

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

Benfang Ruan,[†] James Tsai,^{*} William K. Wilson,^{1,*} and George J. Schroepfer, Jr.^{2,*}

Department of Biochemistry and Cell Biology^{*} and of Department of Chemistry,[†] Rice University, Houston, TX 77005

Abstract Minor aberrant pathways of cholesterol biosynthesis normally produce only trace levels of abnormal sterol metabolites but may assume major importance when an essential biosynthetic step is blocked. Cholesta-5,8-dien-3 β -ol, its $\Delta^{5,7}$ isomer, and other noncholesterol sterols accumulate in subjects with the Smith-Lemli-Opitz syndrome (SLOS), a severe developmental disorder caused by a defective Δ^7 sterol reductase gene. We have explored the formation and metabolism of unsaturated sterols relevant to SLOS by incubating tritium-labeled $\Delta^{5,8}$, $\Delta^{6,8}$, $\Delta^{6,8(14)}$, $\Delta^{5,8(14)}$, and Δ^8 sterols with rat liver preparations. More than 60 different incubations were carried out with washed microsomes or the 10,000 g supernatant under aerobic or anaerobic conditions; some experiments included addition of cofactors, fenpropimorph (a Δ^8 - Δ^7 isomerase inhibitor), and/or AY-9944 (a Δ^7 reductase inhibitor). The tritium-labeled metabolites from each incubation were identified by silver ion high performance liquid chromatography on the basis of their coelution with unlabeled authentic standards, as free sterols and/or acetate derivatives. The $\Delta^{5,8}$ sterol was converted slowly to cholesterol via the $\Delta^{5,7}$ sterol, which also slowly isomerized back to the $\Delta^{5,8}$ sterol. The $\Delta^{6,8}$ sterol was metabolized rapidly to cholesterol by an oxygen-requiring pathway via the $\Delta^{7,9(11)}$, Δ^8 , Δ^7 , and $\Delta^{5,7}$ sterols as well as by an oxygen-independent route involving initial isomerization to the $\Delta^{5,7}$ sterol. The Δ^8 sterol was partially metabolized to $\Delta^{5,8}$, $\Delta^{6,8}$, $\Delta^{7,9(11)}$, and $\Delta^{5,7,9(11)}$ sterols when isomerization to Δ^7 was blocked. The combined results were used to formulate a scheme of normal and aberrant biosynthetic pathways that illuminate the origin and metabolic fate of abnormal sterols observed in SLOS and chondrodysplasia punctata.—Ruan, B., J. Tsai, W. K. Wilson, and G. J. Schroepfer, Jr. *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.* 2000. 41: 1772–1782.

Supplementary key words cholesta-5,8-dien-3 β -ol • Ag⁺-HPLC • microsomes • tritium-labeled substrates • Δ^8 - Δ^7 sterol isomerase • Δ^5 desaturase • Δ^7 reductase • SLOS

Cholesterol biosynthesis is a complex process encompassing a potentially large number of sterol intermediates

(1). Conversion of lanosterol to cholesterol involves saturation of the Δ^{24} bond, demethylation at C-4 and C-14, and double-bond migration to the Δ^5 position. The flexible order of these processes enables certain cells at particular stages of development to produce elevated levels of desmosterol (2, 3) and may play a role in the formation of meiosis-activating 4,4-dimethylsterols in follicular fluid and testes (4, 5). The double-bond shifts subsequent to 14 α -demethylation (1, 6, 7) consist of desaturation of the postulated $\Delta^{8(14)}$ intermediate³ to $\Delta^{8,14}$ (1, 6–8), reduction to Δ^8 (9, 10), isomerization to Δ^7 (11), desaturation to $\Delta^{5,7}$ (12), and reduction to Δ^5 (7, 13, 14). These enzymatic transformations are summarized in **Fig. 1**, which also depicts aberrant intermediates or side products that assume importance only in disease states.

A notable genetic defect leading to the accumulation of cholesterol precursors occurs in the Smith-Lemli-Opitz syndrome (SLOS) (14–22), a severe developmental disorder associated with various mutations in the Δ^7 sterol re-

Abbreviations: Ag⁺, silver ion; BHT, butylated hydroxytoluene; CDP, chondrodysplasia punctata; GC, gas chromatography; HPLC, high performance liquid chromatography; MPLC, medium pressure liquid chromatography; MS, mass spectrometry; MTBE, methyl *tert*-butyl ether; NAD⁺, β -nicotinamide adenine dinucleotide; NADP⁺, β -nicotinamide adenine dinucleotide 2'-phosphate; NMR, nuclear magnetic resonance (spectroscopy); NSL, nonsaponifiable lipids; S₁₀, 10,000 g supernatant (of rat liver); SLOS, Smith-Lemli-Opitz syndrome; TLC, thin-layer chromatography; t_R, retention time.

¹ To whom correspondence should be addressed.

² Senior author; deceased December 11, 1998.

³ Unsaturated C₂₇ 3 β -hydroxysterols are all of 5 α configuration (or Δ^4 or Δ^3) and are designated by their unsaturation as illustrated by the following examples: Δ^0 , 5 α -cholestan-3 β -ol; Δ^5 , cholest-5-en-3 β -ol (cholesterol); Δ^7 , 5 α -cholest-7-en-3 β -ol; Δ^8 , 5 α -cholest-8-en-3 β -ol; $\Delta^{8(14)}$, 5 α -cholest-8(14)-en-3 β -ol; $\Delta^{5,7}$, cholesta-5,7-dien-3 β -ol (7-dehydrocholesterol); $\Delta^{5,8}$, cholesta-5,8-dien-3 β -ol; $\Delta^{5,8(14)}$, cholesta-5,8(14)-dien-3 β -ol; $\Delta^{6,8}$, 5 α -cholesta-6,8-dien-3 β -ol; $\Delta^{6,8(14)}$, 5 α -cholesta-6,8(14)-dien-3 β -ol; $\Delta^{7,9(11)}$, 5 α -cholesta-7,9(11)-dien-3 β -ol; $\Delta^{8,14}$, 5 α -cholesta-8,14-dien-3 β -ol; $\Delta^{5,7,9(11)}$, cholesta-5,7,9(11)-trien-3 β -ol; 19-nor- $\Delta^{5,7,9}$, 19-norcholesta-5,7,9-trien-3 β -ol.

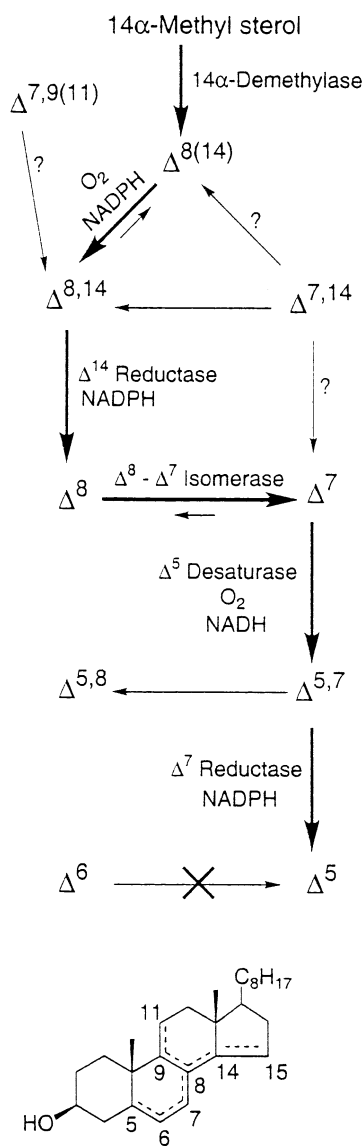


Fig. 1. Known interconversions of unsaturated sterols involved in the late stages of cholesterol biosynthesis. Individual steps in the standard mammalian pathway are denoted by thick arrows, with indications of likely or known requirements for oxygen (O₂) and cofactors (NADPH, NADH). These transformations can occur with or without a Δ^4 bond, and C-4 methyl groups may be present prior to the Δ^8 - Δ^7 isomerization. Highly speculative pathways to aberrant intermediates have been omitted. Atom numbering for designating double-bond positions is indicated on the 3β -hydroxy-C₂₇ sterol structure.

ductase gene (14, 15). Several years ago, Tint et al. (16) discovered that SLOS subjects have markedly elevated blood levels of 7-dehydrocholesterol⁴ and recognized that this was a consequence of a defect in the enzymatic conversion of the $\Delta^{5,7}$ sterol to cholesterol. Other sterols reported in the blood of SLOS subjects include the $\Delta^{5,8}$ (21), $\Delta^{6,8}$ (23, 24), $\Delta^{6,8(14)}$ (25), Δ^7 (24, 26), $\Delta^{8(14)}$ (25), and $\Delta^{5,7,9(11)}$ sterols (27). The $\Delta^{5,7}$, $\Delta^{5,8}$, and $\Delta^{6,8}$ sterols

⁴ In blood and most tissues, unsaturated sterols, including those accumulating in SLOS (18–20), are present mainly in esterified form.

were also detected at low levels in normal blood and at higher levels in patients subjected to ileal resection or cholestyramine treatment (28). Similar accumulation of the $\Delta^{5,7}$, $\Delta^{5,8}$ (29, 30), and Δ^8 (30) sterols has been reported in a rat model after treatment with AY-9944, a Δ^7 reductase inhibitor. Elevated or abnormal levels of other cholesterol precursors have also been observed in gonadal tissue (5), pregnant women (31), and subjects with cerebrotendinous xanthomatosis (32), chondrodysplasia punctata (CDP) (33, 34), and gut resection (35). Some of the deleterious effects of SLOS have been ascribed to oxygenated derivatives of noncholesterol sterols (27), and the $\Delta^{5,7}$ sterol (or an oxygenated derivative thereof) is evidently capable of regulating cholesterol biosynthesis (36). However, relatively little information (28, 37, and references therein) is available on the origin and fate of the accumulating sterols.

A simple and informative system for studying sterol biosynthetic pathways consists of incubating sterol intermediates with washed liver microsomes or the 10,000 g supernatant (S₁₀) of rat liver homogenates. Incubations of ³H- and ¹⁴C-labeled substrates with rat liver preparations under aerobic conditions have shown that the $\Delta^{8(14)}$, $\Delta^{8,14}$, $\Delta^{7,14}$, $\Delta^{7,9(11)}$, Δ^8 , Δ^7 , and $\Delta^{5,7}$ sterols but not the Δ^6 or $\Delta^{5,7,9(11)}$ sterols are convertible to cholesterol (1, 7, 38), and anaerobic incubations with washed microsomes have provided further details of the biosynthetic pathway (Fig. 1) (1, 7, 39). More recently, metabolism of noncholesterol sterols in SLOS has been studied by incubating tritium-labeled Δ^7 or $\Delta^{5,7}$ sterols with microsomes (40) or cultured fibroblasts (41) or by analyzing ¹³C-labeled precursors in cultured rat embryos (30). Because of the presence of endogenous sterol intermediates in microsomal preparations (11, 42), definitive experiments usually require isotopically labeled substrates. The difficulty in preparing labeled substrates and the lack of an effective chromatographic method for resolving unsaturated sterol isomers have been major impediments to detailed studies of aberrant pathways in the late stages of cholesterol biosynthesis.

We described the synthesis of the Δ^8 , $\Delta^{5,8}$, $\Delta^{5,8(14)}$, $\Delta^{6,8}$, and $\Delta^{6,8(14)}$ sterols with a tritium label at the 3 α position (43). These five unsaturated sterols of potential relevance to SLOS were prepared in high radiochemical purity and high specific activity. We have now completed an extensive series of incubations of the five tritiated substrates with washed rat liver microsomes or the S₁₀ fraction. More than 60 different experiments were variously carried out under aerobic or anaerobic conditions, with or without added cofactors (NADP⁺, NAD⁺), and with or without fenpropimorph (a Δ^8 - Δ^7 isomerase inhibitor) or AY-9944 (a Δ^7 reductase inhibitor). The tritium-labeled metabolites from each incubation were identified on the basis of their coelution on silver ion high performance liquid chromatography (Ag⁺-HPLC) (44, 45) with authentic standards, as free sterols and/or acetate derivatives. We have analyzed these results with the aim of understanding the origin and metabolism of unsaturated sterols of potential importance in SLOS.

General methods

Saponification was carried out in an anaerobic glove box (Vacuum Atmospheres, Hawthorne, CA). Reversed-phase HPLC was done on a 5- μ m Customsil ODS column (250 \times 4.6 mm; Custom LC, Houston, TX), using methanol as the solvent (1 ml/min). Sterols were injected in hexane (1–50 μ l), using a Rheodyne (Rohnert Park, CA) 7125 injector. Ag⁺-HPLC columns (250 \times 10 mm, 300 \times 10 mm, 300 \times 3.2 mm, or 300 \times 4.6 mm; 5 μ m, 90- \AA pore size; Alltech, Deerfield, IL) were prepared and used as described previously (44). Semipreparative Ag⁺-HPLC separations were done with 15% acetone in hexane (SS-1), and radio-Ag⁺-HPLC was done on analytical Ag⁺-HPLC columns with the following mobile phases: 9.1% acetone in hexane (SS-2) for the analysis of the free sterols, 3% acetone in hexane (SS-3) for diunsaturated sterol acetates, and 1% acetone in hexane (SS-4) for monounsaturated sterol acetates. Elution of unlabeled sterols was monitored by determining their ultraviolet absorbance at 210 nm. Elution of tritium-labeled sterols was monitored with a β -RAM radioactivity flow detector (IN/US, Tampa, FL) or by taking aliquots for analysis by scintillation spectrometry. Gas chromatography-mass spectrometry (GC-MS) and capillary GC were carried out with 60-m DB-5ms columns on trimethylsilyl or acetate derivatives as described previously (46). ¹H nuclear magnetic resonance (NMR) spectra were acquired as described previously (47) at 25°C in a 1–10 mM solution (CDCl₃) on a Bruker (Billerica, MA) AMX500 spectrometer (500 MHz for ¹H).

Materials

The preparation in high purity of unlabeled 5 α -cholestan-3 β -ol, its unsaturated analogs with double bonds at the Δ^5 , Δ^7 , Δ^8 , $\Delta^8(14)$, Δ^{14} , $\Delta^{5,7}$, $\Delta^{5,8}$, $\Delta^{5,8(14)}$, $\Delta^{5,24}$, $\Delta^{6,8}$, $\Delta^{6,8(14)}$, $\Delta^{7,9(11)}$, $\Delta^{7,14}$, $\Delta^{8,14}$, and $\Delta^{5,7,9(11)}$ positions, and the corresponding acetate derivatives has been described previously (47). [3α -³H]5 α -cholestan-8-en-3 β -ol (64 mCi/mmol), [3α -³H]cholesta-5,8-dien-3 β -ol (60 mCi/mmol), [3α -³H]cholesta-5,8(14)-dien-3 β -ol (60 mCi/mmol), [3α -³H]5 α -cholesta-6,8-dien-3 β -ol (1500 mCi/mmol), and [3 -³H]-5 α -cholesta-6,8(14)-dien-3 β -ol (1500 mCi/ μ mol) were prepared as described previously (43). These sterols showed \geq 99% radiochemical purity (or \geq 97% chemical purity for unlabeled sterols) based on their chromatographic behavior on normal phase and reversed-phase HPLC, Ag⁺-HPLC, GC-MS, and capillary GC. Silica gel (230–400 mesh) and solvents (Omnisolve grade) were obtained from EM Science (Gibbstown, NJ). Butylated hydroxytoluene (BHT; di-*tert*-butyl-4-methylphenol), glucose 6-phosphate, glucose-6-phosphate dehydrogenase, flavin mononucleotide, β -nicotinamide adenine dinucleotide (NAD⁺), and β -nicotinamide adenine dinucleotide 2'-phosphate (NADP⁺) were purchased from Sigma (St. Louis, MO). AY-9944 [*trans*-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride] was obtained from Wyeth-Ayerst Laboratories (St. Davids, PA). Fenpropimorph (*N*-[3-(*p*-*tert*-butylphenyl)-2-methylpropyl]-*cis*-2,6-dimethylmorpholine) was kindly provided by W. Rademacher of BASF (Limburgerhof, Germany).

[4-¹⁴C]cholesta-5,7-dien-3 β -ol was synthesized from [4-¹⁴C]-cholesterol as follows. After purification by medium pressure liquid chromatography (MPLC) on silica gel (100 \times 10 mm i.d. column; elution with acetone–hexane 1:9), [4-¹⁴C]cholesterol [250 μ Ci; \sim 30 mg; single component by radio-thin-layer chromatography (TLC)] was acetylated with pyridine–acetic anhydride 1:1 (1 ml) at room temperature for 24 h, and the crude product was subjected to MPLC (250 \times 10 mm i.d. column; elution with acetone–hexane 2:98). The purified Δ^5 acetate (33 mg, 222 μ Ci) was converted to [4-¹⁴C]7-dehydrocholesteryl acetate by

standard methodology (48) with dibromatin (50 mg) and 2,2'-azobisisobutyronitrile (5 mg) in dry benzene–hexane 1:1 (4 ml), followed by tetrabutylammonium bromide (15 mg) and then tetrabutylammonium fluoride (1.5 ml; 1 M solution in tetrahydrofuran). Polar and nonpolar impurities were removed from the crude [4-¹⁴C] $\Delta^{5,7}$ acetate by MPLC (250 \times 10 mm i.d. column; elution with acetone–hexane 2:98), and the major component (\sim 200 μ Ci) was subjected to Ag⁺-MPLC (43) (500 \times 10 mm i.d. column; elution with acetone–hexane 4:96; 20-ml fraction volumes). As judged by GC-MS, fractions 6–8 contained cholesteryl acetate, fractions 23–41 contained unidentified material, and fractions 65–100 contained [4-¹⁴C] $\Delta^{5,7}$ acetate of >98% purity (trace impurities being attributable to autooxidation or deacetylation). Saponification of a portion of the acetate (26 μ Ci, 3.15 mg) with 10% ethanolic KOH (1 ml) at 70°C for 2 h gave a crude product that was subjected to MPLC (300 \times 10 mm i.d. column; elution with acetone–hexane 5:95; 7-ml fraction volumes). Early-eluting components included cholesta-2,4,6-triene (46), identified by ¹H NMR in fraction 3, and unidentified trienes (fractions 7–8). The desired [4-¹⁴C] $\Delta^{5,7}$ sterol (fractions 10–13) had a specific activity of 2.5 μ Ci/mmol (determined by GC) and showed a single component (>99% purity) by radio-TLC (R_f 0.5; acetone–hexane 1:4), radio-HPLC (t_R 22.3 min; C₁₈ column; elution with water–methanol 2:98), and radio-Ag⁺-HPLC (t_R 62 min; 300 \times 3.2 mm i.d. column; elution with acetone–hexane 1:9). A brief account of this synthesis was published previously (44).

Incubation conditions

The S₁₀ fraction of rat liver homogenate (\sim 26 mg of protein per ml) and washed rat liver microsomes (7 mg of protein per ml) were prepared from female Sprague-Dawley rats (Harlan Sprague-Dawley, Houston TX) as described previously (8, 49). Centrifugation and other operations were done at 4°C, and incubations were carried out the same day. Both preparations contained enzymes for the late stages of cholesterol biosynthesis; unlike the washed microsomes, all S₁₀ homogenates were supplemented with added cofactors (1 mM NAD⁺, 1 mM NADP⁺; including 3 mM glucose 6-phosphate and glucose-6-phosphate dehydrogenase for generating the reduced forms). In some experiments, the inhibitor AY-9944 or fenpropimorph was added to rat liver preparations, followed by incubation at 37°C for 10 min prior to addition of the sterol substrate. Aerobic incubations were carried out in a shaking water bath (80 cycles per min) in the dark for 3 h at 37°C in air. Anaerobic incubations were done similarly except for the use of a modified Warburg flask (125 ml) containing an argon atmosphere. Controls were performed for each sterol substrate under aerobic conditions, using rat liver preparations that had been inactivated by boiling for 10 min. To minimize photolytic degradation of 7-dehydrocholesterol, sterol preparations were protected from light during incubation and workup.

Isolation of incubation products

Incubations were terminated by addition of 15% ethanolic KOH (15 ml) that had been purged with argon for 2 min, followed by addition of BHT (500 μ g) in ethanol (500 μ l). The resulting mixtures were saponified in an anaerobic glove box for 2 h at 70°C under a nitrogen atmosphere, followed by extraction with degassed hexane (4 \times 90 ml). The hexane extracts, containing the nonsaponifiable lipids (NSL), were washed with saturated NaCl solution (50 ml), removed from the anaerobic chamber, dried over anhydrous Na₂SO₄, evaporated to dryness under argon, and dissolved in hexane (1 ml). The following procedures were used to separate the crude NSL into individual sterol components.

Procedure A. [In this procedure only, unlabeled 5 α -cholesta-

6,8-dien-3 β -ol (100 μ g) and cholest-5,7-dien-3 β -ol (200 μ g) were added as carrier mass prior to saponification.] After removal of aliquots for determination of radioactivity, the NSL solution was passed through a short silica gel column (50 \times 5 mm; elution with 3% acetone in hexane) and then subjected to semipreparative Ag⁺-HPLC (250 \times 10 mm column; elution with SS-1; 3 ml/min). Fractions (3 ml) were collected, and aliquots were taken for determination of radioactivity. Fraction sets containing radioactivity (designated as zones) were combined and evaporated to dryness under reduced pressure. The individual ³H zones and the crude NSL aliquot were each mixed with authentic unlabeled free sterols and subjected to analytical Ag⁺-HPLC (300 \times 3.2 mm column). A portion of the material from each zone was acetylated with a mixture of acetic anhydride (500 μ l) and pyridine (500 μ l) for 24 h at room temperature in the dark, followed by addition of water (1 ml) and extraction with methyl *tert*-butyl ether (MTBE) (4 \times 4 ml). The combined MTBE extracts were washed, dried over anhydrous Na₂SO₄, evaporated to dryness under nitrogen, and passed through a short silica gel column (50 \times 5 mm; elution with 3% acetone in hexane). The resulting [³H]acetate derivatives from each zone were mixed with authentic unlabeled sterol acetates and subjected to Ag⁺-HPLC (300 \times 4.6 mm column). The tritiated sterols were identified as described below on the basis of their coelution with authentic standards, both as free sterols and acetate derivatives, with compensation for isotopic fractionation (43).

Procedure B. The crude NSL were chromatographed as described in procedure A except that acetate derivatives were prepared from a crude NSL aliquot instead of from individual zones. Also, Ag⁺-HPLC analyses were performed in the presence of authentic unlabeled sterols or their acetates.

Procedure C. As in procedure A, the crude NSL were analyzed as free sterols (but not acetates) without the addition of unlabeled standards. Individual zones were also evaporated to dryness and analyzed by ¹H NMR and analytical Ag⁺-HPLC.

Procedure D. The crude NSL were chromatographed as free sterols only by Ag⁺-HPLC (300 \times 3.2 mm column) as in procedure C, except that authentic unlabeled standards were added.

Chromatographic identification of metabolites

As described previously (44, 45), Ag⁺-HPLC provided baseline separations of the acetate derivatives of C₂₇ sterols with double bonds in the steroid nucleus at the $\Delta^{8(14)}$, Δ^8 , Δ^7 , Δ^5 , $\Delta^{7,24}$,

$\Delta^{8,24}$, $\Delta^{5,24}$, 19-nor- $\Delta^{5,7,9}$, $\Delta^{5,7,9(11)}$, $\Delta^{5,8(14)}$, $\Delta^{7,9(11)}$, $\Delta^{6,8}$, $\Delta^{5,8}$, $\Delta^{6,8(14)}$, $\Delta^{8,14}$, $\Delta^{7,14}$, $\Delta^{5,7}$, and Δ^{14} positions. The corresponding free sterols also showed good separations (albeit incomplete in some cases) and were eluted in somewhat different order from the acetates (45). The differences in elution order afforded additional confirmation of the identity of each sterol.

The tritium-labeled incubation products were first separated into subclasses by semipreparative Ag⁺-HPLC (1-min fraction intervals). Several zones contained pairs of free sterols having similar mobilities: the Δ^7 and Δ^8 isomers, the $\Delta^{5,8(14)}$ and $\Delta^{7,14}$ isomers, the $\Delta^{5,7,9(11)}$ and $\Delta^{6,8}$ sterols, and the $\Delta^{5,24}$ and $\Delta^{7,9(11)}$ isomers. However, the $\Delta^{5,7,9(11)}$ and $\Delta^{6,8}$ sterols were separable (*t*_R, 21.0 and 23.7 min) on analytical Ag⁺-HPLC, and all four pairs were separable as acetates on Ag⁺-HPLC: the Δ^7 and Δ^8 acetates (*t*_R, 22.6 and 25.2 min; SS-3), the $\Delta^{5,8(14)}$ and $\Delta^{7,14}$ acetates (*t*_R, 24.3 and 73.7 min; SS-4); the $\Delta^{5,7,9(11)}$ and $\Delta^{6,8}$ acetates (*t*_R, 22.8 and 30.3 min; SS-4); and the $\Delta^{5,24}$ and the $\Delta^{7,9(11)}$ acetates (*t*_R, 19.7 and 25.6 min; SS-4). The absolute Ag⁺-HPLC retention times varied somewhat depending on the exact composition of the mobile phase, but the relative retention times were reproducible. As described previously (43), tritium-labeled sterols lagged their unlabeled counterparts by 1–4% on Ag⁺-HPLC. After correction for this isotopic fractionation, free sterols and acetate derivatives were each identified on the basis of coelution of the radioactivity with unlabeled standards. In the case of incubations with the $\Delta^{6,8}$ and $\Delta^{6,8(14)}$ sterols under aerobic conditions, the products were also characterized by ¹H NMR; structures of the unlabeled sterols were assigned on the basis of the identity (± 0.001 ppm) of observed and reported (47) NMR signals.

RESULTS

Overview of incubation results

Tritium-labeled C₂₇ sterols with unsaturation at the Δ^8 , $\Delta^{5,8}$, $\Delta^{5,8(14)}$, $\Delta^{6,8}$, or $\Delta^{6,8(14)}$ position were incubated under both aerobic and anaerobic conditions with rat liver S₁₀ homogenate containing added cofactors, followed by isolation, identification, and quantitation of the metabolites. All five substrates were partially converted to cholesterol under aerobic conditions, whereas no metabolic products were detected in any control experiments. Also, 19-nor-

TABLE 1. Products from incubations of [³ α -³H]5 α -cholest-8-en-3 β -ol with the S₁₀ fraction of rat liver homogenate^a

| Incubation Conditions | Δ^8 | Δ^7 | $\Delta^{5,7}$ | $\Delta^{5,7,9(11)}$ | $\Delta^{5,8}$ | $\Delta^{6,8}$ | $\Delta^{7,9(11)}$ | Δ^5 |
|---|------------|------------|----------------|----------------------|----------------|----------------|--------------------|------------|
| A: Aerobic ^{b,c} | 16 | 8 | 9 | 3 | | | | 64 |
| B: Anaerobic ^d | 77 | 22 | | | | | 1.5 | |
| C: AY-9944, aerobic ^e | 22 | 11 | 59 | 4 | 2 | 1 | | |
| D: 4.4 μ M fenpropimorph, aerobic | 12.5 | 14.5 | 17.5 | 3.4 | 0.8 | 1 | 0.5 | 49 |
| E: 44 μ M fenpropimorph, aerobic | 51 | 13 | 20 | 2 | 2 | 2 | 1 | 8 |
| F: 440 μ M fenpropimorph, AY-9944, aerobic | 93 | | 2 | 0.8 | 1.5 | 3 | | |
| G: 440 μ M fenpropimorph, AY-9944, anaerobic ^f | 96 | | | | | | | |
| H: Boiled control, aerobic ^d | 100 | | | | | | | |

^a All incubations contained added cofactors. Results are expressed as the percentage of each sterol in the radio-labeled nonsaponifiable lipids recovered from incubations. Where indicated, AY-9944 was added at a concentration of 1 μ M.

^b Incubation repeated twice (with comparable results).

^c Similar incubation with washed microsomes and added cofactors gave 86% Δ^8 , 10% Δ^7 , and 4% Δ^5 sterol, whereas a control incubation of boiled microsomes gave 100% Δ^8 sterol.

^d Incubation repeated once (with comparable results).

^e Similar incubation with 70 μ M AY-9944 gave 89% Δ^8 , 10% $\Delta^{5,7}$, and 1% Δ^7 sterol.

^f The only detectable metabolite was a minor unidentified component.

cholesta-5,7,9-trien-3 β -ol, an unusual aromatic steroid that has occasionally been reported to occur in SLOS samples (20, 27) but is more likely a GC artifact (50), was not observed as a product of any incubation as judged by its chromatographic mobility on Ag⁺-HPLC (45).

Various additional conditions were employed to elucidate the metabolism of the Δ^8 , $\Delta^{5,8}$, and $\Delta^{6,8}$ sterols. AY-9944, a potent inhibitor of mammalian Δ^7 reductase and a weak inhibitor of the Δ^8 - Δ^7 sterol isomerase (11), was used to block the reductase reactions. Fenpropimorph was used to inhibit the isomerase, although its potency in rodents and humans is much lower than in yeast (51). Anaerobic conditions were used to block the desaturation of the Δ^7 sterol to the $\Delta^{5,7}$ sterol and other oxygen-requiring reactions. Washed microsomes (lacking cofactors) were used to block reactions requiring NAD(P)H (desaturation and reduction). Altogether, 75 incubations were performed under 61 different sets of conditions. A summary of the incubation products observed for the Δ^8 , $\Delta^{5,8}$, and $\Delta^{6,8}$ sterols under various conditions is presented in Table 1, Table 2, and Table 3, respectively.

Incubations of [3α - ^3H]5 α -cholest-8-en-3 β -ol

Incubations of [3α - ^3H]5 α -cholest-8-en-3 β -ol (100 nmol, 6.6 μCi) in propylene glycol (100 μl) with S₁₀ rat liver homogenate (15 ml) and added cofactors were carried out under a variety of aerobic and anaerobic conditions, indicated in Table 1. Incubation products were isolated and analyzed by procedure A, and the results are summarized in Table 1. Representative semipreparative Ag⁺-HPLC separations of the metabolites under various incubation conditions are shown in Fig. 2. Aerobic conditions produced mainly cholesterol, accompanied by the Δ^8 , Δ^7 , $\Delta^{5,7,9(11)}$, and $\Delta^{5,7}$ sterols (Fig. 2A; Table 1A). Under anaerobic conditions, a major peak comprising a 7:2 mixture of the Δ^8 and Δ^7 sterols and a minor peak identified as the $\Delta^{7,9(11)}$ sterol were observed; no radiolabeled material coeluting with the Δ^5 , $\Delta^{5,7,9(11)}$, or $\Delta^{5,7}$ sterols was detected (Fig. 2B; Table 1B). In the presence of AY-9944, aerobic incubation produced several sterol intermediates [Δ^7 , $\Delta^{5,7}$, $\Delta^{5,7,9(11)}$, $\Delta^{6,8}$, and $\Delta^{5,8}$ sterols] but no cholesterol (Fig. 2D; Table 1C).

A dose-response study of fenpropimorph, performed by incubating the [3α - ^3H] Δ^8 sterol with S₁₀ homogenate in the presence of 4.4, 44, or 440 μM fenpropimorph, was carried out to determine the concentration of fenpropimorph required for inhibition of the Δ^8 - Δ^7 isomerase. As shown in Table 1, 4.4 μM fenpropimorph affected the extent of metabolism only slightly (12.5% recovered Δ^8 sterol; Table 1D), whereas markedly diminished metabolism was observed at higher levels of fenpropimorph (51 and 93% recovered Δ^8 sterol; Table 1E and F). Interestingly, in the presence of 440 μM fenpropimorph and 1 μM AY-9944 under aerobic conditions, minor desaturated products ($\Delta^{5,7}$, $\Delta^{5,7,9(11)}$, $\Delta^{5,8}$, and $\Delta^{6,8}$ sterols) were observed (Fig. 2E; Table 1F), whereas no identifiable metabolites were detected under anaerobic conditions (Table 1G). AY-9944 also inhibits the sterol Δ^8 - Δ^7 isomerase. Although the microsomal

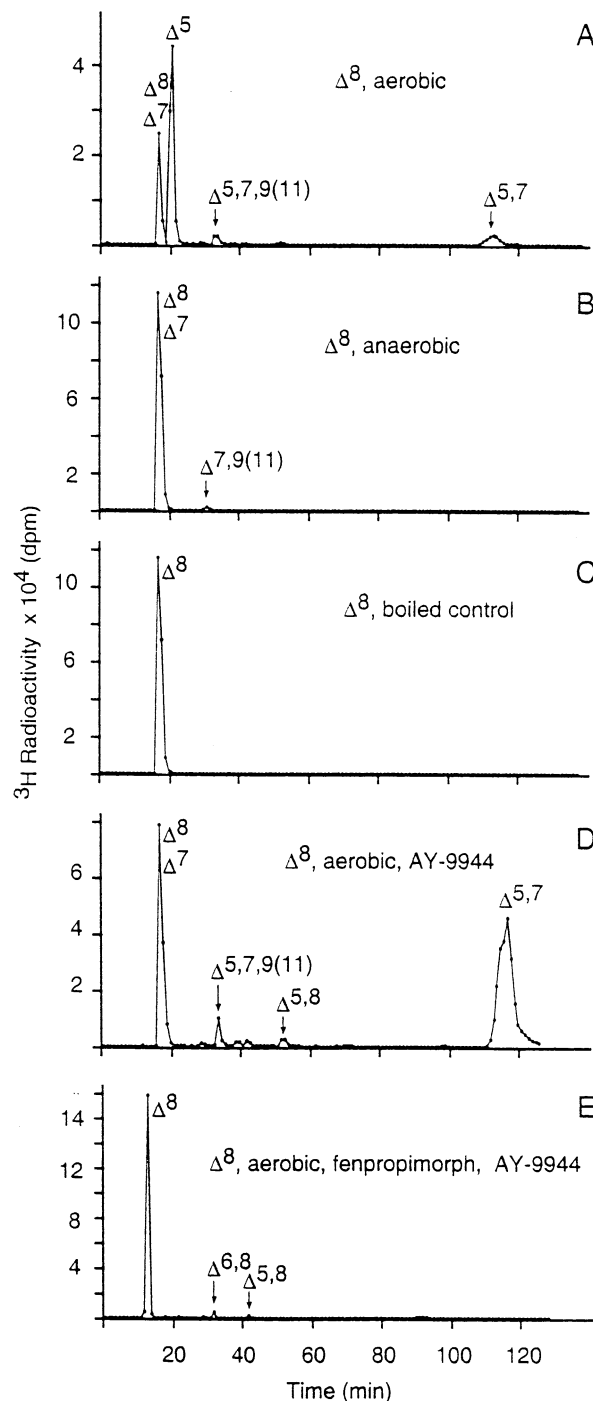


Fig. 2. Radio-Ag⁺-HPLC (300 × 10 mm column; SS-1) of acetate derivatives of the NSL obtained by incubation of [3α - ^3H]5 α -cholest-8-en-3 β -ol with S₁₀ homogenate containing added cofactors. Material from each ^3H peak was subjected to further analysis by analytical Ag⁺-HPLC.

isomerase has been reported to be “particularly sensitive to inhibition by the drug AY-9944” (11), we found that 1 μM AY-9944 completely blocked metabolism by the Δ^7 reductase but had no major effect on the isomerase (Table 1A vs. C). However, 70 μM AY-9944 appeared to strongly inhibit the isomerase (Table 1, footnote e).

TABLE 2. Products from incubations of [3α - ^3H]cholesta-5,8-dien-3 β -ol with rat liver preparations^a

| Incubation Conditions | $\Delta^{5,8}$ | $\Delta^{5,7}$ | Δ^5 |
|--|----------------|----------------|------------|
| A: Washed microsomes, aerobic ^{b,c} | 96 | 4 | |
| B: Washed microsomes, anaerobic ^{b,c} | 95.5 | 4.5 | |
| C: Washed microsomes, anaerobic, cofactors, unlabeled $\Delta^{5,8}$ | 96 | 2 | 4 |
| D: S ₁₀ homogenate, aerobic, cofactors ^{b,d} | 92 | | 6 |
| E: Boiled washed microsomes, aerobic ^b | 100 | | |
| F: Boiled S ₁₀ homogenate, aerobic ^b | 100 | | |

^a Results are expressed as the percentage of each sterol in the radiolabeled nonsaponifiable lipids recovered from incubations.

^b Incubation repeated once (with comparable results).

^c Similar incubations with cofactors gave 1–8% Δ^5 sterol and 0–1% $\Delta^{5,7}$ sterol.

^d Similar incubation of S₁₀ homogenate under aerobic conditions with added AY-9944 (70 μM) gave 100% $\Delta^{5,8}$ sterol.

Incubations of [3α - ^3H]cholesta-5,8(14)-dien-3 β -ol

Incubations of [3α - ^3H]cholesta-5,8(14)-dien-3 β -ol (0.5 nmol, 0.32 μCi) in propylene glycol (100 μl) with S₁₀ homogenate (30 ml) containing added cofactors were carried out under aerobic and anaerobic conditions, followed by analysis using procedure B. Radio- Ag^+ -HPLC analysis of the NSL from the aerobic incubation showed ^3H peaks coeluting with cholesterol (44%) and the starting $\Delta^{5,8(14)}$ sterol (56%), and similar results were observed for the acetate derivatives. Under anaerobic conditions, the ratio of cholesterol to the $\Delta^{5,8(14)}$ sterol was only 3:97. Essentially no radioactivity was associated with other C₂₇ sterols under aerobic or anaerobic conditions, but aerobic incubation with added AY-9944 (70 μM) gave 98% $\Delta^{5,8(14)}$ and 2% $\Delta^{5,7}$ sterol.

Incubations of [3α - ^3H]5 α -cholesta-6,8(14)-dien-3 β -ol

Incubation of [3α - ^3H]5 α -cholesta-6,8(14)-dien-3 β -ol (63 nmol, 95 μCi) in propylene glycol (60 μl) with S₁₀ homogenate containing added cofactors was carried out under anaerobic conditions, followed by analysis by procedure B. Only the unreacted $\Delta^{6,8(14)}$ substrate was recovered from anaerobic incubation, aerobic incubation with added AY-9944 (70 μM), and a boiled control. Aerobic incubation of the [3α - ^3H]5 α -cholesta-6,8(14)-dien-3 β -ol (61 μCi) and unlabeled $\Delta^{6,8(14)}$ sterol (2.5 mg) in propylene glycol (40 μl) was carried out analogously, followed by analysis by procedure C. Semipreparative radio- Ag^+ -HPLC analysis of the NSL gave five zones (A–E), in which the sterols were roughly quantitated and identified (47) by ^1H NMR: zone A (0.3 mg, 1% of ^3H), 44:40:15:2 mixture of Δ^7 , Δ^0 , Δ^8 , and $\Delta^{8(14)}$ sterols; zone B (3% of ^3H), cholesterol; zone C (1 μg , 2% of ^3H), $\Delta^{5,7,9(11)}$ sterol; zone D (1 mg, 1.5% of ^3H), 9:1 mixture of $\Delta^{6,8(14)}$ and $\Delta^{6,8}$ sterols; zone E (0.5 mg, 92% of ^3H), $\Delta^{6,8(14)}$ sterol. The ^3H data from analytical and semipreparative radio- Ag^+ -HPLC indicated formation of the Δ^5 (3%), $\Delta^{5,7,9(11)}$ (2%), $\Delta^{6,8}$ (1.5%), Δ^8 (0.7%), and Δ^7 (0.2%) sterols. The higher levels of Δ^0 and Δ^7 sterols in NMR analyses relative to the ^3H analyses were attributed to endogenous sterols present in the liver homogenate.

Incubation of [3α - ^3H]cholesta-5,8-dien-3 β -ol

Incubations of [3α - ^3H]cholesta-5,8-dien-3 β -ol (90 nmol, 5.4 μCi) in propylene glycol (100 μl) with washed rat liver microsomes (10 ml) were carried out under aerobic condi-

tions, anaerobic conditions, and anaerobic conditions with added cofactors, followed by analysis by procedure B. The incubation products are summarized in **Table 2**, and radio- Ag^+ -HPLC profiles of the acetate derivatives are shown in **Fig. 3**. Aerobic and anaerobic incubations of the [3α - ^3H] $\Delta^{5,8}$ sterol with washed microsomes gave the $\Delta^{5,7}$ sterol as the only product (~4%) (Fig. 3A; Table 2A and B), whereas anaerobic incubation with added cofactors gave both cholesterol (4%) and the $\Delta^{5,7}$ sterol (2%) as products (Fig. 3B; Table 2C). Incubation of the [3α - ^3H] $\Delta^{5,8}$ sterol with the S₁₀ homogenate (15 ml) under aerobic conditions with added cofactors (analysis by procedure A) produced cholesterol

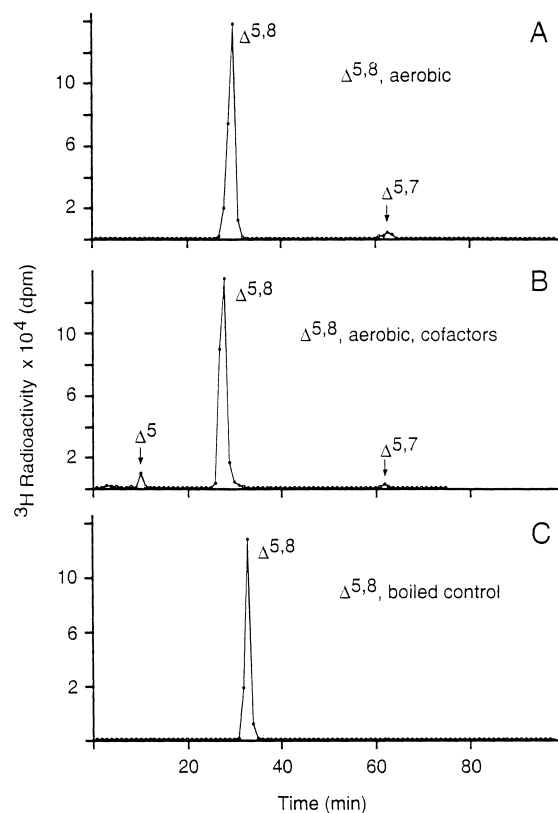


Fig. 3. Radio- Ag^+ -HPLC (300 \times 3.2 mm column; SS-2) of acetate derivatives of the NSL obtained by incubation of [3α - ^3H]cholesta-5,8-dien-3 β -ol with washed rat liver microsomes. Material from each ^3H peak was subjected to further analysis.

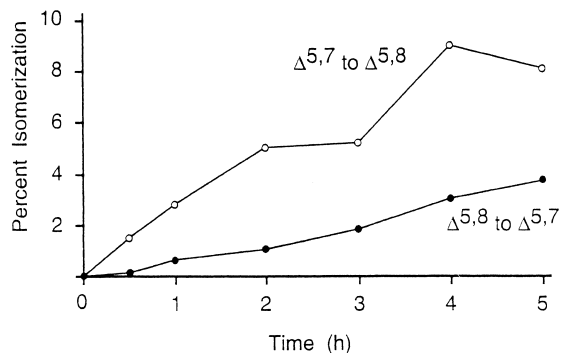


Fig. 4. Percent isomerization of the $3\alpha\text{-}^3\text{H}\text{-}\Delta^{5,8}$ or $4\text{-}^{14}\text{C}\text{-}\Delta^{5,7}$ sterol as a function of incubation time. Open circles represent incubation of the $\Delta^{5,7}$ sterol, and closed circles denote incubation of the $\Delta^{5,8}$ sterol. Sterols were incubated with washed rat liver microsomes (lacking cofactors) under anaerobic conditions.

(6%) but no $\Delta^{5,7}$ sterol (Table 2D). In contrast, an identical incubation of $[3\alpha\text{-}^3\text{H}]5\alpha\text{-cholesta-8-en-3}\beta\text{-ol}$ followed by analysis by procedure A produced 64% conversion of the Δ^8 sterol to cholesterol. This positive control experiment provided evidence that the low conversion of the $\Delta^{5,8}$ sterol to cholesterol was not due to faulty incubation conditions.

Kinetic studies of the enzymatic interconversion of the $\Delta^{5,7}$ and $\Delta^{5,8}$ sterols were carried out by incubating the $[3\alpha\text{-}^3\text{H}]\Delta^{5,8}$ sterol (53 nmol; 590 nCi) or the $[4\text{-}^{14}\text{C}]\Delta^{5,7}$ sterol (29 nmol, 71 nCi) in propylene glycol (40 μl) with washed rat liver microsomes (10 ml) for 0.5, 1, 2, 3, 4, and 5 h, followed by analysis by procedure D. The only products observed were the $\Delta^{5,7}$ and $\Delta^{5,8}$ sterols, the relative amounts of which were determined by radio- Ag^+ -HPLC. The results, shown in **Fig. 4**, indicated slow interconversion of the two sterols.

Incubations of $[3\alpha\text{-}^3\text{H}]5\alpha\text{-cholesta-6,8-dien-3}\beta\text{-ol}$

Incubations of $[3\alpha\text{-}^3\text{H}]5\alpha\text{-cholesta-6,8-dien-3}\beta\text{-ol}$ (20 nmol, 31 μCi) in propylene glycol (100 μl) with washed rat liver microsomes (15 ml) or S_{10} homogenate (15 ml) were carried out under various conditions (**Table 3**), followed by analysis by procedure A. The metabolic products are summarized in Table 3, and semipreparative Ag^+ -HPLC separations of the metabolites are shown in **Fig. 5**.

Aerobic incubation of the $\Delta^{6,8}$ sterol with the S_{10} homogenate gave cholesterol (64%) and minor amounts of the Δ^7 , Δ^8 , $\Delta^{5,7,9(11)}$, and $\Delta^{5,8}$ sterols (**Fig. 5A**; **Table 3A**). Aerobic incubation with washed microsomes gave similar results except that the $\Delta^{5,7,9(11)}$ and the $\Delta^{5,8}$ sterols were absent (**Table 3C**). Anaerobic incubation with S_{10} homogenate produced less cholesterol (24%), and various amounts of the Δ^7 , Δ^8 , $\Delta^{5,7,9(11)}$, $\Delta^{5,8}$, and $\Delta^{7,9(11)}$ sterols (**Fig. 5B**; **Table 3D**). Anaerobic incubation with washed microsomes gave similar results except for the absence of the $\Delta^{5,7,9(11)}$ and the $\Delta^{7,9(11)}$ sterols (**Table 3E**). Additional information was obtained by variously blocking the metabolism of the $\Delta^{6,8}$ sterol. The $\Delta^{7,9(11)}$ sterol was the only significant metabolite observed in anaerobic incubations with washed microsomes (**Fig. 5F**; **Table 3F**), and the $\Delta^{5,7,9(11)}$ sterol was the only detectable metabolite in aerobic incubations with

S_{10} homogenate in the presence of AY-9944 (**Fig. 5E**; **Table 3G** and **H**). When fenpropimorph was used to block isomerization, no conversion of the $\Delta^{6,8}$ sterol to any other sterols was observed (**Fig. 5D**; **Table 3I** and **J**).

In addition, the $[3\alpha\text{-}^3\text{H}]\Delta^{6,8}$ sterol (2 nmol, 3.1 μCi) and unlabeled $\Delta^{6,8}$ sterol (2.2 mg) in propylene glycol (100 μl) were incubated aerobically with S_{10} homogenate (60 ml), followed by analysis by procedure C. The NSL were separated into six zones (A–F) by Ag^+ -HPLC; sterols in each zone were roughly quantitated and identified (47) by ^1H NMR⁵: zone A (0.3 mg, 11% of ^3H), 62:28:10 mixture of Δ^7 , Δ^8 , and Δ^0 sterols; zone B (28% of ^3H), cholesterol; zone C (0.3 mg, 2% of ^3H), 2:1:1 mixture of $\Delta^{7,9(11)}$ and $\Delta^{5,24}$ sterols and isofucosterol; zone D (0.6 mg, 7% of ^3H), 90:8:2 mixture of $\Delta^{6,8}$ and $\Delta^{5,7,9(11)}$ sterols and unknown A; zone E (0.5 mg, 51% of ^3H), 80:2:2 mixture of $\Delta^{6,8}$ and $\Delta^{6,8(14)}$ sterols and unknown A; zone F (1 μg , 2% of ^3H), mainly $\Delta^{5,8}$ sterol. Assuming that isofucosterol and the Δ^0 and $\Delta^{5,24}$ sterols were present in the homogenate prior to incubation, these results are qualitatively compatible with those of the corresponding small-scale incubation.

DISCUSSION

Focusing on the final three steps of cholesterol biosynthesis (**Fig. 1**), we have investigated the origin and metabolism of several aberrant unsaturated sterols. Some of these sterols are present at trace levels in normal human blood (28), but remarkably high concentrations can be produced by certain genetic defects (17). We incubated tritium-labeled Δ^8 , $\Delta^{5,8}$, $\Delta^{6,8}$, $\Delta^{6,8(14)}$, and $\Delta^{5,8(14)}$ sterol substrates with rat liver preparations under a variety of conditions and identified the resulting metabolites on the basis of Ag^+ -HPLC coelution of ^3H radioactivity with unlabeled authentic standards. The high specific activity of the substrates, combined with the exceptional power of Ag^+ -HPLC to resolve unsaturated sterols, generally permitted detection of minor metabolites at a level of 1% (or 2–3% for $\Delta^{5,7}$ and other late-eluting sterols). Identification of the metabolites was facilitated by reported chromatographic mobilities of unsaturated sterols on Ag^+ -HPLC (44, 45), by the availability of authentic standards (47), and by recognition of the effects of isotopic fractionation in argentation chromatography (43, 52). Our results, presented mainly in **Tables 1–3**, consist of the distribution of metabolites observed for each set of conditions. We have used these results to deduce new information about aberrant pathways operating during the last steps of cholesterol biosynthesis. Details of our logic are given below, and our conclusions are summarized in a scheme of aberrant pathways (**Fig. 6**).

Incubation results for the Δ^8 sterol (**Table 1A** and **B**) were compatible with previous findings (1, 7) of its metab-

⁵ NMR data for isofucosterol: δ 0.684 (d, 0.5 Hz), 0.947 (d, 6.6 Hz), 0.976 (d, 6.9 Hz), 0.977 (d, 6.9 Hz), 1.010 (s), 1.590 (dt, 6.8, 1.2 Hz), 2.829 (septet, 6.9 Hz), 5.106 (br q, 6.8 Hz), 5.35 (m). NMR data for unknown A [possibly a $\Delta^{6,8(14),9(11)}$ sterol]: δ 5.768 (dd, 9.7, 3.1 Hz), 5.471 (dd, 9.6, 2.4 Hz), 0.898? (s), 0.637 (s).

TABLE 3. Products from incubations of [3α - ^3H]5 α -cholesta-6,8-dien-3 β -ol with rat liver preparations^a

| Incubation Conditions | $\Delta^{6,8}$ | Δ^8 | Δ^7 | Δ^5 | $\Delta^{5,7,9(11)}$ | $\Delta^{5,8}$ | $\Delta^{7,9(11)}$ |
|---|----------------|------------|------------|------------|----------------------|----------------|--------------------|
| A: S ₁₀ homogenate, aerobic ^b | 20.5 | 8 | 2 | 64 | 4.5 | 1.4 | |
| B: S ₁₀ homogenate, aerobic, unlabeled $\Delta^{6,8}$ ^c | 51 | 7.5 | 3.5 | 28 | 7 | 2 | 2 |
| C: WM, cofactors, aerobic | 18 | 4 | 36 | 42 | | | |
| D: S ₁₀ homogenate, anaerobic ^{b,c} | 30.5 | 3.5 | 15 | 24 | 1 | | 1.4 |
| E: WM, cofactors, anaerobic | 27 | 2 | 41 | 30 | | | |
| F: WM, anaerobic | 93 | | | | | | 7 |
| G: S ₁₀ homogenate, aerobic, 1 μM AY-9944 ^d | 88 | | | | 11 | | |
| H: S ₁₀ homogenate, aerobic, 70 μM AY-9944 | 89 | | | | 8 | | |
| I: S ₁₀ homogenate, aerobic, 1 mM fenpropimorph | 100 | | | | | | |
| J: S ₁₀ homogenate, anaerobic, 1 mM fenpropimorph | 100 | | | | | | |
| K: Boiled S ₁₀ homogenate ^b | 100 | | | | | | |

^a Incubations were done with washed microsomes (WM) or the S₁₀ fraction of rat liver homogenate. All incubations of S₁₀ homogenates contained added cofactors. Results are expressed as the percentage of each sterol in the radiolabeled nonsaponifiable lipids recovered from incubations. Where indicated, AY-9944 was added at a concentration of 1 μM .

^b Incubation repeated twice (with comparable results).

^c $\Delta^{6,8(14)}$ sterol (0.7%) was also detected in the incubation products.

^d Traces (0.5–1.6%) of the $\Delta^{5,7}$ sterol were detected in some cases.

olism to cholesterol via the Δ^7 and $\Delta^{5,7}$ sterols under aerobic conditions and its conversion to the Δ^7 sterol under anaerobic conditions. The $\Delta^{5,8}$ and $\Delta^{6,8}$ sterols were observed at low levels in most aerobic incubations of the Δ^8 sterol, even in the presence of fenpropimorph, but were absent in anaerobic incubations. These results suggest minor aberrant reactions consisting of oxygen-dependent desaturation of the Δ^8 sterol to the $\Delta^{5,8}$ and $\Delta^{6,8}$ sterols. The observed formation of low levels of the $\Delta^{7,9(11)}$ sterol under both aerobic and anaerobic conditions (Table 1B, D, and E) suggested another aberrant pathway consisting of oxygen-independent desaturation of the Δ^8 sterol. Detection of the $\Delta^{7,9(11)}$ sterol in incubations done anaerobically or with added fenpropimorph excluded the possibility of its formation via the $\Delta^{6,8}$ intermediate (see below).

The remaining sterol substrates [$\Delta^{5,8}$, $\Delta^{5,8(14)}$, $\Delta^{6,8}$, and $\Delta^{6,8(14)}$] were also convertible to cholesterol by the rat liver S₁₀ fraction. A surprisingly large portion (56%) of the $\Delta^{5,8(14)}$ sterol was metabolized to cholesterol under aerobic conditions, but only 3% conversion was observed under anaerobic conditions. The oxygen dependence suggests an early desaturation step, and the efficiency of conversion to cholesterol under aerobic conditions excludes the possibility of a $\Delta^{5,8}$ intermediate (see below). Initial metabolism of the $\Delta^{6,8(14)}$ sterol also required oxygen and might likewise involve initial desaturation. Under aerobic conditions, the extent of its conversion to other sterols (8%) was much lower than that of the $\Delta^{5,8(14)}$ sterol (44%).

Considerable interest has focused on the origin of the $\Delta^{5,8}$ sterol, a major species accumulating in SLOS, but not a normal intermediate of cholesterol biosynthesis. An early proposal involving desaturation of the Δ^8 sterol to the $\Delta^{5,8}$ dienol (28) was based on the observation of $\Delta^{5,8}$ and $\Delta^{6,8}$ sterols in normal human blood, but later evidence from SLOS studies suggested formation of the $\Delta^{5,8}$ sterol by isomerization of the $\Delta^{5,7}$ sterol (21, 40). Our results indicate that the $\Delta^{5,8}$ sterol can arise from either source, and both routes may contribute significantly to the trace $\Delta^{5,8}$ sterol levels observed under conditions of normal chole-

sterol biosynthesis. In CDP, which is characterized by a defect of the Δ^8 – Δ^7 isomerase and by Δ^8 plasma levels almost 1,000 times those of the $\Delta^{5,7}$ sterol (34), elevated levels of the $\Delta^{5,8}$ sterol may arise mainly from desaturation of the Δ^8 sterol. In contrast, under SLOS conditions, the $\Delta^{5,7}$ sterol is far more abundant than the Δ^8 sterol, and most of the $\Delta^{5,8}$ sterol may be formed by isomerization from the $\Delta^{5,7}$ sterol. This hypothesis (21) is supported by results of microsomal incubations of the [^3H] Δ^7 sterol (40) and by our observation that incubation of the Δ^8 sterol for 3 h in the presence of AY-9944 produces a 59:2 ratio of $\Delta^{5,7}$ and $\Delta^{5,8}$ sterols (Table 1C), whereas a typical ratio of $\Delta^{5,7}$ to $\Delta^{5,8}$ sterols in SLOS plasma is roughly 3:2 (18, 20, 21).

Our results (Table 2A–D) indicate that the sole metabolic fate of the $\Delta^{5,8}$ sterol is isomerization to the $\Delta^{5,7}$ sterol and subsequent conversion to cholesterol. We also carried out kinetic studies involving separate incubations of the $\Delta^{5,8}$ and $\Delta^{5,7}$ sterols with washed microsomes, which lack cofactors and thus permit only isomerization reactions. The results, shown in Fig. 4, indicate slow isomerization of the $\Delta^{5,8}$ sterol to the $\Delta^{5,7}$ sterol and of the $\Delta^{5,7}$ sterol to the $\Delta^{5,8}$ sterol. The apparent reaction rates depicted in Fig. 4 convey the impression that isomerization of the $\Delta^{5,7}$ sterol is faster than the reverse reaction. That impression is probably correct considering that the $\Delta^{5,8}$ sterol accumulates significantly in SLOS, that the $\Delta^{5,8}$ sterol is initially metabolized only to the $\Delta^{5,7}$ sterol, and that the $\Delta^{5,8}$ sterol arises mainly from the $\Delta^{5,7}$ sterol under SLOS conditions. Assuming that the Δ^8 – Δ^7 isomerase is responsible for the $\Delta^{5,7}$ – $\Delta^{5,8}$ conversion, it is interesting that the isomerase strongly favors the Δ^7 over the Δ^8 sterol in normal sterol biosynthesis⁶ (11), whereas the Δ^8 species appears to be favored when a Δ^5 or Δ^{14} double bond is also present.

Unlike the $\Delta^{5,8}$ sterol, the $\Delta^{6,8}$ sterol is present at low levels in the blood of both SLOS and normal (28) subjects. Our results indicate formation of the $\Delta^{6,8}$ sterol only by de-

⁶ Incubation results in Table 1B suggest that the Δ^8 sterol is favored, but equilibrium may not have been reached in this experiment.

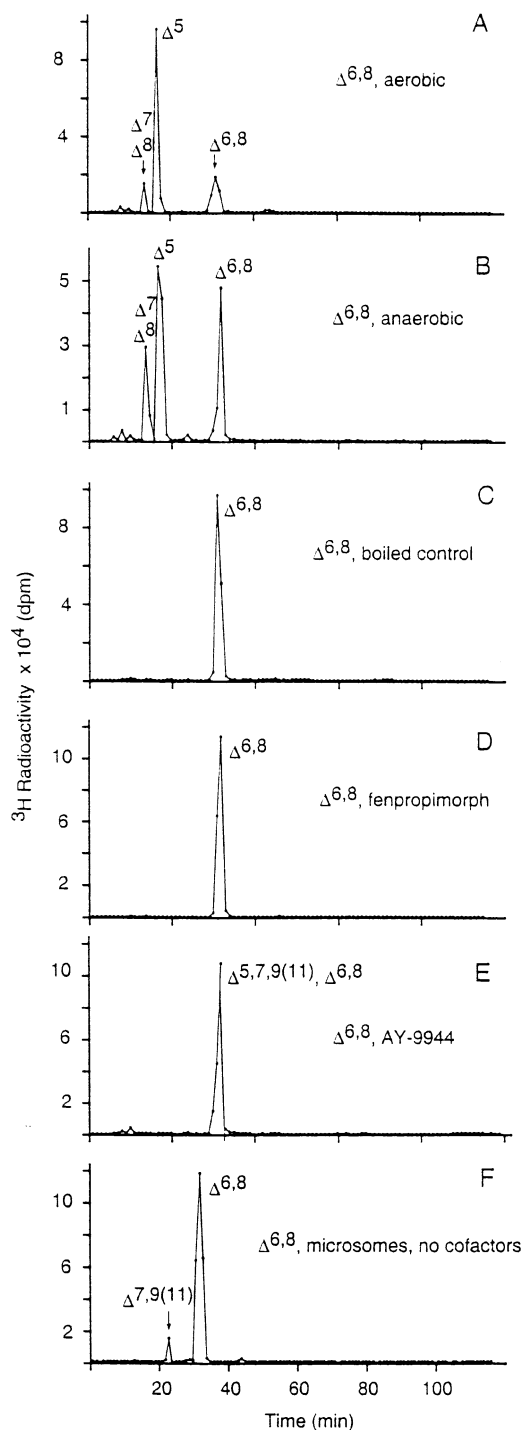


Fig. 5. Radio- Ag^+ -HPLC (300×10 mm column; SS-1) of acetate derivatives of the NSL obtained by incubation of [$3\alpha\text{-}^3\text{H}$]5 α -cholesta-6,8-dien-3 β -ol with S_{10} homogenate with added cofactors (A–E) or washed microsomes lacking cofactors (F). Material from each ^3H peak was subjected to further analysis by analytical Ag^+ -HPLC.

saturation of the Δ^8 sterol (Table 1C–F) and not by isomerization of the $\Delta^{5,8}$ sterol as proposed previously (28). The $\Delta^{6,8}$ sterol was readily metabolized to cholesterol under both aerobic and anaerobic conditions (Table 3A–E). The anaerobic metabolism of the $\Delta^{6,8}$ sterol to cholesterol (Table 3D and E) is most readily explained by direct conver-

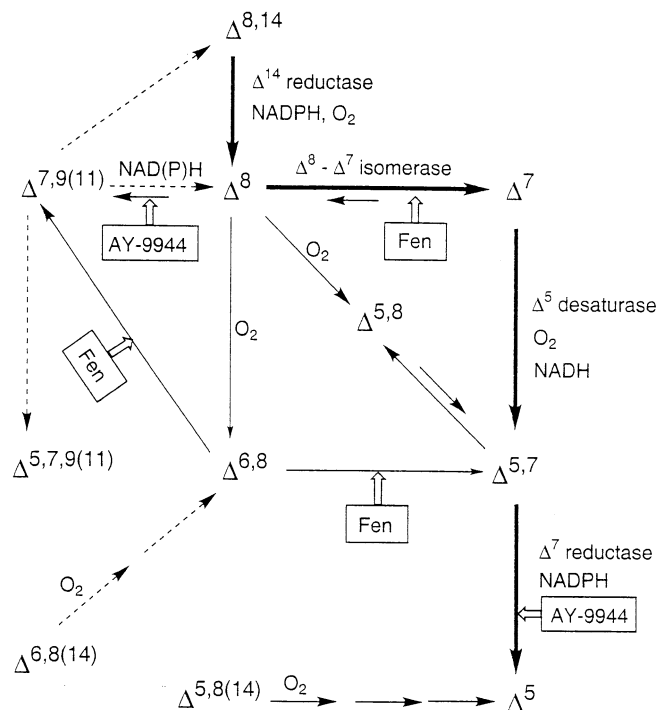


Fig. 6. Aberrant metabolic pathways related to the final steps of cholesterol biosynthesis. Individual steps in the normal conversion of the $\Delta^{8,14}$ sterol to cholesterol are denoted by thick arrows, with indications of requirements for oxygen (O_2) and cofactors (NADPH, NADH). “Fen” and “AY-9944” designate reactions blocked by fenpropimorph ($440 \mu\text{M}$) and AY-9944 ($1 \mu\text{M}$), respectively. Dashed arrows indicate suggested (tentative) transformations. Isomerization of the $\Delta^{7,9(11)}$ to the $\Delta^{8,14}$ sterol was described previously (39).

sion of the $\Delta^{6,8}$ sterol to the $\Delta^{5,7}$ sterol because formation of the $\Delta^{5,7}$ sterol from the Δ^7 or Δ^8 sterols would require oxygen. The observation of Δ^7 , Δ^8 , and $\Delta^{7,9(11)}$ metabolites (Table 3A–E) indicates a second pathway to cholesterol. Anaerobic incubation with washed microsomes produced the $\Delta^{7,9(11)}$ sterol as the only metabolite (Table 3F), suggesting the $\Delta^{7,9(11)}$ sterol as the initial intermediate. The production of Δ^7 and Δ^8 sterols under anaerobic conditions with cofactors (Table 3D and E) pointed to an NAD(P)H-dependent reduction of the $\Delta^{7,9(11)}$ sterol to the Δ^8 sterol, possibly via the $\Delta^{8,14}$ sterol (39).⁷ This proposed oxygen-requiring pathway from the $\Delta^{6,8}$ sterol to cholesterol is consistent with evidence (1, 7) that the Δ^8 and $\Delta^{7,9(11)}$ sterols are convertible to cholesterol via the Δ^7 sterol. The absence of any metabolism of the $\Delta^{6,8}$ sterol in the presence of 1 mM fenpropimorph (Table 3I and J) suggests that the initial metabolic step in each pathway involves a sterol isomerase. Thus, our results indicate two pathways from the $\Delta^{6,8}$ sterol to cholesterol, an oxygen independent pathway

⁷ Our failure to observe any $\Delta^{8,14}$ metabolite raises the possibility of direct reduction to the Δ^8 sterol, perhaps by the Δ^7 reductase. However, small amounts of the late-eluting $\Delta^{8,14}$ sterol might have been overlooked because of its high detection limit (1–2% of total ^3H sterols) on Ag^+ -HPLC. Also, observable amounts of the $\Delta^{8,14}$ intermediate would not be produced if the isomerization from the $\Delta^{7,9(11)}$ sterol (38) is slower than reduction to the Δ^8 sterol.

via initial isomerization to the $\Delta^{5,7}$ sterol and an oxygen dependent pathway via the Δ^8 and Δ^7 sterols. Although not definitive, our data suggest that the low steady-state levels of the $\Delta^{6,8}$ sterol result from its metabolism (Table 3A–E) being much faster than its formation (Table 1C–F).

Concentrations of the $\Delta^{5,7,9(11)}$ sterol in SLOS are somewhat elevated but much lower than levels of the $\Delta^{5,7}$ and $\Delta^{5,8}$ sterols (27). Enzymatic formation of the triene from the $\Delta^{5,7}$ sterol via a peroxide intermediate has been proposed (27). An alternative mechanism involves desaturation of the $\Delta^{5,7}$ or the $\Delta^{7,9(11)}$ sterol, transformations that would likely require cofactors and aerobic conditions. Although our incubations of the $\Delta^{5,7}$ sterol with washed microsomes (lacking cofactors) cannot distinguish between these proposals (Fig. 4 and ref. 44), incubations of the $\Delta^{6,8}$ sterol provide evidence of the formation of the triene from the $\Delta^{7,9(11)}$ sterol. Notably, the triene was the predominant product in aerobic incubations of the $\Delta^{6,8}$ sterol with the S_{10} fraction containing AY-9944 (Table 3G and H), conditions that allow initial isomerization of the $\Delta^{6,8}$ sterol to the $\Delta^{7,9(11)}$ sterol but block subsequent reduction of the $\Delta^{7,9(11)}$ sterol (Fig. 6). Numerous other experiments described in Tables 1–3 also support the proposed route to the triene via the $\Delta^{7,9(11)}$ sterol, although other pathways cannot be excluded. Additional experimentation will be required to determine why the $\Delta^{5,7,9(11)}$ sterol accumulates in SLOS but is present at only trace levels in normal subjects. Parallel to the explanation for elevated $\Delta^{5,8}$ levels in CDP (see above), the high concentration of the $\Delta^{5,7}$ sterol under SLOS conditions may lead to accumulation of the triene at a rate faster than its subsequent metabolism. Alternatively, the triene might be formed mainly via the $\Delta^{7,9(11)}$ sterol, with low Δ^7 reductase activity in SLOS subjects blocking its putative metabolism to cholesterol. Relevant to these hypotheses is the established role of the $\Delta^{7,9(11)}$ sterol as an aberrant intermediate of cholesterol biosynthesis (39) and evidence that, apart from esterification, the $\Delta^{5,7,9(11)}$ sterol is not metabolized by S_{10} rat liver homogenates (38).

Certain limitations in the design of our study should be acknowledged. Most importantly, definitive conclusions regarding relative rates of reactions are elusive because of batch-to-batch variations in the activity of the microsomal enzymes and because of the possibility of selective loss of the 3α -tritium label in some reactions. Also, the multiplicity of potential alternative pathways can prevent unambiguous elucidation of the exact transformations leading from substrate to incubation product. Other concerns include minor species differences that may affect the relative importance of aberrant metabolic pathways in rats and humans and possible differences in the proximity of various enzymes in microsomes relative to the microarchitecture in the native endoplasmic reticulum. Moreover, our blocking of the Δ^7 reductase in the rat model is not exactly equivalent to SLOS. Finally, in view of the multiplicity of SLOS genotypes (14, 15, 22), the relative importance of the aberrant pathways among SLOS subjects may be quite variable. Despite these caveats, our results appear to be internally consistent and compatible with most findings reported by others.

In summary, we have developed effective methods for elucidating aberrant metabolic pathways in the late stages of cholesterol biosynthesis. Using tritium-labeled Δ^8 , $\Delta^{5,8}$, $\Delta^{6,8}$, $\Delta^{6,8(14)}$, and $\Delta^{5,8(14)}$ sterol substrates, we carried out a large number of incubations with rat liver microsomes or the S_{10} fraction and identified the metabolites by their co-elution with authentic standards on Ag^+ -HPLC. On the basis of these extensive results, we have evaluated various proposals to explain the origin and metabolism of the $\Delta^{5,8}$, $\Delta^{6,8}$, $\Delta^{5,7,9(11)}$, and related sterols associated with SLOS and other metabolic disorders. Our conclusions are summarized in a scheme of normal and aberrant pathways of double bond migration (Fig. 6). The findings described herein represent a significant advance in the understanding of the factors affecting the accumulation of noncholesterol sterols in SLOS and other genetic disorders involving defects of cholesterol biosynthesis. **■**

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