Sterol absorption and sterol balance in phytosterolemia evaluated by deuterium-labeled sterols: effect of sitostanol treatment

Dieter Lütjohann,* Ingemar Björkhem,† Uli F. Beil, and Klaus von Bergmann[1],***

Department of Clinical Pharmacology,* University of Bonn, Sigmund-Freud-Strasse 25, 53105 Bonn, Germany; Division of Clinical Chemistry,† Karolinska Institute, Huddinge University Hospital, 14186 Huddinge, Sweden; and Department of Internal Medicine,** University Hospital Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany

Abstract Absorption of dietary cholesterol, campesterol, and sitosterol, cholesterol balance, and fecal excretion of plant sterols were determined in three unrelated patients with phytosterolemia and three healthy volunteers during constant intake of cholesterol and plant sterols using accurate gas–liquid chromatography–mass spectrometry techniques. Each subject received a mixture of [26,26,26,27,27,27-$^2H_6$]cholesterol, [6,7,7-$^2H_3$]sitostanol, and [6,7,7-$^2H_3$]campesterol together with two non-absorbable markers, [5,6,22,23-$^2H_4$]sitostanol and chromic oxide. Feces were collected from days 5 to 7 and absorption of different sterols was calculated from the intestinal disappearance of the different sterols relative to [5,6,22,23-$^2H_4$]sitostanol and chromic oxide. The results obtained by the two markers were not different and the absorption of cholesterol averaged 53 ± 4% for the patients (mean ± SD) and 43 ± 3% for the volunteers. Campesterol absorption averaged 24 ± 4% in patients and 16 ± 3% in healthy volunteers, whereas sitosterol absorption averaged 16 ± 1% and 5 ± 1%, respectively. Cholesterol synthesis expressed by body weight varied considerably in the two groups but appeared to be about 5 times lower in patients than in controls. Administration of a high dose of sitostanol (0.5 g t.i.d.) to two patients was followed by a reduction in cholesterol absorption by 24% and 44%, an increase in fecal output of cholesterol and steroids derived from cholesterol and plant steroids, and a marked reduction of serum cholesterol, campesterol, and sitosterol. Under the conditions used, inhibition of cholesterol absorption by sitostanol was not followed by a significant rise in cholesterol synthesis. The time of observation was, however, too short to allow final conclusion on this. The results show that the absolute difference in absorption rate of different sterols between the patients and healthy volunteers was about the same. As a consequence, increasing hydrophobicity causes a relative decrease of absorption rates. Thus, patients with phytosterolemia seem to have a generally increased absorption of sterols rather than a loss of a specific discriminatory mechanism, and oral administration of sitostanol seems to be an interesting new approach for treatment of phytosterolemia.—**Lütjohann, D., I. Björkhem, U. F. Beil, and K. von Bergmann.** Sterol absorption and sterol balance in phytosterolemia evaluated by deuterium-labeled sterols: effect of sitostanol treatment. *J. Lipid Res.* 1995. **36:** 1763–1773.

Supplementary key words plant sterols · stable isotopes · bile acid excretion

Phytosterolemia, a rare inherited sterol storage disease, was first described in 1974 (1). This disease is characterized by tendon and tuberous xanthomas and by a strong predisposition to premature coronary atherosclerosis (for a review see ref. 2). Increased amounts of phytosterols (plant sterols), such as sitosterol and campesterol as well as the corresponding 5α-stanols, are found in blood, plasma, erythrocytes, and different tissues, especially in the xanthomas and arteries of the affected subjects. Elevated circulating levels of cholesterol and cholestanol are also found in many patients. Increased intestinal absorption of cholesterol, sitosterol, as well as shellfish sterols has been documented in same cases (3–6). According to some studies there is a reduced biliary output of cholesterol, phytosterols, and shellfish sterols (5–7). In addition, the rate of cholesterol synthesis is markedly reduced in patients with phytosterolemia (5, 8–10). The role of the intestinal hyperabsorption of sterols in this metabolic disorder has not been studied systematically for at least

Abbreviations: GLC–MS, gas–liquid chromatography–mass spectrometry; Cr_2O_3, chromic oxide; SIM, selective ion monitoring; TMSi-, trimethylsilyl-; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; ACAT, acyl-CoA:cholesterol acyltransferase.

[1]To whom correspondence should be addressed.

two reasons. First, phytosterolemia is an extremely rare disease, and second, existing methods for the measurement of sterol absorption are usually performed with radioactive labeled tracers. One recent approach for estimating sterol absorption was the introduction of deuterium-labeled tracers and the quantification of these stable isotopes by combined gas–liquid chromatography–mass spectrometry (GLC–MS) (11). In the present work we have synthesized deuterium-labeled campesterol and sitosterol and used them for measurement of absorption of plant sterols in patients with phytosterolemia.

Previous therapeutic approaches for the treatment of elevated serum plant sterols and cholesterol concentrations in phytosterolemia focused on interruption of the enterohepatic circulation of bile acids by cholestyramine or ileal bypass surgery (3, 5, 12–15). Neomycin administration to reduce intestinal sterol absorption failed to affect serum sterol concentrations in one phytosterolemic subject (5). Recent studies from our group have shown that oral administration of sitostanol in children with severe familial hypercholesterolemia causes a reduction in plant sterol concentration that is more pronounced than reduction of serum cholesterol: sitostanol obviously interferes not only with cholesterol absorption, but also with plant sterol absorption (16). As sitostanol is not significantly absorbed in human subjects (17, 18), an attempt was made here to treat phytosterolemic patients with this saturated plant sterol.

METHODS

Human subjects

Three unrelated patients with phytosterolemia, patient 1 (P 1), 48 years old, weight 70 kg, male; P 2, 23 yrs, 75 kg, male; and P 3, 19 yrs, 46 kg, female, participated in the study. Three healthy male volunteers, V 1, 35 yrs, 94 kg; V 2, 27 yrs, 60 kg; and V 3, 29 yrs, 71 kg, served as controls.

Experimental design

Absorption rates of cholesterol, campesterol, and sitosterol together with fecal excretion of neutral and acidic steroids were measured in the patients and healthy volunteers. For this purpose, all subjects were kept for 1 week under metabolic ward conditions and consumed an isocaloric mixed food diet containing 55% carbohydrate, 30% fat, and 15% protein. GLC analysis of dietary steroid content revealed an average intake of 255 mg/day of cholesterol, 27 mg/day of campesterol, and 145 mg/day of sitosterol. Each subject received a mixture of 3 mg [26,26,26,27,27,27-$^2H_6$]cholesterol, 3 mg [6,7,7-$^2H_3$]sitosterol, and 2 mg [6,7,7-$^2H_3$]campesterol t.i.d. for 7 days together with two different fecal markers. The first marker was [$^2H_4$]sitostanol (3 mg t.i.d.), the second was chromic oxide (Cr_2O_3, 60 mg t.i.d.). Total daily feces were collected before marker administration and on days 5, 6, and 7, the exact weight was noted, and the material was immediately frozen at −20°C. All subjects had daily bowel movement. Serum samples were taken on day 0, and days 5 and 7.

Two patients with phytosterolemia (patients 2 and 3) agreed to treatment with sitostanol for 4 weeks. Sitostanol treatment was performed at home and 0.5 g t.i.d. was administered in gelatin capsules. The daily cholesterol intake during the last week of treatment was calculated from a 7-day food diary by a commercial computer program (19). No standard diet was given, both subjects were on their regular, mixed solid-food diet. The average cholesterol intake was 315 mg/day for patient 2, and 270 mg/day for patient 3. During the last week, fecal excretion of neutral and acidic steroids and cholesterol absorption were determined as described previously (11, 19). Serum samples for GLC analysis of steroids were collected on days 0, 14, and 28.

The study was in accordance with the principles of the Helsinki Declaration, the research protocol was approved by the local ethics committee, and all subjects gave informed consent.

Chemicals

Unlabeled compounds. 5α-Cholestane was purchased from Serva Feinbiochemica, Heidelberg, Germany; coprostanol and coprostanone from Steraloid Incorporation, Wilton, NH; cholesterol, sitosterol, and hyodeoxycholic acid from Sigma Chemical Corporation, St. Louis, MO; and sitostanol from Delalande Arzneimittel, Köln, Germany. The purity of the sterols was checked by thin-layer chromatography (TLC) and GLC–MS. Purity was better than 98%, except for sitostanol, which contained 8% of campestanol, and for sitosterol with 40% campesterol. 24-Ethyl-/methylcoprostanol and 24-ethyl-/methylcoprostanone were synthesized from 24-ethyl-/methylcholesterol (sitosterol/campesterol) in two steps. Oppenauer-oxidation using aluminium-tertiary-butoxide (Fluka Chemie AG, Buchs, Switzerland) in dried toluene/acetone led to the corresponding 4-cholesten-3-one products (20). Further reduction by hydrogen gas with platinum-IV-oxide as a catalyst yielded a mixture of 24-ethyl-/methylcoprostanol and -coprostanone. Coprostanols and coprostanones were separated by TLC and the purity of the substances was judged by combined GLC–MS as their trimethylsilyl-(TMSi-)ethers. All other chemicals and solvents used for sample preparation and derivatization were of grade recommended in the literature. Chromic oxide (Cr_2O_3) was supplied by Merck, Darmstadt, Germany.

Labeled compounds. [26,26,26,27,27,27-$^2H_6$]cholesterol and [5,6,22,23-$^2H_4$]sitostanol were obtained from Medical Isotopes Incorporation, Concord, NH. Isotopic purity for [$^2H_6$]cholesterol was higher than 88% and contained less than 0.1% unlabeled compound. The isotopic purity for [$^2H_4$]sitostanol was 30% and contained 5% of unlabeled compound. [6,7,7-$^2H_3$]sitosterol and [6,7,7-$^2H_3$]campesterol were synthesized by a modification of a method published by Corey and Gregoriou (21) and Anagnostopoulos and Fieser (22). GLC–MS analysis of the products, corrected for natural abundance (23), showed an isotopic distribution pattern with 2.63% on M^{+1}, 24.19% on M^{+2}, 67.38% on M^{+3}, and 1.34% on M^{+4} for [6,7,7-$^2H_3$]sitosterol and [6,7,7-$^2H_3$]campesterol, respectively.

Deuterated capsules. We dissolved a mixture (1:1:1.5; w/w/w) of [$^2H_6$]cholesterol, [$^2H_4$]sitostanol, and [$^2H_3$]sitosterol/campesterol (60/40; w/w) in a common commercial plant oil with a plant sterol content lower than all other similar preparations by gently heating and stirring. After complete dissolution, 0.4 ml (containing 3 mg each of [$^2H_6$]cholesterol, [$^2H_4$]sitostanol, and [$^2H_3$]sitosterol, 2 mg of [$^2H_3$]campesterol, and 30 mg of sitostanol) was added to gelatin capsules. These capsules dissolved in the stomach, where the content was released. The capsules were kept at +4°C until use.

Analytical methods

Serum lipid analysis. Serum total steroids and triglycerides were measured enzymatically (24, 25), HDL-steroids were measured enzymatically in the supernatant after precipitation of apolipoprotein B- containing lipoproteins (26), and LDL-steroids were calculated using the Friedewald formula (27).

Analysis of dietary sterols. An exact dietary plan was worked out, containing the amounts of each food compound consumed (weight or volume). In a first study, exactly one tenth of each food compound together with an aliquot of the capsule content (120 µl) were pooled within a day. This mixture was homogenized with water (1:1, w/w), an aliquot of 50 mg was taken, and after addition of 50 µg 5α-cholestane as internal standard (ISTD), the sample was hydrolyzed at 67°C by adding 1 ml 1 N NaOH in 90% ethanol. The neutral sterols were extracted three times with 3 ml n-hexane and after removal of the solvent under a gentle stream of dried nitrogen at 50°C the sterols were converted to trimethylsilyl- (TMSi-)ethers by treatment with 1 ml pyridine–hexamethyldisilazane–trimethylchlorosilane 3:2:1 (v/v/v) at 60°C for 30 min. After removal of the silylating reagents under a nitrogen stream, the residue was dissolved in 1 ml n-hexane and transferred to an autosampler vial. Gas–liquid chromatography was performed on a Hewlett-Packard (HP) 5890 gas chromatograph equipped with an SE 30 capillary column (25 m × 0.32 mm, 0.25 µm phase thickness; Fa. Chromatographie Service, Langerwehe, Germany) connected with a 2-m deactivated, uncoated pre-column (0.53 mm inner diameter). An on-column injection technique was used followed by flame ionization detection. The oven temperature program was as follows: 150°C for 3 min, 30°C/min to 250°C, then 5°C/min to 280°C, where the temperature was kept for 5 min. Hydrogen was used as carrier gas with an inlet pressure of 15 psi. The retention times (minutes) for the different sterols were: 5α-cholestane (ISTD), 13.15; cholesterol, 16.80; campesterol, 18.70; sitosterol 20.65. Peak area integration was done by an HP 3396 A integrator. Concentrations of different steroids were calculated from standard curves prepared from the appropriate compounds.

Cholesterol and non-cholesterol steroids in serum

Fifty µl serum was hydrolyzed, extracted, and silylated as described above after addition of 50 µg 5α-cholestane. GLC analysis was performed on the same system as described previously and the retention times (min) of the interesting serum sterols were: 5α-cholestane (ISTD), 13.15; cholesterol, 16.80; cholestanol, 16.97; desmosterol, 17.56; lathosterol, 17.80; campesterol, 18.73, campestanol 18.93; stigmasterol 19.33; sitosterol, 20.62; sitostanol 20.89; avenasterol, 21.02. Peak area integration was done by an Spectra Physics (SP 4290) integrator, Spectra Physics, San Jose, CA.

Preparation of fecal samples

Total fecal samples from days 0, 5, 6, and 7 were homogenized with distilled water (1:1), and exactly one tenth (weight) was kept frozen for further analysis of neutral and deuterated neutral and acidic steroids, and chromium (19), and for calculation of fecal excretion of neutral and acidic steroids, and sterol absorption (11, 19). Fifty µg 5α-cholestane and 50 µg hyodeoxycholic acid were added to an aliquot (exactly weighed, in the range between 90 and 110 mg) of the homogenate as internal standards for neutral and acidic steroids, respectively. Hydrolysis, extraction, and silylation of fecal neutral steroids were the same as described above for serum and dietary neutral steroids. The aqueous phase of the sample, containing the bile acids, was acidified with 25% HCl to a pH lower than 1.5, and the acidic steroids were extracted three times with 3 ml diethyl ether. The combined ether phases were evaporated to dryness under nitrogen. Methylation was performed by adding 2 ml dried methanol, 1.4 ml dimethoxypropane, and 20 µl concentrated HCl. The samples were mixed thoroughly and allowed to stand at room temperature for at least 1 h. After evaporation to dryness the methylester of the bile acids was additionally derivatized

to TMSi-ethers as described above. Two ml of n-hexane was added, and after centrifugation 1 μl was transferred to an autosampler vial.

Gas–liquid chromatographic analysis of neutral and acidic steroids

Fecal neutral and acidic steroids were analyzed with the same GLC equipment as described above. The following neutral steroids were used for calculation of fecal excretion after separation (min) on the column: 5α-cholestane, 13.15; coprostanol, 15.40; coprostanone, 16.30; cholesterol, 16.80; methylcoprostanol, 17.01; methylcoprostanone, 17.87; ethylcoprostanol 18.56; campesterol 18.73, ethylcoprostanone, 19.95; sitosterol, 20.65; sitostanol, 20.90.

For optimal separation of the relevant acidic compounds, the following temperature program was used: 3 min at 150°C, 30°C/min to 240°C (15 min), then 3°C to a final temperature of 270°C; inlet pressure 15 psi. The main acidic steroids were separated and had the following retention times: isolithocholic acid, 25.61; lithocholic acid 25.90; isochenodeoxycholic acid 25.99; isodeoxycholic acid 27.55, deoxycholic acid, 27.67; chenodeoxycholic acid 28.58; cholic acid, 28.82; hyodeoxycholic acid (ISTD), 29.05; ursodeoxycholic acid 29.70; 7-ketolithocholic acid, 30.71.

Quantification and calculation of neutral and acidic steroid excretion using chromic oxide or [$^2H_4$]sitostanol as fecal marker

Chromium was determined according to Calvert with some modifications (28). The concentration of [$^2H_4$]sitostanol was determined by a combination of GLC and GLC–MS. We carried out measurements of concentrations of natural TMSi-ethers of cholesterol, sitosterol, and campesterol together with their corresponding 5β-stanols (coprostanol) and 5β-stanones (coprostanone) and sitostanol by GLC using 5α-cholestane as internal standard. The absolute concentration of [$^2H_4$]sitostanol was calculated by the percental distribution pattern on M^{+4} calculated by GLC–MS, as described in the next paragraph, and the daily neutral and acidic steroid excretion finally calculated according to the following equation:

$$\text{Neutral / acidic steroids (mg / day)} = \frac{\text{Neutral / acidic steroids (mg / g sample)}}{\text{Cr (mg / g sample) or [}^2\text{H}_4\text{]sitostanol (mg / g sample)}} \times \text{Cr or [}^2\text{H}_4\text{]sitostanol (mg / day)}$$

Gas–liquid chromatography–mass spectrometry

The absorption of cholesterol, campesterol, and sitosterol was calculated after GLC and GLC–MS quantification of natural and deuterated cholesterol, coprostanol, coprostanone, campesterol, methylcoprostanol, methylcoprostanone, sitosterol, ethylcoprostanol, and ethylcoprostanone, and deuterated sitostanol as marker. GLC–MS was performed on a Hewlett-Packard (HP 5970) quadrupole type mass spectrometer with an HP 5890 gas chromatograph and an HP 7698 A automatic sample injector. The steroids were separated on an HP 1 dimethylsilicone capillary column (12 m × 0.2 mm, 0.25 μm phase thickness). Helium was used as carrier gas with a column head pressure of 35 kPa. A splitless type of injection system was used. One μl of the sample was injected. The injector temperature was 270°C. The oven temperature was set to an initial value of 180°C during 1.5 min after sample injection and then increased at a rate of 19°C/min to a final temperature of 300°C and immediately raised again with a rate of 10°C/min to a final temperature of 300°C, where the temperature was kept constant for 8.5 min. Electron impact ionization was applied with 70 eV of ionizing energy. The mass spectrometer was operated in the selective ion monitoring (SIM) mode and several ions of the same steroid were detected within a short time interval, corresponding to the degree of labeling of the sterol. The selected ions were monitored at least 20 scans as a minimum sampled over the peak of the eluting compound. The ions used for analysis (*m/z*) and acquisition time intervals (minutes) for the compounds were as follows: coprostanol: [$^2H_6$]coprostanol, 370–376, 10.20–10.40 min; coprostanone: [$^2H_6$]coprostanone, 386–392, 10.40–10.75 min; cholesterol: [$^2H_6$]cholesterol, 458–464, 10.75–11.10 min; methylcoprostanol: [$^2H_3$]methylcoprostanol, 384–387, 11.10–11.50 min; methylcoprostanone: [$^2H_3$]methylcoprostanone, 400–403, 11.50–11.85 min; ethylcoprostanol: [$^2H_3$]ethylcoprostanol, 398–401, 11.85–12.00 min; campesterol: [$^2H_3$]campesterol, 472–475, 12.00–12.45 min; ethylcoprostanone: [$^2H_3$]ethylcoprostanone, 414–417, 12.45–12.80 min; sitosterol: [$^2H_3$]sitosterol, 486–489, 12.80–13.00 min; sitostanol: [$^2H_4$]sitostanol, 488–492, 13.00–13.15 min. The electron ionization energy was 70 eV. Mass spectra of all steroids (TMSi-ethers of 3β-hydroxy steroids) were obtained by scanning between 100 and 500 *m/z*.

Calculation of sterol absorption

The absorption rates of the different sterols were calculated from the constant ratio of deuterated cholesterol, campesterol, and sitosterol to the orally administered [$^2H_4$]sitostanol or chromic oxide and their disappearance during intestinal transit relative to the non-absorbable markers ([$^2H_4$]sitostanol or chromic oxide). For this purpose the amounts (areas) of natural and deuterated sterols (M^+, M^{+1}, M^{+2}, ...M^{+x}) and their corresponding metabolites (coprostanols and copro-

stanones) were measured by GLC–MS. With the use of %-isotopic distribution the amount of deuterated steroids could be quantified from the amounts, quantitated before by GLC of the same sample. The values obtained for days 5, 6, and 7 were finally corrected for the natural isotopic abundance on M^{+x} for unlabeled steroids on day 0, before administration of the markers. Results obtained by these measurements were then used for the calculation of sterol absorption rates using [$^2H_4$]sitostanol or chromic oxide as non-absorbable markers. The following equation was applied:

$$\% \text{ absorption of } S_{1\text{-}3} = 100 \times \left[1 - \frac{\text{fecal}\left(\frac{A_{1\text{-}3} + B_{1\text{-}3} + C_{1\text{-}3}}{\text{marker } D_{1,2}} \right)}{\text{dietary}\left(\frac{A_{1\text{-}3}}{\text{marker } D_{1,2}} \right)} \right] \qquad \textit{Eq. 1)}$$

where S_1 = cholesterol, S_2 = campesterol (24-methylcholesterol), S_3 = sitosterol (24-ethylcholesterol); and A to D are the concentrations of: A_1 = [$^2H_6$]cholesterol, B_1 = [$^2H_6$]coprostanol, C_1 = [$^2H_6$]coprostanone; A_2 = [$^2H_3$]campesterol, B_2 = [$^2H_3$]methylcoprostanol, C_2 = [$^2H_3$]methylcoprostanone; A_3 = [$^2H_3$]sitosterol, B_3 = [$^2H_3$]ethylcoprostanol, C_3 = [$^2H_3$]ethylcoprostanone; D_1 = [$^2H_4$]sitostanol, D_2 = Cr_2O_3.

Statistical analysis

We expressed our results as mean ± standard deviation (SD) using an IBM computer model PS/2-80 with statistical software SPSS$^+$. When not otherwise mentioned, the Student's *t*-test for unpaired observations was used.

RESULTS

Serum lipoproteins

In **Table 1** serum lipoproteins for patients and volunteers are given. The concentrations of serum lipids were very constant during a week, and no tendencies could be seen. Patients with phytosterolemia had higher concentrations of total steroids, LDL steroids, and triglycerides, but lower levels of HDL steroids. Plasma LDL/HDL ratio averaged 4.6 in the patients and was 4.2 times higher than in controls.

TABLE 1. Serum lipoproteins in three patients with phytosterolemia (P) and three normolipemic, healthy volunteers (V)

Subject	Total Steroids	Triglycerides	LDL Steroids	HDL Steroids
	mg/dl (mean ± SD)			
P 1	218 ± 7	84 ± 9	166 ± 7	35 ± 1
P 2	385 ± 60	121 ± 81	311 ± 47	50 ± 4
P 3	247 ± 8	189 ± 66	155 ± 17	56 ± 5
V 1	186 ± 12	50 ± 7	100 ± 6	58 ± 4
V 2	174 ± 6	46 ± 3	83 ± 4	79 ± 2
V 3	139 ± 3	57 ± 2	50 ± 3	81 ± 3

The concentrations of all steroid fractions were performed enzymatically and reflect the sum of all steroids. Results represent the mean of three measurements during 1 week.

Serum sterols

Results of GLC analysis of serum sterols are summarized in **Table 2.** Individual concentrations of plant sterols showed a wide variation in patients, but the percent of plant sterols was constant and averaged almost 20%. Of all plant sterols sitosterol had the highest concentration in patients whereas campesterol was highest in volunteers. The ratio of sitosterol to campesterol in patients was extremely constant (x = 1.73 ± 0.06). The amounts of saturated plant sterols and of cholestanol were also markedly increased in patients. It is of interest to note that the ratios for different sterols between patients and volunteers increased from 1.3 for cholesterol to 34 for campesterol, to 118 for sitosterol. In contrast to plant sterols, cholesterol precursors (desmosterol and lathosterol) were markedly lower in patients with phytosterolemia compared with the concentrations in healthy volunteers.

TABLE 2. Serum cholesterol and non-cholesterol steroids in three patients with phytosterolemia (P) and three normolipemic, healthy volunteers (V) measured by gas–liquid chromatography

Subject	Cholesterol	Cholestanol	Desmosterol	Lathosterol	Campesterol	Campestanol	Stigmasterol	Sitosterol	Sitostanol	Avenasterol
	mg/dl (mean ± SD)									
P1	184 ± 10	2.12 ± 0.03	0.02 ± 0.01	0.07 ± 0.02	12.93 ± 0.02	1.53 ± 0.13	1.25 ± 0.03	22.10 ± 0.61	2.66 ± 0.07	3.84 ± 0.11
P 2	274 ± 36	4.94 ± 0.06	0.02 ± 0.01	0.03 ± 0.01	24.10 ± 0.26	3.90 ± 0.20	1.93 ± 0.05	42.40 ± 0.73	7.00 ± 0.12	4.39 ± 0.08
P 3	206 ± 9	1.90 ± 0.03	0.03 ± 0.01	0.02 ± 0.01	10.90 ± 0.27	1.63 ± 0.16	1.02 ± 0.03	20.50 ± 0.43	3.15 ± 0.07	3.98 ± 0.08
V 1	200 ± 9	0.14 ± 0.02	0.18 ± 0.01	0.53 ± 0.04	0.38 ± 0.04	n.d.	0.05 ± 0.01	0.23 ± 0.03	n.d.	0.05 ± 0.02
V 2	166 ± 10	0.07 ± 0.01	0.11 ± 0.01	0.21 ± 0.02	0.40 ± 0.02	n.d.	0.03 ± 0.01	0.21 ± 0.02	n.d.	0.04 ± 0.03
V 3	134 ± 8	0.03 ± 0.01	0.10 ± 0.01	0.17 ± 0.01	0.63 ± 0.01	n.d.	0.05 ± 0.01	0.29 ± 0.01	n.d.	0.06 ± 0.02

Results represent the mean of three measurements during 1 week; n.d. = not detectable.

Fecal sterol excretion of neutral and acidic steroids

The fecal recovery of chromic oxide and [$^2H_4$]sitostanol was not different between normal subjects and patients with phytosterolemia and was over 96% for both markers in all participants of the study. Therefore, only minor differences in fecal excretion of neutral, acidic, and plant steroids were found when calculated by the two different markers and on the average there was no difference in the results (**Table 3**). The relatively small variations in fecal excretion of the steroids during the last 3 days of the week seem to suggest that the subjects were near a steady state. Fecal excretion of cholesterol and steroids derived from cholesterol were significantly ($P < 0.05$) lower in patients. Cholesterol synthesis expressed per kg body weight appeared to be 5.3 times lower in patients (2.4 ± 0.6 mg/kg $\times$ d^{-1}) than in controls (12.9 ± 5.8 mg/kg $\times$ d^{-1}). Due to the high interindividual variation, this difference was not significant from a statistical point of view ($P > 0.05$). Bile acid synthesis was not significantly different in the two groups ($P > 0.05$).

Sterol absorption

The absorption rates of cholesterol, campesterol, and sitosterol are given in **Table 4.** As noted for fecal excretion of steroids, we found minor differences in the calculated absorption rate when using [$^2H_4$]sitostanol and chromic oxide as markers with a tendency to lower absorption rates using chromium as marker. Sterol absorption with use of [$^2H_4$]sitostanol has a potential to be more accurate because the results are obtained from simultaneous measurements on one sample only. With chromic oxide as marker, separate samples of the same aliquot must be used for analysis of deuterium-labeled steroids and chromic oxide. Cholesterol absorption was somewhat higher in patients, but still in the normal range (11). As expected, absorption of plant sterols was markedly higher in patients. Whereas cholesterol absorption was only about 1.2 times higher in patients than in controls, campesterol and sitosterol absorption were 1.4 and 2.7 times higher, respectively.

Treatment with sitostanol

Administration of sitostanol (0.5 g t.i.d.) for 4 weeks in patients 2 and 3 resulted in a reduction of cholesterol absorption from 58% to 44% (-24%) and from 54% to 30% (-44%), respectively. The decrease in dietary cholesterol absorption was followed not only by increased fecal output of cholesterol and steroids derived from cholesterol, but also by a marked increase in fecal excretion of plant steroids (**Table 5**). There was only a minor difference between cholesterol synthesis before and after treatment with sitostanol (54 mg/day for patient 2 and 46 mg/day for patient 3). The reduction in cholesterol absorption and probably also in plant sterol absorption resulted in a marked decline in all serum sterols, except for campestanol and sitostanol (**Table 6**). No marked differences could be seen between the results for desmosterol and lathosterol before and during treatment with sitostanol.

DISCUSSION

Methodology

The methodology used during the present investigation has obvious merits in comparison to that used in previous studies on sterol absorption. The deuterated cholesterol, sitosterol, and campesterol should be ideal markers for determination of the degree of absorption in relation to the nonabsorbable markers. The ratio between deuterated and nondeuterated steroids can be

TABLE 3. Results of fecal excretion of steroids derived from cholesterol, acidic steroids, plant steroids, and cholesterol synthesis in three patients with phytosterolemia (P) and three normolipemic, healthy volunteers (V)

	Cholesterol[a]		Acidic Steroids		Cholesterol Synthesis		Campesterol[b]		Sitosterol[c]	
Subject	A[d]	B[d]	A	B	A	B	A	B	A	B
	mg/day (mean ± SD)									
P1	162 ± 44	173 ± 49	218 ± 43	231 ± 47	125 ± 47	149 ± 45	16 ± 3	17 ± 4	109 ± 9	101 ± 11
P 2	244 ± 51	188 ± 54	207 ± 19	261 ± 13	196 ± 42	194 ± 38	15 ± 7	14 ± 5	104 ± 3	114 ± 9
P 3	129 ± 28	111 ± 13	259 ± 54	224 ± 26	133 ± 44	81 ± 18	11 ± 3	8 ± 1	91 ± 6	98 ± 12
V 1	1130 ± 27	1243 ± 307	775 ± 109	880 ± 274	1651 ± 85	1868 ± 68	21 ± 4	25 ± 3	142 ± 7	138 ± 4
V 2	814 ± 82	624 ± 84	330 ± 70	253 ± 43	889 ± 76	623 ± 84	19 ± 3	23 ± 6	138 ± 9	133 ± 5
V 3	405 ± 98	373 ± 72	305 ± 57	278 ± 33	456 ± 95	397 ± 83	23 ± 2	21 ± 1	134 ± 3	137 ± 6

Each value represents the mean of 3 consecutive days.
[a]Fecal excretion of "Cholesterol" is the sum of cholesterol, coprostanol, and coprostanone.
[b]Fecal excretion of "Campesterol" is the sum of campesterol, methylcoprostanol, and methylcoprostanone.
[c]Fecal excretion of "Sitosterol" is the sum of sitosterol, ethylcoprostanol, and ethylcoprostanone.
[d]A = calculated by [$^2H_4$]sitostanol as fecal marker; B = calculated by chromic oxide as marker.

TABLE 4. Comparison of cholesterol, campesterol, and sitosterol absorption in three patients (P) and three normolipemic, healthy volunteers (V) calculated by two different methods (A, B)

	Absorption					
	Cholesterol		Campesterol		Sitostosterol	
Subject	A[a]	B[a]	A	B	A	B
	% (*mean* ± *SD*)					
P1	56 ± 4	47 ± 4	29 ± 5	22 ± 4	18 ± 5	17 ± 4
P 2	58 ± 8	51 ± 2	26 ± 4	18 ± 7	16 ± 2	16 ± 4
P 3	53 ± 3	53 ± 1	25 ± 3	26 ± 3	15 ± 1	15 ± 2
Mean ± SD	56 ± 3	50 ± 3	27 ± 2	22 ± 4	16 ± 2	16 ± 1
V 1	46 ± 2	44 ± 2	18 ± 2	14 ± 4	6 ± 1	5 ± 1
V 2	45 ± 1	39 ± 5	19 ± 2	15 ± 6	6 ± 1	5 ± 2
V 3	43 ± 4	39 ± 3	19 ± 4	12 ± 3	5 ± 3	4 ± 1
Mean ± SD	45 ± 2	41 ± 3	19 ± 1	14 ± 2	6 ± 1	5 ± 1
	$P < 0.005$		$P < 0.005$		$P < 0.005$	

Values represent the mean of three measurements during 1 week.
[a]A = calculated by [$^2H_4$]sitostanol as fecal marker; B = calculated by chromic oxide as marker.

measured with a very high degree of accuracy by combined gas–liquid chromatography–mass spectrometry (for a review, see ref. 29). The fact that the results obtained with Cr_2O_3 as a nonabsorbable marker were similar to those obtained with deuterated sitostanol as nonabsorbable marker demonstrates the reliability of the method and indicates that sitostanol is not absorbed to a significant degree in patients with phytosterolemia. It should be pointed out that the degree of absorption measured is restricted to the absorption of the specific deuterated sterol species administered. The present techniques for measuring the absorption rate of plant sterols have the advantage of providing estimates of absorption over several days, whereas previous studies in patients with phytosterolemia only measured a single dose of radioactive sitosterol and cholesterol (1, 3).

Degree of absorption of sterols in phytosterolemia

The present study has focused on discriminating between the absorption of different sterols (cholesterol, campesterol, and sitosterol) in phytosterolemic patients and normolipidemic controls. The absorption rates of different sterols have only occasionally been performed in humans. Using an intestinal perfusion method, our laboratory has recently shown that increasing the side-chain of cholesterol without altering the Δ5 double-bond decreases the absorbability of sterols in humans (30). Thus, the absorption of cholesterol is higher than that of campesterol and absorption of campesterol is higher than that of sitosterol. We confirmed these results here with the deuterated sterols using two different nonabsorbable markers. The degree of absorption of cam-

TABLE 5. Fecal excretion of cholesterol and steroids derived from cholesterol, and plant steroids, and cholesterol absorption in two patients with phytosterolemia before and after 1 month of sitostanol administration (0.5 g t.i.d.)

	Patient 2		Patient 3	
Day	Control	Sitostanol	Control	Sitostanol
	mean ± *SD*			
Cholesterol[a] [mg/day]	244 ± 51	279 ± 16	129 ± 28	200 ± 44
Acidic steroids [mg/day]	207 ± 19	287 ± 14	259 ± 54	249 ± 32
Cholesterol intake [mg/day]	255	315	255	270
Cholesterol synthesis[b] [mg/day]	196 ± 42	251 ± 17	133 ± 44	179 ± 37
Plant steroids [mg/day]	199 ± 3	246 ± 69	102 ± 5	297 ± 36
Cholesterol absorption [%]	58 ± 8	44 ± 3	54 ± 3	30 ± 7

Each value represents the mean of 3 consecutive days.
[a]Fecal excretion of "cholesterol" is the sum of cholesterol, coprostanol, and coprostanone.
[b]"Cholesterol synthesis" is the sum of "Cholesterol" and "Acidic steroids" minus "Cholesterol intake".

TABLE 6. Serum steroid concentrations in two patients with phytosterolemia during 1 month of treatment with sitostanol (0.5 g t.i.d.)

	Patients 2			Patients 3		
Day	0	14	28	0	14	28
Cholesterol [mg/dl]	297	224	186	206	144	134
Cholestanol [mg/dl]	4.7	4.0	3.0	1.9	1.4	1.4
Ratio[a]	1.6	1.8	1.6	0.9	1.0	1.0
Campesterol [mg/dl]	34.7	26.0	21.9	10.9	9.1	8.2
Ratio	11.7	11.6	11.8	5.3	6.3	6.1
Campestanol [mg/dl]	3.8	3.4	3.2	1.6	1.8	1.9
Ratio	1.3	1.5	1.7	0.8	1.3	1.4
Sitosterol [mg/dl]	42.8	32.9	28.4	20.5	16.7	15.4
Ratio	14.4	14.7	15.3	10.0	11.6	11.5
Sitostanol [mg/dl]	6.9	7.0	7.2	6.1	5.4	5.3
Ratio	2.3	3.1	3.8	3.0	3.9	4.0

[a] "Ratio" = (sterol [mg/dl]/cholesterol [mg/dl] × 100).

pesterol in phytosterolemia has never been reported. As expected, absorption of all sterols was significantly higher in patients with phytosterolemia than in healthy controls ($P < 0.05$). Most interesting, however, was the fact that the absolute difference in absorption rates between the phytosterolemic patients and the healthy controls was about the same for each of the three sterols measured. Whereas absorption of campesterol in phytosterolemic patients was 1.7 times higher than the absorption of sitosterol, the normal subjects had a 3.2 times higher absorption rate. This could explain why serum campesterol concentrations in normal subjects exceed concentrations of sitosterol, despite the lower dietary intake, as well as the possibility that sterols could have different biliary excretory potentials. This is not the case in phytosterolemic subjects.

Under the conditions, and with the specific technique used here, our phytosterolemic patients absorbed about 16% of the ingested sitosterol. Shulman et al. (4) measured an intestinal absorption of this sterol in the range of 22–30% in one patient, and Miettinen (5) found an absorption of 19% in his patient. Three patients were reported by Salen et al. (3, 31) to absorb 28% and 68% and 34%, respectively (3). To what extent the above differences are dependent upon the methodology used is, however, difficult to evaluate. In any case, cholesterol, campesterol, and sitosterol absorption were very constant in the three unrelated patients in the present study.

Circulating steroids

The pattern and levels of the different phytosterols in the circulatory system of our patients were similar to those previously reported (2, 32). In a group of 14 patients, the mean sitosterol level was reported to be 35 mg/dl with a range of 14 to 42 mg/dl (12). In our patients, the levels varied between 21 and 42 mg/dl, and averaged 28 mg/dl. The concentrations of the 5α-saturated derivatives were also present in levels similar to those previously reported (12, 13, 33, 34). The mean cholestanol level in the reported group of phytosterolemic patients was 4.2 mg/dl, which is about fortyfold higher than normal. Cholestanol levels in our three patients varied between 1.9 and 4.9 mg/dl, and averaged 3.0 mg/dl. About 50% of phytosterolemic patients have been reported to have moderately higher levels of serum cholesterol (32). Our three patients had cholesterol levels from 184 to 274 mg/dl. The LDL/HDL ratio averaged 4.6, indicating an elevated atherogenic risk.

The levels of sitosterol in the serum of our phytosterolemic patients were about 100-fold higher than the corresponding levels in the healthy subjects. The 3-fold higher intestinal absorption of sitosterol in the phytosterolemic patients can hardly explain this marked difference. Thus, phytosterolemic patients must also have a reduced secretion of all sterols from the liver into the bile. Indeed, recent studies have shown that patients with phytosterolemia have a sluggish turnover (3, 7, 31). Such an inability for biliary excretion of sterols may be directly linked to the basic metabolic defect or it may be secondary to the high circulating levels of plant sterols (35). Plant sterols in bile were not directly measured in the present study. In the patient described by Miettinen (5), the total secretion of sterols in bile was less than 50% of controls. Low biliary concentrations of sitosterol, resulting in low sitosterol to cholesterol ratios, have been reported in several patients with phytosterolemia (5–7).

Excretion of bile acids

The three patients that we studied with respect to bile acid excretion into the feces seemed to have a slightly reduced excretion (Table 3). This reduction was not significant from a statistical point of view, however. In

the patient described by Miettinen (5), bile acid excretion was quantitatively normal or slightly higher than in the controls. It has been shown that sitosterol depresses the cholesterol 7α-hydroxylase activity in both rat and human liver microsomes (36–38). In two patients with phytosterolemia, the cholesterol 7α-hydroxylase activity was reduced about 70% of controls.

Cholesterol synthesis

In a careful study by Nguyen et al. (9) it was calculated that the overall cholesterol synthesis in phytosterolemic patients was reduced to about 30–50% of controls. In mononuclear leukocytes isolated from patients with phytosterolemia, the HMG-CoA reductase activity was reduced to about 30% of the control level and associated with about 65% less enzyme protein than in control preparations. The hepatic HMG-CoA reductase activity was less than 20% in patients as compared to controls (8, 9). The present work confirms previous reports that cholesterol synthesis is reduced in phytosterolemia (5, 8–10). Cholesterol synthesis in the present study was about 6-fold higher in the controls than in the phytosterolemic patients. Serum lathosterol levels, which have been shown to reflect HMG-CoA reductase activity in humans (39), were about 10-fold higher in the controls than in the phytosterolemic patients.

Therapeutic approaches to phytosterolemia

Patients with phytosterolemia should be treated with a diet low in cholesterol and with the lowest possible amount of plant sterols (7, 40, 41). Cholestyramine may also be used in combination with the diet, as cholestyramine causes a greater than expected reduction of plasma sterols (5, 12–14). A reduction of plasma sterols could also be achieved by ileal bypass surgery (3, 12). Neomycin, which blocks cholesterol absorption, has been suggested as an alternative to cholestyramine but was administered to only one patient, with no obvious therapeutic reduction in serum sterols (5). Previous studies from our group have indicated that sitostanol is not absorbed at all (11, 18, 19) and is, therefore, a very powerful substance to reduce intestinal cholesterol absorption (16–18). Studies in children with severe familial hypercholesterolemia treated with sitostanol showed a 27% reduction in serum cholesterol but a 50% reduction in campesterol and sitosterol, probably by inhibition of their absorption. These results prompted us to try sitostanol in patients with phytosterolemia to reduce the evaluated plant sterol concentrations. Indeed, administration of sitostanol in a dose of 0.5 g t.i.d. was followed by reduced cholesterol absorption, increased fecal output of cholesterol and plant sterols, and a marked reduction in serum sterols. To our surprise, the reduction in campesterol and sitosterol was of the same order of magnitude as the reduction of cholesterol. During treatment with sitostanol the two phytosterolemic patients seemed to be unable to increase their endogenous cholesterol synthesis, confirmed by the fecal output of the steroids derived from cholesterol. However, it is not justified to make final conclusions, because of the short duration of the treatment.

Basic defect in phytosterolemia

The most obvious defects in phytosterolemia are the increased intestinal absorption of neutral sterols confirmed for three sterols in the present study, the reduced biliary secretion of phytosterols and cholesterol, and probably a decreased synthesis of cholesterol. It is difficult to understand how a mutation in one specific enzyme system can result in all these deficiencies. The first two defects lead to a marked expansion of the pool of sitosterol and other phytosterols. Nguyen et al. (9) recently suggested that the primary metabolic defect in phytosterolemia is inadequate cholesterol synthesis due to reduced formation of HMG-CoA reductase followed by hyperabsorption and retention of all sterols. Gregg et al. (6) proposed that phytosterolemia results from a general loss of discrimination between various sterols for esterification (6). According to this hypothesis increased mucosal esterification of sitosterol leads to increased absorption, while increased esterification in the liver prevents secretion of the sterol into the bile. There is no evidence, however, of alteration in any specific mucosal acyl-CoA:cholesterol acyltransferase (ACAT) activity in this disease, and sitosterol does not stimulate the ACAT reaction in macrophages (42). Thus, at the present stage of knowledge, the basic defect in phytosterolemia remains to be established. It seems evident that more knowledge about the normal mechanism(s) for the intestinal absorption and biliary excretion of sterols must be acquired before this disease can be explained at a molecular level. However, the inhibition of sterol absorption by low dose of sitostanol to reduce serum cholesterol and plant sterols seems effective and merits further studies over a longer period of time.

Excellent technical assistance of Mr. Manfred Held, Mrs. Edith Carnott, Mrs. Heike Klein, Mrs. Susanne Akbar, and secretarial assistance of Mrs. M. Zerlett are gratefully acknowledged. This study was supported by research grants from the Paul-Martini-Stiftung, Bonn, Germany, and from the Doktor-Robert-Pfleger-Stiftung, Bamberg, Germany.

Manuscript received 12 January 1995 and in revised form 19 April 1995.

REFERENCES

1. Bhattacharyya, A. K., and W. E. Connor. 1974. β-Sitosterolemia and xanthomatosis. A newly described lipid

storage disease in two sisters. *J. Clin. Invest.* **53:** 1033–1043.
2. Björkhem, I., and S. Skrede. 1989. Familial diseases with storage of sterols other than cholesterol: cerebrotendinous xanthomatosis and phytosterolemia. *In* The Metabolic Basis of Inherited Disease. 6th edition. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill Book Co., New York. 1283–1302.
3. Salen, G., V. Shore, G. S. Tint, T. Forte, S. Shefer, I. Horak, E. Horak, B. Dayal, L. Nguyen, A. K. Batta, F. T. Lindgren, and P. O. Kwiterovich, Jr. 1989. Increased sitosterol absorption, decreased removal, and expanded body pools compensate for reduced cholesterol synthesis in sitosterolemia with xanthomatosis. *J. Lipid Res.* **30:** 1319–1330.
4. Shulman, R. S., A. K. Bhattacharyya, W. E. Connor, and D. S. Fredrickson. 1976. β-Sitosterolemia and xanthomatosis. *N. Engl. J. Med.* **294:** 472–483.
5. Miettinen, T. A. 1980. Phytosterolemia, xanthomatosis and premature atherosclerotic arterial disease: a case with high plant sterol absorption, impaired sterol elimination and low cholesterol synthesis. *Eur. J. Clin. Invest.* **10:** 27–35.
6. Gregg, R. E., W. E. Connor, D. S. Lin, and H. B. Brewer, Jr. 1986. Abnormal metabolism of shellfish sterols in a patient with sitosterolemia and xanthomatosis. *J. Clin. Invest.* **77:** 1864–1872.
7. Bhattacharyya, A. K., W. E. Connor, D. S. Lin, M. M. McMurry, and R. S. Shulman. 1991. Sluggish sitosterol turnover and hepatic failure to excrete sitosterol into bile cause expansion of body pool sitosterol in patients with sitosterolemia and xanthomatosis. *Atheroscler. Thromb.* **11:** 1287–1294.
8. Nguyen, L., S. Shefer, G. Salen, G. C. Ness, G. S. Tint, F. G. Zaki, and I. Rani. 1990. A molecular defect in hepatic cholesterol biosynthesis in sitosterolemia and xanthomatosis. *J. Clin. Invest.* **86:** 923–931.
9. Nguyen, L., G. Salen, S. Shefer, G. S. Tint, V. Shore, and G. C. Ness. 1990. Decreased cholesterol biosynthesis in sitosterolemia with xanthomatosis: diminished mononuclear leukocyte 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and enzyme protein associated with increased low-density lipoprotein receptor function. *Metab. Clin. Exp.* **39:** 436–443.
10. Lin, H. J., C. Wang, G. Salen, K. C. Lam, and T. K. Chan. 1983. Sitosterol and cholesterol metabolism in a patient with coexisting phytosterolemia and cholestanolemia. *Metabolism.* **32:** 126–133.
11. Lütjohann, D., C. O. Meese, J. R. Crouse III, and K. von Bergmann. 1993. Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in humans. *J. Lipid Res.* **34:** 1039–1046.
12. Salen, G., P. O. Kwiterovich, Jr., S. Shefer, G. S. Tint, I. Horak, V. Shore, B. Dayal, and E. Horak. 1985. Increased plasma cholestanol and 5α-saturated plant sterol derivatives in subjects with sitosterolemia and xanthomatosis. *J. Lipid Res.* **26:** 203–209.
13. Whitington, G. L., J. B. Ragland, S. M. Sabesin, and L. B. Kuiken. 1980. Neutralsterolemia and xanthomas. *Circulation.* **59:** 11–33 (abstract).
14. Nguyen, L. B., M. Cobb, S. Shefer, G. Salen, G. C. Ness, and G. S. Tint. 1991. Regulation of cholesterol biosynthesis in sitosterolemia: effects of lovastatin, cholestyramine, and dietary sterol restriction. *J. Lipid Res.* **32:** 1941–1948.
15. Belamarich, P. F., R. J. Deckelbaum, T. J. Starc, B. E. Dobrin, G. S. Tint, and G. Salen. 1990. Response to diet and cholestyramine in a patient with sitosterolemia. *Pediatrics.* **86:** 977–981.
16. Becker, M., D. Staab, and K. von Bergmann. 1993. Treatment of severe familial hypercholesterolemia in childhood with sitosterol and sitostanol. *J. Pediatrics.* **122:** 292–296.
17. Heinemann, T., O. Leiss, and K. von Bergmann. 1986. Effect of low dose sitostanol on serum cholesterol in patients with hypercholesterolemia. *Atherosclerosis.* **61:** 219–223.
18. Heinemann, T., G. A. Kullak-Ublick, B. Pietruck, and K. von Bergmann. 1991. Mechanism of action of plant sterols on inhibition of cholesterol absorption. *Eur. J. Clin. Pharmacol.* **40 Suppl. 1:** 59–63.
19. Czubayko, F., B. Beumers, S. Lammsfuss, D. Lütjohann, and K. von Bergmann. 1991. A simplified micro-method for quantification of fecal excretion of neutral and acidic sterols for outpatient studies in humans. *J. Lipid Res.* **32:** 1861–1867.
20. Vogel, A. I. 1956. Practical Organic Chemistry including Qualitative Organic Analysis. 3rd edition. Longmann, Green and Co., London, NY. 886–888.
21. Corey, E. J., and G. A. Gregoriou. 1959. Stereospecific syntheses of the 7-deuterio- and 7-tritiocholesterols. The mechanism of enzyme-catalyzed hydroxylation at a saturated carbon atom. *J. Am. Chem. Soc.* **81:** 3127–3133.
22. Anagnostopoulos, C. E., and L. F. Fieser. 1954. Nitration of unsaturated steroids. *J. Am. Chem. Soc.* **76:** 532–536.
23. Biemann, K. 1962. Mass Spectrometry: Organic Chemical Applications. McGraw-Hill Book Company, New York. 204–207.
24. Siedel, J., E. O. Hägele, J. Ziegenhorn, and A. W. Wahlefeld. 1983. Reagent for the enzymatic determination of serum total cholesterol with impaired lipolytic efficiency. *Clin. Chem.* **29:** 1075–1080.
25. Wahlefeld, A. W. 1974. Triglycerides determination after enzymatic hydrolysis. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Verlag Chemie, Weinheim. 1831–1835.
26. Burstein, M., H. R. Schemick, and R. Martin. 1970. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.* **11:** 583–587.
27. Friedewald, W. T., R. J. Levy, and D. S. Fredrickson. 1972. Estimation of the centrifugation of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **18:** 499–509.
28. Calvert, R. J., E. S. Kritchevsky, E. Einhorn, O. M. Klurfeld, and D. Kritchevsky. 1989. An impaired method for oxidation of chromium (III) oxide-containing fecal samples by using sodium peroxide fusion. *Am. J. Clin. Nutr.* **49:** 901–903.
29. Björkhem, I. 1979. Selected ion monitoring. *Rev. Clin. Lab. Sci.* **11:** 63–105.
30. Heinemann, T., G. Axtmann, and K. von Bergmann. 1993. Comparison of intestinal absorption of cholesterol with different plant sterols in man. *Eur. J. Clin. Invest.* **23:** 827–831.
31. Salen, G., G. S. Tint, S. Shefer, V. Shore, and L. Nguyen. 1992. Increased sitosterol absorption is offset by rapid elimination to prevent accumulation in heterozygotes with sitosterolemia. *Arterioscler. Thromb.* **12:** 563–568.

32. Salen, G., S. Shefer, L. Nguyen, G. C. Ness, G. S. Tint, and V. Shore. 1992. Sitosterolemia. *J. Lipid Res.* **33:** 945–955.
33. Skrede, B., I. Björkhem, O. Bergesen, H. J. Kayden, and S. Skrede. 1985. The presence of 5α-sitostanol in the serum of a patient with phytosterolemia, and its biosynthesis from plant steroids in rats with bile fistula. *Biochim. Biophys. Acta.* **836:** 368–375.
34. Dayal, B., G. S. Tint, A. K. Batta, J. Speck, A. K. Khachdurian, S. Shefer, and G. Salen. 1982. Identification of 5α-stanols in patients with sitosterolemia and xanthomatosis: stereochemistry of the protonolysis of steroidal organoboranes. *Steroids.* **40:** 233–243.
35. Clayton, P. T., A. Bowron, K. A. Mills, A. Massond, M. Casteels, and P. J. Milla. 1993. Phytosterolemia in children with parenteral nutrition-associated cholestatic liver disease. *Gastroenterology.* **105:** 1806–1813.
36. Boberg, K. M., J. E. Åkerlund, and I. Björkhem. 1989. Effect of sitosterol on the rate-limiting enzymes in cholesterol synthesis and degradation. *Lipids.* **24:** 9–12.
37. Shefer, S., S. Hauser, V. Lapar, and E. H. Mosbach. 1973. Regulatory effects of sterols and bile acids on hepatic 3-hydroxy-3-methylglutaryl CoA reductase and cholesterol 7α-hydroxylase in the rat. *J. Lipid Res.* **14:** 573–580.
38. Shefer, S., G. Salen, L. Nguyen, A. K. Batta, V. Packin, G. S. Tint, and S. Hauser. 1988. Competive inhibition of bile acid synthesis by endogenous cholestanol and sitostanol in sitosterolemia and xanthomatosis. *J. Clin. Invest.* **82:** 1833–1839.
39. T. A. Miettinen, R. S. Tilvis, and Y. A. Kesäniemi. 1990. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am. J. Epidemiol.* **131:** 20–31.
40. Bhattacharyya, A. K., and W. E. Connor. 1978. Familial diseases with storage of sterols other than cholesterol: cerebrotendinous xanthomatosis, and β-sitosterolemia and xanthomatosis. *In* The Metabolic Basis of Inherited Disease. 4th edition. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors, McGraw-Hill Book Company, New York. 656–687.
41. Kuksis, A., J. J. Myher, L. Marai, J. A. Little, R. G. McArthur, and D. A. K. Roncari. 1986. Fatty acid composition of individual plasma steryl esters in phytosterolemia and xanthomatosis. *Lipids.* **21:** 371–377.
42. Tabas, I., S. J. Feinmark, and N. Beatini. 1989. The reactivity of desmosterol and other shellfish- and xanthomatosis-associated sterols in the macrophage sterol esterification. *J. Clin. Invest.* **84:** 1713–1721.