Down-regulation of cholesterol biosynthesis in sitosterolemia: diminished activities of acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA synthase, reductase, squalene synthase, and 7-dehydrocholesterol Δ^7-reductase in liver and mononuclear leukocytes

Akira Honda,[1] Gerald Salen, Lien B. Nguyen, G. Stephen Tint, Ashok K. Batta, and Sarah Shefer

Departments of Medicine and Liver Center, the University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark, NJ 07103, and Veterans Affairs Medical Center, East Orange, NJ 07018

Abstract Sitosterolemia is a recessively inherited disorder characterized by abnormally increased plasma and tissue plant sterol concentrations. Patients have markedly reduced whole body cholesterol biosynthesis associated with suppressed hepatic, ileal, and mononuclear leukocyte 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-controlling enzyme in cholesterol biosynthetic pathway, coupled with significantly increased low density lipoprotein (LDL) receptor expression. To investigate the mechanism of down-regulated cholesterol biosynthesis, we assayed several other key enzymes in the cholesterol biosynthetic pathway including acetoacetyl-CoA thiolase, HMG-CoA synthase, squalene synthase, and 7-dehydrocholesterol Δ^7-reductase activities in liver and freshly isolated mononuclear leukocytes from four sitosterolemic patients and 19 controls. Hepatic acetoacetyl-CoA thiolase, HMG-CoA synthase, reductase, and squalene synthase activities were significantly decreased ($P < 0.05$) -39%, -54%, -76%, and -57%, respectively, and 7-dehydrocholesterol Δ^7-reductase activity tended to be lower (-35%) in the sitosterolemic compared with control subjects. The reduced HMG-CoA synthase, reductase, and squalene synthase activities were also found in mononuclear leukocytes from a sitosterolemic patient. Thus, reduced cholesterol synthesis is caused not only by decreased HMG-CoA reductase but also by the coordinate down-regulation of entire pathway of cholesterol biosynthesis. These results suggest that inadequate cholesterol production in sitosterolemia is due to abnormal down-regulation of early, intermediate, and late enzymes in the cholesterol biosynthetic pathway rather than a single inherited defect in the HMG-CoA reductase gene.—**Honda, A., G. Salen, L. B. Nguyen, G. S. Tint, A. K. Batta, and S. Shefer.** Down-regulation of cholesterol biosynthesis in sitosterolemia: diminished activities of acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA synthase, reductase, squalene synthase, and 7-dehydrocholesterol Δ^7-reductase in liver and mononuclear leukocytes. *J. Lipid Res.* 1998. **39:** 44–50.

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Sitosterolemia (1) is a rare recessive inherited disorder characterized clinically by tendon and tuberous xanthomas, accelerated atherosclerosis, hemolytic episodes, and recurrent arthritis and arthralgias. Biochemically, concentrations of plant sterols (sitosterol, campesterol, stigmasterol, avenosterol) and 5α-saturated stanols are markedly elevated in virtually all tissues but brain, while plasma cholesterol levels may be normal or only moderately increased (1, 2). Plasma and tissue plant sterols are not synthesized endogenously in humans including sitosterolemic subjects (3, 4), but are derived entirely from the diet. Sitosterolemic patients absorb between 15–60% of dietary sitosterol compared to less than 5% absorbed in normal subjects. Further, plant sterols are preferentially excreted by the liver in normal subjects but are retained along with cholesterol in sitosterolemic patients (3–8). Thus, increased intestinal plant sterol absorption is combined with slow hepatic removal to cause abnormally enlarged plant sterol tissue and plasma pools in this disease.

Another key biochemical feature of sitosterolemia is

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; SRE-1, sterol regulatory element 1.

[1]To whom correspondence should be addressed.

extremely reduced whole body cholesterol synthesis measured by sterol balance, isotope kinetic, and mevalonic acid excretion methods (4, 6, 7, 9). We also reported that the activity, mass, and mRNA level of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-controlling enzyme in cholesterol biosynthetic pathway, were markedly reduced and associated with enhanced expression of low density lipoprotein (LDL) receptor binding in liver (10), ileum (11) and freshly isolated mononuclear leukocytes (12–14) from sitosterolemic patients. Attempts to stimulate endogenous cholesterol synthesis by bile acid malabsorption (cholestyramine or ileal bypass surgery) or a low sterol diet did not stimulate inhibited HMG-CoA reductase activity in mononuclear leukocytes although plasma cholesterol and sitosterol levels declined markedly (14). These observations suggested that cholesterol biosynthesis was abnormally down-regulated in sitosterolemia and augmented LDL receptor function provided sterols that could not be synthesized by cells. However, the mechanism(s) of abnormal down-regulation of HMG-CoA reductase, increased plant sterol absorption, and hepatic sterol retention have not been elucidated.

In this report, we assayed several key enzymes in the early, intermediate, and late cholesterol biosynthetic pathway (**Fig. 1**) in liver and freshly isolated mononuclear leukocytes from four sitosterolemic patients (two unrelated families) and 19 controls. Acetoacetyl-CoA thiolase and HMG-CoA synthase catalyze the formation of the substrate HMG-CoA; HMG-CoA reductase transforms HMG-CoA to mevalonic acid and is considered the rate-controlling enzyme in the pathway. Squalene synthase is involved in the cyclization of squalene and is the first product totally committed to the cholesterol biosynthetic pathway, while the last enzyme, 7-dehydrocholesterol Δ^7-reductase reduces 7-dehydrocholesterol to yield cholesterol. The results indicate that in sitosterolemia, all enzyme activities in cholesterol biosynthesis were inhibited and are not coordinately regulated with increased LDL receptor function.

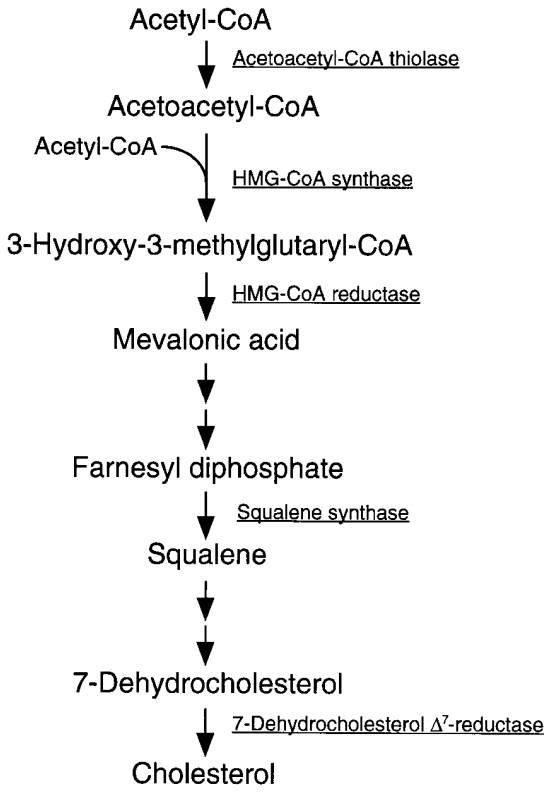

Fig. 1. Cholesterol biosynthetic pathway from acetyl-CoA.

MATERIALS AND METHODS

Clinical

We studied four patients in whom a diagnosis of sitosterolemia was established by clinical and biochemical criteria (15). Patients KC, TC, and KCN are female siblings age 30, 34, and 36 years, and complete clinical and biochemical descriptions have been presented elsewhere (15–17). Patient GB is a 35-year-old man who is unrelated to the three sisters and has been described previously (14). Liver biopsies were obtained from each (four) patient during ileal bypass surgery for prevention of rapidly developing atherosclerosis. The patients had not been under drug treatment for at least 2 months before surgery. Control liver specimens were from 19 healthy people who died unexpectedly and whose livers became available because no suitable recipient for liver transplantation could be found (National Institutes of Health contract NO1-DK-62274). All liver tissues were stored at −70°C until used. Mononuclear leukocytes were obtained before and after ileal bypass surgery from patient KC, after ileal bypass surgery from TC and KCN, and after cholestyramine treatment (15 g/day) from GB. Control mononuclear leukocytes were from five healthy volunteers. Blood was collected at 9:00 am after a 12-h fast. During this study all patients were on a free-living American diet that contains 400–500 mg/day cholesterol and 100–150 mg/day plant sterols as estimated from daily food diaries. Informed consent was obtained from the sitosterolemic subjects, and the experimental protocol was approved by the human studies committees at the UMDNJ-New Jersey Medical School, Newark, NJ and VA Medical Center, East Orange, NJ.

Chemicals

Acetyl-CoA, acetoacetyl-CoA, CoA, squalene, farnesol, cholesterol (5-cholesten-3β-ol), and [1-^{3}H]farnesyl diphosphate were purchased from Sigma Chemical Co. (St. Louis, MO), and 7-dehydrocholesterol (5, 7-cholestadien-3β-ol), tetrabutylammonium hydroxide (40% aqueous solution), and phosphoric acid were from Aldrich Chemical Co., Inc. (Milwaukee, WI). [1-^{14}C] acetyl-CoA, [3-^{14}C]HMG-CoA, and [4-^{14}C]cholesterol were obtained from Amersham Co. (Arlington Heights, IL), and [2-^{14}C]mevalonolactone was from New England Nuclear (Boston, MA). [1,2-^{3}H]7-dehydrocholesterol was synthesized as described previously (18).

Determination of plasma sterol concentrations

Plasma sterol concentrations were measured by capillary gas–liquid chromatography as described previously (13).

Preparation of microsomal and cytosolic fractions from liver and mononuclear leukocytes

Mononuclear leukocytes (lymphocytes + monocytes) were isolated from 30–60 ml of venous blood as described previously (14). Microsomes and cytosol (100,000 *g* supernatant) from liver and mononuclear leukocytes were prepared by differential ultracentrifugation (18). The cytosolic fractions were dialyzed for 24 h at 4°C against a 1,000-fold excess of 20 mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA and 0.5 mM dithiothreitol (19) to inhibit contaminated HMG-CoA lyase activity. Microsomal protein concentrations and dialyzed cytosolic protein fractions were determined by the Lowry method (20).

Assay of acetoacetyl-CoA thiolase activity

The activity of cytosolic acetoacetyl-CoA thiolase was determined by spectrophotometric assay (21, 22). The reaction mixture (final volume 1 ml) consisted of 100 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA, 0.12 mM acetoacetyl-CoA, 90 μM CoA, and 50 μg of cytosolic protein. The mixture was preincubated for 3 min at 25°C, after which the reaction was started by adding CoA and was incubated for 3 min. The activities were measured in the direction of cleavage of acetoacetyl-CoA, but are expressed here in the direction of formation of acetoacetyl-CoA (23). The velocity in the direction of formation of acetoacetyl-CoA was calculated by multiplying the velocity in the direction of cleavage by 0.033 (21).

Assay of HMG-CoA synthase activity

Cytosolic HMG-CoA synthase activity was assayed according to the method of Scharnagl et al. (24) with minor modifications. The composition of the reaction mixture (final volume 100 μl) was 100 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA, 60 μM acetoacetyl-CoA, 400 mM [^{14}C]acetyl-CoA (28,000 dpm/nmol), and 100 μg of dialyzed cytosolic protein. To inhibit contaminated hepatic mitochondrial HMG-CoA synthase activity, 20 mM $MgCl_2$ was also added to the incubation mixture for liver. The mixture was preincubated for 2 min at 37°C and the reaction was initiated by the addition of dialyzed cytosolic fraction. After 5 min for liver and 15 min for mononuclear leukocytes, the reaction was stopped by adding 200 μl of 200 mM TBAP buffer (pH 5.5) dissolved in methanol–water 3:2 containing 15 nmol of HMG-CoA. The incubated mixture was then centrifuged for 1 min at 10,000 rpm in a Beckman microfuge, and was kept on ice until analyzed. An aliquot (100 μl) of the supernatant was injected onto a μ-Bondapak C_{18} column (3.9 × 300 mm, Waters Corp., Milford, MA) equilibrated with 50 mM tetrabutylammonium phosphate buffer (pH 5.5) dissolved in methanol–water 43:57, and the column was eluted with the same solvent at a rate of 1.3 ml/min. The HMG-CoA-containing fraction was collected, evaporated under N_2 at 90°C, and the radioactivity was measured.

Assay of HMG-CoA reductase activity

The activity of microsomal total HMG-CoA reductase was measured as described previously by Nguyen et al. (10). Briefly, 50–200 μg of microsomal protein was preincubated for 5 min at 37°C in a final volume of 150 μl buffer (pH 7.4) containing a NADPH generating system and [^{3}H]mevalonolactone (40,000 dpm) as internal recovery standard. The reaction was started with the addition of 30 nmol [^{14}C]HMG-CoA and stopped after 10 min for liver and 30 min for mononuclear leukocytes with the addition of 20 μl 6 N HCl. After localization, the products were separated by thin-layer chromatography, and mevalonolactone was measured by dual-label liquid scintillation counting.

Assay of squalene synthase activity

The assay of microsomal squalene synthase activity was based on the methods of Shechter et al. (25), Eilenberg et al. (26), and Faust et al. (27). The reaction mixture (final volume 200 μl) consisted of 100 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 5 mM $MgCl_2$, 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM DTT, 2 mM NADPH, 10 μM [^{3}H]farnesyl diphosphate (90,000 dpm/nmol), and 20–100 μg of microsomal protein. The reaction was started by the addition of substrate and stopped after 15 min at 37°C by adding 0.8 ml of hexane–acetone 1:1. After addition of 100 μl of ethanol containing 100 μg of squalene and [^{14}C] cholesterol (3,500 dpm) as an internal recovery stan-

dard, the lipids were extracted with hexane and separated by thin-layer chromatography developed with hexane. Spots corresponding to squalene (R_f = 0.5) and cholesterol (R_f = 0.0) were scraped and measured by liquid scintillation counting.

Assay of 7-dehydrocholesterol Δ^7-reductase activity

The activity of microsomal 7-dehydrocholesterol Δ^7-reductase was assayed according to the method as described previously by Shefer et al. (18). [^{3}H]7-dehydrocholesterol (30 nmol, 8,000 cpm) was solubilized with 6 μl of a 33% solution of β-cyclodextrin (Pharmatec, Inc., Alachua, FL) and incubated in a final volume of 150 μl buffer (pH 7.3) containing an NADPH generating system. The reaction was initiated by the addition of 0.25 mg of microsomal protein supplemented with 0.5 mg of bovine serum albumin. The reaction was stopped after 30 min at 37°C with the addition of 2 ml of methylene chloride–ethanol 5:1. After adding 150 μl of water to the mixture, the products were extracted, separated by argentation TLC, and radioactivity was measured by liquid scintillation counting.

Statistics

Data are reported here as the mean ± SEM. The statistical significance of differences between the results in the different groups was elevated by the Student's two-tailed *t* test or by Mann-Whitney two-tailed test, and significance was accepted at the level of $P < 0.05$.

RESULTS

Fasting plasma sterols and 5α-stanols concentrations in the four untreated sitosterolemic patients and 20 healthy controls are presented in **Table 1**. Cholesterol concentrations in patients were moderately elevated, while plant sterols and 5α-stanols concentrations were increased more than 100-fold and 30-fold, respectively, compared with controls.

Table 2 lists hepatic activities of acetoacetyl-CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, squalene synthase, and 7-dehydrocholesterol Δ^7-reductase in controls and sitosterolemic patients. HMG-CoA reductase activity, the rate-controlling enzyme in cholesterol biosynthetic pathway, was decreased −76% ($P < 0.05$) in four sitosterolemic patients compared with controls. Similarly, the activities of acetoacetyl-CoA thiolase, the initiating enzyme in the cholesterol biosynthetic pathway, HMG-CoA synthase that precedes HMG-CoA reductase, and squalene synthase, intermediate in the pathway were decreased in parallel with HMG-CoA reductase −39% ($P < 0.05$), −54% ($P < 0.05$), and −57% ($P < 0.05$), respectively, compared with control subjects. Mean activity of 7-dehydrocholesterol Δ^7-reductase, the last enzyme in the cholesterol biosynthetic pathway, tended to be lower (−35%) than in controls.

TABLE 1. Plasma concentrations of cholesterol, plant sterols, and 5α-stanols

Subjects	Cholesterol	Plant Sterols[a]	5α-Stanols[b]
		mg/dl	
Sitosterolemia			
KC	202	22	8.3
TC	233	31	11
KCN	184	23	6.3
GB	277	21	4.5
Mean ± SEM	224 ± 20[c]	24 ± 2[c]	7.5 ± 1.4[c]
Controls			
Mean ± SEM (n = 20)	180 ± 5	0.22 ± 0.20	0.20 ± 0.20

[a]Plant sterols consisted of sitosterol and campesterol.
[b]5α-Stanols consisted of 5α-cholestanol, 5α-sitostanol, and 5α-campestanol.
[c]$P < 0.01$ vs. Controls.

Table 3 shows HMG-CoA synthase, HMG-CoA reductase, and squalene synthase activities in freshly isolated mononuclear leukocytes from patient KC before and after ileal bypass surgery. Untreated HMG-CoA synthase and reductase activities were 55% and 48% lower, respectively, than the control mean, which was below the 95% confidence limits. After ileal bypass surgery that produced bile acid malabsorption, the activities of HMG-CoA synthase, reductase, and squalene synthase remained paradoxically reduced, and all were below the lower limits of the 95% confidence interval for untreated control mean. In three other patients, we have measured the three enzyme activities in mononuclear leukocytes after ileal bypass surgery (TC and KCN) or cholestyramine treatment (GB). HMG-CoA synthase, reductase, and squalene synthase activities were 9.4 ± 2.0, 5.1 ± 0.6, and 7.8 ± 1.0 pmol/min per mg protein, respectively, and were not up-regulated compared with untreated control activities. Thus, bile acid malabsorption does not stimulate early and intermediate cholesterol biosynthesis in the sitosterolemic individuals.

DISCUSSION

The results of this investigation extend our understanding of the abnormal down-regulation of the cholesterol biosynthetic pathway in sitosterolemic subjects. Early, intermediate, and late enzymatic reactions were inhibited in the liver and mononuclear leukocytes from four patients (three sisters and an unrelated male). The data are consistent with our previous observations that the conversion of [^{14}C]mevalonic acid to choles-

TABLE 2. Hepatic activities of acetoacetyl (AcAc)-CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, squalene synthase, and 7-dehydrocholesterol (7-DHC) Δ^7-reductase in control and sitosterolemic subjects

Subjects	AcAc-CoA Thiolase	HMG-CoA Synthase	HMG-CoA Reductase	Squalene Synthase	7-DHC Δ^7-Reductase
			pmol/min/mg protein		
Sitosterolemia					
KC	6369	195	10	12	219
TC	6666	119	18	84	642
KCN			12	89	619
GB	5676	171	37	27	100
Mean ± SEM	6237 ± 293[a]	162 ± 22[a]	19 ± 6[a]	53 ± 20[a]	395 ± 138
Controls					
Mean ± SEM (n)	10210 ± 564 (18)	356 ± 45 (11)	78 ± 23 (19)	122 ± 14 (19)	605 ± 50 (10)

[a] $P < 0.05$ vs. Controls.

terol in a sitosterolemic liver slice was 50% less than controls (10). Therefore, the reduced formation of cholesterol that is characteristic of patients with sitosterolemia (4, 6, 7, 9), relates to the inhibition of the entire cholesterol biosynthetic pathway including HMG-CoA reductase, the rate-controlling enzyme in the pathway. Thus, an attempted therapeutic treatment by feeding mevalonic acid was unsuccessful in patient TC (G. Salen and S. Shefer, unpublished result) because of the inhibition of enzymatic reactions after the formation of mevalonic acid.

These results also emphasize the coordinate down-regulation of these key enzymes in sitosterolemia and argue against the possibility of a single inherited gene defect involving these enzymes as it is not likely that the genes for these enzymes in cholesterol biosynthesis are simultaneously defective. The relationship between enhanced sterol absorption, the loss of intestinal sterol discrimination, hepatic sterol retention, and the down-regulating of cholesterol biosynthesis that underlies this disease remains unknown. However, beneficial treatment can be achieved by inducing bile acid malabsorption which stimulates new bile acid synthesis by up-regulation cholesterol 7α-hydroxylase. Plasma sterol concentrations declined markedly compared with similarly treated hypercholesterolemic subjects, and remained low (9, 15, 16, 28) because cholesterol biosynthesis in the liver and mononuclear leukocytes could not up-regulate to produce additional cholesterol as in normals. We have taken advantage of this mechanism to treat these patients and induced long term clinical benefits that include disappearance of xanthomas, diminished aortic stenosis, decreased frequency in angina pectoris and arthralgia attacks (28, 29).

With regard to the abnormal regulation of the enzymes in the cholesterol biosynthetic pathway, sitosterol is probably not important as a feedback inhibitor of these enzymes. Recently, it was demonstrated that sitosterol did not down-regulate hepatic activities of HMG-CoA reductase (30, 31) or HMG-CoA synthase (A. Honda, G. Salen, and S. Shefer, unpublished results) in a rat model where sitosterol was administered intravenously so that concentrations in the liver and tissues were similar to those found in sitosterolemic subjects. Also Brown and Goldstein (32) and Field, Born, and Mathur (33) reported that sitosterol was less effective than cholesterol in down-regulating HMG-CoA reductase activity in cultured human skin fibroblasts and in human intestinal CaCo-2 cells, respectively.

A major unexplained relationship in sitosterolemic patients concerns the up-regulation of LDL receptor binding and down-regulation of the entire cholesterol

TABLE 3. Activities of HMG-CoA synthase, HMG-CoA reductase, and squalene synthase in mononuclear leukocytes from control and sitosterolemic subjects

Subjects	HMG-CoA Synthase	HMG-CoA Reductase	Squalene Synthase
		pmol/min/mg/protein	
Sitosterolemia			
KC (untreated)	4.5	6.2	4.9
KC (after ileal bypass)	1.5	6.0	3.7
Controls (untreated)			
Mean ± SEM (n)	10.0 ± 1.0 (5)[a]	12.0 ± 1.0 (11)[a]	6.4 ± 1.0 (5)[a]

[a] 95% confidence invtervals for control means of HMG-CoA synthase, reductase, and squalene synthase activities are 7.1 to 12.9, 9.6 to 14.4, and 3.8 to 9.0 pmol/min/mg protein, respectively.

biosynthetic pathway. The coordinate regulation of HMG-CoA synthase, HMG-CoA reductase, squalene synthase, and LDL receptor has been generally accepted because the promoter regions of the genes encoding these enzymes and LDL receptor have already been cloned and characterized, and contain sterol regulatory element 1 (SRE-1) sequence (34, 35). Low intracellular sterol levels result in proteolysis of SRE-1 binding protein in the endoplasmic reticulum, and the mature form of the protein enters the nucleus, binds to the SRE-1, and simultaneously activates transcription of HMG-CoA synthase, HMG-CoA reductase, squalene synthase, and LDL receptor genes (36, 37). In contrast, high intracellular sterol levels prevent the proteolysis and inactivate transcription. Thus, normally all these enzymes and LDL receptors are coordinately regulated in the same direction in response to changing levels of cellular cholesterol. In sitosterolemic patients, hepatic cholesterol levels are less than controls (10, 31). Our finding of significantly reduced HMG-CoA synthase, HMG-CoA reductase, and squalene synthase in the sitosterolemic livers which previously were shown to have enhanced LDL receptor binding (10) points to defective coordinate regulation of both pathways. Moreover, mononuclear leukocytes continue to take up LDL sterols even though cell sterol concentrations are 2- to 3-times greater in sitosterolemic than control cells (12). As a result, the sitosterolemic monocytes are engorged with sterols to resemble foam cells which may contribute to the acceleration of atherosclerosis in this disease.

In summary, five key enzymes in cholesterol biosynthesis were down-regulated in the liver of four sitosterolemic subjects from two unrelated families. The results support the contention that reduced cholesterol biosynthesis is caused by inhibition of the entire cholesterol biosynthetic pathway and not by a single enzyme defect.

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