained with informed consent of the subjects or their parents, and the studies of human samples were performed with institutional approval. Most samples (C–G, I–O, and S–U) consisted of leftover material from blood drawn for diagnostic purposes.

Procedures for processing and analysis of blood samples

Procedure 1. Procedure 1 was used for samples A and B. To the SLOS plasma or erythrocyte sample (about 10 ml) was added a solution of freshly purified [4-14C]cholesterol (1.25 μCi; 55 mCi/mmol) in toluene (100 μl) and BHT (500 μg) in ethanol (0.5 ml). The resulting mixture was transferred to an anaerobic glove box containing nitrogen, mixed with a 15% solution of KOH in 95% ethanol (25 ml, deoxygenated), and heated at 70°C for 2 h. To the reaction was added water (25 ml), followed by extraction with deoxygenated hexane (3 × 240 ml). The hexane extracts were washed with brine (120 ml), dried over Na_2SO_4, and evaporated to dryness under reduced pressure. The residue, comprising the nonsaponifiable lipids (NSL), was dissolved in toluene (10 ml), and aliquots were taken for the measurement of radioactivity, 1H NMR, GC, and GC-MS. The NSL were applied to a 2:1 silicic acid-Super Cel column (1 m × 10 mm; elution with toluene, followed by diethyl ether-toluene 2:8 after fraction 129; 17-ml fraction volumes). On the basis of elution of the 14C label in fractions 59–70, fractions 59–129 were combined, and aliquots were taken for measurement of radioactivity, NMR, GC, GC-MS, and Ag+-HPLC. Later fractions containing polar material were also analyzed by GC and NMR. The C_{27} sterols were acetylated by treatment with [3H]acetic anhydride (25 mCi; 1.25 mCi/mmol) in dry pyridine (0.8 ml) for 24 h at room temperature in the dark. The [3H]acetate derivatives of the C_{27} sterols were partially purified on a 2:1 silicic acid-Super Cel column (1 m × 20 mm; elution with toluene), and the contents of fractions containing 3H and 14C were subjected to alumina-$AgNO_3$ MPLC (1 m × 10 mm column; elution with toluene-hexane 1:9, followed by toluene-hexane 1:3 after fraction 100 and toluene-hexane 4:6 after fraction 230; 20-ml fraction volumes). On the basis of the resulting radioactivity profile, fractions were combined into zones, which were subjected to NMR, GC, GC-MS, and Ag+-HPLC analyses, including GC and Ag+-HPLC calibration with authentic standards.

Procedure 2. Procedure 2 was used for samples C–G. Plasma samples (about 1 ml) were processed as in procedure 1 to obtain the NSL, which were then partially separated into polar and non-polar fractions on silica gel [50 × 5 mm column; stepwise elution with hexane (2 ml), acetone-hexane 5:95 (20 ml), and acetone (5 ml)]. The polar fractions were subjected to GC-MS and NMR analysis. After removal of aliquots for measurement of radioactivity, GC-MS, and 1H NMR, the non-polar fractions, containing the C_{27} sterols and other cholesterol precursors, were

separated on normal-phase HPLC (SS-2, CL-2) into several fraction sets, designated as zones. Each zone was analyzed by GC-MS and 1H NMR, followed by further separation on Ag+-HPLC. The resulting subfractions, each of which contained at most two sterols, were also analyzed by GC-MS and 1H NMR.

Procedure 3. Normal blood samples P–R (40 ml each) were drawn, immediately cooled on ice, and centrifuged to remove the erythrocytes. The plasma samples were processed as in procedure 1 to obtain the NSL, except that F_7-cholesterol (102 μg) in toluene (200 μl) was added. As in procedure 2, the NSL were purified on silica gel and separated by normal-phase HPLC (SS-3, CL-2) into zones. An early zone containing mainly cholesterol and a later zone containing trace-level sterols were each analyzed by GC-MS and NMR and then subfractionated on Ag+-HPLC, followed by additional GC-MS and NMR analyses.

Procedure 4. The remaining samples (H–O and S–U) were saponified as described in procedure 1 (without chromatography or addition of [14C]cholesterol) to give the NSL. Relative amounts of the sterols were estimated from the 1H NMR spectrum of the NSL, and the total amount of sterols was based on GC-MS analysis. Portions of these samples were also subjected to Folch extraction (dropwise addition of plasma to 20 volumes of methanol-chloroform 2:1). After vortex mixing, the chloroform layer was separated and evaporated to a residue, which was analyzed by 1H NMR.

RESULTS

Nine SLOS blood samples (A–G) and three normal plasma samples (P–R) were subjected to detailed analysis for C_{27} sterols. After saponification of plasma or erythrocytes under oxygen-free conditions, normal-phase chromatography, Ag+-HPLC, and GC were used to separate, identify, and quantitate the individual sterol components. Separations were guided by the known (27–29) chromatographic behavior of the sterols, which is summarized in **Fig. 2**. Sterol preparations were chromatographed as either the free sterols or 3H-labeled acetates, which were analyzed at each stage of derivatization and purification by GC-MS, GC, and, in most cases, Ag+-HPLC and 1H NMR. This thorough characterization at each step allowed monitoring for potential artifact formation and for losses during processing. Loss of material was also monitored by measuring levels of [14C]cholesterol (added initially).

Several variations of processing and derivatization (procedures 1–4) were used. For the 20-ml SLOS blood samples (A and B), 3H-labeled acetate derivatives were prepared to facilitate the quantitation of minor components; **Fig. 3** summarizes the experimental design, which was derived from methodology used to analyze oxysterols in plasma (38). To minimize processing losses, a simplified procedure was used for the 1-ml SLOS blood samples C–G, which were fractionated by normal-phase HPLC and subfractionated by Ag+-HPLC. For the normal blood samples P–R, which contained almost exclusively cholesterol, a small amount of F_7-cholesterol was added prior to sample processing to permit accurate quantitation of the trace-level sterols by GC-MS and HPLC. Analysis of SLOS samples H–O and normal samples S–U was limited to 1H NMR of the NSL and Folch extracts.
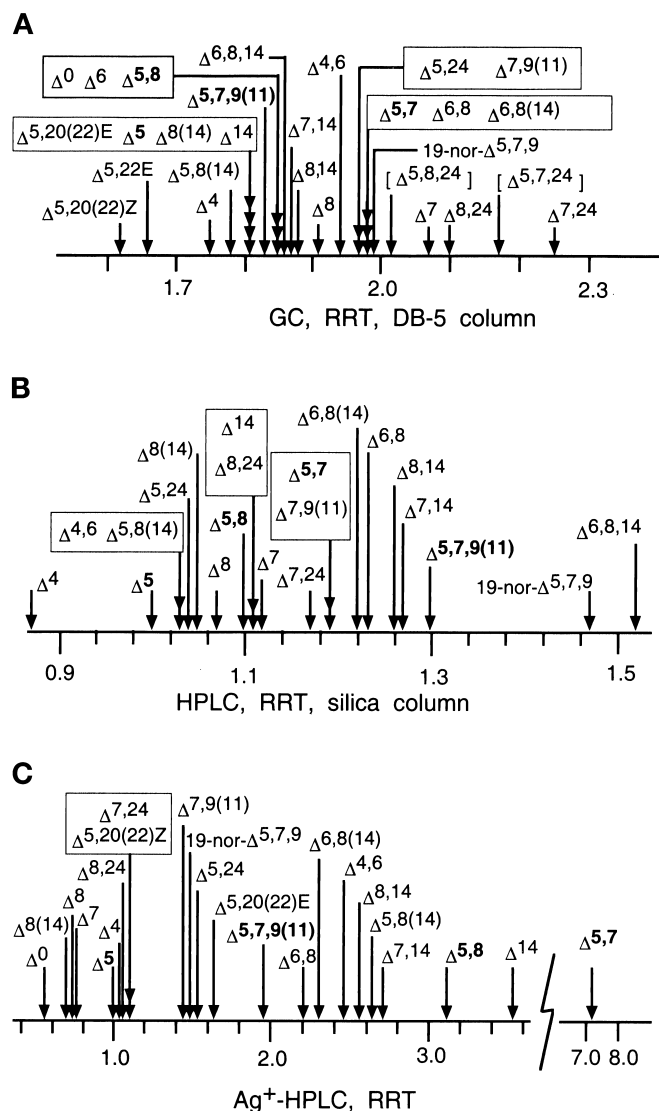
Fig. 2. Chromatographic behavior of C_{27} sterols on GC (A), normal-phase HPLC (B), and Ag^+-HPLC (C) on a scale of relative retention time (RRT) to 5α-cholestane (A) or cholesterol (B and C). The chromatographic mobilities shown are for free sterols (or TMS ethers for GC) and are derived from data presented in refs. (27) and (28), and similar data are available for acetates (27–29). Conditions: GC, 60-m DB-5 column at 250°C (isothermal); HPLC, silica column, isocratic elution with acetone–hexane 3:97; Ag^+-HPLC, isocratic elution with acetone–hexane 1:10. The RRT scale in (A) can be converted to retention indices by using data in ref. (27). Values in brackets in (A) denote predicted mobilities (27). The RRT scale gives a distorted impression of separating power, which is highest for Ag^+-HPLC because the sterols are eluted over a much wider span than on GC or normal-phase HPLC.

Identification and quantitation of sterols

Sterols were identified by matching their chromatographic (Ag^+-HPLC, GC) and spectral (MS, NMR) properties with those of authentic standards (27–29, 31). This kind of structural evidence is given in Ref. (39) for the Δ^5, $\Delta^{5,7}$, $\Delta^{5,8}$, and $\Delta^{5,7,9(11)}$ sterols. These sterols, composing >98% of total sterols (except in sample H), were isolated in substantial amounts as individual components and
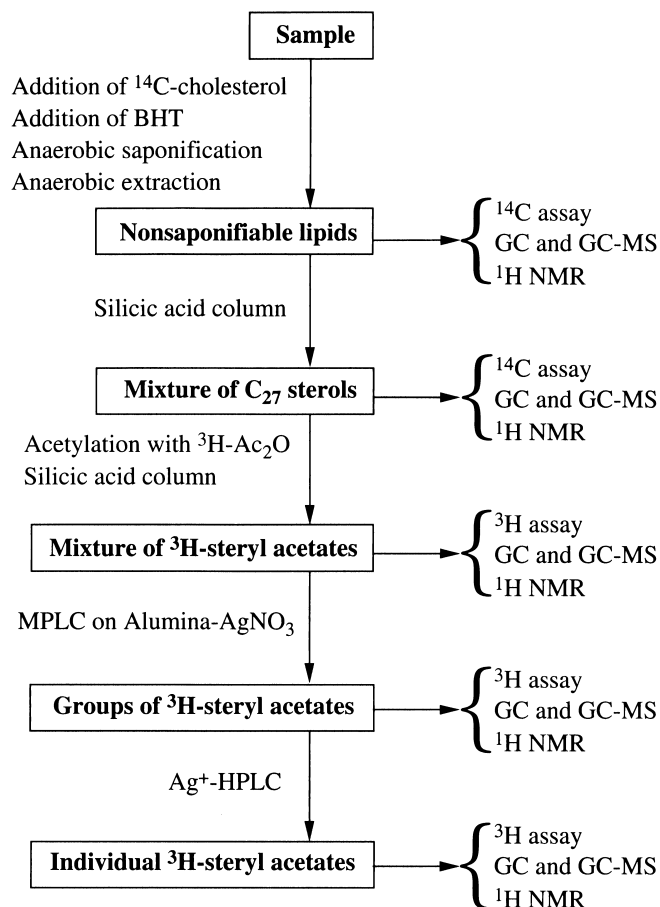

Fig. 3. Experimental design for the isolation and characterization of unsaturated C_{27} sterols in blood (illustrated for procedure 1). This comprehensive analysis, with multiple assays carried out at each stage of purification, was performed for SLOS samples A and B (erythrocytes and plasma).

characterized by GC, GC-MS, Ag^+-HPLC, ^1H NMR, and (in most cases) ^{13}C NMR. ^1H NMR spectra of the $\Delta^{5,8}$ and $\Delta^{5,7,9(11)}$ sterols purified from SLOS blood are shown in **Fig. 4**. Structural evidence for the minor sterols was based on less exhaustive characterization, which is summarized in **Table 1**. Nevertheless, the highly distinctive NMR chemical shifts, together with the characteristic mass spectral data and Ag^+-HPLC mobilities, provided strong evidence for the assigned structures. NMR spectra of selected minor sterols are shown in Fig. 4C–4E.

Absolute amounts of the major sterols were determined at each processing step by GC quantitation against an added internal standard, and the relative amounts of all sterols were measured by ^1H NMR (**Fig. 5**). Quantitation was more difficult for the minor sterols, which were rarely detectable by GC-MS of the NSL. Although one or more NMR signals of several minor sterols in the NSL mixture were resolved at 500 MHz, signal overlap precluded quantitation and unequivocal identification of most trace-level sterols. However, chromatographic fractionation of the NSL provided partially purified sterols that could be identified reliably by ^1H NMR, GC-MS, and Ag^+-HPLC and quantitated in some cases against other components. The
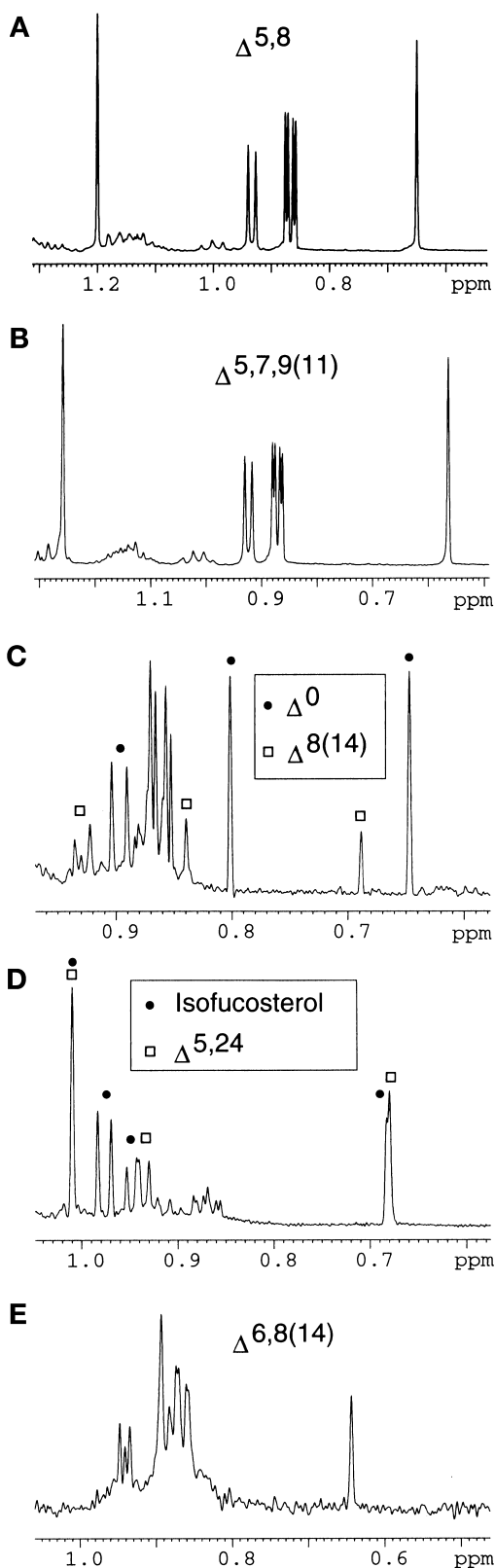
Fig. 4. ^{1}H NMR spectra of noncholesterol sterols isolated from blood of SLOS and normal subjects. A: $\Delta^{5,8}$ acetate from plasma sample A; B: $\Delta^{5,7,9(11)}$ acetate from erythrocyte sample B; C: 3:1 mixture of the Δ^0 and $\Delta^{8(14)}$ sterols from sample Q; D: 55:45 mixture of the $\Delta^{5,24}$ sterol and isofucosterol from sample P; E: $\Delta^{6,8(14)}$ sterol from sample G. The 500-MHz NMR spectra were processed with slight to moderate Gaussian apodization; only the methyl region is shown.

Δ^0, Δ^7, and $\Delta^{6,8(14)}$ sterols were obtained in $\geq 90\%$ purity, but characterization and limited quantitation were possible from simple mixtures. The Δ^8, $\Delta^{8(14)}$, $\Delta^{5,24}$, $\Delta^{6,8}$, and $\Delta^{6,8(14)}$ sterols were observed at low levels in most of samples A–G as acetates and/or free sterols.

Detailed analysis of C_{27} sterols as [3H]acetates in SLOS blood samples A and B

A portion (9 ml) of erythrocyte sample B was processed according to procedure 1. ^{1}H NMR showed a 67:23:9:1:0.3 ratio of the Δ^5, $\Delta^{5,7}$, $\Delta^{5,8}$, $\Delta^{5,7,9(11)}$, and Δ^7 sterols, and GC quantitation indicated a similar ratio for the major sterols. The recovery of added ^{14}C in the crude NSL fraction was 99.6%. A sample of purified cholesterol (27 mg) processed in identical fashion as the erythrocyte sample showed 99.0% recovery of added ^{14}C and 97.7% recovery of unlabeled cholesterol. Silicic acid-Super Cel chromatography of the crude NSL led to a 94% recovery of sterols, which were present in essentially unchanged proportions as judged by GC-MS and ^{1}H NMR. The absence of ^{14}C label in the polar fractions indicated that negligible autoxidation occurred during sample processing, and GC-MS and ^{1}H NMR analyses showed no detectable oxysterols in the polar fraction (detection limit, about 10 μM).

Acetylation of the nonpolar NSL fraction provided [3H]acetate derivatives (84 μCi of ^{3}H) in 86% yield, showing a 74:17:9 ratio by GC of the Δ^5, $\Delta^{5,7}$, and $\Delta^{5,8}$ sterols, suggesting selective loss of $\Delta^{5,7}$ material during acetylation and chromatographic purification. (Much of the $\Delta^{5,7}$ sterol was also lost during acetylation of plasma samples B and G, but such losses were not observed for all samples.) MPLC of the [3H]acetates on alumina-AgNO$_3$ gave six fraction sets: zone I (fractions 5–16), zone II (fractions 17–25), zone III (fractions 99–110), zone IV (fractions 111–118), zone V (fractions 119–134), and zone VI (fractions 221–254), showing ^{3}H yields of 2.6%, 55.7%, 0.9%, 1.2%, 5.9%, and 13.9%, respectively (total recovery of ^{3}H, 80%).

Each zone was analyzed by GC, GC-MS, Ag$^+$-HPLC, and ^{1}H NMR, and zones containing multiple sterol components were further purified by Ag$^+$-HPLC. Zone I (1.7 μCi; 0.21 mg) showed a 67:23:7:1 ratio of Δ^5, Δ^7, Δ^0, and Δ^8 acetates by ^{1}H NMR and a 62:29:9 ratio of Δ^5, Δ^7, and Δ^0 acetates by GC-MS. After subfractionation on Ag$^+$-HPLC (SS-4, CL-6, 1-ml fraction volumes), ^{1}H NMR, GC-MS, and Ag$^+$-HPLC analyses showed the following: fractions 6–10, 94:6 mixture of Δ^0 and $\Delta^{8(14)}$ acetates; fractions 20–23, 98:2 mixture of Δ^7 and Δ^8 acetates; fractions 36–41, Δ^5 acetate. Zone II (38 μCi; 12.8 mg) contained the Δ^5 acetate as a single component ($\geq 99\%$ purity). ^{1}H NMR analysis of zone III (0.6 μCi; 0.005 mg) indicated a mixture of unidentified components. Zone IV (0.8 μCi; 0.10 mg) showed a 2:1 mixture of $\Delta^{5,7,9(11)}$ and $\Delta^{5,8}$ acetates by GC, GC-MS, and NMR. After further purification on Ag$^+$-HPLC (SS-3, CL-6, 1-ml fraction volumes), ^{1}H NMR, GC-MS, and Ag$^+$-HPLC analyses showed the following: fractions 12–15, a mixture of Δ^5- and Δ^7-plant steryl acetates containing ~15% $\Delta^{5,7,9(11)}$ acetate; fractions 16–20,

TABLE 1. Concentration ranges and structural evidence for the $\Delta^{5,7,9(11)}$ sterol and minor sterols found in SLOS and normal blood

	Concentration[a]	¹H NMR Signals[b]				Ag⁺-HPLC[c]	GC-MS[d]
		H-18	H-19	H-21	Other		
	$\mu g/ml$					t_R (min) or RRT	t_R (min) or RRT
Δ^0 sterol	1–5	0.647	0.801	0.897	3	RRT, 0.54	44.8 (44.2)
Δ^0 acetate	1–5	0.646	0.817	0.897	8		51.9 (51.6)
Δ^7 sterol	1–5	0.534	0.796	0.920	6	t_R 8.7 (8.5)	46.1 (46.2)
Δ^7 acetate	1–5	0.533	0.811	0.920	6	t_R 14.4 (14.2)	57.7 (57.6)
Δ^8 sterol	~0.1	0.609	0.950	0.923	0	RRT, 0.77 (0.73)	45.9 (46.5)
Δ^8 acetate	~0.1	0.606	0.963		0	t_R 13.6 (13.1)	52.6 (53.3)
$\Delta^{8(14)}$ sterol	~0.03	0.840	0.689	0.929	1	RRT, 0.73 (0.69)	45.1 (45.3)
$\Delta^{8(14)}$ acetate	~0.03	0.838	0.704	0.929	1	t_R 8.5 (8.5)	49.7 (50.4)
$\Delta^{5,8(14)}$ sterol		0.877	0.888	0.941	2	RRT, 2.64 (2.65)	38.5 (~39)
$\Delta^{5,24}$ sterol		0.680	1.010	0.935	7		42.8 (43.0)
$\Delta^{6,8}$ sterol	0.1–1	0.576	0.793	0.939	3		1.06 (1.09, Δ^5)
$\Delta^{6,8(14)}$ sterol	~0.3	0.644	0.894	0.941	2		1.06 (1.09, Δ^5)
$\Delta^{7,24}$ sterol		0.536					1.07 (1.09, Δ^7)
$\Delta^{7,24}$ acetate						RRT, 1.07 (1.11, $\Delta^{8,24}$)	2.20 (2.21, $\Delta^{8,24}$)
$\Delta^{5,7,9(11)}$ sterol	0.1–40	0.566	1.246	0.922	10		48.0 (48.9)
$\Delta^{5,7,9(11)}$ acetate[e]	0.1–40	0.563	1.256	0.922	23	t_R 21.5 (22.5)	50.2 (49.9)

[a] Approximate concentration range observed for normal and SLOS samples; higher concentrations of the $\Delta^{6,8}$ and $\Delta^{5,7,9(11)}$ sterols were observed only in SLOS samples.

[b] The observed NMR chemical shifts generally matched literature values (31) to ±0.001 ppm. The column labeled "Other" gives the number of additional NMR signals observed.

[c] Comparison of observed chromatographic mobilities on Ag⁺-HPLC with those of authentic standards. Relative retention times (RRT) are referred to cholesterol unless otherwise specified.

[d] GC-MS retention times of authentic standards (or RRT values and the reference sterol) are given in parentheses; the modest precision resulted from effects of column overloading. The following mass spectral data closely matched those of authentic standards and were compatible with published data (27): Δ^0 TMS ether, m/z 460 (47), 445 (50), 370 (20), 355 (20), 351 (85), 325 (51), 215 (100); Δ^0 acetate, m/z 430 (21), 370 (87), 355 (44), 316 (33), 257 (31); Δ^7 TMS ether, m/z 458 (100), 443 (19), 368 (40), 353 (26), 303 (20), 255 (52); Δ^7 acetate, m/z 428 (84), 413 (18), 368 (100), 353 (44), 255 (90), 229 (34), 213 (55); Δ^8 TMS ether, m/z 458 (100), 443 (20), 368 (50), 353 (33), 329 (23), 255 (12); Δ^8 acetate, m/z 428 (100), 368 (50), 351 (38), 255 (34), 229 (40), 213 (68); $\Delta^{8(14)}$ acetate, m/z 428 (100), 413 (20), 368 (63), 353 (63), 316 (16), 255 (49), 229 (36), 213 (55); $\Delta^{5,8(14)}$ TMS ether, m/z 456 (100), 441 (23), 366 (56), 351 (86), 343 (21), 325 (37), 253 (67), 227 (22), 199 (44), 129 (34); $\Delta^{5,24}$ TMS ether, m/z 456 (34), 441 (31), 372 (18), 366 (32), 351 (28), 343 (67), 327 (45), 255 (13), 253 (37), 129 (100); $\Delta^{7,24}$ TMS ether, m/z 456 (40), 441 (33), 343 (100), 255 (19); $\Delta^{7,24}$ acetate, m/z 426 (26), 411 (30), 351 (10), 342 (17), 313 (100), 255 (20), 253 (17), 213 (26); $\Delta^{5,7,9(11)}$ TMS ether, m/z 454 (16), 439 (10), 364 (100), 349 (25), 251 (26); $\Delta^{5,7,9(11)}$ acetate, m/z 424 (4), 364 (100), 349 (62), 251 (46), 235 (29), 209 (65).

[e] In additional characterization of the $\Delta^{5,7,9(11)}$ acetate from SLOS samples, UV data (λ_{max} 324, shoulders at 312 and 340) and behavior in the Liebermann-Burchard color test were similar to those of an authentic standard.

$\Delta^{5,7,9(11)}$ acetate; fractions 23–27, $\Delta^{5,8}$ acetate. Zone V (4.0 μCi; 0.866 mg) contained mainly the $\Delta^{5,8}$ and $\Delta^{5,7,9(11)}$ acetates (95:2 ratio). Zone VI (9.4 μCi; 2.51 mg) consisted of the $\Delta^{5,7}$ acetate. These results are summarized in **Table 2**. Analysis of plasma samples A and B and erythrocyte sample A gave similar results, details of which are described in the Ph.D. thesis of B. Ruan (39).[5]

Effects of cholesterol feeding therapy on sterol levels

Data in Table 2 allow comparison of sterol levels before (A) and after (B) cholesterol feeding therapy in plasma and erythrocytes from an adult SLOS subject. Erythrocytes showed no change in cholesterol levels but an approximately 50% decrease in levels of $\Delta^{5,7}$, $\Delta^{5,8}$, and $\Delta^{5,7,9(11)}$ sterols after therapy. By contrast, noncholesterol sterol concentrations were essentially unchanged in plasma, while cholesterol levels increased slightly. Although these limited data preclude generalizations, our results are consistent with a report by Linck et al. (40), indicating that cholesterol supplementation (in small

children) produces only minor effects on plasma sterol levels after 4–8 weeks but marked improvements after several months.

Analysis of C₂₇ sterols in SLOS plasma samples C–G

SLOS plasma samples C–G were processed by procedure 2. Detailed results are presented herein for sample G (900 μl, 940 mg). The NSL showed a 69:18:11:1.1:0.4 mixture of Δ^5, $\Delta^{5,7}$, $\Delta^{5,8}$, $\Delta^{5,7,9(11)}$, and Δ^7 sterols (1.90 mg/ml total sterols) by ¹H NMR (Fig. 5), GC, and GC-MS, with a 99.7% recovery of added [¹⁴C]cholesterol after saponification. Semipreparative normal-phase HPLC (SS-2, CL-2) of the NSL (**Fig. 6**) gave seven zones (I–VII). In addition, no early cholesterol biosynthesis intermediates, such as farnesol or squalene, were detected by GC analysis of the early-eluting material (t_R 2–30 min).

Zone I (t_R 37–40 min), a 999:1 mixture of Δ^5 and Δ^0 sterols by NMR, was further separated on Ag⁺-HPLC into an early fraction containing mainly the Δ^0 sterol (by ¹H NMR and GC-MS) and a later fraction consisting of cholesterol. Zone II (t_R 40–42 min), a 57:26:6:6:3:2 mixture of $\Delta^{5,8}$, Δ^5, $\Delta^{5,8(14)}$, $\Delta^{8(14)}$, $\Delta^{5,24}$, and Δ^8 sterols by NMR, was subfractionated on Ag⁺-HPLC (SS-1, CL-5) as follows: zone II-1 (t_R 7–8 min), 3:1 mixture of $\Delta^{8(14)}$ and Δ^8 sterols by GC-MS, NMR, and Ag⁺-HPLC; zone II-2 (t_R 10–

5 In ref. (39), plasma samples A–F are designated as plasma samples A and B and small plasma samples A, B, D, and C, respectively. Samples P–R are designated as plasma samples A–C from normal subjects. Samples G–O and S–U are not described in ref. (39).
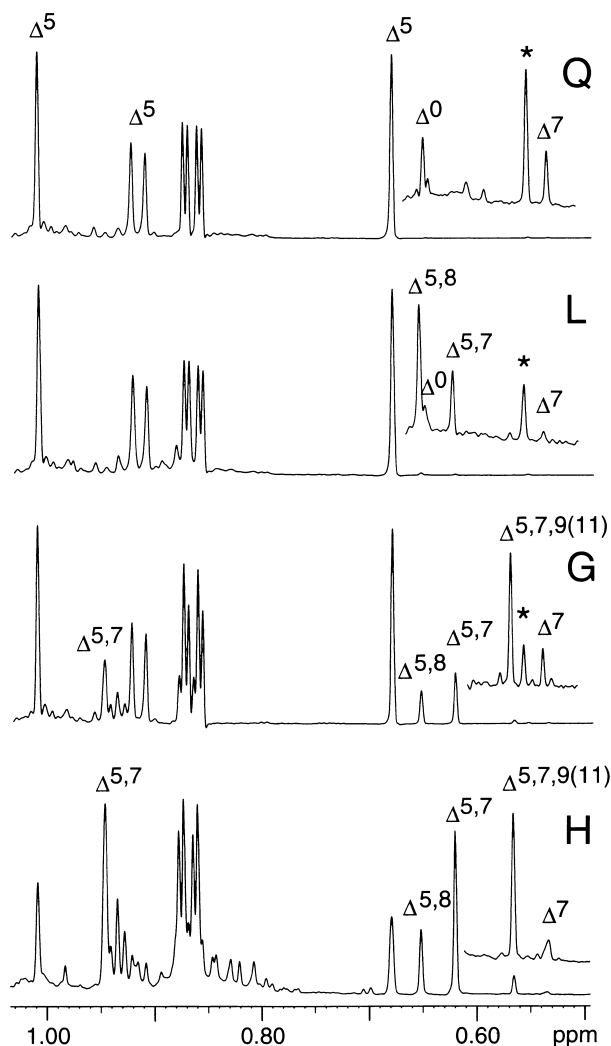
Fig. 5. ^{1}H NMR spectra of the NSL from blood of a normal subject (Q) and SLOS subjects with mild (L), moderate (G), and severe (H) symptoms. NMR analyses were done on the NSL from plasma (samples Q, L, and G) or whole blood (sample H). The 500-MHz NMR spectra were processed with slight Gaussian apodization; only the upfield portion of the methyl region is shown (δ 1.03–0.5). For readability, assignments are shown only for selected signals. The upfield ^{13}C satellite of cholesterol is marked by an asterisk.

TABLE 2. Concentrations of C_{27} sterols observed in erythrocyte and plasma samples from SLOS and normal subjects[a–d]

Sample	Concentration						
	Δ^5	$\Delta^{5,7}$	$\Delta^{5,8}$	$\Delta^{5,7,9(11)}$	Δ^7	Δ^0	$\Delta^{6,8}$
	$\mu g/ml$						
Whole blood sample, SLOS							
H	100	364	182	40.9	5.2	*2.3*	0.9
Erythrocyte samples, SLOS							
A	710	205	90	10.1	5.0	*2.0*	1.0
B	740	122	45	5.3	2.1	0.8	1.2
Plasma samples, SLOS							
A	810	134	99	10.1	**	**	**
B	1,010	145	100	10.1	2.5	*1.9*	0.6
C	910	89	68	8.0	3.7	*0.9*	1.0
D	640	112	67	13.2	2.6	0.6	0.9
E	590	199	77	30.4	4.6	*3.7*	1.5
F	590	118	88	8.8	*1.8*	**	1.5
G	1,180	303	227	19.5	5.5	0.8	1.8
I	920	78	73	4.0	*0.9*	1.4	*0.7*
J	1,240	6.4	8.9	*0.4*	2.7	3.1	*
K	1,170	77	69	3.8	1.8	2.2	*
L	1,490	17.5	8.4	1.0	1.3	2.6	*
M	1,430	70	69	5.1	2.3	2.5	*0.4*
N	1,860	9.5	10.2	*0.3*	1.5	4.1	*
O	1,830	7.1	12.0	*0.7*	0.9	3.1	*
Normal plasma samples							
P	1,640	0.04	0.16	*0.1*	1.6	3.6	0.05
Q	2,280	0.10	0.53	0.10	4.5	4.4	0.13
R	2,380	0.14	0.46	*0.1*	5.6	3.7	0.15
S	1,280	*	*	*	1.0	2.4	*
T	1,490	*0.3*	*	*	2.4	5.8	*0.3*
U	1,860	*0.3*	*	*	3.0	2.2	*

[a] Relative levels of sterols were determined primarily from upfield methyl signal intensities in the ^{1}H NMR spectrum of the NSL. Absolute cholesterol levels were measured by GC-MS; the value for sample H is approximate.

[b] Samples A and B were collected from the same subject before (A) and after (B) 1 month of cholesterol feeding therapy.

[c] Components not detected are designated by * (0.1- to 0.8-µg/ml detection limit) or ** (1- to 8-µg/ml detection limit). Italics indicate quantitation based on a weak signal.

[d] Values for the Δ^7 sterol may include $\Delta^{7,24}$ material.

11 min), Δ^5 sterol by GC-MS and Ag^{+}-HPLC; zone II-3 (t_R 14–15 min), $\Delta^{5,24}$ sterol by GC-MS and Ag^{+}-HPLC; zone II-4 (t_R 22–24 min), unidentified sterol, GC-MS, t_R 0.91 relative to the $\Delta^{8(14)}$ TMS ether; m/z 456 (69), 441 (31), 366 (100), 351 (82), 327 (11), 253 (37), 129 (36); zone II-5 (t_R 26–28 min), $\Delta^{5,8(14)}$ sterol by GC-MS, NMR, and Ag^{+}-HPLC; zone II-6 (t_R 31–34 min), $\Delta^{5,8}$ sterol by GC-MS, accompanied by the 19-nor-$\Delta^{5,7,9}$ artifact. Zone III (t_R 42–44 min), a 97:3 mixture of $\Delta^{5,8}$ and Δ^7 sterols by NMR, was subfractionated on Ag^{+}-HPLC as follows: zone III-1 (t_R 8–9 min), Δ^7 sterol by GC-MS, NMR, and Ag^{+}-HPLC); zone III-2 (t_R 29–35 min), mixture including a major component with GC-MS showing t_R 1.09 relative to cholesterol (TMS ethers), m/z 456 (100), 441 (32), 366 (10), 351 (14), 343 (6), 255 (9), 229 (21), 107 (46); possibly the $\Delta^{8(14),24}$ sterol; zone III-3 (t_R 29–35

min), $\Delta^{5,8}$ sterol by GC-MS, accompanied by the 19-nor-$\Delta^{5,7,9}$ artifact. Zone IV (t_R 45–47 min) contained the $\Delta^{5,7}$ sterol (>99% purity) by GC-MS, NMR, and Ag^{+}-HPLC. Zone V (t_R 46–48 min), a 3:1:1 mixture of $\Delta^{5,7}$, $\Delta^{6,8}$, and $\Delta^{6,8(14)}$ sterols, was subfractionated on Ag^{+}-HPLC (SS-1, CL-5) as follows: zone V-1 (t_R 21–22 min), $\Delta^{6,8}$ sterol by NMR and Ag^{+}-HPLC; zone V-2 (t_R 22–23.5 min), $\Delta^{6,8(14)}$ sterol by GC-MS, NMR, and Ag^{+}-HPLC; zone V-3 (t_R 70 min), $\Delta^{5,7}$ sterol by Ag^{+}-HPLC. Zone VI (t_R 49–50 min) was a 97:3 mixture of $\Delta^{5,7,9(11)}$ and $\Delta^{5,7}$ sterols by NMR. Zone VII (t_R 54–60 min), corresponding to the t_R of the 19-nor-$\Delta^{5,7,9}$ sterol, contained unidentified C_{27} trienols by GC-MS, but no trace of the nortriene was detected by NMR or GC-MS.

As an adjunct to the analyses described in procedure 2, an aliquot from combined zones I–VII was acetylated with [^{3}H]acetic anhydride and pyridine (24 h, 22°C) for additional quantitation of the C_{27} sterols. The crude acetates
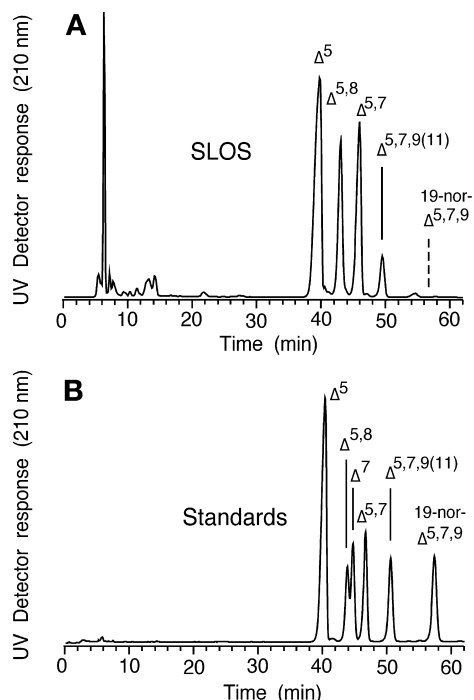
Fig. 6. Normal-phase HPLC chromatograms showing (A) separation of the major sterols in SLOS sample G (NSL) and (B) separation of authentic standards of the Δ^5, $\Delta^{5,8}$, Δ^7, $\Delta^{5,7,9(11)}$, and 19-nor-$\Delta^{5,7,9}$ sterols. HPLC conditions: SS-2, CL-2, UV detection at 210 nm. The HPLC peak intensities are affected by the UV detector response, which is low for cholesterol and high for the 19-nor-$\Delta^{5,7,9}$ sterol (entirely absent in the SLOS sample).
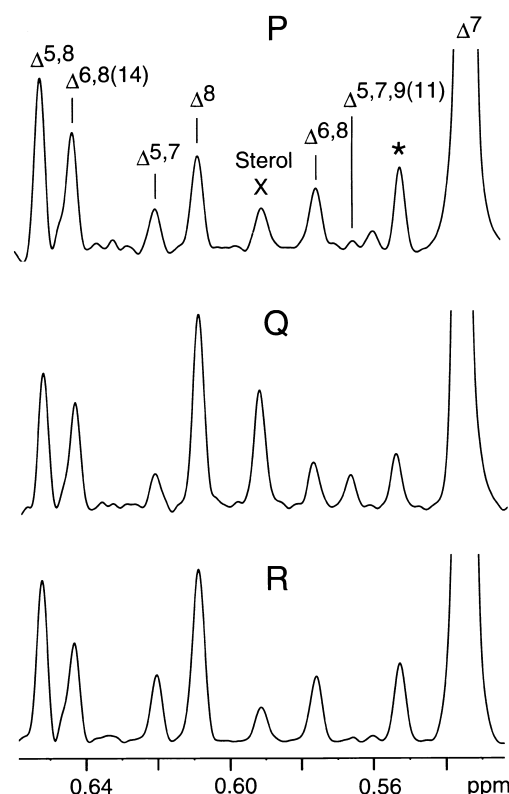

Fig. 7. ^1H NMR spectra showing trace-level unsaturated sterols in normal human blood samples P, Q, and R. The NMR samples represent the NSL fraction eluted after cholesterol on normal-phase HPLC and contain sterols eluted between the $\Delta^{8(14)}$ and $\Delta^{5,7,9(11)}$ sterols (see Fig. 2B). In samples P and R, the $\Delta^{5,7,9(11)}$ sterol was eluted in a later HPLC fraction. The 500-MHz NMR spectra were processed with slight Gaussian apodization and baseline correction. Only a small portion of the methyl region is shown (δ 0.66–0.52); comparison with spectra in Fig. 5 gives a perspective on the magnitude of vertical and horizontal expansion. The upfield ^{13}C satellite of cholesterol is marked by an asterisk.

were purified on silica gel (SS-4, 500 × 10 mm column). On the basis of ^3H analysis, fractions 10–14 were combined, mixed with authentic standards of unlabeled C_{27} sterols, and subjected to radio-Ag$^+$-HPLC (SS-3, CL-6). Resolved peaks for the Δ^5, $\Delta^{5,7}$, $\Delta^{5,8}$, and $\Delta^{5,7,9(11)}$ sterols showed a relative ratio of 73:13.5:12.6:1.2. Additional radio-Ag$^+$-HPLC (SS-4, CL-6) of the monoenes showed the Δ^5, Δ^7, $\Delta^{8(14)}$, Δ^8, and Δ^0 sterols in a 98:1.0:0.3:0.2:0.15 ratio. Similar radio-Ag$^+$-HPLC (SS-3, CL-6) of the dienes showed the $\Delta^{5,8}$, $\Delta^{5,7,9(11)}$, $\Delta^{6,8(14)}$, and $\Delta^{6,8}$ sterols in a ratio of 88:9.4:1.5:1.3 (the $\Delta^{5,7}$ sterol was excluded from the quantitation). On the basis of a combination of these results and quantitations by GC-MS and NMR, the concentrations of sterols in sample G are given in Table 2. Similar data in Table 2 for samples C–F are based on ^3H, GC, and NMR quantitation, described in (39).

C_{27} sterols observed in normal blood samples P–R

Normal blood samples P–R were processed by procedure 3. Detailed results are presented for sample Q. The NSL from 12.14 g of plasma showed by GC-MS a single component corresponding to cholesterol (TMS ether), and GC quantification against added 5α-cholestane showed a total sterol level of 27.6 mg (27.5 mg, 69 μg, and 58 μg of Δ^5, Δ^0, and Δ^7 sterols). ^1H NMR revealed 0.2% each of the Δ^0 and Δ^7 sterols and signals of several other trace-level sterols (Fig. 5 and **Fig. 7**). The NSL were separated by normal-phase HPLC into zone I (fractions 51–57, mainly cholesterol) and zone II (fractions 58–70). NMR and GC-MS analysis of zone II showed Δ^5 (70%), Δ^7 (9.6%), Δ^0 (trace; also in zone I), Δ^8 (1.3%), $\Delta^{8(14)}$ (1.0%), $\Delta^{5,7}$ (0.2%), $\Delta^{5,8}$ (1.0%), $\Delta^{5,24}$ (~1.5%), $\Delta^{6,8}$ (0.3%), $\Delta^{6,8(14)}$ (0.7%), $\Delta^{5,7,9(11)}$ ($\geqslant$0.2%), sterol X (0.9%), sterol Y (0.9%), F_7-cholesterol (15.6%), but no trace of the $\Delta^{7,9(11)}$ sterol (<0.04%). Except for $\Delta^{8(14)}$ and Δ^0 sterols (also present in zone I), concentrations of the minor sterols were calculated from the amount of added F_7-cholesterol standard (102 μg).

Subfractionation of zone I on Ag$^+$-HPLC (SS-4, CL-3) gave the following components (amounts roughly estimated from the NMR sensitivity): fractions 7–20 (2 μg), 3:1 mixture of Δ^0 and $\Delta^{8(14)}$ sterols; fractions 26–30, Δ^5 sterol; fractions 34–37 (6 μg total), 4:3:3 mixture of Δ^5, $\Delta^{5,24}$, and isofucosterol. Subfractionation of zone II on Ag$^+$-HPLC (SS-1, CL-5) gave the following sterols: fraction 8 (0.3 μg), Δ^0 sterol; fraction 9 (10 μg), 84:12:7 mixture of Δ^7, Δ^8, and $\Delta^{8(14)}$ sterols; fractions 10–12 (50 μg), Δ^5 sterol; fractions 13 and 14 (20 μg), 95:5 mixture of F_7-

cholesterol and $\Delta^{5,24}$ sterol; fractions 17 and 18 (0.5 μg), sterol X;[6] fractions 20 and 21 (1 μg), 6:5:2 mixture of sterol Y,[6] $\Delta^{6,8(14)}$, and $\Delta^{6,8}$ sterols; fractions 23–25 (2 μg), $\Delta^{5,8}$ sterol; fractions 38 and 39 (0.5 μg), sterol Z.[6] Samples P and R showed similar amounts of these minor sterols (Fig. 7), which were characterized by GC-MS and ^{1}H NMR [described in detail in ref. (39)]. In addition, the $\Delta^{7,24}$ sterol was observed by GC-MS in samples P and R, and the $\Delta^{5,8(14)}$ sterol was observed as a minor component in a subfraction of sample R. The distribution of sterols in the three normal plasma samples is summarized in Table 2.

Other analyses of C[27] sterols (samples H–O and S–U)

SLOS whole blood sample H, SLOS plasma samples I–O, and normal plasma samples S–U were saponified to provide the NSL, which were analyzed for sterol content by ^{1}H NMR (procedure 4). Although several trace-level sterol components could not be quantitated accurately because of signal overlap at 500 MHz, this simple analysis provided reliable data for most sterol species of interest (Fig. 5). One sample (H) from a severely affected SLOS subject, who was fed a plant lipid emulsion intravenously, also contained sitosterol (55 μg/ml), stigmasterol (14 μg/ml), campesterol (14 μg/ml), dihydrobrassicasterol (11 μg/ml), and unidentified components (10–50 μg/ml; NMR singlets at δ 1.109, 0.983, 0.918, 0.706); other blood samples contained much less (or none) of these substances.

NMR analysis of Folch extracts: relative amounts of esters and free sterols

Crude Folch extracts of samples I–L were analyzed by ^{1}H NMR. Despite the presence of large amounts of triacylglyerols and other lipids, the methyl region of the NMR spectrum (δ 0.5–1.2) contained mainly sterol resonances, and the δ 0.5–0.84 region was virtually free of nonsterol interference. The H-18 signals of the Δ^5, $\Delta^{5,7}$, and $\Delta^{5,8}$ sterols were broadened owing to the presence of both free sterol and ester species in the Folch mixture, the H-18 ester signals being shifted 0.001–0.003 ppm upfield (31). Relative amounts of free sterol and ester could be estimated by peak deconvolution or from heights of resolution-enhanced peaks. Similar estimates were obtained by comparing intensities of the well-separated H-19 ester and free sterol signals, which, however, were overlapped with methine multiplets. We found ~75% of the cholesterol to be esterified in both SLOS and normal blood samples. The $\Delta^{5,8}$ sterol was ~70% esterified, but the $\Delta^{5,7}$ sterol was only ~50% esterified. These NMR measurements showing differences in extent of esterification among the SLOS sterols are compatible with data obtained by comparing sterol levels in NSL and Folch extracts (10).

### Unsaturated sterols not observed in normal or SLOS blood samples

3β-Hydroxysterols with unsaturation at the following positions were not detected in any normal or SLOS plasma or erythrocyte samples: Δ^4 (<30 μg/ml), Δ^6 (<2 μg/ml), Δ^{14} (<2 μg/ml), $\Delta^{4,6}$ (<1 μg/ml), $\Delta^{7,14}$ (<1 μg/ml), $\Delta^{7,9(11)}$ (<1 μg/ml), $\Delta^{8,14}$ (<3 μg/ml), $\Delta^{8,24}$ (<2 μg/ml), $\Delta^{6,8,14}$ (<3 μg/ml), and 19-nor-$\Delta^{5,7,9}$ sterol (<0.5 μg/ml). The stated detection limits were derived from representative ^{1}H NMR spectra of the NSL. Most of the undetected sterols are not highly labile and would not be expected to decompose during saponification or subsequent processing. However, some of these sterols are intermediates of cholesterol synthesis and are likely present at trace levels, particularly in liver. Difficulties in resolving distinctive NMR signals of minor components having a Δ^{24} bond contributed to our inability to detect the $\Delta^{5,7,24}$ and $\Delta^{5,8,24}$ sterols, which are likely minor constituents of SLOS blood. Nevertheless, the lack of observable NMR signals in NSL material at δ 1.683 and 1.602 (H-26 and H-27 of Δ^{24} sterols) indicates that Δ^{24} sterols in aggregate compose much less than 1% of total sterols in SLOS blood.

Because of the considerable attention given to the 19-nor-$\Delta^{5,7,9}$ sterol in the SLOS literature (9, 18–21, 25, 26, 30, 44), we prepared authentic samples of the nortriene and its acetate derivative and determined their chromatographic and spectral properties. Using this information, we examined SLOS samples for the presence of the nortriene by normal-phase HPLC, Ag^{+}-HPLC, GC, GC-MS, and NMR. HPLC (Fig. 6) and NMR analyses indicated no trace of the nortriene in SLOS or normal blood. Particularly sensitive were NMR analyses based on the absence of any NMR signal at δ 0.588, which falls in a largely unobscured spectral region. As expected (30), the nortriene was observed by GC-MS in fractions containing the $\Delta^{5,8}$ sterol, but NMR analysis of the same fractions indicated complete absence of the nortriene. Moreover, no nortriene was observed by GC-MS or NMR in HPLC fractions corresponding to the chromatographic mobility of the nortriene.

DISCUSSION

This study describes the rigorous analysis of C[27] sterols present in blood from 6 normal subjects and 13 SLOS patients, comprising 1 severe case and 12 mild to moderate cases. The key results are summarized in Table 2 and **Fig. 8**. As expected (1–5, 8), the $\Delta^{5,7}$ and $\Delta^{5,8}$ sterol levels were markedly elevated in SLOS relative to normal subjects. The elevation for the $\Delta^{5,7}$ sterol ranged from roughly 100-

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[6] Characterization of sterol X: ^{1}H NMR (tentative), δ 5.26 (m), 0.945 (s), 0.903 (d, 6.5 Hz), 0.867 (d, 6.6 Hz), 0.863 (d, 6.6 Hz), 0.592 (s); GC-MS of TMS ether (t_R 1.50 and 0.98 relative to 5α-cholestane and Δ^5 TMS ether), m/z 458 (100), 443 (23), 368 (11), 353 (76), 274 (21), 260 (12), 255 (15), 213 (24), 105 (28); possibly the $\Delta^{9(11)}$ sterol. Characterization of sterol Y: ^{1}H NMR (tentative), δ 5.813 (ddt, 6.1, ~2, ~1.5 Hz), 5.773 (ddd, 6.0, 3.2, 1.4 Hz), 5.381 (dt, 5.2, 2 Hz), 1.030 (s), 0.914 (d, 6.6 Hz), 0.874 (d, 6.6 Hz), 0.870 (d, 6.6 Hz), 0.765 (s); GC-MS of TMS ether (t_R 1.40 and 0.94 relative to 5α-cholestane and Δ^5 TMS ether), m/z 456 (100), 441 (21), 366 (44), 351 (47), 343 (24), 327 (13), 290 (48), 253 (70), 200 (69), 159 (74), 145 (62), 129 (56); possibly the $\Delta^{5,11}$ sterol. Characterization of sterol Z: ^{1}H NMR (tentative), δ 5.405 (m), 5.182 (m), 1.023 (s), 0.930 (s), 0.928 (d, 6.2 Hz), 0.871 (d, 6.6 Hz), 0.869 (d, 6.6 Hz); GC-MS of TMS ether (t_R 1.55 and 0.99 relative to 5α-cholestane and Δ^5 TMS ether), m/z 456 (20), 441 (13), 366 (15), 351 (13), 343 (57), 327 (7), 253 (100), 211 (15), 129 (38); possibly the $\Delta^{5,14}$ sterol. Sterols containing unsaturation at $\Delta^{9(11)}$ (41), $\Delta^{5,11}$ (42), and $\Delta^{5,14}$ (43) have been described previously.
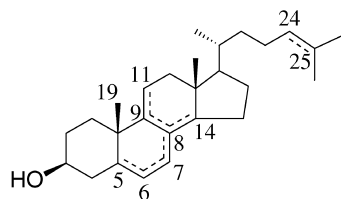
Sterols relevant to SLOS

Markedly elevated
in SLOS

$\Delta^{5,7}$

$\Delta^{5,8}$

$\Delta^{5,7,9(11)}$

Depressed in SLOS

Δ^5

Low levels in SLOS
and normal blood

Δ^0	$\Delta^{5,24}$
Δ^7	$\Delta^{5,8(14)}$
Δ^8	$\Delta^{6,8}$
$\Delta^{8(14)}$	$\Delta^{6,8(14)}$
	$\Delta^{7,24}$

Not present in blood

19-nor-$\Delta^{5,7,9}$

Fig. 8. Summary of sterols relevant to SLOS. Levels of the Δ^7 and $\Delta^{6,8}$ sterols appeared to be modestly elevated in SLOS relative to normal blood.

fold in mild SLOS cases to about 10,000-fold in the severely affected subject. This large variation in SLOS sterol levels stems from differences in genotype, age, diet, rate of growth, and other factors. Consistent with previous reports (7, 45), our data also indicate marked variation in the relative concentrations of the dienes, the $\Delta^{5,7}$ levels ranging from 60% to 270% of the $\Delta^{5,8}$ levels. The lowest $\Delta^{5,7}:\Delta^{5,8}$ ratios were generally found in mild cases of SLOS and in normal subjects. The $\Delta^{5,7}$ and $\Delta^{5,8}$ sterols undergo slow enzymatic interconversion (32) that apparently allows a significant buildup of the $\Delta^{5,8}$ sterol when the $\Delta^{5,7}$ sterol is produced faster than it can be metabolized. When the rate of sterol synthesis diminishes, the $\Delta^{5,8}$ sterol can be slowly metabolized via the $\Delta^{5,7}$ sterol to cholesterol. The proportions of $\Delta^{5,7}$ and $\Delta^{5,8}$ sterols may also depend on their relative rates of esterification, rates of incorporation into lipoproteins and membranes, the clearance rate of sterols from plasma (20), and the microarchitecture of the endoplasmic reticulum (e.g., proximity of the Δ^8–Δ^7 isomerase, Δ^5 desaturase, and DHCR7). Notably, the Δ^5, $\Delta^{5,7}$, and $\Delta^{5,8}$ sterols were esterified to different extents in both our results and findings of another study (10).

SLOS subjects also showed elevated levels of the $\Delta^{5,7,9(11)}$ sterol, for which we describe the first set of definitive measurements. The $\Delta^{5,7,9(11)}$ sterol concentrations in SLOS samples ranged from 0.02% to 5% of total sterols (median, 1%) and were elevated up to 1,000-fold over normal levels. The $\Delta^{5,7,9(11)}$ sterol has been reported previously in SLOS blood (19) and corresponds by GC retention time (27) to "sterol III" (8, 10) and by GC-MS to

"trienol I" (25) of early reports in which the $\Delta^{5,8}$ and $\Delta^{5,7,9(11)}$ sterols were resolved on polyethylene glycol GC columns. More recently, SLOS sterols have been analyzed with rugged nonpolar GC columns, on which the $\Delta^{5,7,9(11)}$ sterol is poorly resolved from the Δ^5 and $\Delta^{5,8}$ sterols (27). On the basis of our identification of "sterol III" as the $\Delta^{5,7,9(11)}$ sterol, reported (8) proportions of the $\Delta^{5,7}$, $\Delta^{5,8}$, and $\Delta^{5,7,9(11)}$ sterols appear to be relatively constant in a variety of tissues.

Two known (46) minor constituents of blood are the Δ^7 and Δ^0 sterols, which we observed in normal plasma at concentrations an order of magnitude higher than those of the $\Delta^{5,7}$, $\Delta^{5,8}$, and $\Delta^{5,7,9(11)}$ sterols. In normal and SLOS blood samples, levels of the Δ^7 and Δ^0 sterols spanned similar ranges (0.05%–0.6% and 0.1%–0.4% of total sterols, respectively) but were not correlated with each other. Relative to normal samples, SLOS blood contained slightly depressed levels of the Δ^0 sterol, a cholesterol metabolite whose levels are negatively related to cholesterol synthesis and bile production but positively related to sterol absorption (47). The Δ^7 levels were perhaps modestly elevated, at least in more severe SLOS cases. Mildly elevated levels of the Δ^7 sterol have been noted previously in SLOS plasma (14, 16) and amniotic fluid (14, 17). This elevation is not attributable to the known (48–53) correlation of Δ^7 levels with the rate of sterol synthesis, which is depressed in SLOS subjects (20, 45). Because of interrelationships among Δ^7 levels, sterol synthesis, and rate of growth, Δ^7 levels in SLOS children are quite variable and will likely defy simple explanations.

Both normal and SLOS samples also contained trace levels of several cholesterol precursors and aberrant sterols, including the Δ^8, $\Delta^{8(14)}$, $\Delta^{5,24}$, $\Delta^{6,8}$, $\Delta^{6,8(14)}$, and $\Delta^{7,24}$ sterols, and the $\Delta^{5,8(14)}$ sterol was found in SLOS plasma. Relative to normal subjects, SLOS patients showed a modest elevation in levels of the $\Delta^{6,8}$ and possibly other sterols. Some of these sterols have been reported previously in SLOS samples (1, 8, 11, 16, 17, 26), but, except for the $\Delta^{5,24}$ sterol (8), the limited analyses carried out would not likely permit the detection or identification of these trace-level sterols in the presence of vastly higher concentrations of Δ^5, $\Delta^{5,7}$, $\Delta^{5,8}$, and $\Delta^{5,7,9(11)}$ sterols. More rigorous analyses of minor sterols have been described for normal human blood, which is known to contain Δ^0, Δ^7 (5.7 μM), Δ^8, $\Delta^{5,7}$ (0.14 μM), $\Delta^{5,8}$, $\Delta^{6,8}$ (0.18 μM), and $\Delta^{5,24}$ sterols (48–54). Most of these sterols were partially isolated by multiple chromatographic purifications and identified with the aid of spectral methods and authentic standards, but no single study incorporates a comprehensive determination of normal blood sterols. Our data are compatible with these reported analyses and indicate the presence of several C_{27} sterols previously undocumented in normal human blood, including the $\Delta^{8(14)}$, $\Delta^{6,8(14)}$, $\Delta^{5,7,9(11)}$, and unidentified sterols. In addition, some samples contained trace levels of sitosterol, stigmasterol, isofucosterol, and other sterols of apparent dietary origin. Blood from a severely affected SLOS subject contained substantially elevated levels of sitosterol and other plant sterols from intravenous feeding of a plant lipid emulsion. Micromolar

levels of numerous unidentified components in this and other samples suggest a greater complexity of aberrant pathways in sterol synthesis than recognized previously (32).

Our analyses of SLOS and normal blood samples indicated no trace of the 19-nor-$\Delta^{5,7,9}$ sterol, which has been reported by multiple research groups (9, 18–21, 25, 26) in amounts up to 10% of total sterols. Apart from an unconfirmed HPLC peak assignment (21), the nortriene has been observed in SLOS samples only by GC-MS of preparations containing the $\Delta^{5,8}$ sterol, which partially decomposes to the nortriene under the thermal conditions found in most GC injectors (30). Despite this evidence that the nortriene is a GC artifact, reports (20, 21, 26) persist of its presence and possible deleterious effects in SLOS, and its origin in SLOS samples continues to remain controversial (44). In both normal and SLOS samples, we routinely observed the 19-nor-$\Delta^{5,7,9}$ sterol during GC-MS analysis of fractions containing the $\Delta^{5,8}$ sterol, but ^{1}H NMR spectra of the same fractions indicated complete absence of the nortriene. Samples showing the 19-nor-$\Delta^{5,7,9}$ sterol by GC-MS corresponded to normal-phase HPLC or Ag$^+$-HPLC fractions that should not contain the nortriene, based on its known (28) chromatographic mobility (Fig. 2). Notably, our nortriene detection limit of 0.05% of total sterols is vastly lower than the reported levels of the nortriene (9, 18–21, 25, 26). These combined observations constitute compelling evidence that the nortriene is not present in SLOS or normal blood.

The sensitive and selective methods used herein also indicated that many other sterols are either absent in plasma or present at low concentrations. The Δ^6, Δ^{14}, $\Delta^{4,6}$, $\Delta^{7,14}$, $\Delta^{7,9(11)}$, $\Delta^{8,14}$, and $\Delta^{8,24}$ sterols, which have NMR signals in uncluttered portions of the upfield methyl region, could be excluded at relatively low detection limits (<3 µg/ml). These undetected sterols include several cholesterol precursors, which are almost certainly present at trace levels in plasma and await quantitation by more sensitive methods. Our data also pointed to the absence of 5,8α-epidioxy-5α-cholest-6-en-3β-ol, which has been suggested to be present in SLOS blood (44). Although the epiperoxide was not detected in the polar chromatographic fractions, its potential lability during processing was not excluded.

Apart from exogenous plant sterols, the noncholesterol sterols observed in SLOS and normal blood can be rationalized as biosynthetic intermediates or products of minor aberrant pathways. The Δ^8, Δ^7, $\Delta^{5,7}$, and $\Delta^{5,24}$ sterols are known cholesterol precursors (55). Many, but not all (56), experimental results suggest that the $\Delta^{8(14)}$ sterol is an intermediate of cholesterol synthesis (55), and our detection of the $\Delta^{8(14)}$, $\Delta^{5,8(14)}$, and $\Delta^{6,8(14)}$ sterols supports this proposal. In a study of aberrant pathways of cholesterol biosynthesis in rat liver preparations (32), a small portion of the Δ^8 sterol was desaturated to $\Delta^{5,8}$ and $\Delta^{6,8}$ sterols. The $\Delta^{5,8}$ sterol was metabolized slowly to cholesterol via the $\Delta^{5,7}$ sterol, and the $\Delta^{6,8}$ sterol was rapidly converted to cholesterol under aerobic conditions, a process that cannot involve the slow-metabolizing $\Delta^{5,8}$ sterol. Rat

liver incubations of the $\Delta^{6,8}$ sterol with a DHCR7 inhibitor (1 µM AY-9944) produced only the $\Delta^{5,7,9(11)}$ sterol (32). Thus, a significant metabolic flux through the $\Delta^{6,8}$ sterol could account for the elevated $\Delta^{5,7,9(11)}$ levels in SLOS. Alternately, the triene might arise mainly from desaturation of the $\Delta^{5,7}$ sterol, other minor aberrant pathways (32), or a hydroperoxide intermediate (19).

Our analytical approach for elucidating the metabolic complexity of SLOS is much more elaborate than the simple techniques suitable for large-scale screening and the GC-MS methods used clinically for the biochemical diagnosis of SLOS (8, 10, 12, 14–17). Although GC-MS analyses produce the 19-nortriene artifact and do not resolve the $\Delta^{5,7,9(11)}$ sterol on nonpolar columns (Fig. 2), this methodology generally distinguishes normal from SLOS subjects and furnishes a measure of the severity of the disorder. Unlike normal-phase and reversed-phase HPLC, which can provide better separations of the major SLOS sterols (19, 28), GC-MS quantitation of C$_{27}$ sterols rarely suffers from interference by nonsteroidal contaminants from the biological matrix. An attractive alternative to GC-MS is ^{1}H NMR, which features superior resolution of the major SLOS sterols without artifact formation. Unlike GC retention times, NMR chemical shifts are highly reproducible (31) and require no calibration with sterol standards. Moreover, NMR analyses can be done directly on the Folch extract, thus furnishing a rapid and sensitive method for biochemical diagnosis.

In contrast to these simple diagnostic methods, our manuscript is directed primarily toward a comprehensive analysis of SLOS sterols. Our results indicate that no single analytical technique provides an adequate picture of the complex mixture of sterols commonly observed in samples of biological origin. Major SLOS sterols can be resolved on normal-phase HPLC (28), reversed-phase HPLC (19, 28), Ag$^+$-HPLC (28, 29), and GC on polyethylene glycol columns (10, 27), but the chromatographic peaks of the major sterols conceal numerous minor components. Assigning the structure of these minor sterols with any measure of confidence requires combinations of multiple chromatographic and spectral techniques in conjunction with the use of authentic standards to calibrate GC-MS and HPLC data.

Beginning with a complex mixture of NSL, we isolated sterols by a variety of procedures, with analyses conducted at each stage of purification. Except for some loss of the $\Delta^{5,7}$ sterol during acetylation, the distribution of major sterols was essentially unchanged, whether the analysis was done on the crude Folch extract, the crude NSL fraction, free sterols isolated by normal-phase HPLC or Ag$^+$-HPLC, or acetates isolated by alumina-AgNO$_3$ MPLC. Sterol mixtures were resolved into individual components on multiple chromatographic systems chosen for their diversity of selectivities and their ability to resolve numerous unsaturated sterols (Fig. 2). The most effective strategy involved resolution of sterols into major groups on normal-phase HPLC followed by separations on Ag$^+$-HPLC and GC. After isolation, the major sterols were identified unequivocally by multiple methods, including GC, GC-MS, Ag$^+$-

HPLC, normal-phase HPLC, and ^{1}H and ^{13}C NMR. Most minor sterols were only partially purified and were identified by GC-MS, Ag$^+$-HPLC, and a limited set of ^{1}H NMR signals, data that were nevertheless often definitive. Quantitation of the sterols by GC (flame ionization detection), GC-MS (total ion current), ^{1}H NMR (signal intensity), and scintillation counting (radioactivity of [^{3}H]acetates) gave similar results for the major sterols. The general agreement of the data from this diverse set of analytical methods supports the overall validity of the results.

In summary, an eclectic combination of chromatographic and spectral methods was used to isolate, identify, and quantitate sterols in blood of SLOS and normal subjects. Our results show that only three noncholesterol sterols accumulate significantly in SLOS: the $\Delta^{5,7}$, $\Delta^{5,8}$, and $\Delta^{5,7,9(11)}$ sterols. Blood of both normal and SLOS subjects also contained Δ^0 and Δ^7 sterols and traces of numerous other sterols, including biosynthetic intermediates and products of minor aberrant pathways that may attain significance under abnormal metabolic conditions. Our results confirm that the 19-nor-$\Delta^{5,7,9}$ sterol is a GC artifact and not a component of normal or SLOS blood. The overall findings described herein have important implications for the understanding of SLOS and provide a methodological model for analyzing sterols accumulating in other disorders of cholesterol biosynthesis (1, 5, 6, 57). 🔒

REFERENCES

1. Kelley, R. I., and R. C. M. Hennekam. 2000. The Smith-Lemli-Opitz syndrome. *J. Med. Genet.* **37:** 321–335.
2. Porter, F. D. 2000. RSH/Smith-Lemli-Opitz syndrome: a multiple congenital anomaly/mental retardation syndrome due to an inborn error of cholesterol biosynthesis. *Mol. Genet. Metab.* **71:** 163–174.
3. Battaile, K. P., and R. D. Steiner. 2000. Smith-Lemli-Opitz syndrome: the first malformation syndrome associated with defective cholesterol synthesis. *Mol. Genet. Metab.* **71:** 154–162.
4. Waterham, H. R., and R. Wanders. 2000. Biochemical and genetic aspects of 7-dehydrocholesterol reductase and Smith-Lemli-Opitz syndrome. *Biochim. Biophys. Acta.* **1529:** 340–356.
5. Opitz, J. M. 1999. RSH (so-called Smith-Lemli-Opitz) syndrome. *Curr. Opin. Pediatr.* **11:** 353–362.
6. Moebius, F. F., B. U. Fitzky, and H. Glossmann. 2000. Genetic defects in postsqualene cholesterol biosynthesis. *Trends Endocrinol. Metab.* **11:** 106–114.
7. Yu, H., M. H. Lee, L. Starck, E. R. Elias, M. Irons, G. Salen, S. B. Patel, and G. S. Tint. 2000. Spectrum of Δ^7-dehydrocholesterol reductase mutations in patients with the Smith-Lemli-Opitz (RSH) syndrome. *Hum. Mol. Genet.* **9:** 1385–1391.
8. Tint, G. S., M. Seller, R. Hughes-Benzie, A. K. Batta, S. Shefer, D. Genest, M. Irons, E. Elias, and G. Salen. 1995. Markedly increased tissue concentrations of 7-dehydrocholesterol combined with low levels of cholesterol are characteristic of the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **36:** 89–95.
9. Salen, G., S. Shefer, A. K. Batta, G. S. Tint, G. Xu, A. Honda, M. Irons, and E. R. Elias. 1996. Abnormal cholesterol biosynthesis in the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **37:** 1169–1180.
10. Tint, G. S., M. Irons, M. R. Elias, A. K. Batta, R. Frieden, T. S. Chen, and G. Salen. 1994. Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. *N. Engl. J. Med.* **330:** 107–113.
11. Batta, A. K., G. S. Tint, G. Salen, S. Shefer, M. Irons, and E. R. Elias. 1994. Identification of 7-dehydrocholesterol and related sterols in patients with Smith-Lemli-Opitz syndrome. *Am. J. Med. Genet.* **50:** 334.
12. Tint, G. S., G. Salen, A. K. Batta, S. Shefer, M. Irons, E. R. Elias, D. N. Abuelo, V. P. Johnson, M. Lambert, R. Lutz, C. Shanen, C. A. Morris, G. Hoganson, and R. Hughes-Benzie. 1995. Correlation of severity and outcome with plasma sterol levels in variants of the Smith-Lemli-Opitz syndrome. *J. Pediatr.* **127:** 82–87.
13. Batta, A. K., G. S. Tint, S. Shefer, D. Abuelo, and G. Salen. 1995. Identification of 8-dehydrocholesterol (cholesta-5,8-dien-3β-ol) in patients with Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **36:** 705–713.
14. Cunniff, C., L. E. Kratz, A. Moser, M. R. Natowicz, and R. I. Kelley. 1997. Clinical and biochemical spectrum of patients with RSH/Smith-Lemli-Opitz syndrome and abnormal cholesterol metabolism. *Am. J. Med. Genet.* **68:** 263–269.
15. Kelley, R. I. 1994. Quantification of neutral sterols by gas chromatography/mass fragmentography for diagnosis of inborn errors of sterol metabolism. *Am. J. Med. Genet.* **50:** 335.
16. Kelley, R. I. 1995. Diagnosis of Smith-Lemli-Opitz syndrome by gas chromatography/mass spectrometry of 7-dehydrocholesterol in plasma, amniotic fluid and cultured skin fibroblasts. *Clin. Chim. Acta.* **236:** 45–58.
17. Rossiter, J. P., K. J. Hofman, and R. I. Kelley. 1995. Smith-Lemli-Opitz syndrome: prenatal diagnosis by quantification of cholesterol precursors in amniotic fluid. *Am. J. Med. Genet.* **56:** 272–275.
18. Batta, A. K., G. Salen, G. S. Tint, and S. Shefer. 1995. Identification of 19-nor-5,7,9(10)-cholestatrien-3β-ol in patients with Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **36:** 2413–2418.
19. De Fabiani, E., D. Caruso, M. Cavalieri, M. G. Kienle, and G. Galli. 1996. Cholesta-5,7,9(11)-trien-3β-ol found in plasma of patients with Smith-Lemli-Opitz syndrome indicates formation of sterol hydroperoxide. *J. Lipid Res.* **37:** 2280–2287.
20. Steiner, R. D., L. M. Linck, D. P. Flavell, D. S. Lin, and W. E. Connor. 2000. Sterol balance in the Smith-Lemli-Opitz syndrome: reduction in whole body cholesterol synthesis and normal bile acid production. *J. Lipid Res.* **41:** 1437–1447.
21. Ness, G. C., D. Lopez, O. Borrego, and E. Gilbert-Barness. 1997. Increased expression of low-density lipoprotein receptors in a Smith-Lemli-Opitz infant with elevated bilirubin levels. *Am. J. Med. Genet.* **68:** 294–299.
22. Natowicz, M. R., and J. E. Evans. 1994. Abnormal bile acids in the Smith-Lemli-Opitz syndrome. *Am. J. Med. Genet.* **50:** 364–367.
23. Shackleton, C. H. L., E. Roitman, L. E. Kratz, and R. I. Kelley. 1999. Midgestational maternal urine steroid markers of fetal Smith-Lemli-Opitz (SLO) syndrome (7-dehydrocholesterol 7-reductase deficiency). *Steroids.* **64:** 446–452.
24. Shackleton, C. H. L., E. Roitman, and R. I. Kelley. 1999. Neonatal urinary steroids in Smith-Lemli-Opitz syndrome associated with 7-dehydrocholesterol reductase deficiency. *Steroids.* **64:** 481–490.
25. Wolf, C., F. Chevy, J. Pham, M. Kolf-Clauw, D. Citadelle, N. Mulliez, and C. Roux. 1996. Changes in serum sterols of rats treated with 7-dehydrocholesterol-Δ^7-reductase inhibitors: comparison to levels in humans with Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **37:** 1325–1333.
26. Llirbat, B., C. Wolf, F. Chevy, D. Citadelle, G. Bereziat, and C. Roux. 1997. Normal and inhibited cholesterol synthesis in the cultured rat embryo. *J. Lipid Res.* **38:** 22–34.
27. Gerst, N., B. Ruan, J. Pang, W. K. Wilson, and G. J. Schroepfer, Jr. 1997. An updated look at the analysis of unsaturated C$_{27}$ sterols by gas chromatography and mass spectrometry. *J. Lipid Res.* **38:** 1685–1701.
28. Ruan, B., N. Gerst, G. T. Emmons, J. Shey, and G. J. Schroepfer, Jr. 1997. Sterol synthesis. A timely look at the capabilities of conventional and silver ion high performance liquid chromatography for the separation of C$_{27}$ sterols related to cholesterol biosynthesis. *J. Lipid Res.* **38:** 2615–2626.
29. Ruan, B., J. Shey, N. Gerst, W. K. Wilson, and G. J. Schroepfer, Jr. 1996. Silver ion high pressure liquid chromatography provides unprecedented separation of sterols: application to the enzymatic formation of cholesta-5,8-dien-3β-ol. *Proc. Natl. Acad. Sci. USA.* **93:** 11603–11608.

30. Ruan, B., J. Pang, W. K. Wilson, and G. J. Schroepfer, Jr. 1996. Concerning the thermolability of cholesta-5,8-dien-3β-ol, a sterol that accumulates in blood and tissues in a human genetic developmental disorder. *Bioorg. Med. Chem. Lett.* **6:** 2421–2424.

31. Wilson, W. K., R. M. Sumpter, J. J. Warren, P. S. Rogers, B. Ruan, and G. J. Schroepfer, Jr. 1996. Analysis of unsaturated C_{27} sterols by nuclear magnetic resonance spectroscopy. *J. Lipid Res.* **37:** 1529–1555.

32. Ruan, B., W. K. Wilson, J. Tsai, and G. J. Schroepfer, Jr. 2000. Aberrant pathways in the late stages of cholesterol biosynthesis in the rat. Origin and metabolic fate of unsaturated sterols relevant to the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **41:** 1772–1782.

33. Swaminathan, S., W. K. Wilson, F. D. Pinkerton, N. Gerst, M. Ramser, and G. J. Schroepfer, Jr. 1993. Inhibitors of sterol synthesis. Chemical synthesis of 3β-hydroxy-25,26,26,26,27,27,27-heptafluoro-5α-cholest-8(14)-en-15-one and 25,26,26,26,27,27,27-heptafluoro-cholesterol and their effects on 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured mammalian cells. *J. Lipid Res.* **34:** 1805–1823.

34. Pascal, R. A., C. L. Farris, and G. J. Schroepfer, Jr. 1980. Sterol synthesis. Medium pressure chromatography of C_{27} sterol precursors of cholesterol on alumina-silver nitrate columns. *Anal. Biochem.* **101:** 15–22.

35. Frantz, I. D., Jr., A. G. Davidson, E. Dulit, and M. L. Mobberley. 1957. The state of esterification of the sterols in rat skin. *J. Biol. Chem.* **226:** 139–144.

36. Van der Gen, A., J. Lakeman, M. A. M. P. Gras, and H. O. Huisman. 1964. Addition to steroid polyenes. I. Reaction of 7- dehydrocholesteryl acetate with diethyl diazodicarboxylate. *Tetrahedron.* **20:** 2521–2530.

37. Moriarty, R. M., and H. E. Paaren. 1981. Synthesis of C_{19}-functionalized 7-dehydrocholesteryl derivatives. Photochemical transformation to vitamin D_3 analogues. *J. Org. Chem.* **46:** 970–977.

38. Kudo, K., G. T. Emmons, E. W. Casserly, D. P. Via, L. C. Smith, J. St. Pyrek, and G. J. Schroepfer, Jr. 1989. Inhibitors of sterol synthesis. Chromatography of acetate derivatives of oxygenated sterols. *J. Lipid Res.* **30:** 1097–1111.

39. Ruan, B. 2000. Chemical, Biochemical, and Bioanalytical Studies of Sterols and Isoprenoids. Smith-Lemli-Opitz Syndrome, Langer-Giedion Syndrome, Activation of Meiosis, Nuclear Orphan Receptor LXRα and Cytochrome P450s . PhD Dissertation. Rice University, Houston, TX [*Diss. Abs. Int.-B.* **61–04**: 1720].

40. Linck, L. M., D. S. Lin, D. Flavell, W. E. Connor, and R. D. Steiner. 2000. Cholesterol supplementation with egg yolk increases plasma cholesterol and decreases plasma 7-dehydrocholesterol in Smith-Lemli-Opitz syndrome. *Am. J. Med. Genet.* **93:** 360–365.

41. Morisaki, M., and N. Ikekawa. 1984. High pressure liquid chromatography of sterol benzoates. *Chem. Pharm. Bull.* **32:** 865–871.

42. Alam, M. S., N. Chopra, M. Ali, M. Niwa, and T. Sakae. 1994. Ursane and sterol derivatives from *Pleuchea lanceolata. Phytochemistry.* **37:** 521–524.

43. Withers, N. W., M. C. Kokke, W. Fenical, and C. Djerassi. 1982. Sterol patterns of cultured zooxanthellae isolated from marine invertebrates: synthesis of gorgosterol and 23-desmethylgorgosterol by aposymbiotic algae. *Proc. Natl. Acad. Sci. USA.* **79:** 3764–3768.

44. Gaoua, W., F. Chevy, C. Roux, and C. Wolf. 1999. Oxidized derivatives of 7-dehydrocholesterol induce growth retardation in cultured rat embryos: a model for antenatal growth retardation in the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **40:** 456–463.

45. Honda, M., G. S. Tint, A. Honda, G. Salen, S. Shefer, A. K. Batta, Y. Matsuzaki, and N. Tanaka. 2000. Regulation of cholesterol biosynthetic pathway in patients with the Smith-Lemli-Opitz syndrome. *J. Inherit. Metab. Dis.* **23:** 464–474.

46. Nikkila, K., S. Riikonen, M. Lindfors, and T. A. Miettinen. 1996. Serum squalene and noncholesterol sterols before and after delivery in normal and cholestatic pregnancy. *J. Lipid Res.* **37:** 2687–2695.

47. Miettinen, T. A., R. S. Tilvis, and Y. A. Kesaniemi. 1989. Serum cholestanol and plant sterol levels in relation to cholesterol metabolism in middle-aged men. *Metabolism.* **38:** 136–140.

48. Kempen, H. J., J. F. Glatz, J. A. Gevers Leuven, H. A. van der Voort, and M. B. Katan. 1988. Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans. *J. Lipid Res.* **29:** 1149–1155.

49. Miettinen, T. A. 1985. Cholesterol precursors and their diurnal rhythm in lipoproteins of patients with jejuno-ileal bypass and ileal dysfunction. *Metabolism.* **34:** 425–430.

50. Björkhem, I., T. Miettinen, E. Reihner, S. Ewerth, B. Angelin, and K. Einarsson. 1987. Correlation between serum levels of some cholesterol precursors and activity of HMG-CoA reductase in human liver. *J. Lipid Res.* **28:** 1137–1143.

51. Duane, W. C. 1995. Serum lathosterol levels in human subjects reflect changes in whole body cholesterol synthesis induced by lovastatin but not dietary cholesterol. *J. Lipid Res.* **36:** 343–348.

52. Axelson, M., B. Angelin, C. G. Hillebrant, E. Reihner, and C. Einarsson. 1998. The level of 7-dehydrocholesterol in plasma reflects the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the human liver. *Biochim. Biophys. Acta.* **1394:** 153–157.

53. Matthan, N. R., M. Raeini-Sarjaz, A. H. Lichtenstein, L. M. Ausman, and P. J. Jones. 2000. Deuterium uptake and plasma cholesterol precursor levels correspond as methods for measurement of endogenous cholesterol synthesis in hypercholesterolemic women. *Lipids.* **35:** 1037–1044.

54. Axelson, M. 1991. Occurrence of isomeric dehydrocholesterols in human plasma. *J. Lipid Res.* **32:** 1441–1448.

55. Schroepfer, G. J., Jr. 1982. Sterol biosynthesis. *Annu. Rev. Biochem.* **51:** 555–585.

56. Paik, Y. K., J. T. Billheimer, R. L. Magolda, and J. L. Gaylor. 1986. Microsomal enzymes of cholesterol biosynthesis from lanosterol: solubilization and purification of sterol 8-isomerase. *J. Biol. Chem.* **261:** 6470–6477.

57. Herman, G. E. 2000. X-linked dominant disorders of cholesterol biosynthesis in man and mouse. *Biochim. Biophys. Acta.* **1529:** 357–373.