

Inverse relationship between plasma cholestanol concentrations and bile acid synthesis in sitosterolemia

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Abstract We investigated the relationship between plasma cholestanol (5 α -dihydrocholesterol) concentrations and the activity and mRNA levels of cholesterol 7 α -hydroxylase, the rate-controlling enzyme for bile acid synthesis, in three female sitosterolemic homozygotes. In this lipid storage disease, large amounts of plant sterols and cholestanol accumulate because of hyperabsorption and endogenous synthesis, respectively. Plasma cholestanol concentrations were 14 times greater in the three sitosterolemic homozygotes than the mean for five control subjects. To investigate the cholestanol biosynthetic pathway, tracer doses of two putative precursors, [1,2-³H]4-cholesten-3-one and [4-¹⁴C]7 α -hydroxycholesterol were injected intravenously into a homozygote, and radioactivity was sought in cholestanol, bile acids, cholesterol, and sitosterol fractions isolated from plasma and bile. Tritium was concentrated only in cholestanol; neither cholesterol, sitosterol nor bile acids were derived from [1,2-³H]4-cholesten-3-one. In contrast, bile acids were labeled exclusively with ¹⁴C from [4-¹⁴C]7 α -hydroxycholesterol; no ¹⁴C radioactivity was detected in cholestanol. Mathematical analysis of specific activity versus time curves for [³H]cholestanol revealed very slow decay, large exchangeable pools, and enhanced synthesis in the sitosterolemic homozygote. Measurements of cholesterol 7 α -hydroxylase activity were 39% lower in whole liver microsomes from three sitosterolemic homozygotes that contained 19% plant sterols as compared to the mean value for six control microsomal specimens that contained 0.1% plant sterols. Removal of the excess plant sterols from the microsomes, in vitro, normalized microsomal cholesterol 7 α -hydroxylase activity in the homozygotes but did not affect enzyme activity in the controls. Equal amounts of cholesterol 7 α -hydroxylase mRNA were detected in the livers of both control and sitosterolemic subjects. Bile acid malabsorption after ileal bypass surgery stimulated cholesterol 7 α -hydroxylase activity 78% in sitosterolemic whole liver microsomes and reduced plasma cholesterol, sitosterol, and cholestanol levels 61%, 55% and 91%, respectively, producing a pronounced decrease in the cholestanol/cholesterol ratio without changing the sitosterol/cholesterol ratio. These results demonstrate that increased cholestanol is synthesized from 4-cholesten-3-one and not 7 α -hydroxycholesterol in sitosterolemia. Enhanced pools and plasma concentrations are related inversely to hepatic cholesterol 7 α -hydroxylase activity. Competitive inhibition of cholesterol 7 α -hydroxylase by the large microsomal plant sterol pool diverts cholesterol into cholestanol. Alternatively, stimulating cholesterol 7 α -hydroxylase activity after ileal bypass surgery markedly diminished plasma

cholestanol levels. We propose that increased cholestanol is synthesized in sitosterolemic subjects because cholesterol 7 α -hydroxylase is competitively inhibited by the large microsomal sitosterol pool so that cholesterol that cannot be transformed to bile acids is available as substrate for cholestanol formation. —Salen, G., A. K. Batta, G. S. Tint, S. Shefer, and G. C. Ness. Inverse relationship between plasma cholestanol concentrations and bile acid synthesis in sitosterolemia. *J. Lipid Res.* 1994. 35: 1878–1887.

Supplementary key words cholesterol 7 α -hydroxylase activity and mRNA levels • cholesterol • sitosterol

Small amounts of cholestanol, the 5 α -dihydro derivative of cholesterol, accompany cholesterol in almost every mammalian tissue. Recently, extraordinary quantities of cholestanol were discovered in subjects with two rare lipid storage diseases, cerebrotendinous xanthomatosis (CTX) and sitosterolemia (1–4) and in patients with cholestatic liver diseases (5). In the former condition (CTX), progressive neurologic dysfunction, cataracts, atherosclerosis, pulmonary insufficiency, and xanthomatous deposits in brain and tendons develop. Plasma and most tissue cholestanol levels are moderately elevated (2% of total sterols) with greater enrichment in brain, nerve, xanthomas, and bile where from 10 to 50% of the tissue sterols are cholestanol (5–7). Increased cholestanol production is related to defective bile acid synthesis (1, 6, 8). Treatment with chenodeoxycholic acid suppresses abnormal bile acid synthesis and reduces cholestanol synthesis and plasma levels in CTX (5, 9, 10).

Abbreviations: cholestanol, 5 α -cholestan-3 β -ol; cholesterol, 5-cholesten-3 β -ol; CTX, cerebrotendinous xanthomatosis; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; HMG-CoA, hydroxymethylglutaryl coenzyme A; TMS, trimethylsilyl.

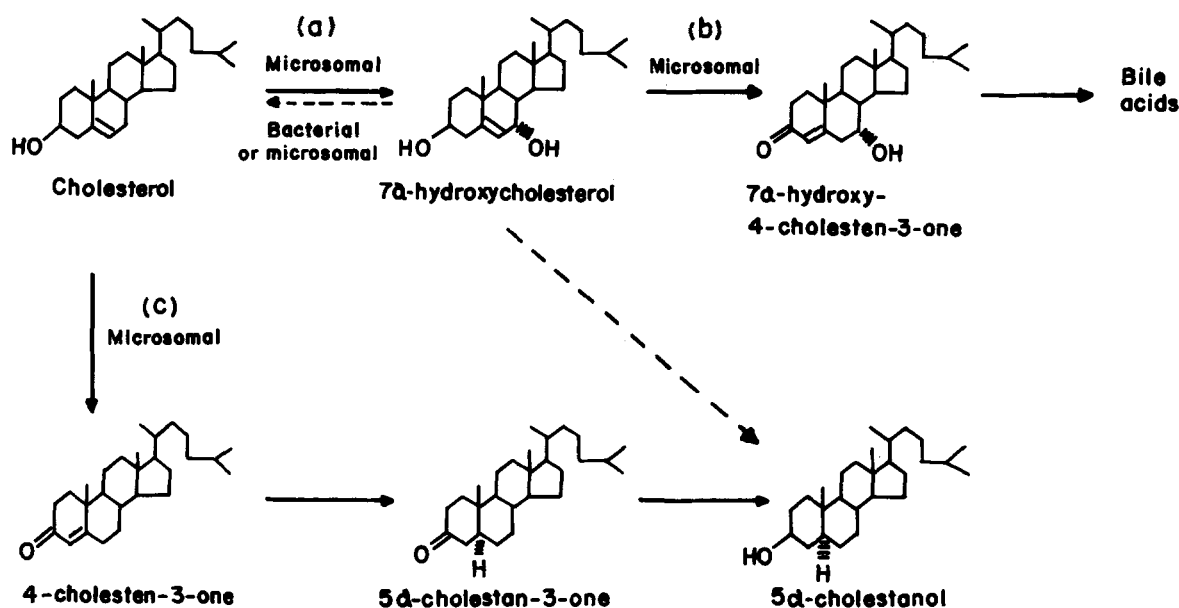


Fig. 1. Biosynthetic pathways for cholestanol and bile acids. Early reactions in the transformation of cholesterol to bile acids are catalyzed by the microsomal enzymes, cholesterol 7 α -hydroxylase (a) and 7 α -hydroxycholesterol-3 β -hydroxy- $\Delta^{4,5}$ -steroid dehydrogenase-isomerase (b). The 7-dehydroxylation step is a well-known bacterial reaction but also may be carried out by reversal of microsomal cholesterol 7 α -hydroxylase (a) as indicated by the dashed arrow (\rightarrow). The key step in the transformation of cholesterol to cholestanol involves the formation of 4-cholesten-3-one by the microsomal enzyme 3 β -hydroxy- $\Delta^{4,5}$ -steroid dehydrogenase-isomerase (c). The reaction may be catalyzed by the same microsomal enzyme active in bile acid synthesis (b). The possibility that either 7 α -hydroxycholesterol or 7 α -hydroxy-4-cholesten-3-one is transformed directly to cholestanol in CTX also exists (21).

In sitosterolemia, tendon xanthomas, hemolytic episodes, painful joints, and accelerated coronary atherosclerosis often complicated by lethal myocardial infarctions are the principal clinical manifestations (1, 3, 4, 11). Large quantities of plant sterols (campesterol and sitosterol) are found together with 5 α -stanols (cholestanol, 5 α -campestanol, and 5 α -sitostanol) in all tissues except brain (2, 4, 11). Although enhanced intestinal absorption combined with impaired sterol elimination account for the high levels of unsaturated plant sterols in sitosterolemia (12–17), the increased deposits of cholestanol probably result from endogenous biosynthesis as diets contain only very small quantities of cholestanol (2).

According to the classical pathway (**Fig. 1**), cholestanol is formed from cholesterol via 4-cholesten-3-one and 5-cholesten-3-one (18–20). The conversion of cholesterol to 4-cholesten-3-one (**Fig. 1c**) is considered the rate-determining step and the reaction is catalyzed enzymatically by a 3 β -hydroxy- $\Delta^{4,5}$ -steroid dehydrogenase-isomerase¹ located in hepatic microsomes (20). Similar hepatic microsomal 3 β -hydroxy- $\Delta^{4,5}$ -steroid dehydro-

genase-isomerase activity is required for bile acid synthesis in the transformation of 7 α -hydroxycholesterol to 7 α -hydroxy-4-cholesten-3-one (**Fig. 1b**). This latter compound is the last intermediate common to both the cholic acid and chenodeoxycholic acid biosynthetic pathways. It has not been established in humans whether hepatic microsomal 7 α -hydroxycholesterol-3 β -hydroxy- $\Delta^{4,5}$ -steroid dehydrogenase-isomerase active in bile acid synthesis is the same enzyme that catalyzes the conversion of cholesterol to 4-cholesten-3-one (18).

Recently, an alternative pathway for cholestanol biosynthesis that includes 7 α -hydroxycholesterol as an intermediate has been suggested in CTX by Skrede et al. (21). These investigators (22) postulate that because hepatic cholesterol 7 α -hydroxylase activity is increased and the conversion of distal bile acid precursors is blocked in CTX (1), excess 7 α -hydroxycholesterol accumulates and is partly converted to cholestanol. Other putative intermediates in the alternative pathway include 7 α -hydroxy-4-cholesten-3-one and 4,6-cholestadien-3-one (21).

In this study, we investigated the formation of cholestanol in a sitosterolemic homozygote where abundant cholestanol was detected in plasma. Our objective was to examine the pathway by testing the transformation of tracer doses of two putative precursors, 4-cholesten-3-one and 7 α -hydroxycholesterol, into cholestanol and bile acids. Mathematical analysis of a specific activity versus time decay curve for cholestanol gave a quantitative esti-

¹The microsomal 3 β -hydroxy- $\Delta^{4,5}$ -steroid dehydrogenase-isomerase activity referred to in this paper is believed to be carried out by a single enzyme. However, as the enzyme has not been isolated and purified, it is possible that two separate microsomal enzymes carry out these reactions: a microsomal 3 β -hydroxysteroid dehydrogenase in combination with a microsomal $\Delta^{4,5}$ -3-oxosteroid isomerase.

mate of cholestanol production (synthesis) that was correlated with hepatic microsomal cholesterol 7 α -hydroxylase activity measured in three sitosterolemic homozygotes and mRNA levels determined in one subject. The latter enzyme catalyzes the conversion of cholesterol to 7 α -hydroxycholesterol, the rate-controlling step in bile acid biosynthesis. Further, the effect of ileal bypass surgery on plasma cholestanol levels and hepatic microsomal cholesterol 7 α -hydroxylase activity in these sitosterolemic subjects was examined.

METHODS

Studies were conducted in three homozygous sitosterolemic sisters. Patient KeC is a 20-year-old woman who shows xanthomas on the extensor tendons of the hands and an aortic systolic murmur. KCN is her 26-year-old sister and manifests an aortic systolic murmur, coronary atherosclerosis, Achilles tendon, and tuberous xanthomas. TC is a 24-year-old sister with an aortic systolic murmur and recurrent bouts of arthritis. The homozygous brother of these sisters died of an acute myocardial infarction at age 17 (11). Because of symptomatic coronary atherosclerosis as determined by coronary angiography and the inability to tolerate bile acid binding resins, ileal bypass surgery was performed in KCN in September 1985, TC in March 1989, and in KeC in November, 1992. No post-operative complications occurred. Detailed descriptions of these patients have appeared elsewhere (1). Five age-matched female subjects who were healthy served as control subjects for plasma sterol and stanol determinations.

All subjects were fed regular diets that contained approximately 45% of the calories as carbohydrates, 15% protein, and 40% fat; cholesterol intake was approximately 500 mg/day and sitosterol intake was 100 mg/day. The caloric consumption was adjusted to maintain constant weight throughout each study.

A surgical liver biopsy (approximately 1 g) was obtained from each sitosterolemic subject during ileal bypass surgery. Nine months later, a percutaneous needle liver biopsy (58 mg) was performed in KCN. After diagnostic histologic evaluation, a portion of the liver tissue was used for measurements of sterol concentrations, composition, and microsomal cholesterol 7 α -hydroxylase activity. For controls, six liver specimens were obtained from the Liver Tissue Procurement and Distribution System, University of Minnesota Hospital, Minneapolis, MN, (NIH contract No. DK-62274) which makes livers available when no suitable liver transplant recipient can be found.

The experimental protocols were approved by the Human Study Committees of the Veterans Affairs Medical Center, East Orange, NJ and the University of Medicine

and Dentistry of New Jersey-New Jersey Medical School, Newark, NJ.

Experimental design

Patient KeC (sitosterolemia) received 9.1 μ Ci of [1,2- 3 H]4-cholesten-3-one and 3.8 μ Ci of [4- 14 C]7 α -hydroxycholesterol. The radioactive steroids were dissolved in 1 ml ethanol, dispersed in 150 ml physiologic saline, and then immediately infused intravenously. Specimens of plasma were obtained at 2, 7, and 11 days and of bile 7 days after pulse-labeling for the isolation of cholestanol, cholesterol, sitosterol, and bile acids that were purified and assayed for radioactivity and mass. Additional plasma specimens from which only cholestanol was isolated for specific activity determination were taken on days 27, 55, 60, and 67 after pulse-labeling. The demonstration of 3 H or 14 C in cholestanol, cholesterol, or bile acids would indicate that the radioactive steroids were precursors in the pathway. However, it is important to emphasize that the transformation of an injected tracer into a product does not necessarily define the pathway or prove the quantitative importance of the intermediate. However, the absence of label in the end product indicates that the tracer probably is not a precursor in the pathway provided the injected tracer mixes with its endogenous metabolic pool.

In separate experiments, cholestanol turnover and exchangeable pools were calculated from mathematical analysis of the normalized cholestanol specific activity versus time decay curve in the sitosterolemic subject (KeC) (14–17). Similar estimates reported previously (8) in a CTX subject and five healthy subjects served as control values for comparison. Normalized specific activity is calculated by dividing the measured specific activity (dpm/mg) on the day isolated after pulse-labeling by the specific activity at 0 time multiplied by 100.

Hepatic cholesterol 7 α -hydroxylase activity was measured in whole microsomes and in acetone-treated microsomes to remove all endogenous sterols from the three sitosterolemic homozygote and six transplant donor specimens (23, 24). Acetone treatment removes virtually all endogenous sterols, therefore, the microsomes were reconstituted with optimum amounts of cholesterol and cofactors to perform the assay for cholesterol 7 α -hydroxylase activity. Using separate aliquots, microsomal sterol concentrations and composition were determined before and after acetone treatment.

Steroid analysis

Specimens of plasma (3–10 ml) or bile (1 ml) were saponified with 1 N NaOH, and the neutral sterols were extracted with hexane. After the solvent was evaporated, the sterols were separated on thin-layer plates coated with AgNO₃-Silica Gel G (10% w/w), and developed in chloro-

form-acetone 97:3 (vol/vol) at 4°C. The fractions (cholesterol, R_f 0.30 and cholestanol, R_f 0.35) were identified and eluted separately with ethyl ether. Individual sterols and stanols from each fraction were further purified by high performance liquid chromatography (HPLC) (Waters Associates ALC 201 system Milford, MA) equipped with a model 401 refractive index detector and a radial-pack μ Bondapak C₁₈ reversed phase column (10 μ particle size). The mobile phase consisted of methanol-chloroform-water 90:2:10 (v/v/v), at a flow rate of 2 ml/min, operating pressure 2000 psi. The HPLC retention volume for cholesterol was 18.8 ml, cholestanol 20.2 ml, sitosterol 24.8 ml, and 7 α -hydroxycholesterol 16.2 ml.

A measured portion of each fraction isolated by HPLC was assayed for radioactivity, and the mass was quantitated by capillary gas-liquid chromatography (GLC) as the TMS-ether derivative on a 25-m column internally coated with CP Wax 57 CB (Chrompack, Bridgewater, NJ) (24). The coefficients of variation calculated for cholesterol, sitosterol, and cholestanol were 8.4%, 6.1%, and 20.1%, respectively, in untreated plasma and 10%, 15%, 24%, respectively, after ileal bypass surgery.

Bile acids were isolated from the bile after rigorous saponification in 3 N NaOH (20). The deconjugated bile acids were methylated and purified by TLC on plates coated with Silica Gel H developed in chloroform-methanol 70:25:5 (v/v/v). The R_f value for methyl cholate was 0.30, and for methyl deoxycholate, 0.60. An aliquot of each fraction was assayed for radioactivity and the mass was quantitated by capillary GLC as TMS-ether derivatives on a 25-m capillary column internally coated with CP Sil 5 CB (Chrompack, Bridgewater, NJ). 5 α -Cholestane was added as an internal standard. About 0.1 μ g of sterols or bile acids was injected for GLC analysis.

Synthesis of labeled precursors

[1,2-³H]4-cholesten-3-one. [1,2-³H]cholesterol (5 mg, 50 μ Ci) was dissolved in acetone (1 ml) and cooled to 0°C. Jones' reagent (0.1 ml, prepared by adding 2.2 gram chromium trioxide into 2.8 ml concentrated H₂SO₄ and water to a final volume of 10 ml) was added and the solution was warmed to room temperature (15 min). The reaction mixture was then diluted with water and extracted with n-hexane. The hexane layer was washed with water, dried over anhydrous sodium sulfate, and solvent was evaporated to dryness at 40°C under N₂. The white residue of [1,2-³H]4-cholesten-3-one was crystallized from methanol as colorless microscopic needles (yield 3 mg; melting point = 79–80°C, sp act 10 μ Ci/mg) and was found to be greater than 98% pure as determined by TLC (solvent system: chloroform, R_f 0.50).

[4-¹⁴C]7 α -hydroxycholesterol. A solution of [4-¹⁴C]cholesterol (15 mg, 100 μ Ci) in pyridine (0.5 ml) and acetic anhydride (0.5 ml) was allowed to stand overnight at room

temperature and was then diluted with 10 ml ice-cold water. The precipitated [14-¹⁴C]cholesterol acetate was filtered, washed with water, and dried. The solid was dissolved in acetic acid (4 ml) at 50°C and 20 mg chromium trioxide was added over a period of 5 min. The contents were stirred for 30 min at 50°C, then cooled and diluted with 25 ml cold water. The precipitate was filtered, washed with water, dried, and crystallized from methanol to yield about 4 mg of [4-¹⁴C]7-ketocholesterol acetate. The ketoacetate was dissolved in 5 ml methanol by warming and then cooled to about 35°C, and 4 mg sodium borohydride was added with stirring. The reaction mixture was allowed to stand overnight at room temperature; then 1 ml of 10% aqueous KOH was added and the contents were refluxed for 30 min. The methanol was evaporated at 55–60°C under a current of N₂ with simultaneous addition of water. The reaction mixture was cooled to 10°C and filtered. The white solid obtained was washed with water and dried. The product showed two spots on TLC when developed in ethyl ether: R_f 0.40 and 0.55, respectively. The slower moving compound was found to be identical with authentic 7 α -hydroxycholesterol whereas the faster moving compound migrated with authentic 7 β -hydroxycholesterol. [4-¹⁴C]7 α -hydroxycholesterol was obtained from this mixture by preparative TLC (solvent system, ethyl ether). It was found to be greater than 99% pure as determined by TLC (melting point = 185°C) with a final sp act of 6.3 μ Ci/mg.

Northern blot analysis

Total RNA from samples of frozen liver was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method. Portions of frozen liver, 0.2 g, were homogenized in 2 ml of TRI reagent (room temperature) purchased from Molecular Research Center, Cincinnati, OH, using a Polytron Tissue Disrupter at full speed for 5–10 sec. After 5 min, 0.4 ml chloroform was added, the sample was mixed vigorously and centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase was removed and 1 ml isopropanol was added. The samples were mixed and allowed to stand at room temperature for 5–10 min, then centrifuged at 12,000 g for 10 min at 4°C. The pelleted RNA was then washed in 75% ethanol. The total RNA pellet was dissolved in 100 μ l diethylpyrocarbonate-treated water. Poly A⁺ RNA was isolated by oligo (dT) cellulose chromatography. The relative levels of cholesterol 7 α -hydroxylase mRNA were quantitated by Northern blotting analysis using a Rad Roller hybridization oven from Hoefer Scientific Instruments. β -Actin (HH Cl 89, American Type Culture Collection, Rockville, MD) served as the internal standard. The cDNA for rat liver cholesterol 7 α -hydroxylase, 7 α 6, was a gift from Dr. J. Y. L. Chiang, Northeastern Ohio University, Rootstown, OH.

Radioactivity assay

Measured portions of the purified sterols, stanols, or bile acids were dissolved in toluene phosphor (4.2% Liquiflor, New England Nuclear, Boston, MA) and assayed for radioactivity in a Beckman Model LS-250 liquid scintillation system (Beckman Instruments, Inc., Fullerton, CA). The efficiencies for counting ^3H and ^{14}C were 51% and 71%, respectively. Samples were counted up to 100 min to record significant differences between the sample and background.

Cholesterol 7α -hydroxylase activity

Hepatic cholesterol 7α -hydroxylase activity was assayed by the isotope incorporation method according to Nicolau et al. (23) and Shefer et al. (24). In this technique, whole microsomes were isolated from liver specimens by differential ultracentrifugation and incubated with [^{14}C]cholesterol and co-factors for 30 min at 30°C . Methylene chloride-ethanol 5:1 (v/v) was added to stop the reaction and the product, [^{14}C] 7α -hydroxycholesterol, was isolated by TLC on Silica Gel G plates developed in ethyl ether at R_f 0.40. Microsomal proteins are determined on a separate aliquot (25). Enzyme activity was expressed as pmol 7α -hydroxycholesterol formed/mg microsomal protein per min.

To eliminate the competing substrate effect of microsomal plant sterols on cholesterol 7α -hydroxylase activity, microsomes were treated with ice-cold acetone to remove all endogenous sterols. The microsomal protein was reconstituted with only cholesterol and optimal amounts of co-factors and assayed for cholesterol 7α -hydroxylase activity as described above (24).

Determination of plasma or hepatic microsomal sterols

One milliliter of plasma or known aliquots of untreated or acetone-treated microsomal suspensions were refluxed

with 1 N NaOH (plasma) or 25% KOH in 95% ethanol for 3 h. Sterols were extracted with hexane and analyzed by capillary GLC on a 25-m fused silica capillary column internally coated with CP Wax 57 CB. The retention times relative to the internal standard, 5α -cholestane, were: cholestanol 1.71, cholesterol 1.86, and sitosterol 2.86.

Statistical analysis

Statistical significance was tested by the Student's t test.

RESULTS

Plasma cholesterol, sitosterol, and cholestanol concentrations are reported in **Table 1** for three sitosterolemic homozygotes and five female age-matched control subjects. In the untreated sitosterolemic subjects, plasma contained high levels of cholesterol, large quantities of sitosterol, and increased amounts of cholestanol. Control plasma contained virtually all cholesterol with only trace amounts of sitosterol and cholestanol. After ileal bypass surgery, mean values for plasma cholesterol declined 61% and sitosterol 55% while cholestanol decreased 91% in the three sitosterolemic homozygotes.

In **Table 2** are listed the plasma cholestanol/cholesterol and sitosterol/cholesterol ratios. In the three sitosterolemic homozygotes, the cholestanol/cholesterol ratio was 34 times greater and the sitosterol/cholesterol ratio 273 times larger than controls. After ileal bypass surgery, the mean cholestanol/cholesterol ratio declined 74%, while the sitosterol/cholesterol ratio did not change significantly in two homozygotes and, actually rose in subject TC. Thus, bile acid malabsorption after ileal bypass surgery decreased cholestanol disproportionately relative to cholesterol, while sitosterol declined in parallel with cholesterol.

TABLE 1. Plasma sterols and 5α -stanol concentrations^a

Subjects	n ^b	Cholesterol	Cholestanol	Sitosterol
			<i>mg/dl ± SD</i>	
Controls				
Five, female age-matched		187 ± 5	0.2 ± 0.2	0.2 ± 0.1
Sitosterolemic homozygotes				
KeC, untreated	5	220 ± 25	4.7 ± 1.00	21 ± 1.3
KeC, 9 months post ileal bypass	5	80 ± 4 ^c	0.28 ± 0.07 ^c	9.4 ± 0.8 ^c
KCN, untreated	5	217 ± 15	2.1 ± 0.4	25.2 ± 2.0
KCN, 8 yr post ileal bypass	5	92 ± 16 ^c	0.22 ± 0.03 ^c	9.1 ± 2.0 ^c
TC, untreated	5	235 ± 17	1.50 ± 0.3	21 ± 0.9
TC, 4 yr post ileal bypass	5	90 ± 7 ^c	0.21 ± 0.07 ^c	11.1 ± 1.6 ^c

^aOther sterols and 5α -stanols, campesterol, stigmasterol, avenasterol, 5α -campestanol, and 5α -sitostanol, were also present in the plasma of sitosterolemic homozygotes.

^bNumber of weekly determinations.

^c $P < 0.0001$, untreated versus treated.

TABLE 2. Plasma stanol and sterol ratios

Subjects	n ^a	Cholestanol/Cholesterol	Sitosterol/Cholesterol
Controls, five		0.0011 ± 0.0012	0.0011 ± 0.0012
Sitosterolemic homozygotes			
KeC, untreated	5	0.021 ± 0.007	0.095 ± 0.017
KeC, 9 months post ileal bypass	5	0.0035 ± 0.0011 ^b	0.118 ± 0.016 ^c
KCN, untreated	5	0.0097 ± 0.0025	0.116 ± 0.017
KCN, 8 yr post ileal bypass	5	0.0024 ± 0.0007 ^b	0.099 ± 0.039 ^c
TC, untreated	5	0.0064 ± 0.0017	0.089 ± 0.010
TC, 4 yr post ileal bypass	5	0.0023 ± 0.0012 ^b	0.123 ± 0.027 ^d

^aNumber of weekly determinations.

^b*P* < 0.005, homozygote: untreated versus ileal bypass.

^cNot significant: homozygote, untreated versus ileal bypass.

^d*P* < 0.0005, homozygote: untreated versus ileal bypass.

Clinically, ileal bypass surgery produced marked improvement in symptoms: the aortic stenosis murmurs have diminished, tendon and tuberous xanthomas are smaller, and attacks of arthritis and arthralgias no longer occur.

Two years before the surgery, homozygote KeC received a mixture of [1,2-³H]4-cholesten-3-one and [4-¹⁴C]7 α -hydroxycholesterol intravenously and cholestanol, cholesterol, and sitosterol were isolated and purified from three plasma specimens, and cholic acid from a sample of bile. The purity of the cholestanol, cholesterol, and sitosterol fractions was confirmed by HPLC and capillary GLC. In **Table 3** are given the mass and radioactivity of the sterols, cholestanol, and cholic acid and the calculated specific activities (dpm/mg). Cholestanol was highly labeled with tritium derived from [1,2-³H]4-cholesten-3-one with almost all the label concentrated in the 5 α -stanol; only traces of tritium were detected in cholesterol and none in bile acids. In contrast, cholic acid that was isolated from bile was highly radioactive from [4-¹⁴C]7 α -hydroxycholesterol. No ¹⁴C radioactivity was detected in cholestanol or cholesterol. These results demonstrate that 4-cholesten-3-one was converted efficiently into cholestanol and 7 α -hydroxycholesterol was transformed only

into bile acids. Thus, in the sitosterolemic homozygote with high plasma cholestanol levels, 7 α -hydroxycholesterol was not a precursor.

The normalized [³H]cholestanol specific activity versus time decay curve was constructed from seven determinations obtained over 67 days. The results are shown in **Fig. 2** along with normalized cholestanol decay curves published previously (8) in a CTX homozygote and the mean curve for five control subjects for comparison. As noted earlier, cholestanol decayed similarly and more rapidly in the CTX and control subjects than in the sitosterolemic homozygote. The turnover curves were divided into two exponentials and analyzed mathematically according to a two-pool model (**Table 4**). The half-life of the first exponential $t_{1/2}^1$ was nearly 4 times longer and the half-life of the second exponential $t_{1/2}^2$ was more than 10 times longer, for the sitosterolemic homozygote than for the control and CTX subjects. The daily production rate (PR_A), which is equivalent to synthesis because human diets contain virtually no cholestanol (<2 mg/day) (8), was 10 times greater than controls and twice that found in the CTX subject where cholestanol is also overproduced. More importantly, the total exchangeable cholestanol pool (M_A + M_B) was extremely large and

TABLE 3. ³H and ¹⁴C specific activities after pulse labeling with [1,2-³H]4-cholesten-3-one and [4-¹⁴C]7 α -hydroxycholesterol

Days after Pulse-Labeling	Plasma									Bile		
	Cholestanol			Cholesterol			Sitosterol			Cholic Acid		
	mg ^a	³ H	¹⁴ C	mg ^a	³ H	¹⁴ C	mg ^a	³ H	¹⁴ C	mg ^a	³ H	¹⁴ C
		dpm/mg				dpm/mg				dpm/mg		
2	0.5	1,500	—	8.1	35	—	1.3	—	—	—	—	—
4	0.4	1,000	—	7.5	90	—	1.2	—	—	3.5	—	340,000
11	0.5	700	—	7.9	—	—	0.9	—	—	—	—	—

(—) Not detected.

^aRepresents the mass of purified sterol, 5 α -stanol, or bile acid that was isolated from plasma or bile for determination of specific activity (dpm/mg).

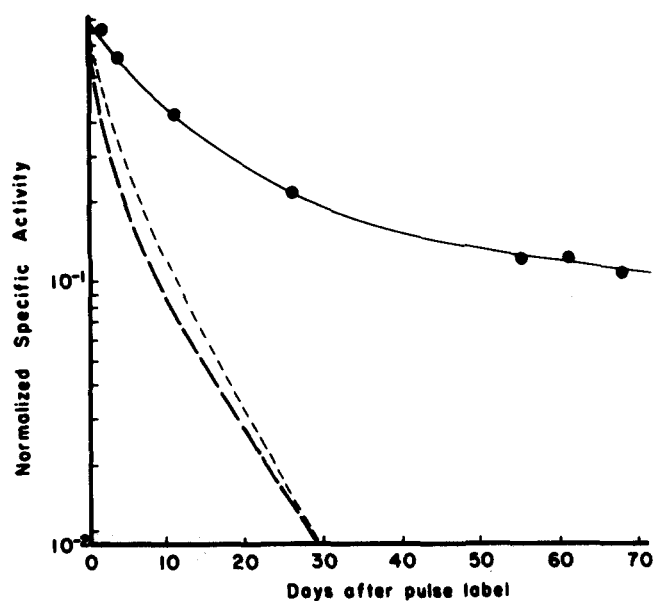


Fig. 2. Specific activity decay curves for cholestanol in sitosterolemic (—), CTX (---), and control subjects (· · ·). The specific activity decay of [³H]cholestanol was rapid in the control and CTX subjects (8) but was much slower in the sitosterolemic homozygote.

more than 95 times and 110 times greater, respectively, than in the CTX or control subjects (8). Thus, increased cholestanol synthesis coupled to slow elimination accounts for the huge exchangeable pools in the sitosterolemic homozygote.

Hepatic microsomal cholesterol 7 α -hydroxylase activity was assayed in liver specimens from three sitosterolemic homozygotes before treatment and in one homozygote (KCN) 9 months after ileal bypass surgery (Table 5). For comparison, microsomal cholesterol 7 α -hydroxylase activity was measured in liver specimens (from six healthy transplant donors who died unexpectedly) where no suitable recipients could be found. Cholesterol 7 α -hydroxylase activity in the whole microsomes that contained 19% plant sterols from three sitosterolemic homozygotes was approximately 40% lower than the mean value for the six control liver specimens ($P < 0.001$). Removal of the endogenous sterols from the microsomes by acetone treatment and reconstituting the protein with only cholesterol

and optimal co-factors increased cholesterol 7 α -hydroxylase activity more than 60% to normal levels ($P < 0.05$) in the homozygotes but did not change control values. The coefficient of variation for the enzyme assays are included in Table 5 and show consistently reproducible activities for the controls and sitosterolemic subjects. The results indicate that the enlarged plant sterol microsomal pool competitively inhibited enzyme activity in the three homozygotes.

In Fig. 3 are shown the Northern blots for cholesterol 7 α -hydroxylase and β -actin (internal standard) mRNAs isolated from specimens of normal and sitosterolemic (KeC) liver. Only a single transcript of equal intensity for cholesterol 7 α -hydroxylase mRNA at 2.1 kb was detected in both liver specimens. Thus, cholesterol 7 α -hydroxylase transcription was similar in the control and sitosterolemic livers.

Nine months after ileal bypass surgery, cholesterol 7 α -hydroxylase activity in whole microsomes from the sitosterolemic homozygote (KCN) had increased 78% (Table 5). Although, the percutaneous liver biopsy specimen was too small to measure microsomal sterol concentrations, bile acid malabsorption produced coincidentally a pronounced decrease in plasma sitosterolemia concentrations (Table 1).

DISCUSSION

The results of this investigation demonstrate an inverse relationship between elevated cholestanol plasma concentrations and synthesis, and reduced hepatic microsomal cholesterol 7 α -hydroxylase activity in three sitosterolemic homozygotes. In these subjects, hepatic cholesterol 7 α -hydroxylase activity was inhibited competitively by the large plant sterol pool which amounted to 19% of the total microsomal sterols (Table 5). Removal of the endogenous sterols from the microsomes by acetone treatment and reconstituting the protein with cholesterol and optimal co-factors restored cholesterol 7 α -hydroxylase activity to control levels (Table 5) in the homozygotes but did not significantly increase cholesterol 7 α -hydroxylase activity further in the control microsomes where only trace

TABLE 4. Cholestanol kinetic parameters

Diagnosis	Sitosterolemia	CTX	Controls n = 5
			mean \pm SD
$t_{1/2}^A$ Half-life of first exponential A (days ⁻¹)	6.8	1.8	1.3 \pm 0.4
$t_{1/2}^B$ Half-life of second exponential B (days ⁻¹)	80	5.8	7.0 \pm 2.8
PR _A production rate synthesis, (mg/day)	83	48	11.8 \pm 6.0
M _A (mg)	5500	148	48 \pm 23
M _B (mg)	3500	67	41 \pm 26

TABLE 5. Hepatic microsomal cholesterol 7 α -hydroxylase activity: effect of ileal bypass

Condition	Cholesterol 7 α -hydroxylase ^a	Coefficient of Variation	Endogenous microsomal sterols
	<i>pmol/mg protein/min</i>	%	<i>μmol/mg (% plant sterols)</i>
Controls (n = 6)	17.5 ± 1.6	9.1	60(0.1) ^b
Controls (n = 6) (acetone-treated microsomes, reconstituted assay system)	18.0 ± 1.5	12.6	0.48(-) ^c
Sitosterolemia (n = 3) (whole microsomes)	10.7 ± 2.4 ^d	22.4	68(19) ^b
Sitosterolemia (n = 3) (acetone-treated microsomes, reconstituted assay system)	17.2 ± 1.4 ^e	8.5	0.54(-) ^c
Sitosterolemia (KCN) (whole microsomes, 9 months after ileal bypass surgery)	19.0 ^b		

^aMean ± SD.^bAverage of two determinations.^cNone detected.^d*P* < 0.001, whole microsomes: controls versus sitosterolemia.^e*P* < 0.05, sitosterolemia: whole microsomes versus acetone-treated microsomes.

amounts of plant sterols normally are detected. Further, equal amounts of cholesterol 7 α -hydroxylase mRNA were detected by Northern blot analysis (Fig. 3) in both the sitosterolemic and control livers which indicated that enzyme protein formation was similar in these subjects. This finding supports our contention that competitive inhibition of the enzyme by the enlarged microsomal plant sterol substrate pool was responsible for reduced cholesterol 7 α -hydroxylase activity. As bile acid formation represents the major quantitative pathway for the catabolism of cholesterol, and may be reduced in sitosterolemia (16, 26, 27), the accumulation of microsomal sitosterol that competitively inhibits the 7 α -hydroxylation of cholesterol (Table 5) can also increase plasma cholesterol levels seen in this disease (1, 4, 26, 27) and in rats infused intravenously (28). Conversely, stimulating bile acid synthesis (cholesterol 7 α -hydroxylase activity) after ileal bypass surgery (Table 5) profoundly decreased plasma

cholesterol and sitosterol concentrations and returned plasma cholestanol to almost normal levels (Table 1), (27, 29, 30). We have interpreted the greater reduction in plasma cholestanol relative to cholesterol after ileal bypass surgery to mean diminished cholestanol synthesis, whereas the proportional decline in plasma cholesterol and sitosterol levels with bile acid malabsorption more likely reflects the increased expression of LDL receptors and almost parallel transformation of both sterols to bile acids (16, 26, 27, 29, 30). Therefore, increasing the conversion of microsomal sterols to their 7 α -hydroxy derivatives and bile acids reduces the availability of microsomal cholesterol to be transformed to 4-cholesten-3-one and cholestanol. It is important to note that although cholestanol can be 7 α -hydroxylated (19), no allo bile acids have been detected in the bile from the three sitosterolemic subjects (unpublished observation). Moreover, the greater than expected reduction in plasma cholesterol levels and constancy of the sitosterol/cholesterol ratio result from the failure to up-regulate cholesterol biosynthesis in these homozygotes (27, 29, 30). Normally, cholesterol synthesis is stimulated by interruption of the enterohepatic circulation of bile acids (29–32). However, in sitosterolemia, HMG-CoA reductase is fundamentally suppressed (33) and cannot be increased by reduced hepatic bile acid flux (29, 30). As a result, body cholesterol and sitosterol pools including the plasma compartment are utilized for bile acid synthesis. We believe this rapid mobilization of plasma and tissue sterols is responsible for clinical improvement.

Plasma cholestanol concentrations are markedly elevated in all sitosterolemic homozygotes (2, 4) to levels found in CTX, another disease where cholestanol accumulates in tissues. As diets contain only small amounts, (2, 8), increased tissue cholestanol arises from endogenous hyperproduction, a conclusion supported by quantitative estimates of cholestanol synthesis determined

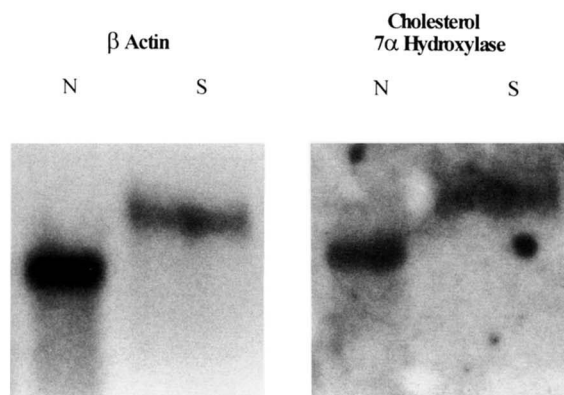


Fig. 3. Northern blot of β -actin and cholesterol 7 α -hydroxylase mRNAs. A single transcript of cholesterol 7 α -hydroxylase mRNA at 2.1 kb was detected in the control (N) and sitosterolemic (S) specimens.

by mathematical analysis of the specific activity versus time decay curve (Table 4). Moreover, the extremely slow elimination of cholestanol from the body as evidenced by the prolonged half-lives of the two exponentials of the specific activity decay curves (Table 4) also contributes to the expansion of the cholestanol pool in the sitosterolemic homozygote. It is noteworthy that prolonged cholestanol turnover resembles the slow elimination of cholesterol and sitosterol in these homozygotes (14–17) and indicates that both stanols and sterols are retained in this disease.

We have previously studied the mechanism by which sitosterol competitively blocks microsomal cholesterol 7 α -hydroxylase (24). Because sitosterol is structurally similar to cholesterol, differing only in an extra ethyl substituent at C-24, it is chemically attracted to the enzyme but is more slowly 7 α -hydroxylated. Similar results showing the competitive inhibition of rat microsomal cholesterol 7 α -hydroxylase by sitosterol have been reported by Boberg, Åkerlund, and Björkhem (28). The unused microsomal cholesterol is available to be converted to cholestanol. After ileal bypass surgery, microsomal cholesterol 7 α -hydroxylase activity increased due both to the induction of more enzyme activity by interruption of the enterohepatic circulation of bile acids and the partial removal of the competing cholesterol analogues from the microsomes (24). As a result, microsomal cholesterol was transformed more efficiently to bile acids and less was available as substrate for conversion to cholestanol.

With regard to the biosynthetic pathway, cholesterol is the precursor of cholestanol (18–20, 34). This investigation further demonstrates that cholestanol was formed efficiently from [1,2-³H]4-cholesten-3-one but not from [4-¹⁴C]7 α -hydroxycholesterol in the sitosterolemic homozygote. The high specific activity of [¹⁴C]cholic acid attests that exogenous injected [4-¹⁴C]7 α -hydroxycholesterol was taken up by the liver, mixed with the endogenous hepatic 7 α -hydroxycholesterol pool and was transformed into bile acids. The absence of tritium from the injected [1,2-³H]4-cholesten-3-one in the cholic acid emphasizes the requirement for a 7 α -hydroxy group in precursors for bile acid synthesis (20). More importantly, the lack of [¹⁴C]cholestanol in the sitosterolemic homozygote, where large quantities of cholestanol are formed, argues against the 7-dehydroxylation of 7 α -hydroxycholesterol or its product, 7 α -hydroxy-4-cholesten-3-one, as a step in the biosynthesis of cholestanol, as has been suggested to occur in CTX (21, 35). Moreover, in contrast to CTX where 7 α -hydroxycholesterol is transformed to cholestanol (21) and stimulating cholesterol 7 α -hydroxylase by cholestyramine treatment increases plasma cholestanol concentrations and synthesis (8), increasing cholesterol 7 α -hydroxylase activity is associated with reduced cholestanol plasma levels and formation in sitosterolemia. Further, Koopman et al. (5) have noted increased cholestanol concentrations in serum from patients

with cholestasis where bile acid synthesis also is inhibited. Thus, interfering with the normal conversion of microsomal cholesterol to bile acids may result in enhanced plasma cholestanol concentrations.

In summary, large plasma and tissue cholestanol concentrations in sitosterolemic homozygotes were related to increased synthesis combined with reduced removal and associated with the competitive inhibition of cholesterol 7 α -hydroxylase by the enlarged microsomal sitosterol pool. Stimulating cholesterol 7 α -hydroxylase activity promotes the elimination of cholesterol as bile acids, and diminishes the formation and plasma concentrations of cholestanol. ■

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