Increased plasma cholestanol and 5α-saturated plant sterol derivatives in subjects with sitosterolemia and xanthomatosis

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Abstract We have measured plasma sterol composition in 14 subjects with sitosterolemia and xanthomatosis. In addition to elevated plasma phytosterol (campesterol 16 ± 7 mg/dl and sitosterol 35 ± 16 mg/dl) and normal to moderately high cholesterol levels (258 ± 96 mg/dl), concentrations of 5α-saturated stanols, cholestanol, 5α-campestanol, and 5α-sitostanol were at least 10 times greater than controls. Diets contained plentiful quantities of cholesterol and plant sterols, but only trace amounts of cholestanol (<2 mg/day) and no detectable 5α-campestanol and 5α-sitostanol, which indicated that the 5α-saturated stanols were formed endogenously. Treatment with cholestyramine reduced plasma cholesterol and phytosterol levels by 45% and 5α-saturated stanols by 55%. These results indicate that abnormally high plasma concentrations of cholestanol, 5α-campestanol, and 5α-sitostanol are found in subjects with sitosterolemia and xanthomatosis, and that treatment with cholestyramine effectively reduced elevated plasma sterol levels. —**Salen, G., P. O. Kwiterovich, Jr., S. Shefer, G. S. Tint, I. Horak, V. Shore, B. Dayal, and E. Horak.** Increased plasma cholestanol and 5α-saturated plant sterol derivatives in subjects with sitosterolemia and xanthomatosis. *J. Lipid Res.* 1985. **26:** 203–209.

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Sitosterolemia with xanthomatosis is a rare inherited lipid storage disease that was described first in 1974 by Bhattacharyya and Connor (1). The major clinical manifestations include tendon and tuberous xanthomas that involve the Achilles tendons, extensor tendons of the hand and the skin of the elbows and knees, recurrent arthritis and arthralgias of the knees and ankle joints, and premature atherosclerosis. Chemically, increased amounts of plant sterols, such as campesterol and sitosterol, (**Fig. 1**) are present in the plasma, erythrocytes, and xanthomas, while plasma cholesterol levels are normal to slightly elevated (2). Hyperapobetalipoproteinemia is often present (3). Although plant sterols are present in abundant amounts in most American diets, only small quantities of sitosterol (less than 1 mg/dl) can be detected in normal plasma (4). Limited intestinal absorption combined with enhanced biliary excretion presumably keeps plasma plant sterol concentrations low in normal subjects (4). In contrast, intestinal absorption of sitosterol in patients with sitosterolemia and xanthomatosis was from 5 to 10 times greater than in controls and was associated with reduced clearance of sitosterol and cholesterol from the plasma (1, 5, 6). Thus two factors, increased absorption coupled with reduced removal, apparently lead to the enhanced tissue deposits of plant sterols and cholesterol in this disease. However, not all of the biochemical defects are known.

Recently, uncertainty has arisen concerning plasma sterol composition in patients with sitosterolemia with xanthomatosis. In the original description, Bhattacharyya and Connor (1) found only cholesterol and unsaturated sterols, campesterol, stigmasterol, and sitosterol in the plasma of two subjects. Further, only Δ^5-unsaturated sterols were detected in the plasma of the patient reported by Shulman, et al. (7) and the five patients recently studied by Kwiterovich et al. (3). However, other investigators (Whitington et al. (8), Khachadurian and Salen (9), and Wang et al. (10)) have discovered that subjects with typical clinical and chemical features of sitosterolemia and xanthomatosis (tendon xanthomas, arthritis, premature atherosclerosis, increased plasma cholesterol

Abbreviations: GLC, gas-liquid chromatography; RRT, relative retention time; LDL, low density lipoproteins; HDL, high density lipoproteins.

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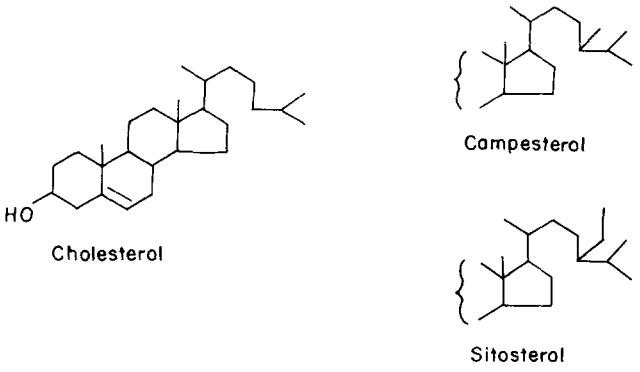

Fig. 1 Structures of major unsaturated sterols present in sitosterolemic plasma.

and plant sterol concentrations) have, in addition, markedly elevated plasma levels of cholestanol, 5α-campestanol, and 5α-sitostanol (**Fig. 2**), the respective 5α-saturated derivatives of cholesterol, campesterol, and sitosterol.

Therefore, to determine whether increased 5α-saturated stanols are a constant biochemical abnormality in this disease, plasma sterols were measured by gas–liquid chromatography in fourteen affected subjects. It has been previously reported that no cholestanol or 5α-saturated plant sterol derivatives were detected in the plasma of eight of these subjects although previous analytic methods may not have been sufficiently sensitive (1, 5, 7).

In addition, the effect of cholestyramine, a resin that binds bile acids and promotes their excretion in feces, was evaluated on plasma sterol concentrations in four subjects. Cholestyramine treatment enhances the conversion of cholesterol to bile acids in liver and often diminishes plasma cholesterol levels (11). Our results indicate that increased amounts of cholestanol, 5α-campestanol, and 5α-sitostanol were present in the plasma of *all* subjects with sitosterolemia and xanthomatosis. Cholestyramine treatment reduced elevated plasma sterol concentrations in this disease.

METHODS

Subjects

Studies were conducted in fourteen subjects (**Table 1**) in whom a diagnosis of sitosterolemia and xanthomatosis was established by clinical and biochemical criteria. Complete clinical and biochemical descriptions have been presented elsewhere (2). There were five male and nine female subjects. Three subjects (R. C., C. L., and L. B.) have died of acute myocardial infarctions. At postmortem, R. C. had extensive coronary and thoracic aorta atherosclerosis.

All subjects ate regular food. Blood was collected into tubes containing solid EDTA after an overnight fast of at least 12 hr. No subject was taking medication except during the cholestyramine treatment period. Plasma sterol concentrations were determined in ten normolipidemic individuals of the same age range and served as control values.

Chemical

Plasma sterol concentrations were measured by gas–liquid chromatography (GLC) according to the method of Ishikawa et al. (12). Briefly, plasma (1 ml) was saponified in 1 N NaOH for 1 hr. The neutral sterols were extracted with hexane and the solvent was evaporated. Neutral sterols (approximately 100 μg) were redissolved in 200 μl of hexane containing 140 μg of 5α-cholestane as an internal standard, and 3 μl was analyzed by GLC. The underivatized free sterols were separated on 180 cm × 4 mm glass columns packed with 1% SP-1000 on 80/100 mesh Gas Chrom Q (Supelco Inc., Bellefonte, PA) without prior purification by argentation thin-layer chromatography. A Hewlett Packard, Model 5830, gas chromatograph equipped with a flame ionization detector was operated at the following conditions: column temperature 230°C, flame detector 260°C, flash heater 250°C, N_2 flow 30 cc/min. The retention times (RRT) relative to 5α-cholestane for ten consecutive determinations were: cholestanol 6.3 ± 0.1, cholesterol 7.1 ± 0.1, 5α-campestanol 8.3 ± 0.1, campesterol 9.2 ± 0.1, 5α-sitostanol 10.2 ± 0.1, and sitosterol 11.3 ± 0.2. Continuous use of the SP-1000 column at these conditions is associated with shortening of the sterol retention times and periodic monitoring with reference standards was necessary. The identity of the sterols was checked by co-chromatography with authentic reference sterols and was confirmed by mass spectroscopy according to Dayal et al. (13). The mass of each sterol was corrected for analytic losses by recovery of known quantities of reference standards. A typical chromatogram that illustrates the separation of the sterols on SP-1000 is given in **Fig. 3**.

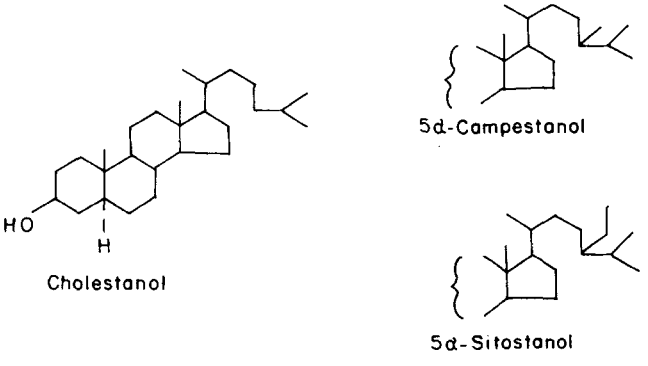

Fig. 2 Structures of 5α-saturated stanols present in plasma of sitosterolemic subjects.

TABLE 1. Plasma sterol concentration

Patient	Age	Sex	Cholesterol	Cholestanol	Campesterol	Campestanol	Sitosterol[b]	Sitostanol
	yr				*mg/dl*			
Ki. C. (n = 10)[a]	24	F	245 ± 39	6.7 ± 1.1	12 ± 1.1	2.3 ± 0.4	20 ± 2.3	4.2 ± 1.1
Ke. C. (n = 10)	18	F	202 ± 25	4.7 ± 1.0	8 ± 3.1	1.4 ± 0.2	14 ± 4.1	2.2 ± 0.7
T. C. (n = 10)	22	F	233 ± 12	3.8 ± 1.4	10 ± 5.1	1.9 ± 1.0	21 ± 8.3	5.4 ± 2.5
R. C. (n = 10)	16	M	249 ± 39	7.5 ± 2.4	13 ± 1.5	2.6 ± 0.9	20 ± 5.5	3.9 ± 1.1
C. L.	52	M	134	1.6	13	1.5	27	3.0
M. M.	7	M	202	1.2	12	1.6	26	3.1
L. H. O.	32	F	207	2.5	10	1.9	28	2.0
R. H.	30	F	368	3.6	29	9.0	65	8.0
P. M.	41	F	169	1.2	18	7.0	42	4.0
P. Z.	22	M	324	3.9	27	3.0	60	6.0
M. Z.	20	M	271	3.8	19	2.0	42	4.0
R. S.	32	F	256	3.1	15	1.0	29	3.0
L. B.	24	F	482	11	24	4.0	56	6.0
J. B.	38	F	336	4.9	20	1.2	45	3.2
Mean ± SD			258 ± 96	4.2 ± 2.7	16 ± 7	2.9 ± 2.3	35 ± 16	4.1 ± 1.7
% of Total sterols			80.5	1.3	5.0	0.9	11	1.3
% of Respective Δ^5-derivative				1.6		18		13
Control (10)			187 ± 29	0.4 ± 0.2			0.3 ± 0.3	

[a]Ten consecutive monthly determinations; average of two measurements given for remaining subjects.
[b]Small quantities of stigmasterol (24-ethyl-5,22-cholestadien-3β-ol) accompanied campesterol and sitosterol but only amounted to 1-3% of the sitosterol mass.

In separate experiments, the Δ^5-sterols, cholesterol, campesterol, and sitosterol were separated from their 5α-dihydro derivatives cholestanol, 5α-campestanol, and 5α-sitostanol by argentation thin-layer chromatography, and were then quantitated as their trimethylsilyl ether derivatives by gas-liquid chromatography on 180 cm × 4 mm glass columns packed with 3% QF-1 (Applied Science Lab, State College, PA). The retention times relative to 5α-cholestane of the trimethylsilyl ether derivatives are: cholesterol 1.73, cholestanol 1.85, campesterol 2.52, 5α-campestanol 2.66, sitosterol 3.03, and 5α-sitostanol 3.17. It is necessary to separate the unsaturated sterols from their 5α-saturated derivatives by argentation thin-layer chromatography because only small differences exist between the unsaturated and 5α-saturated sterol peak retention times on QF-1 (2). However, quantitative results by the two independent methods agreed within ± 10%.

Lipoproteins were separated by the method of Havel, Eder, and Bragdon (14). After separation of the low density and high density lipoprotein fractions, the proportions of free and esterified sterols were measured in each fraction (11).

RESULTS

Plasma concentrations of campesterol and sitosterol (Table 1) were markedly elevated in all fourteen clinically affected subjects, consistent with previous findings that this is the major biochemical determinant in establishing the diagnosis of this condition (1-10). Plasma cholesterol levels were elevated in seven of fourteen subjects, which was a finding of importance. Approximately 80% of the unsaturated sterols were cholesterol and 16% were plant sterols. Of the remaining plasma sterols, cholestanol concentrations were increased in all subjects and the mean

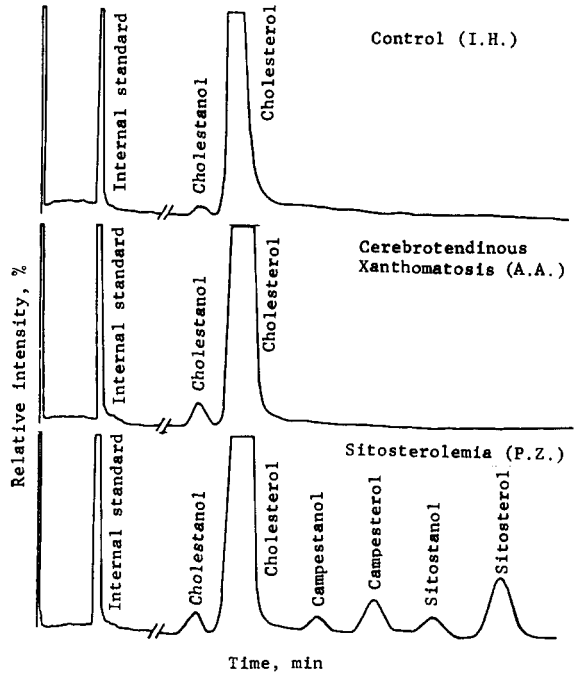

Fig. 3 Gas-liquid chromatograms of underivatized sterols present in plasma of control, cerebrotendinous xanthomatosis, and sitosterolemic patients that illustrate the separation of the Δ^5-unsaturated sterols from their 5α-saturated analogs on SP 1000 column.

value was 10-fold higher than the mean for plasma cholestanol determined in ten normolipidemic subjects. Similarly, high concentrations of 5α-campestanol and 5α-sitostanol were present in all subjects. These 5α-saturated stanols were not found in the plasma of the normolipidemic controls. Interestingly, cholestanol, although increased, amounted to only 1.6% of the plasma cholesterol level, while 5α-campestanol and 5α-sitostanol represented 18% and 13% of their respective unsaturated derivatives.

In three affected and two control subjects, plasma lipoproteins were fractionated by sequential ultracentrifugation and the sterol composition was determined. The results are given for low density lipoproteins (LDL) in **Table 2** and for high density lipoproteins (HDL) in **Table 3**, along with the proportions of esterified sterols. In the control LDL, only small amounts of cholestanol (0.4%) were found. Plant sterols or their saturated analogs were not detected. In contrast, substantial amounts of campesterol, sitosterol, and the 5α-saturated stanols, cholestanol, 5α-campestanol, and 5α-sitostanol were present in sitosterolemic LDL. Of interest, the proportion of esterified plant sterols and 5α-saturated stanols was lower than for cholesterol in this fraction (Table 2). In sitosterolemic HDL, cholesterol concentrations were lower while cholestanol levels were similar to controls. Both campesterol and sitosterol were present, but only trace quantities of their 5α-saturated analogs were detected. The proportion of sterol esters was comparable to controls. Thus, increased amounts of plant sterols and 5α-saturated stanols are carried mainly unesterified in LDL and are a constant biochemical abnormality in patients with sitosterolemia. High density lipoproteins transport proportionally less plant sterols and 5α-saturated stanols than LDL in these subjects.

The average daily intake of dietary sterols and calories consumed by members of the C-family are listed in **Table 4.** The cholesterol and plant sterol intakes were determined by GLC on food that was set aside, mixed together with water, and homogenized; aliquots were analyzed for sterol composition. Cholesterol and the plant sterols were present in plentiful amounts since no attempt was made to restrict the diet during this period of observation. However, cholestanol constituted only about 0.5% of the dietary cholesterol consumed while 5α-saturated plant sterol analogs could not be detected. Thus, only trace quantities of cholestanol and no 5α-saturated plant sterol derivatives were found in the diet of this family.

In separate experiments, the effect of cholestyramine was evaluated on plasma sterol concentrations in four sitosterolemic subjects and the results are given in **Table 5.** Prior to treatment, plasma sterol concentrations were typical for these subjects with elevated levels of cholesterol, campesterol, sitosterol, and their respective 5α-saturated analogs cholestanol, 5α-campestanol, and 5α-sitostanol. After treatment with up to 12 g/day of cholestyramine for at least 1 month, plasma sterol concentrations declined markedly in all subjects; cholesterol and phytosterol levels decreased 45%, while 5α-saturated sterols decreased 55%. In two subjects (Ki. C. and Ke. C), plasma sterol concentrations rose substantially 1 month after cholestyramine was discontinued.

DISCUSSION

The results of these studies confirm and extend our knowledge regarding the biochemical defects in the lipid disorder sitosterolemia with xanthomatosis. All symptomatic subjects had increased campesterol and sitosterol concentrations associated with normal to moderately elevated plasma cholesterol levels, which are expected in this disease (1). In addition, plasma concentrations of the 5α-saturated stanols, cholestanol, 5α-campestanol, and 5α-sitostanol, which are the respective 5α-dihydro analogs of cholesterol, campesterol, and sitosterol (Fig. 1), were also in high concentration and were present in eight subjects where previously these stanols were not detected (1, 3, 7). However, the earlier measurements of plasma sterols in these subjects were made by GLC on columns (1% SE 30) that would not separate 5α-saturated stanols from their respective unsaturated sterol derivatives, and thus these sterols would be missed.

TABLE 2. Low density lipoproteins sterol composition

Patient	Cholesterol	Cholestanol	Campesterol	Campestanol	Sitosterol	Sitostanol
			mg/dl (% ester)			
Ke. C.	120(71)	7.1(54)	12.0(10)	2.0(10)	16(14)	4.0(11)
R. C.	220(70)	7.0(13)	12.0(40)	1.9(11)	21(46)	3.2(9)
M. M.	90(71)	3.5(51)	16.0(26)	1.6(13)	32(20)	3.5(17)
Mean	143(71)	5.9(39)	13.0(25)	1.8(11)	23(27)	3.6(12)
Control (n = 2)	76(70)	0.3(66)				

TABLE 3. High density lipoproteins sterol composition

Patient	Cholesterol	Cholestanol	Campesterol	5α-Campestanol	Sitosterol	5α-Sitostanol
			mg/dl (% ester)			
Ke. C.	45(82)	0.9(62)	0.2		3.3(88)	
R. C.	26(76)	0.7(63)	0.6		1.9	
M. M.	37(79)	0.6(62)	0.3		6.3(83)	
Mean	36(79)	0.7(62)	0.4		3.5(85)	
Control (n = 2)	49(84)	0.5(60)				

The absolute identification of the 5α-saturated stanols was based upon co-chromatographic comparison with known reference standards that were prepared chemically from their respective unsaturated derivatives by a hydroboration-protonolysis sequence as described by Dayal et al. (13). The structures of the sterols also were confirmed by mass spectroscopy (13, 15). Thus, elevated plasma levels of cholestanol, 5α-campestanol, and 5α-sitostanol are additional biochemical abnormalities in this rare lipid storage disease and these 5α-stanols along with phytosterols are deposited in virtually every tissue in the same proportion as plasma.

It was noted that, like cholesterol and unsaturated plant sterols, the 5α-saturated stanols were transported mainly by low density lipoproteins. Further, plant sterols and 5α-saturated stanols in sitosterolemic low density lipoproteins were less esterified than cholesterol, which suggests that hepatic acylcoenzyme A:cholesterol acyltransferase and plasma lechithin:cholesterol acyltransferase may be sterol-specific. In contrast, sitosterolemic high density lipoproteins transported less cholesterol with only slightly increased amounts of cholestanol and plant sterols. However, the relative proportion of cholesterol and cholestanol esters was similar to control. These results suggest that less sterol may be transported by high density lipoproteins.

In regard to the mechanism of sterol accumulation in this condition, it is well-established that enhanced intestinal absorption of the unsaturated plant sterols (campesterol and sitosterol) occurs and, perhaps in combination with reduced removal, leads to their retention in the body (1, 5, 7). Since most diets include a plentiful supply of vegetables that contain these sterols, the availability for absorption is widespread. Indeed, analysis of the diet of the C-family (Table 4) showed that almost 200 mg of phytosterols and 400 mg of cholesterol per 2000/calories were consumed each day. Thus, these individuals (C-family) ate food rich in cholesterol and plant sterols that were similar in quantity to that found in most American diets. Importantly, only about 2 mg of cholestanol and trace quantities of the saturated plant sterol derivatives, 5α-campestanol and 5α-sitostanol, were present in the daily diet. Furthermore, in previous studies, we have determined that normal individuals synthesize about 12 mg of cholestanol each day (16). Therefore, the majority of the cholestanol and virtually all of 5α-campestanol and 5α-sitostanol that are found in these subjects probably were produced endogenously.

It is now established that cholestanol is formed from cholesterol via the ketonic intermediate, 4-cholesten-3-one (17, 18), and that liver microsomes contain the 3β-hydroxy $\Delta^{4,5}$ steroid dehydrogenase that catalyzes this reaction. In contrast, skin fibroblasts from both normal subjects and those with cerebrotendinous xanthomatosis when grown in sterol-deficient media do not produce cholestanol, although cholesterol biosynthesis is normal (19). Thus, the liver appears to be essential for the formation of cholestanol. It is very likely that 5α-campestanol and 5α-sitostanol are derived from campesterol and sitosterol via an analogous pathway.

Treatment with cholestyramine reduced saturated and unsaturated plasma sterol concentrations in all treated subjects. Plasma concentrations of unsaturated sterols declined about 44%, while the corresponding 5α-saturated stanols diminished about 57%. Thus, treatment produced a greater effect on saturated sterol levels. Cholestyramine acts by promoting the intestinal loss of bile acids, thereby increasing hepatic bile acid formation from cholesterol (11). As a result, more plasma cholesterol is utilized for bile acid synthesis and plasma levels decline.

TABLE 4. Diet sterol composition

Substance	Amount/Day
Diet	
Calories	2000
Carbohydrate	225 g
Protein	75 g
Fat	90 g
Sterols	
Cholesterol	400 mg
Campesterol	65 mg
Stigmasterol	5 mg
Sitosterol	130 mg
Cholestanol	2 mg
5α-Campestanol	not detected
5α-Sitostanol	not detected

TABLE 5. Effect of cholestyramine on plasma sterol concentrations

Patient	Treatment (Duration)	Cholesterol	Cholestanol	Campesterol	5α-Campestanol	Sitosterol	5α-Sitostanol
				mg/dl			
Ki. C.	None	363	4.0	22	6	44	5
	Cholestyramine (1 month)	192	1.6	13	4	28	1
	None	270	2.2	19	5	36	4
Ke. C.	None	250	2.5	20	5	38	5
	Cholestyramine (1 month)	172	1.0	9	1.4	25	2
	None	234	2.2	18	4.4	34	4
L. H. O.	None	207	2.5	10	2	28	2
	Cholestyramine (12 months)	126	0.5	6.1	0.7	20	1.7
R. H.	None	368	3.6	29	9	65	8
	Cholestyramine (12 months)	125	0.8	8	2	24	2.0

Further, because of the enhanced fecal losses, bile acid concentrations in the upper intestine may fall sufficiently to interfere with intestinal sterol absorption. Both mechanisms may play a role in reducing the plasma concentrations of cholesterol, plant sterols, and 5α-stanols. In addition, the activation of cholesterol 7α-hydroxylase, the rate-controlling enzyme in bile acid synthesis which occurs with cholestyramine treatment, may increase the formation of 7α-hydroxylated sterols committed for bile acid synthesis, thereby reducing the intrahepatic pool of sterols available for conversion into 5α-stanols.

It is of considerable interest that subjects with both sitosterolemia and xanthomatosis and cerebrotendinous xanthomatosis exhibit common features: xanthomatosis, premature atherosclerosis, and high plasma cholestanol levels (2). However, in sitosterolemia, increased plant sterols and 5α-saturated stanols are found, no neurologic dysfunction has been noted, and cholestyramine treatment lowered plasma sterol levels, including cholestanol, markedly. In contrast, during cholestyramine treatment, plasma cholestanol levels in cerebrotendinous xanthomatosis increased 4-fold. Clearly, these two conditions in which plasma cholestanol is high are different.

In summary, increased plasma cholestanol and 5α-saturated plant stanol derivatives, 5α-campestanol and 5α-sitostanol, were found in the plasma of all fourteen subjects with sitosterolemia and xanthomatosis. Since diet contained little cholestanol and virtually no saturated stanols, absorption of these compounds probably was not responsible for their plasma accumulation. It is proposed that increased endogenous synthesis from the corresponding unsaturated sterols, cholesterol, campesterol, and sitosterol occurs. Treatment with cholestyramine reduced plasma sterol concentrations. The importance of this abnormality is not known. Whether its significance is limited to a biochemical marker of this disease or whether 5α-stanols contribute to the development of the accelerated atherosclerosis which is the major clinical feature remains to be determined.

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