Markedly Inhibited 7-Dehydrocholesterol-Δ^7-Reductase Activity in Liver Microsomes from Smith-Lemli-Opitz Homozygotes

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Abstract

We investigated the enzyme defect in late cholesterol biosynthesis in the Smith-Lemli-Opitz syndrome, a recessively inherited developmental disorder characterized by facial dysmorphism, mental retardation, and multiple organ congenital anomalies. Reduced plasma and tissue cholesterol with increased 7-dehydrocholesterol concentrations are biochemical features diagnostic of the inherited enzyme defect. Using isotope incorporation assays, we measured the transformation of the precursors, [3α-^{3}H]lathosterol and [1,2-^{3}H]7-dehydrocholesterol into cholesterol by liver microsomes from seven controls and four Smith-Lemli-Opitz homozygous subjects. The introduction of the double bond in lathosterol at C-5[6] to form 7-dehydrocholesterol that is catalyzed by lathosterol-5-dehydrogenase was equally rapid in controls and homozygotes liver microsomes (120±8 vs 100±7 pmol/mg protein per min, P = NS). In distinction, the reduction of the double bond at C-7 [8] in 7-dehydrocholesterol to yield cholesterol catalyzed by 7-dehydrocholesterol-Δ^7-reductase was nine times greater in controls than homozygotes microsomes (365±23 vs 40±4 pmol/mg protein per min, $P < 0.0001$). These results demonstrate that the pathway of lathosterol to cholesterol in human liver includes 7-dehydrocholesterol as a key intermediate. In Smith-Lemli-Opitz homozygotes, the transformation of 7-dehydrocholesterol to cholesterol by hepatic microsomes was blocked although 7-dehydrocholesterol was produced abundantly from lathosterol. Thus, lathosterol 5-dehydrogenase is equally active which indicates that homozygotes liver microsomes are viable. Accordingly, microsomal 7-dehydrocholesterol-Δ^7-reductase is inherited abnormally in Smith-Lemli-Opitz homozygotes. (*J. Clin. Invest.* 1995. 96:1779–1785.) Key words: abnormal cholesterol biosynthesis • 7-dehydrocholesterol • Smith-Lemli-Opitz syndrome

Introduction

According to current theory, (Fig. 1) mevalonic acid is the first precursor in the cholesterol biosynthetic pathway that is almost totally committed to cholesterol synthesis. It is formed from the reduction of 3-hydroxy-3-methylglutaryl (HMG)[1] CoA (1, 2). The reaction is catalyzed by the microsomal enzyme, HMG-CoA reductase, and is considered rate determining as the activity of the enzyme varies under feedback control. Unused substrate, HMG-CoA, can be converted to fatty acids and CO_2 and does not accumulate (3, 4). Subsequently, mevalonic acid is transformed to isopentenyl pyrophosphate and two molecules joined into geranyl pyrophosphate with a third molecule added to form farnesyl pyrophosphate. Squalene arises from the condensation of two molecules of farnesyl pyrophosphate and is then cyclized into lanosterol, a 30 carbon sterol. In the reactions from lanosterol to form cholesterol, three methyl groups are eliminated at carbons C-4, C-4′, and C-14, the double bonds in the side chain at C-24 [25], and in ring B at C-8 [9] are reduced, and a double bond inserted at C-5 [6] (5, 6). Lathosterol and 7-dehydrocholesterol are the last two precursors in the pathway before cholesterol.

Recently, we have described an abnormality in late cholesterol biosynthesis in subjects with the Smith-Lemli-Optiz syndrome (7, 8). Homozygotes with this recessively inherited condition cannot form cholesterol efficiently, and accumulate the final precursor, 7-dehydrocholesterol, in plasma and tissues (Fig. 1). As a result of deficient biosynthesis, organs are deprived of cholesterol and contain substantial quantities of 7-dehydrocholesterol so that development is hindered and critical functions are impaired. Smith-Lemli-Opitz homozygotes manifest facial dysmorphic features and congenital anomalies affecting multiple organs particularly the brain, where cholesterol normally constitutes 25% of the myelin, and substantial amounts are present in gray matter. All are mentally retarded (9, 10). We postulated the inherited enzyme defect involves 7-dehydrocholesterol-Δ^7-reductase, that catalyzes the conversion of 7-dehydrocholesterol to cholesterol (Fig. 1) which is the penultimate reaction in the biosynthetic pathway (7, 8, 11–13). In support of this reasoning, rats treated with BM 15.766, an inhibitor of 7-dehydrocholesterol-Δ^7-reductase became deficient in cholesterol and accumulated 7-dehydrocholesterol in all tissues reproducing the biochemical abnormalities as seen in Smith-Lemli-Opitz homozygotes (14, 15).

To prove that 7-dehydrocholesterol-Δ^7-reductase is abnormal in Smith-Lemli-Opitz homozygotes, the final two reactions in the cholesterol biosynthetic pathway were investigated (Fig.

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1. *Abbreviations used in this paper:* GLC, gas-liquid chromatography; HMG, hydroxymethyl glutaryl; TMS, trimethylsilyl. The following names are also used: cholesterol, 5-cholesten-3β-ol; 7-dehydrocholesterol, 5,7-cholestadien-3β-ol; 8-dehydrocholesterol, 5,8-cholestadien-3β-ol; lathosterol, 7-cholesten-3β-ol; 8-cholestenol, 8-cholesten-3β-ol; lanosterol, 4,4,14-trimethyl 8(9), 24(25)-cholestadien-3β-ol. 24-dihydrocholanosterol, 4,4,14-trimethyl-8(9)-cholesten-3β-ol.

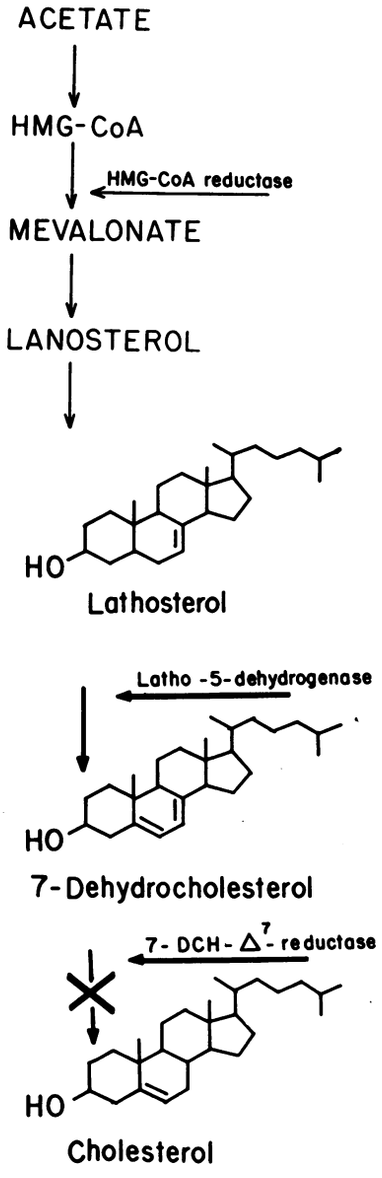

Figure 1. Flow diagram showing key intermediates in the pathway for the formation of cholesterol from acetate. HMG-CoA reductase is considered the rate-controlling enzyme that regulates the formation of mevalonic acid, the first almost totally committed precursor in the pathway. Lanosterol the first cyclized sterol is also noted. Two key reactions in late cholesterol biosynthesis are emphasized: the conversion of lathosterol to 7-dehydrocholesterol catalyzed by lathosterol-5-dehydrogenase, and the transformation of 7-dehydrocholesterol to cholesterol catalyzed by 7-dehydrocholesterol-Δ^7-reductase. The suspected abnormal reaction between 7-dehydrocholesterol and cholesterol in the Smith-Lemli-Opitz syndrome is denoted by a cross.

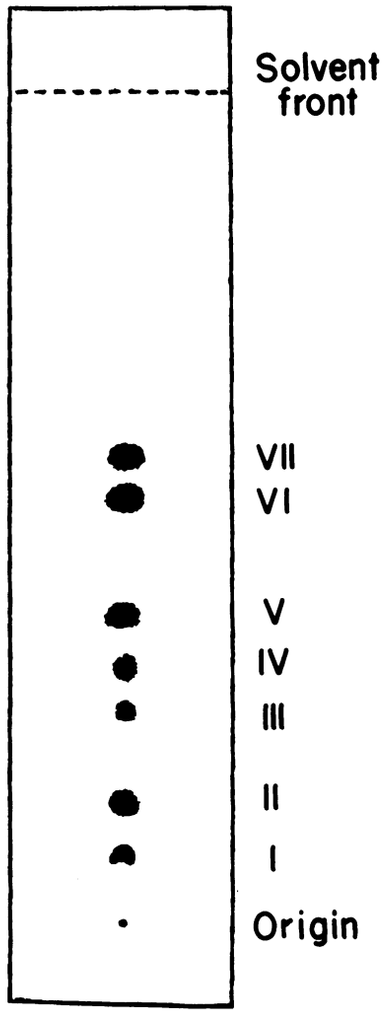

Figure 2. The separation of precusors from cholesterol on Silicia Gel G plates containing Ag NO_3. The following R_f values were observed: I, 7-dehydrocholesterol, 0.09; II, 8-dehydrocholesterol, 0.14; III, desmosterol, 0.27; IV, cholesterol, 0.31; V, lathosterol, 0.37; VI, lanosterol, 0.51; and VII, 24(25)-dihydrolanosterol, 0.56. Solvent system/chlorform/acetone, 57:2, vol/vol.

1). The introduction of a double bond at C-5[6] in lathosterol to form 7-dehydrocholesterol catalyzed by lathosterol 5-dehydrogenase followed by the reduction of the double bond at C-7[8] catalyzed by 7-dehydrocholesterol-Δ^7-reductase to yield cholesterol were measured in hepatic microsomes from seven human transplant donors where suitable recipients could not be located and four Smith-Lemli-Opitz homozygotes. The results confirm that lathosterol and 7-dehydrocholesterol are precursors in the pathway of cholesterol biosynthesis, and that hepatic microsomal 7-dehydrocholesterol-Δ^7-reductase activity is deficient in Smith-Lemli-Opitz homozygotes.

Methods

Clinical

Studies were conducted with liver tissues that were obtained from four female Smith-Lemli-Opitz homozygotes with 46, XX chromosome patterns. The homozygotes were unrelated and exhibited the characteristic clinical phenotype that included microcephaly, sloping forehead, ptosis, flat nasal bridge, low set posteriorly rotated ears, micrognathia, polydactaly, and syndactyly of the second and third toes (9, 10). Severe neurologic dysfunction with mental retardation and decreased muscle tone were evident. In a 6-mo-old subject, who failed to thrive, a gastric feeding tube was inserted surgically, and a liver biopsy was obtained for histology and liver enzyme analysis. Three other homozygotes suffered from severe congenital organ anomalies which included atrial and ventricular septal defects and died at 37 h, 3 wk, and 3 mo, respectively, after birth. Post mortem examinations were performed promptly and liver tissue was frozen immediately and maintained at −70°C for enzyme measurements.

Control liver specimens were obtained from seven (three males and four females) healthy human donors (ages 1–5 yr) when no suitable transplant recipients for their livers were found through the Liver Procurement Program, University of Minnesota Medical School, Minneapolis, MN (LTPADS, National Institutes of Health Contract No 1-DK62274). All specimens were frozen and stored at −70°C until analyzed.

Blood was collected in tubes containing EDTA and the erythrocytes were separated from plasma by centrifugation at 5,000 *g* for 10 min. Plasma was refrigerated at 4°C until analyzed.

The experimental protocol was approved by the human studies committees at the VA Medical Center, East Orange, NJ, and UMD-New Jersey Medical School, Newark, NJ.

Preparation of [^{3}H]-substrates

[3α-^{3}H]Lathosterol. [3α-^{3}H]Lathosterol was prepared from unlabeled lathosterol (Aldrich Chemical Co., Milwaukee, WI) by chromium trioxide oxidation of the 3β-hydroxyl group to form 7-cholesten-3-one. The 3-oxo-derivative was reduced with [^{3}H]sodium borohydride (DuPont-NEN Research Products, Boston, MA) to form [3α-^{3}H]lathosterol. The compound was isolated by preparative TLC on Silica Gel H plates developed with chloroform/acetone (97:3; vol/vol, R_f 0.35) and was > 98% pure; specific activity was 830 cpm/nmol.

[1,2-^{3}H]7-Dehydrocholesterol was synthesized from [1,2-^{3}H]-cholesterol (DuPont-NEN Research Products). The [1,2-^{3}H]cholesterol was acetylated with acetic anhydride in pyridine and the acetate was refluxed for 2 min with 1,3-dibromo-5,5-dimethylhydantoin in *n*-hexane. The solvent was evaporated, and the residue dissolved in *o*-xylene, and heated at 160°C for 3 h in the presence of triethyl phosphite. After the evaporation of the solvent, the product was saponified in 1 N NaOH and purified by reversed-phase high performance liquid chromatography on a Waters Associates (Milford, MA) radial pak, μ Bondapak C_{18} reversed phase column (100 × 8 mm I.D., 5 μM particle size) using ethanol/methanol/water (160:40:10, vol/vol/vol) as the developing solvent system at a flow rate of 2 ml/min according to Holick et al. (16, 17). [1,2-^{3}H]7-Dehydrocholesterol was recovered at retention volume of 15.3 ml and was > 99% pure when examined by argentation TLC (10% silver nitrate in Silica Gel G [wt/wt]) developed with chloroform/acetone, 57:2, vol/vol, R_f 0.09, (Fig. 2). The sp act was 330 cpm/nmol. Aliquots of [1,2-^{3}H]7-dehydrocholesterol were stored under argon in sealed tinfoil covered ampules at −70°C until used for enzyme assay.

Sterol analysis. Aliquots from plasma and liver microsomes were saponified in 1 N NaOH and the neutral sterols extracted with *n*-hexane. 5α-Cholestane was added as an internal quantitative standard and the neutral sterols were converted to TMS ether derivatives by the addition of Sil Prep (Alltech Associates, Deerfield, IL). Measurements were made by capillary gas liquid chromatography (GLC) according to Tint et al. (7). The trimethylsilyl (TMS) ether derivatives were injected onto a 25-m capillary column internally coated with CP-WAX 57 CB (Chrompack, Inc., Raritan, NJ). The retention times relative to 5α-cholestane (7.8 min) of the TMS-ether of cholesterol was 1.66; TMS-ether of 7-dehydrocholesterol was 2.26; and TMS-ether of 8-dehydrocholesterol was 1.89. The structures of 7-dehydrocholesterol, 8-dehydrocholesterol, and cholesterol were confirmed by mass-spectroscopy (7, 18).

Preparation of liver microsomes. Aliquots of liver (50–500 mg) were homogenized in 0.25 M sucrose solution. Microsomes were prepared from the whole homogenate by differential ultracentrifugation at 10,000 *g* for 20 min to remove the nuclei, cell debri, and mitochondria, followed by centrifugation of the supernatant at 100,000 *g* for 90 min. The microsomal pellet was removed, suspended in buffer containing 100 mM K_2HPO_4, pH 7.3; 1 mM EDTA; 5 mM DTT; 50 mM KCl; and 20% glycerol and the protein content determined by the Lowry method (19). The 100,000 *g* supernatant was retained and the protein concentration also determined (19) to be used in the enzyme assays.

Assay of lathosterol 5-dehydrogenase activity. [3α-^{3}H]Lathosterol (30 nmol, 25,000 cpm) was solubilized with 6 μl of a 33% solution of β-cyclodextrin (2-hydroxypropyl-β-cyclodextrin; Pharmatec, Inc., Alachua, FL) and incubated in a final volume of 150 μl that contained: phosphate buffer, 100 mM K_2HPO_4, pH 7.3; 0.1 mM EDTA; 1 mM DTT; 30 mM nicotinamide; NADPH generating system: 3.4 mM $NaDP^+$, 30 mM glucose 6-phosphate, and 0.3 IU glucose 6-phosphate dehydrogenase. The reaction was initiated by the addition of 0.1 mg of microsomal protein supplemented with 0.5 mg of cytosol protein (100,000 *g* supernatant). The mixture was incubated in air at 37°C for periods ranging from 5 to 60 min. The reaction was stopped by adding 50 μl aqueous NaOH (50%) so that the final mixture contained 12.5% NaOH. The reaction products were extracted with ethyl acetate and separated by argentation TLC on plates containing $AgNO_3$ in Silica Gel G developed with chloroform/acetone (57:2, vol/vol). The individual sterols: lathosterol, R_f 0.37; 7-dehydrocholesterol, R_f 0.09; 8-dehydrocholesterol, R_f 0.14; and cholesterol, R_f 0.31 were scraped into counting vials. A representative argentation thin-layer chromatogram that illustrates the separated sterols is shown in Fig. 2. Radioactivity was measured by liquid scintillation spectroscopy by dissolving the sterols in Ecolume (ICN Radiochemicals, Irvine, CA). The mass of each product was calculated from the recovered radioactivity divided by the initial specific activity of the substrate. The enzyme specific activity was measured after 5 min incubation when both curves were still linear and expressed as picomole-s of product (formed) per milligram of microsomal protein per minute.

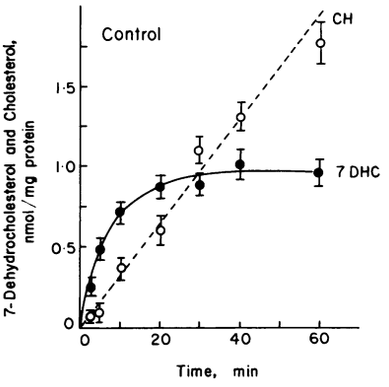

Figure 3. Conversion of [3α-^{3}H]lathosterol to 7-dehydrocholesterol (*7DHC*) and cholesterol (*CH*) by control microsomes. Each point represents the mean±SD for seven subjects. 7-Dehydrocholesterol was produced rapidly after the reaction was started, and its mass increased linearly over 5 min. Cholesterol was not formed abundantly until after 10 min and then was synthesized linearly from newly formed 7-dehydrocholesterol over the next 50 min. A precursor–product relationship was demonstrated between 7-dehydrocholesterol and cholesterol.

Crystallization of reaction products: [^{3}H]7-dehydrocholesterol and [^{3}H]cholesterol. After 60 min incubation, the reaction products, [^{3}H]7-dehydrocholesterol and [^{3}H]cholesterol, were isolated individually from the incubation mixture by preparative argentation TLC. Each radioactive compound was taken up in 2 ml of chloroform and washed once with 1 ml water to remove residual silver nitrate, and then evaporated to dryness. About 10 mg of unlabeled authentic cholesterol or 7-dehydrocholesterol (Aldrich Chemical Co.) were added to the tube containing the respective labeled compound. The sterols were dissolved in 1 ml boiling methanol and allowed to stand at room temperature for 2 h at which time newly formed crystals were filtered and washed with 1 ml cold methanol. After drying under N_2 at 60°C for 2 h, the crystals were weighed, dissovled in 2 ml methanol, and the radioactivity in an aliquot (100 μl) was determined by scintillation spectroscopy. The remaining solution was concentrated to 0.5 ml and allowed to stand at room temperature for 2 h. The newly formed crystals were filtered, weighed, dissolved in 2 ml methanol, and radioactivity again determined on a 100-μl aliquot by scintillation spectroscopy. A third crystallization was repeated similarly.

Assay of 7-dehydrocholesterol-Δ^7-reductase activity. [1,2-^{3}H]7-Dehydrocholesterol (30 nmol, 10,000 cpm) was solubilized in 6 μl of 33% aqueous cyclodextrin and incubated in a final volume of 150 μl containing: phosphate buffer, 100 mM K_2HPO_4, pH 7.3; 1 mM DTT; 30 mM nicotinamide; 0.1 mM EDTA, NADPH generating system: 3.4 mM $NADP^+$, 30 mM glucose-6-phosphate; and 0.3 IU glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of 0.1 mg of microsomal protein supplemented with 0.5 mg cytosolic protein (100,000 *g* supernatant) and continued for 90 min. The reaction was stopped by adding 50 μl of 50% aqueous NaOH. The final products, cholesterol (R_f 0.31) and 8-dehydrocholesterol (R_f 0.14) were separated from 7-dehydrocholesterol (R_f 0.09) by argentation TLC (Fig. 2) developed with chloroform/acetone, 57:2, vol/vol. Radioactivity was measured by liquid scintillation spectroscopy. The enzyme specific activity was calculated from the mass of product formed after 60 min incubation

Table I. Plasma and Liver Sterol Concentrations

	SLO	Control
Plasma	$n = 4$	$n = 7$
Total sterols±SD (mg/dl)	36±4*	150±32
Cholesterol (%)	20.1	99.9
7-Dehydrocholesterol (%)	79.9‡	trace
Liver microsomes		
Total sterols±SD (μg/mg)	15.8±0.9§	21.1±1.6
Cholesterol (%)	37.4	99.9
7-Dehydrocholesterol (%)	62.6‡	trace

* $P < 0.001$ as compared to controls. ‡ This fraction contained about 30% 8-dehydrocholesterol. § $P < 0.0002$ as compared to controls. SLO, Smith-Lemli-Opitz.

and was expressed as picomole or nanomole product/milligram microsomal protein per minute.

Statistics: Data were expressed as mean±SD. Differences were analyzed statistically by the unpaired Student's t test and by comparing confidence intervals for the means (20).

Results

In Table I the plasma and liver microsomal sterol concentrations and percent composition for seven human controls and four Smith-Lemli-Opitz homozygotes are listed. Cholesterol was the only sterol that could be measured in the plasma and liver microsomes from control subjects. Trace amounts of 7-dehydrocholesterol were barely detected but could not be quantitated by capillary GLC. In distinction, total plasma sterol levels were markedly reduced in the Smith-Lemli-Opitz homozygotes where cholesterol constituted only 20% of the total sterols, which amounted to 7.2 mg/dl. The remainder was composed chiefly of 7-dehydrocholesterol with 8-dehydrocholesterol also present (18). Liver microsomal sterol concentrations also were reduced in the specimens from the Smith-Lemli-Opitz homozygotes with cholesterol accounting for ~ 37% of the sterols; the remaining sterols were composed mainly of 7-dehydrocholesterol with 8-dehydrocholesterol also detected.

These results confirm that cholesterol biosynthesis in the Smith-Lemli-Opitz homozygotes was interrupted as evidenced by markedly reduced plasma and liver microsomal cholesterol concentrations and the substantial accumulation of the precursor, 7-dehydrocholesterol (7, 8).

Shown in Fig. 3 is the transformation of [3α-^{3}H]lathosterol, the intermediate that precedes 7-dehydrocholesterol in the pathway (Fig. 1), to cholesterol by liver microsomes from 7 control subjects. Radioactive 7-dehydrocholesterol was formed almost immediately after adding the microsomal enzyme preparation to the [3α-^{3}H]lathosterol substrate. The production of newly synthesized [^{3}H]7-dehydrocholesterol increased linearly over the first 5 min after which labeled cholesterol appeared in the reaction mixture and the slope of the [^{3}H]7-dehydrocholesterol curve flattened. The cholesterol mass rose linearly for the final 50 min and intersected with the curve for 7-dehydrocholesterol at its maximum revealing a precursor–product relationship between the two sterols. No 8-dehydrocholesterol was detected in the seven control microsomal incubations.

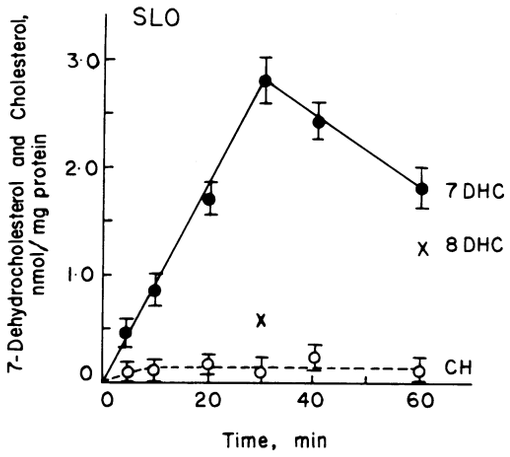

Figure 4. Conversion of [3α-^{3}H]lathosterol to 7-dehydrocholesterol (*7DHC*) and cholesterol (*CH*) by Smith-Lemli-Optiz liver microsomes. Mean±SD are presented for four subjects. 7-Dehydrocholesterol was produced abundantly over 30 min while virtually no cholesterol was formed. A small quantity of 8-dehydrocholesterol (*8DHC*) first appeared at 30 min with increased amounts at 60 min.

Presented in Figure 4 is the conversion of [3α-^{3}H]-lathosterol to cholesterol by liver microsomes from four Smith-Lemli-Opitz homozygotes. Although 7-dehydrocholesterol was produced efficiently and its mass increased linearly for 30 min, little cholesterol was formed by the Smith-Lemli-Opitz microsomes during the entire 1 h-incubation. As a consequence, these microsomes accumulated three times more 7-dehydrocholesterol by 30 min than controls. The reaction rates for lathosterol 5-dehydrogenase that catalyzed the conversion of lathosterol to 7-dehydrocholesterol were calculated after 5 min of incubation when both product curves were linear and were almost equal in the control and Smith-Lemli-Opitz homozygotes microsomes: 120±8 pmol/mg protein per min compared to 100±7 pmol/mg protein per min (Table II). Thus, the abundant formation of 7-dehydrocholesterol and similarity of lathosterol 5-dehydrogenase activities in both microsomal preparations are evidence that Smith-Lemli-Opitz liver microsomes are as viable as controls.

At the completion of 60 min, the radioactive products (cholesterol and 7-dehydrocholesterol) were isolated individually from a control incubation by argentation TLC and the radioactive sterols combined with ~ 10 mg of either authentic cholesterol or 7-dehydrocholesterol. Each sterol product was crystallized sequentially three times from methanol. The specific activities (cpm/mg) after each crystallization are reported in Table

Table II. Microsomal Enzyme Activities

Enzyme	SLO	Control
	$n = 4$	$n = 7$
	pmol/mg protein per min±SD	
Lathosterol-5-dehydrogenase	100±7	120±8
7-Dehydrocholesterol-Δ^7-reductase	40±4*	365±4

* $P < 0.0001$ as compared to controls. SLO, Smith-Lemli-Opitz.

Table III. Sterol-specific Activities After Crystallization

Sterols	Crystallization	mg	cpm/mg
7-Dehydrocholesterol	1st	5.4	8,200
	2nd	3.2	8,100
	3rd	2.1	8,100
Cholesterol	1st	5.1	8,600
	2nd	2.7	8,600
	3rd	1.4	8,600

III and remained constant. This finding indicated that isolated radioactive 7-dehydrocholesterol and cholesterol were pure and were not contaminated with other radioactive material.

Another noteworthy point is that a new sterol, radioactive 8-dehydrocholesterol, appeared in the Smith-Lemli-Opitz reaction mixtures (Fig. 4) at 30 min and increased in concentration at 60 min which coincided with the decline in 7-dehydrocholesterol levels. Since cholesterol formation was minimal and 8-dehydrocholesterol was separated cleanly from other sterols by argentation TLC (Fig. 2), radioactive 8-dehydrocholesterol was newly synthesized from 7-dehydrocholesterol probably by the induction of a microsomal enzyme that becomes active after 30 min incubation and isomerizes the double bond at C-7[8] to C-8[9].

To specifically measure 7-dehydrocholesterol-Δ^7-reductase activity, microsomes were prepared from seven controls and four Smith-Lemli-Opitz homozygotes livers and incubated with synthetic [1,2-^{3}H]7-dehydrocholesterol under optimal conditions for 90 min (Fig. 5). Cholesterol was formed efficiently and linearly over 90 min from [1,2-^{3}H]7-dehydrocholesterol by the control liver microsomes. In distinction, Smith-Lemli-Opitz liver microsomes barely produced cholesterol. After 30 min and 60 min, when both reactions were linear, 11.8 and 9.1 times more cholesterol were formed, respectively, by controls than homozygotes liver microsomes. As a consequence, Smith-Lemli-Opitz liver microsomal 7-dehydrocholesterol-Δ^7-reductase activity determined after 60 min was 1/9 as active as control ($P < 0.0001$), although lathosterol 5-dehydrogenase activities were about equal in both microsomal preparations (Table II). Thus, the accumulation of 7-dehydrocholesterol and reduced cholesterol levels in the plasma and tissues of Smith-Lemli-Opitz homozygotes can be attributed to the severe deficiency of 7-dehydrocholesterol-Δ^7-reductase activity inherited in liver microsomes.

Discussion

The results of this investigation conclusively demonstrate that 7-dehydrocholesterol-Δ^7-reductase activity is inhibited markedly in liver microsomes from four Smith-Lemli-Opitz homozygotes. In these subjects, who exhibited typical clinical phenotypes with facial dysmorphism, mental retardation, and multiple congenital organ anomalies, the biochemical abnormalities of low plasma and tissue cholesterol combined with elevated 7-dehydrocholesterol concentrations can be explained by the inability to convert 7-dehydrocholesterol to cholesterol because of deficient microsomal 7-dehydrocholesterol-Δ^7-reductase activity that is inherited. Moreover, these findings support the

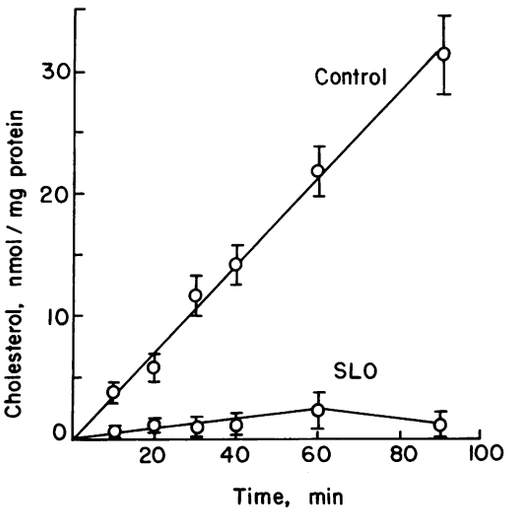

Figure 5. Conversion of [1,2-^{3}H]7-dehydrocholesterol to cholesterol by control ($n = 7$) and Smith-Lemli-Opitz ($n = 4$) liver microsomes. Mean±SD are plotted. Cholesterol was produced linearly over 90 min in the control incubations while little cholesterol was formed from 7-dehydrocholesterol by the Smith-Lemli-Opitz microsomes.

role of 7-dehydrocholesterol as the penultimate precursor in the cholesterol biosynthetic pathway (21). No desmosterol or its immediate precursor 5,7,24-cholestatrien-3β-ol were detected. These Δ^{24} precursors would have been separated and quantitated by the argentation TLC (Fig. 2, *compound III*), and capillary GLC systems used for sterol measurements. Although the absence of desmosterol or its 7-dehydro precursor does not rule out a role for these Δ^{24}-sterols in the cholesterol biosynthetic pathway, it argues for the earlier reduction of the double bond at C-24, in lanosterol to form 24[25]-dihydrolanosterol in the cholesterol biosynthetic pathway as was shown to occur in another inherited inborn error disease with active cholesterol biosynthesis: cerebrotendinous xanthomatosis (22, 23). However, it is important to emphasize that the formation of desmosterol would require active 7-dehydrocholesterol-Δ^7-reductase and if defective, as in Smith-Lemli-Opitz homozygotes, we would expect the accumulation of 5,7,24-cholestatrien-3β-ol which did not occur. Both lathosterol and 7-dehydrocholesterol which follow lanosterol were shown to be key intermediates in the cholesterol biosynthetic pathway (11–13, 24), and control liver microsomes rapidly and efficiently transformed both sterols to cholesterol. Therefore, we believe that most cholesterol biosynthesis occurs via 7-dehydrocholesterol and not desmosterol.

In the conversion of lathosterol to cholesterol, the reaction proceeded via 7-dehydrocholesterol and was catalyzed by the enzyme, lathosterol 5-dehydrogenase, which is located in liver microsomes. It was, therefore, noteworthy that [^{3}H]lathosterol was transformed equally well to 7-dehydrocholesterol by both control and Smith-Lemli-Opitz microsomes (Figs. 3 and 4); and that the reaction rates or specific activities of microsomal lathosterol 5-dehydrogenase were similar in control and Smith-Lemli-Opitz microsomes (Table II). The fact that lathosterol 5-dehydrogenase activities were nearly equal is evidence that Smith-Lemli-Opitz hepatic microsomes were as viable as controls.

With respect to the assay for lathosterol 5-dehydrogenase

activity, oxygen was required and 100,000 *g* supernatant (cytosolic) proteins were necessary to stabilize the microsomal enzyme and solubilize the substrate for maximum enzyme activity especially when small quantities of microsomal protein were used. Similar conditions were reported by Dempsey et al. for the assay of rat liver microsomal lathosterol 5-dehydrogenase (25). However, it is important to emphasize that no enzyme activity was detected in the cytosolic 100,000 *g* protein fraction alone. The microsomal enzyme catalyzes the insertion of a double bond at C-5[6] in lathosterol to form 7-dehydrocholesterol (Fig. 1) and according to Gaylor et al. reacts stereospecifically with lathosterol and will not transform the putative cholesterol precursor, Δ^8-cholestenol, (5, 6). Control microsomes continued the reaction sequence and converted newly formed 7-dehydrocholesterol to cholesterol, indicative of active microsomal 7-dehydrocholesterol-Δ^7-reductase activity. This reaction became evident 10 min after incubation when the supply of newly synthesized 7-dehydrocholesterol, its substrate, became saturating and the formation of the product, cholesterol, increased linearly for the next 50 min with a precursor-product relationship demonstrated between 7-dehydrocholesterol and cholesterol. In contrast, Smith-Lemli-Opitz microsomes were unable to complete the reaction sequence and little cholesterol was produced so that newly formed 7-dehydrocholesterol accumulated in homozygotes microsomes which after 30 min contained three times more 7-dehydrocholesterol than controls (Figs. 3 and 4). Interestingly, the concentration of 7-dehydrocholesterol declined after 30 min and a new sterol, [^{3}H]8-dehydrocholesterol, appeared with increased amounts at 60 min in the Smith-Lemli-Opitz microsomal reaction mixtures (18). We believe that 8-dehydrocholesterol was newly formed from 7-dehydrocholesterol because of the induction of a microsomal enzyme that isomerizes the double bond at C-7[8] to C-8[9] and becomes active when 7-dehydrocholesterol can not be converted to cholesterol. This reaction would explain the presence of 8-dehydrocholesterol in the plasma and tissues of the Smith-Lemli-Opitz homozygotes and its absence from controls (Table I). However, preliminary evidence suggests that 8-dehydrocholesterol is not a direct precursor of cholesterol, although Paik et al. have shown that the isomerase catalyzed reaction is reversible (24). Control microsomes did not produce detectable quantities of 8-dehydrocholesterol.

It is also important to emphasize that the radioactive products formed from a microsomal incubation with [^{3}H]lathosterol, 7-dehydrocholesterol, and cholesterol were isolated individually by preparative argentation TLC (Fig. 2) and each sterol crystallized three times. The constant specific activities (Table III) indicated that no other radioactive contaminants were present in these newly formed sterols.

With respect to the assay of 7-dehydrocholesterol-Δ^7-reductase activity, control microsomes formed cholesterol from 7-dehydrocholesterol efficiently and linearly for 90 min (Fig. 5). In contrast, Smith-Lemli-Opitz microsomes were barely able to produce cholesterol so that after 30 and 60 min when the formation of cholesterol in both reactions was linear, control microsomes produced substantially more cholesterol (Fig. 5) and the microsomal activity (pmol/mg protein per min) of 7-dehydrocholesterol-Δ^7-reductase was ninefold higher in controls (Table II). For optimum assay conditions, nicotinamide, NADPH, and cytosolic (100,000 *g*) proteins were all required to maximize enzyme activity when small amounts of microsomal protein were used as the enzyme source. Again, 7-dehydrocholesterol-Δ^7-reductase activity was not present in the cytosolic protein.

Finally, it is necessary to point out that the small amount (1.4 nmol) of endogenous 7-dehydrocholesterol present in 0.1 mg of microsomal protein from the Smith-Lemli-Opitz liver reduced the final specific activity of the radioactive substrate 4.6% and was corrected for in the final calculation of cholesterol product formed.

Recently, Honda et al. reported decreased formation of cholesterol and the accumulation of 7-dehydrocholesterol in cultured fibroblasts from Smith-Lemli-Opitz homozygotes (26). This indicated that the enzyme defect could be detected in other tissues from homozygotes.

In summary, these results demonstrate that the deficiency of cholesterol and accumulation of 7-dehydrocholesterol in the plasma and tissues of Smith-Lemli-Opitz homozygotes result from markedly decreased 7-dehydrocholesterol-Δ^7-reductase activity. This enzyme is located in hepatic microsomes, requires NADPH and soluble cytosolic proteins for stability and solubilization of substrate and is 1/9 as active in Smith-Lemli-Opitz homozygotes as controls.

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