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Effect of high plant sterol-enriched diet and cholesterol absorption inhibitor, SCH 58235, on plant sterol absorption and plasma concentrations in hypercholesterolemic wild-type Kyoto rats

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Abstract

Background and Aims: Plant sterols are widely distributed in human diet but are poorly absorbed so that their plasma levels are very low. However, when fed in large amounts, they lower plasma cholesterol levels by interfering with cholesterol absorption. We have studied the effect of 4 weeks of feeding a chow diet supplemented with 1% plant sterols [brassicasterol (6.3%), campesterol (28.5%), stigmasterol (15.6%) and sitosterol (49.6%)], with or without SCH 58235 (a derivative of ezetimibe), 30 mg/kg per day, known to suppress intestinal cholesterol absorption, on plasma, tissue, biliary, and fecal sterols in Wistar and wild-type Kyoto (WKY) rats, and their metabolism by intestinal bacteria.

Methods: After 2 weeks of feeding control or experimental diet, rats were given [3α-^{3}H]sitosterol intravenously and [4-^{14}C]sitosterol by mouth, and blood was collected after 1, 2, 3, and 5 days after labeling to determine sitosterol absorption. Feces were collected during the last 3 days and freeze dried. At the end of feeding, bile fistulas were created in 3 rats of each strain and bile was collected for 1 hour. All rats were then sacrificed and plasma and liver were collected for sterol measurements and activities of hepatic HMG-CoA reductase, cholesterol 7α-hydroxylase, and cholesterol 27-hydroxylase.

Results: Wild-type Kyoto rats were hypercholesterolemic compared to Wistar rats and had increased plant sterols in the plasma. Plasma cholesterol tended to be lower in WKY rats after feeding with plant sterol-enriched diet whereas plant sterol levels rose to approximately 31% of plasma sterols in WKY and 14% in Wistar rats. However, brassicasterol and stigmasterol, with a double bond at C-22, constituted less than 3.5% of total plasma plant sterols. After feeding, biliary plant sterols increased 2.25-fold in Wistar and 1.5-fold in WKY rats, suggesting less hepatic clearance in WKY rats. SCH 58235 feeding significantly increased plasma as well as biliary cholesterol levels in both the untreated and plant sterol-fed WKY rats, and the plasma plant sterols showed a tendency to increase but did not reach significant level. Intestinal bacteria in both rat strains metabolized all plant sterols to mainly the 5β-H-stanols. However, the C-22 double bond was stable to bacterial degradation. Intestinal absorption of sitosterol and cholesterol was increased 1.5- and 1.3-fold, respectively, in the WKY rats as compared to the Wistar rats, and plant sterol feeding lowered absorption of these sterols in both strains. Absorption of both these sterols was also lowered in SCH 58235-treated rats in both strains and was further lowered when SCH 58235 and plant sterols were simultaneously fed. The activity of the rate-limiting enzyme, HMG-CoA reductase, was increased 1.57-fold in Wistar rats and 1.27-fold in WKY rats that were fed plant sterols as compared to untreated rats.

Conclusions: (1) Plant sterol absorption was increased whereas hepatic elimination of all sterols was diminished in WKY rats accounting for elevated cholesterol and plant sterol levels. (2) The 1% plant sterol-enriched diet tended to lower plasma cholesterol levels whereas SCH 58235 feeding significantly increased plasma cholesterol levels in the WKY rats. (3) Intestinal absorption of sterols with C-22 double bond is diminished and the side-chain double bond is resistant to intestinal bacteria.

1. Introduction

Plant sterols, campesterol and sitosterol, are structurally similar to cholesterol, differing only in the structure of the side-chain, with campesterol having a methyl group at C-24 and sitosterol having an ethyl group at C-24 (Fig. 1).

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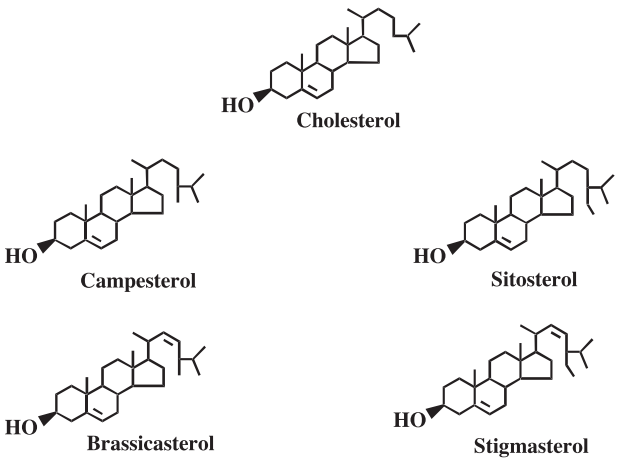

Fig. 1. Structures of cholesterol and plant sterols.

However, despite this small structural difference, plasma levels of plant sterols are very low, although approximately 250 mg/d are consumed in human diets [1-6] and even feeding with large doses of plant sterols increases their plasma levels to less than 2-fold only [7]. Recent studies have shown that the genes, ABCG5 and ABCG8, located in the enterocyte work together to limit intestinal absorption by promoting the secretion of cholesterol and plant sterols back to the intestinal lumen and hepatic secretion of cholesterol [8-10]. On the other hand, plant sterols are preferentially secreted into the bile relative to cholesterol by hepatic ABCG5/8, which further diminish body pools of plant sterols [11]. In the liver, plant sterols are poor substrates for 7α-hydroxylation, so they are secreted into the bile unchanged [12-14]. In the intestine, plant sterols compete with cholesterol for micellar solubilization and thereby interfere with cholesterol absorption [15]. There has been considerable interest recently in plant sterols as agents to reduce plasma cholesterol.

Although cholesterol is the major sterol present in animals, plants generally contain a mixture of sterols, with campesterol and sitosterol predominating. Most commercial sources of plant sterols also have significant proportions of the 22-dehydro derivatives of these 2 plant sterols, viz, brassicasterol and stigmasterol (Fig. 1). Although there are several reports on the intestinal absorption and metabolism of campesterol and sitosterol [1,16,17], little is known about the fate of brassicasterol and stigmasterol in human beings and animals [18]. In this report, we fed 2 species of rats, Wistar and wild-type Kyoto (WKY), with a plant sterol mixture that contains all 4 of these plant sterols, and studied the incorporation of the plant sterols into plasma, secretion into bile, effect on cholesterol synthesis, and their subsequent metabolism by intestinal bacteria. Our aim was to see if the high dose of plant sterols would lower plasma cholesterol levels in the hypercholesterolemic WKY rats and to study the mechanism of enrichment of plant sterols in their plasma. We also wanted to study the effect of side-chain unsaturation in campesterol and sitosterol on plasma incorporation and intestinal metabolism in these rats.

The WKY rat is derived from Wistar rats and was found to have elevated plant sterol levels on regular rat chow. Scoggan et al [19] and Yu et al [20] have recently shown that WKY rats possess a homozygous guanidine to thymine transversion in exon 12 of the Abcg5 gene, that is involved with limiting plant sterol absorption and results in the substitution of a conserved glycine residue for a cysteine amino acid in the extracellular loop between the fifth and sixth membrane-spanning domains of the half-transporter, sterolin-1.

Recent evidence supports the presence of a specific transporter that facilitates the movement of cholesterol from bile acid micelles into the brush border membrane of enterocytes [21]. This mechanism of cholesterol transport has been exploited as a therapeutic target in the development of a new class of compounds, 2-azetidinones, which is shown to lower cholesterol absorption [22,23]. Ezetimibe and its derivative, SCH 58235, are the most potent 2-azetidinones and are considered to work by selectively inhibiting the intestinal uptake and absorption of dietary and biliary cholesterol at the brush border of the small intestine [24]. Cholesterol is thereby excluded from enterocytes and confined to the intestinal lumen for subsequent excretion. Although a lot of clinical studies have been carried out that show the beneficial effect of ezetimibe alone or in combination with statins to lower plasma cholesterol levels, its effect on plant sterol absorption is not clearly defined [25-30]. In this report, we have also studied the effect of SCH 58235 alone and in combination with plant sterols on the absorption and plasma incorporation of plant sterols in the 2 rat species.

2. Experimental

2.1. Materials

The plant sterol mixture used in the feeding studies was derived from soybeans and was supplied by Unilever, Toronto, Ontario. SCH 58235 was a gift from Merck and Co. Reference standards of sterols for gas-liquid chromatography (GLC) and gas chromatography-mass spectrometry were obtained from Steraloids, Newport, RI. Sil-Prep (hexamethyldisilazane/trimethylchlorosilane/pyridine, 3:1:9) used for preparation of trimethylsilyl (TMS) ether derivatives of the sterols was purchased from Alltech Associates, Deerfield, IL. All other chemicals and solvents were purchased from Aldrich Co., Milwaukee, WI, and Sigma, St Louis, MO.

2.2. Animals

Two strains of 7-week-old male rats, the Wistar and WKY rats, were used in this study and were purchased from Charles River Laboratories, Newfield, NJ. Rats from each strain were divided into 4 groups of 6 rats each: group A was fed basic rat chow; group B was fed basic rat chow and was given in the

morning 30 mg/kg SCH 58235 (7.5 mg suspended in 10.5 mL liposyn) via gavage; group C was given rat chow fortified with 1% plant sterols and was given in the morning 30 mg/kg SCH 58235 via gavage; and group D was given rat chow fortified with 1% plant sterols. Each rat consumed approximately 20 to 30 g/d of basic rat chow or chow supplemented with plant sterols. After 2 weeks of feeding plant sterol-rich diet, all rats were given [3α-^{3}H]sitosterol intravenously and [23-^{14}C]sitosterol by mouth, and 120 μL of blood was collected via tail puncture on days 1, 2, 3, and 5 after labeling. The blood sample was taken in a vial containing 1 mL absolute ethanol and 3 mL of *n*-hexane, vortexed for 2 min, and centrifuged at 1000 × *g* for 5 minutes. The clear colorless supernatant was subjected to radioactivity counting. Feeding was discontinued for Wistar rats in groups B and C, and these rats were sacrificed. The feeding regimen was continued in the other rats for 2 more weeks. Feces from untreated rats of each strain and rats in group D were collected during the last 3 days and collectively freeze dried. On the last day of plant sterol feeding, 3 rats from each strain, either on basic rat chow or on the experimental diet, were anesthetized and bile fistulas were created and bile was collected for 1 h. All rats were then sacrificed, blood was drawn via heart puncture, and plasma was collected and stored at −20°C for sterol analysis, whereas liver was collected from untreated rats and rats in group D from both strains and immediately stored at −70°C for sterol measurement and determination of enzyme activities.

In a separate experiment, 4 groups each of 6 Wistar and 6 WKY rats were used to measure cholesterol absorption: group A was fed basic rat chow; group B was fed basic rat chow fortified with 1% cholesterol; group C was fed basic rat chow and was given in the morning 30 mg/kg SCH 58235 (7.5 mg suspended in 10.5 mL liposyn) via gavage; and group D was given rat chow fortified with 1% plant sterols. After 2 weeks of feeding experimental diets, all rats were given [3α-^{3}H]cholesterol intravenously and [4-^{14}C]cholesterol by mouth, and 120 μL of blood was collected on days 1, 2, 3, and 5 after labeling and radioactivity was measured as described above.

The animal protocol was approved by the Subcommittee on Animal Studies at the Veterans Affairs Medical Center and by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey.

2.3. Animal diet

Basic rat chow (Charles River) contained 0.21 mg/g plant sterols. The experimental diet was prepared at Charles River by mixing 2% by weight of plant sterol mixture. The sterol compositions in the basic and experimental chow are given in Table 1.

Table 1
Major sterols in basic rat chow and rat chow fortified with 1% plant sterol mixture

Sterol	Basic chow (mg/g)	1% Plant sterol-fortified chow (mg/g)
Cholesterol	0.14	0.16
Cholestanol	0.03	–
Brassicasterol	–	0.62
Campesterol	0.03	2.81
Stigmasterol	0.01	1.54
Sitosterol	0.15	4.90
Sitostanol	0.02	0.33

Coprostanol (20 μg in 100 μL ethyl acetate) was taken in a screw-cap vial and solvent was evaporated. The chow (20 mg) was then added followed by addition of Sil-Prep (400 μl), and the contents were heated at 55°C for 30 minutes. After centrifugation at 1000 × *g* for 5 minutes, 2 μL of the clear supernatant was injected directly onto the GLC column on a chemically bonded fused silica CP-Sil-5 CB capillary column (25 m × 0.22 mm ID). Helium was used as the carrier gas and the following gas chromatography operating conditions were used: injector and detector temperatures were 260°C and 290°C, respectively. After injection, oven temperature was kept at 100°C for 2 min, and then programmed at a rate of 35°C/min to a final temperature of 268°C.

2.4. Methods

Trimethylsilyl ether derivatization of sterols and stanols: Aliquots of the biologic extracts that contained the sterols (and stanols) were treated with 100 μL of Sil-Prep for 30 minutes at 55°C. The solvents were evaporated at 55°C under nitrogen, the reaction product was dissolved in 100 μL hexane, and the aliquot was used for GLC [31].

2.5. Gas liquid chromatography

A Hewlett-Packard model 6890 gas chromatograph equipped with a flame ionization detector and an injector with a split/splitless device for capillary columns was used for all separations. The chromatographic column consisted of a chemically bonded fused silica CP-Sil-5 CB (stationary phase, 100% dimethylsiloxane) capillary column (25 m × 0.22 mm ID) (Chrompack, Raritan, NJ), and helium was used as the carrier gas [31]. The GC operating conditions were as follows: injector and detector temperatures were 260°C and 290°C, respectively. After injection, oven temperature was kept at 100°C for 2 min, and then programmed at a rate of 35°C/min to a final temperature of 268°C. The retention times of various sterols and 5α- and 5β-stanols are given in Table 4.

2.6. Gas liquid chromatography-mass spectrometry

Mass spectra of the various sterols, bile acids and fatty acids, whenever needed, were carried out on a Hewlett-Packard Model 5972A mass-selective detector coupled to a Model 6890 gas chromatograph using a 25-m CP-Sil-5 CB capillary column.

2.7. Sterol determination

2.7.1. Plasma, tissue, and bile

Plasma (100 μL), bile (50 μL) or liver, kidney or heart (50-100 mg, exactly weighed) plus internal standard (coprostanol, 20 μg in 200 μL ethanol) were taken in 10 mL of 1 N ethanolic sodium hydroxide and heated at 70°C for 1 hour. After cooling, the neutral sterols and stanols were extracted

with hexane (3 × 30 mL). Hexane was evaporated under vacuum at 40°C and the residual product was transferred to a small vial and subjected to TMS ether formation. After evaporation of excess reagent at 50°C under a current of nitrogen, the residue was dissolved in 100 μL of hexane and 1 μL was injected onto the GLC column.

2.7.2. Stool

(i) As TMS ether derivatives: Freeze-dried homogenized feces (10-15 mg exactly weighed) was taken in a small screw-cap vial and heated with Sil-Prep (200 μL) at 55°C for 30 minutes. After centrifugation at 1000 × *g* for 10 min, 2 μL of the clear supernatant was subjected to GLC [32].

(ii) As acetate derivatives: Freeze-dried homogenized feces (10-15 mg exactly weighed) was taken in a small screw-cap vial and heated with pyridine (100 μL) plus acetyl chloride (100 μL) at 100°C for 30 minutes. After centrifugation at 1000 × *g* for 10 min, 2 μL of the clear supernatant was subjected to GLC.

2.7.3. Identification of fecal sterol metabolites

Approximately 200 mg of freeze-dried homogenized feces from rats that were given the plant sterol diet was refluxed with chloroform (10 mL) for 1 hour to extract sterols. After cooling, the chloroform was decanted and the residual feces were again refluxed with 10 mL of chloroform, and the total chloroform extract was evaporated to dryness. The residue was heated for 1 hour at 65° with 5% methanolic sodium hydroxide (5 mL). Most of the methanol was evaporated under nitrogen at 50°C, and the residual solution (approximately 1 mL) was dissolved in diethyl ether (10 mL) and washed with water (4 × 3 mL). Ether was evaporated and the residue was treated with pyridine/acetic anhydride as described above for the preparation of acetate derivatives. The product thus obtained was dissolved in 0.3 mL chloroform and subjected to preparative thin-layer chromatography (TLC) in a solvent system of benzene/hexane, 3:7 (v/v). Reference standards of cholesterol acetate and cholestanone were applied together as one spot on the side of the TLC plate, and the plate was developed to approximately 17 cm from the origin. After keeping at room temperature for 15 minutes, the TLC plate was carefully covered with aluminum foil so that a 1.5-cm-wide strip of silica gel, where the reference standards were developed, was exposed. This portion of the silica gel was sprayed with 20% sulfuric acid/3.5% phosphoric acid in isopropanol followed by careful heating to visualize the spots (R_f value of cholesterol acetate, 0.75, and of cholestanone, 0.5). The silica gel from the plate where the fecal sterols were developed was then collected in 2 bands: band 1 contained silica gel from 1 cm above the origin to 1 cm above the region where the spot due to cholestanone was observed, and band 2 contained silica gel above band 1 up to the solvent front. Silica gel in both bands was eluted with 30 mL of chloroform/methanol, 9:1 (v/v). Solvents were evaporated and the residue was dissolved in 0.3 mL chloroform and a 1- to 3-μL aliquot was used for GLC and gas chromatography-mass spectrometry.

2.7.4. Assays for HMG-CoA reductase, cholesterol 7α-hydroxylase, and cholesterol 27-hydroxylase activities

Hepatic microsomes and mitochondria were prepared by differential ultracentrifugation [33], and protein levels were determined according to Lowry et al [34]. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in the microsomes was determined according to literature [35]. Cholesterol 7α-hydroxylase activity was measured in the microsomes after extraction of endogenous lipids in acetone and reconstitution of the microsomal protein with cholesterol and optimal amounts of cofactors by the isotope incorporation method of Shefer et al [36]. Mitochondrial 27-hydroxylase activity was determined by an isotope incorporation method as described before [37].

2.7.5. Intestinal sitosterol and cholesterol absorption

The dual isotope ratio method [38,39], originally described by Zilversmit and Hughes, was used. [3α-^{3}H]sitosterol or [3α-^{3}H]cholesterol (1 μCi, in 0.2 mL of 20% liposyn) was injected intravenously and [23-^{14}C]sitosterol or [4-^{14}C]cholesterol (2 μCi, in 0.5 mL of 20% liposyn) was given by gavage in the morning to each rat and 120 μL blood was obtained on days 1, 2, 3, and 5 as described in the Experimental section. The blood sample was taken in a vial containing 1 mL of absolute ethanol and 3 mL of *n*-hexane, vortexed for 2 min, and centrifuged at 1000 × *g* for 5 minutes. The clear colorless supernatant was subjected to radioactivity counting. Percent absorption was calculated by determining the mean ^{14}C/^{3}H ratio in the blood and dividing by the ideal ratio:

$$\%\ \text{absorption} = \frac{[^{14}\text{C}/^{3}\text{H}]\ \text{blood}}{[^{14}\text{C}/^{3}\text{H}]\ \text{Ideal}} \times 100$$

The ideal ratio is the total oral dose/total injected dose and this ratio would be obtained in the blood if 100% of the oral [^{14}C]radioactivity would be absorbed. Mean absorption values for days 1, 2, 3, and 5 were obtained and values reported are a mean for 6 rats in each group.

2.8. Statistics

Data are reported as the mean ± standard deviation. The statistical significance of difference among the results in different groups was evaluated by the Student *t* test (unpaired) and significance was accepted at the level of $P < .05$.

3. Results

The hyperabsorbing WKY rats were used in this study and were compared to the Wistar rats used as controls. A

Table 2
Plasma sterols in Wistar and WKY rats before and after feeding experimental diets for 1 month

Treatment	Cholesterol	Campesterol	Stigmasterol (mg/dL)	Sitosterol	Sitostanol	% Plant sterols
Wistar rats						
Untreated	50.6 ± 6.0	1.0 ± 0.3	–	1.1 ± 0.3	–	3.98
Plant sterols	55.0 ± 10.2*	5.2 ± 1.4**	0.1 ± 0.1	3.7 ± 1.1**	0.1 ± 0.03	14.20
WKY rats						
Untreated	71.8 ± 6.3***	2.80 ± 0.30***	0.30 ± 0.10	4.30 ± 0.40***	0.50 ± 0.05	9.9
SCH 58235	103.5 ± 13.5**	2.12 ± 0.21*	0.25 ± 0.10	3.26 ± 0.55*	0.40 ± 0.13	5.5
SCH 58235 + plant sterols	88.5 ± 15.2*	9.16 ± 1.84**	1.00 ± 0.49	9.64 ± 2.06**	1.32 ± 0.53	19.3
Plant sterols	56.6 ± 8.6*	13.1 ± 2.4**	0.4 ± 0.1	11.4 ± 2.7**	0.6 ± 0.1	31.1

Six rats of each strain were used for each feeding regimen. Plasma (100 μL) was saponified with 1N ethanolic sodium hydroxide, sterols were extracted with *n*-hexane, and aliquot was used as the trimethylsilyl ether derivatives for quantitation by GLC as described under Table 1. Values are reported as mean ± SD.

* P = NS when compared to untreated rats of same strain.

** $P < .05$ when compared to untreated rats of same strain.

*** $P < .05$ when compared to untreated Wistar rats.

goal of this study was to see if plant sterols and SCH 58235 would interfere with cholesterol absorption and lower plasma cholesterol levels in the WKY rats that absorb increased amounts of cholesterol and plant sterols. The experimental diets were well tolerated by all animals and they gained weight normally. The Wistar rats weighed 350 ± 50 g at the start of the study and 550 ± 60 g at the end of the study and ate an average of 30 g chow per day whereas the WKY rats (mean weight, 250 ± 40 g at the start of study and 346 ± 38 g at end of the study) consumed an average of 20 g chow per day.

Table 2 shows plasma cholesterol levels in the WKY and Wistar rats. The WKY rats had higher plasma cholesterol levels as compared to Wistar rats at baseline (71.8 ± 6.3 vs 50.6 ± 6.0 mg/dL; $P < .05$). The WKY rats had elevated plant sterol levels in their plasma that were 2.5- to 3-fold more abundant than in the plasma of Wistar rats (Table 2). Plant sterol levels rose 2- to 5-fold from pretreatment levels in plasma from rats in both species after feeding plant sterol-rich diet for 1 month. However, the plant sterol levels tended to be lower in rats treated with SCH 58235, a phenomenon also observed when ezetimibe was added to the plant sterol diet. Further, it was seen that whereas plasma cholesterol levels were virtually unchanged after plant sterol feeding in Wistar rats, plasma cholesterol levels declined in WKY rats from their elevated pretreatment levels (56.6 ± 8.6 from 71.8 ± 6.3 mg/dL, $P < .05$). This suggested that the increased intake of dietary plant sterols could produce a cholesterol-lowering effect in WKY rats. Feeding with SCH 58235, on the other hand, increased plasma cholesterol levels in the untreated rats as well as rats treated with plant sterols (Table 2). Consistent with earlier findings that campesterol was absorbed more efficiently than sitosterol [18,38], we found that in both Wistar and WKY rats, proportions of plasma campesterol were higher than those of sitosterol, although the diet contained one third more sitosterol than campesterol. On the other hand, the Δ^{22}-unsaturated derivatives of campesterol and sitosterol, viz, brassicasterol and stigmasterol, were barely detected in plasma from Wistar rats and constituted only 3.0% to 3.4% of total plant sterols in the plasma from the WKY rats, although they made up 22% of the total plant sterols in the

Table 3
Biliary sterols in Wistar and WKY rats before and after feeding experimental diets for 1 month

Treatment	Cholesterol	Cholesterol[a] precursors	Campesterol (μg/mL) bile	Stigmasterol	Sitosterol	% Plant sterols
Wistar rats						
Untreated	70.0 ± 6.3	0.6 ± 0.3	3.7 ± 0.9	–	5.0 ± 0.8	11.05
Plant sterols	71.4 ± 7.8*	0.8 ± 0.5*	9.1 ± 1.9**	1.4 ± 0.3	9.1 ± 1.9**	21.54
WKY rats						
Untreated	75.2 ± 6.1	1.3 ± 0.4	3.8 ± 0.2	–	2.9 ± 0.1	8.2
SCH 58235	87.3 ± 7.2*	4.2 ± 1.3**	3.6 ± 0.1*	–	3.4 ± 0.3*	7.4
SCH 58235 + plant sterols	68.2 ± 3.9*	4.9 ± 1.7**	7.9 ± 1.5**,***	1.2 ± 0.8	5.0 ± 0.5**,***	17.1
Plant sterols	44.4 ± 5.4**	2.2 ± 0.5**	6.0 ± 1.2**	1.2 ± 0.6	6.0 ± 1.2**	22.9

Bile fistula was created in 3 male rats of each strain, on basic rat chow, or on the last day of feeding of the experimental diet, and bile was collected for 1 hour (0.8 mL of bile was obtained from Wistar rats and 0.45 mL of bile was obtained from WKY rats). Bile (100 μL) was heated for 1 hour at 70°C with 2 mL of 1N ethanolic sodium hydroxide. Sterols were extracted with *n*-hexane and converted into trimethylsilyl ether derivatives and a one hundredth aliquot was injected onto the GLC column. Gas-liquid chromatography operating conditions used were as described under Table 1. Each analysis was carried in duplicate and values reported are average for each treatment. Values are reported as mean ± SD.

[a] Desmosterol + lathosterol.

* P = NS when compared to untreated rats of same strain.

** $P < .05$ when compared to untreated rats of same strain.

*** $P < .05$ when compared to WKY rats that were fed SCH 58235 alone.

diet. Thus, the Δ^{22}-unsaturated sterols are poorly incorporated into plasma as compared to their saturated analogs.

Biliary sterols in both rat species are reported in Table 3. The pretreatment plant sterol levels in the Wistar rats were higher than the WKY rats (8.7 μg/mL bile from Wistar rats vs 6.7 μg/mL bile from WKY rats). However, in spite of large increase in plasma plant sterols in the WKY rats, the proportion of biliary plant sterols did not increase over that found for the Wistar rats that were fed the plant sterol-enriched diet (Wistar rats, 2.25-fold increase, and WKY rats, 2.2-fold increase). These data suggest that hepatic clearance of plant sterols is less in the WKY rats as compared to the Wistar rats. Although barely detected in the untreated rats of both species, stigmasterol constituted 7% and 9.1% of the plant sterols secreted in the bile after feeding the plant sterol-rich diet. Due to a small number of bile samples available, we cannot say if stigmasterol is secreted more efficiently in the bile than sitosterol, but proportion of stigmasterol as compared to sitosterol was greater in the bile than plasma. SCH 58235 seemed to have a slight lowering effect on biliary plant sterol secretion, both in untreated rats and rats that were also fed plant sterols. However, the differences were not significant. Cholesterol precursors (desmosterol and lathosterol) were found to be somewhat increased in untreated WKY rats as compared to the Wistar rats. However, these sterols were significantly increased after plant sterol feeding to WKY rats, but not Wistar rats (Table 3). SCH 58235 feeding with or without plant sterol feeding also significantly increased the levels of cholesterol precursors in the bile.

Fecal sterol pattern was very complex because of the formation of a large number of bacterial metabolites of the ingested plant sterols. To characterize the fecal sterols and their metabolites, fecal sterols were isolated and converted to their acetate derivatives. The ketonic compounds were then separated from the bulk of the sterol and stanol acetates by preparative TLC, and each fraction was then analyzed by gas-liquid chromatography-mass spectrometry. The various sterols and their metabolites that were characterized are reported in Table 4. The 5α- and 5β-H derivatives of cholesterol, brassicasterol, campesterol, stigmasterol, and sitosterol were all identified [39]. The corresponding 5β-H-3-one and traces of 4-en-3-one and 4,6-dien-3-one derivatives of cholesterol, campesterol, and

Table 4
Sterols and their bacterial metabolites identified in feces from Wistar and WKY rats after feeding 1% plant sterol-enriched diet

Steroid[a]	GLC retention time[b]	Pertinent mass-ion fragments
Coprostanol	19.60	215, 257, 355, 370 (M^+-60)
Epi-coprostanol	19.97	215, 257, 355, 370 (M^+-60)
Cholesterol	21.80	213, 255, 260, 353, 368 (M^+-60)
Cholestanol	21.95	215, 257, 353, 370, 430 (M^+)
Coprostanone	17.90	231, 316, 353, 371, 386 (M^+)
Cholest-4-en-3-one	21.55	124, 229, 261, 342, 384 (M^+-60)
Cholesta-4,6-dien-3-one	22.39	136, 227, 247, 269, 382 (M^+-60)
5β-Δ^{22}-Campestanol	21.18	215, 257, 315, 339, 344, 382, 442 (M^+)
Brassicasterol[c]	23.54	213, 255, 365, 380 (M^+-60)
5α-Δ^{22}-Campestanol	23.75	215, 257, 315, 339, 344, 382, 442 (M^+)
24-Methyl coprostanol	23.24	215, 257, 369, 384 (M^+-60)
24-Methyl epi-coprostanol[c]	23.63	215, 257, 369, 384 (M^+-60)
Campesterol	26.43	213, 255, 274, 367, 382 (M^+-60)
Campestanol	26.58	215, 257, 369, 384, 444 (M^+)
24-Methyl-coprostanone	20.94	231, 330, 367, 385, 400 (M^+)
24-Methyl-cholest-4-en-3-one	25.63	124, 229, 275, 356, 398 (M^+)
24-Methyl-cholesta-4,6-dien-3-one	26.68	136, 228, 261, 269, 396 (M^+)
5β-Δ^{22}-Stigmastanol	24.63	215, 257, 315, 344, 353, 396, 456 (M^+)
Stigmasterol[c]	27.81	213, 255, 379, 394 (M^+-60)
5α-Δ^{22}-Campestanol	28.23	215, 257, 315, 344, 353, 396, 456 (M^+)
24-Ethyl coprostanol	27.18	215, 257, 383, 398 (M^+-60)
24-Ethyl epi-coprostanol[c]	27.74	215, 257, 383, 398 (M^+-60)
Sitosterol	30.95	213, 255, 288, 381, 396 (M^+-60)
Sitostanol	31.19	215, 257, 383, 398, 458 (M^+)
24-Ethyl-coprostanone	24.07	231, 344, 381, 399, 414 (M^+-60)
24-Ethyl-cholest-4-en-3-one	29.86	124, 229, 289, 370, 412 (M^+-60)
24-Ethyl-cholesta-4,6-dien-3-one	31.16	136, 227, 269, 410 (M^+-60)

The sterols and their metabolites were extracted with chloroform from 200 μl of freeze-dried feces, converted into their acetate derivatives with acetic anhydride/pyridine at room temperature, and subjected to preparative TLC in a solvent system of benzene/hexane, 7:3, as described under the Experimental section. The purified fractions (bands 1 and 2) containing the various steroid mixtures were subjected to gas-liquid chromatography-mass spectrometry.

[a] Structures were determined either by direct comparison of the mass spectra with those of reference standards or by mass spectral fragmentation pattern.

[b] Capillary GLC of the products from bands 1 and 2 was carried out on a CP-Sil-5CB capillary column as described under the Experimental section.

[c] These compounds showed similar retention times on GLC and were separated by subjecting the product in band 2 to a second preparative TLC when the sterols with 5β-H appeared as a band slightly below the band due to sterols with 5α-H and the Δ^5-sterols. Products in these 2 bands were isolated and subjected to GLC and gas-liquid chromatography-mass spectrometry.

Table 5
Major sterols and their metabolites in rat feces before and after feeding 1% plant sterol-enriched diet

Steroid	Wistar rats (mg/d)		WKY rats (mg/d)	
	Untreated	Plant sterol fed	Untreated	Plant sterol fed
Coprostanol	2.65 ± 1.00	5.80 ± 3.20	2.46 ± 1.22	3.60 ± 1.55
Epi-coprostanol	0.57 ± 0.30	2.00 ± 1.05	0.41 ± 0.30	0.40 ± 0.30
Cholesterol	4.50 ± 1.65	5.42 ± 2.80*	2.20 ± 1.10	3.05 ± 1.05*
Cholestanol	0.10 ± 0.10	0.28 ± 0.20	0.09 ± 0.05	0.30 ± 0.20
Coprostanone	0.10 ± 0.10	0.86 ± 0.56	0.06 ± 0.06	0.38 ± 0.20
Δ^{22}-5β-Campestanol	ND	2.00 ± 0.65	ND	1.05 ± 0.35
Brassicasterol	ND	8.02 ± 2.55	ND	4.00 ± 1.55
Δ^{22}-5α-Campestanol	ND	0.40 ± 0.25	ND	0.87 ± 0.35
24-Methyl coprostanol	0.59 ± 0.30	4.00 ± 1.55	0.84 ± 0.44	2.50 ± 1.01
Campesterol	0.82 ± 0.30	65.0 ± 15.0	0.46 ± 0.22	38.05 ± 10.10
Campestanol	0.24 ± 0.12	2.80 ± 1.35	0.18 ± 0.10	3.30 ± 1.45
24-Methyl coprostanone	Trace	2.00 ± 1.05	Trace	2.00 ± 1.05
Δ^{22}-5β-Stigmastanol	0.51 ± 0.35	4.00 ± 1.05	0.50 ± 0.50	2.00 ± 0.50
Stigmasterol	0.37 ± 0.15	22.24 ± 4.50	0.08 ± 0.08	10.90 ± 1.85
Δ^{22}-5α-Stigmastanol	ND	1.08 ± 0.85	ND	0.60 ± 0.35
24-Ethyl coprostanol	1.42 ± 0.40	10.86 ± 3.55	1.34 ± 0.25	7.25 ± 1.85
Sitosterol	2.76 ± 1.20	104.3 ± 28.5	1.10 ± 0.25	69.20 ± 15.0
Sitostanol	0.59 ± 0.25	5.00 ± 1.85	0.30 ± 0.20	6.70 ± 2.50
24-Ethyl-coprostanone	0.13 ± 0.13	2.60 ± 1.55	Trace	0.80 ± 0.40

Feces were collected during the last 3 days from either untreated rats or from rats that were fed the plant sterol-enriched diet. Freeze-dried homogenized feces (10-15 mg exactly weighed) was taken in a screw-cap vial and heated with Sil-Prep (200 μl) at 55°C for 30 minutes. After centrifugation at 1000 × g for 10 min, 2 μl of the clear supernatant was subjected to GLC. Values are given as milligrams per day dry feces and are reported as mean ± SD.

* P = NS when compared to untreated rats.

sitosterol were also identified [40]; however, the ketonic derivatives of brassicasterol and stigmasterol could not be detected. The daily outputs of the major sterols and their fecal metabolites in both rat species before and after feeding the plant sterol diet were measured and are given in Table 5. All sterols were found to be present mainly in the unesterified form and were quantitated via direct trimethylsilylation of the dried fecal samples. There was no significant qualitative or quantitative difference between the sterol outputs in the 2 rat strains. Coprostanol, 24-methyl-coprostanol, Δ^{22}-5β-stigmastanol, and 24-ethyl-coprostanol were the major metabolites of the Δ^5-sterols, cholesterol, campesterol, stigmasterol, and sitosterol, and were present in proportions similar to the corresponding Δ^5-sterols in all animals. Brassicasterol was absent from regular rat chow and was not detected in the feces of any animal fed regular diets. However, when the plant sterol-fortified diet was given, brassicasterol and both its 5α- and 5β-H derivatives were readily identified in all fecal samples (Table 5). We could not confirm in our experiment if the intestinal bacteria also saturated the Δ^{22}-double bond in brassicasterol and stigmasterol, because the bacterial products would then become identical with those obtained from campesterol and sitosterol. However, if such a reduction did take place, it would be of a minor

Table 6
Hepatic enzyme activities in Wistar and WKY rats before and after feeding 1% plant sterol-enriched diet

Rat	(pmol/mg protein per minute)			Hepatic sterols (μg/ml)[a]	
	HMG-CoA	CYP 7A1[b]	CYP 27A[c]	Cholesterol	Plant sterols
Wistar					
Untreated	74.4 ± 11.9	23.4 ± 2.5	21.3 ± 3.6	2447 ± 327	99 ± 12
Plant sterol-fed	116.8 ± 5.4*	30.2 ± 3.7**	28.4 ± 2.8**	2190 ± 460**	330 ± 56*
WKY					
Untreated	73.0 ± 10.7	18.7 ± 1.4	16.5 ± 2.1	2120 ± 214	241 ± 35
Plant sterol-fed	92.6 ± 5.5**	22.4 ± 1.6**	19.5 ± 1.4**	1392 ± 189*	603 ± 65*

Livers were collected immediately after rats were sacrificed, stored at −70°C, and used for measurement of enzyme activities. Each analysis was carried out in duplicate and average was used. Values are reported as mean ± SD.

[a] Approximately 100 mg of liver tissue was exactly weighed and heated for 1 hour at 70°C with 2 mL of 1 N ethanolic sodium hydroxide. Sterols were extracted with n-hexane and converted into trimethylsilyl ether derivatives, and a one hundredth aliquot was injected onto the GLC column. Gas-liquid chromatography operating conditions used were as described under Table 1.

[b] Cholesterol 7α-hydroxylase.

[c] Cholesterol 27-hydroxylase.

* P < .05 when compared to untreated rats from same strain.

** P = NS when compared to untreated rats from same strain.

nature, because the total proportion of brassicasterol and stigmasterol and their 5α- and 5β-H metabolites compared to the total plant sterols in the feces was 19% to 21% as compared with 22% in the plant sterol-fortified diet.

The activity of the rate-limiting enzyme for cholesterol synthesis (HMG-CoA reductase) in the liver was found to be similar in both rat species at baseline and increased 1.57- and 1.27-fold, respectively, in the Wistar and the WKY rats after feeding the plant sterol-enriched diet (Table 6). The greater increase in the Wistar rats apparently reflects reduced suppression of cholesterol absorption by plant sterol feeding in these rats as compared to the WKY rats (Table 8). The activity of cholesterol 7α-hydroxylase, reflecting classic bile acid synthesis, was 20% lower in the WKY rats at baseline and rose 19.8% after plant sterol feeding in both species of rats. Cholesterol 27-hydroxylase activity that reflects alternative (acidic) bile acid synthesis was slightly decreased in WKY rats at baseline (P = NS) and also rose after plant sterol feeding (Table 6).

The intestinal absorption of sitosterol and cholesterol was determined in untreated Wistar and WKY rats as well as in rats after feeding SCH 58235, plant sterols/cholesterol, or SCH 58235 + plant sterol diets by the plasma dual isotope ratio method of Zilversmit. Thus, tritium-labeled sterol was infused intravenously and ^{14}C-labeled sterol was given by gavage to rats that had been fed the control or the experimental diets for 2 weeks, and the ^{14}C/^{3}H ratio in the plasma was determined 1, 2, 3, and 5 days after labeling while the diets were continued to be fed. Percent absorption in each rat was calculated by determining the mean ^{14}C/^{3}H ratio in the blood and dividing by the ideal ratio (total oral dose/total injected dose). The rate of sterol absorption was found to be linear over the 5-day period. It was determined that in the untreated rats, where plant sterol absorption would be maximum, the absorption of ^{14}C-labeled sitosterol was greater in WKY rats than Wistar rats (12.6% vs 8.5%). Sitosterol absorption was reduced after feeding to the rats the plant sterol-rich diet, and 8.2% of the ^{14}C-labeled sitosterol was absorbed in the WKY rats, whereas 5.3% was absorbed in the Wistar rats (Table 7). On the other hand, SCH 58235 feeding significantly lowered plant sterol absorption both in untreated rats as well as rats fed plant sterols (2.2- and 2.7-fold decreased in untreated Wistar and WKY rats, respectively, and 2.8- and 3.2-fold decreased from pretreatment levels in Wistar and WKY rats, respectively, that were also fed plant sterols). There was a further lowering of 1.8- and 2.1-fold of sitosterol absorption in Wistar and WKY rats that were fed plant sterols when SCH 58235 was added in the diet (Table 7). It is possible that the lowering of sitosterol absorption may be in part due to the competitive effect of the other plant sterols present in the plant sterol-enriched rat chow that was fed to these rats. However, because the total amount of sitosterol fed is proportionately decreased, this effect is likely to be small.

Table 7
Percent absorption of sitosterol in Wistar and WKY rats treated with SCH 58235 and plant sterol-enriched diet

Rat	Sitosterol absorption (%)			
	Untreated	SCH 58235	SCH 58235 + plant sterol	Plant sterol
Wistar	8.5 ± 0.5	3.9 ± 0.2*	3.0 ± 0.2*	5.3 ± 0.1**
WKY	12.6 ± 1.3**	4.7 ± 0.4*	4.0 ± 0.3*	8.0 ± 0.7***

Rats were fed basic rat chow or experimental diet for 2 weeks and were then given 1 μCi of ^{3}H-labeled sitosterol intravenously and 2 μCi of ^{14}C-labeled sitosterol via gavage. Blood was taken on days 1, 2, 3, and 5, red cells were removed by addition of ethanol and centrifugation, and clear liquid was subjected to scintillation counting as described under Experimental section. Values are reported as mean ± SD.

* $P < .05$ when compared to untreated rats of the same species.

** $P < .05$ when compared to untreated Wistar rats.

*** $P < .05$ when compared to Wistar rats fed plant sterol diet.

Table 8
Percent absorption of cholesterol in Wistar and WKY rats treated with SCH 58235 and cholesterol and plant sterol-enriched diet

Rat	Cholesterol absorption (%)			
	Untreated	Cholesterol	SCH 58235	Plant sterol
Wistar	48 ± 6	17 ± 2*	12 ± 3*	18 ± 2*
WKY	64 ± 7**	21 ± 1*	12 ± 3*	21 ± 1*

Rats were fed basic rat chow or experimental diet for 2 weeks and were then given 1 μCi of ^{3}H-labeled cholesterol intravenously and 2 μCi of ^{14}C-labeled cholesterol via gavage. Blood was taken on days 1, 2, 3, and 5, red cells were removed by addition of ethanol and centrifugation, and clear liquid was subjected to scintillation counting as described under Experimental section. Values are reported as mean ± SD.

* $P < .05$ when compared to untreated rats of the same species.

** $P < .05$ when compared to untreated Wistar rats.

Intestinal absorption of cholesterol in the 2 rat strains after various treatment regimens is given in Table 8. Cholesterol absorption was increased in the WKY rats as compared to the Wistar rats (64% vs 48%, $P < .05$), and it was proportionately reduced in both Wistar and WKY rats after cholesterol, plant sterol, and SCH 58235 feeding, the effect being more pronounced in the WKY rats (Table 8).

4. Discussion

We have confirmed in these studies that the WKY rats are hypercholesterolemic as compared to the Wistar rats. Our results also confirm that these rats accumulate plant sterols in plasma and liver. Feeding a plant sterol-rich diet that contained almost 50 times more plant sterols resulted in significantly greater plasma plant sterol levels in both rat species (14.2% vs 3.98% plant sterols in Wistar rats at baseline, and 31.1% vs 9.9% plant sterols in WKY rats) (Table 2). On the other hand, whereas plasma cholesterol levels did not appreciably change in Wistar rats, they were 21% lowered in WKY rats after feeding 1% plant sterols for 1 month (P = NS). The hepatic cholesterol levels were significantly reduced after plant sterol feeding to WKY rats (Table 6). It may be mentioned that a plant sterol mixture was fed to the rats (49.6% sitosterol, 28.5% campesterol, and 21.9% Δ^{22}-unsaturated plant sterols) and the effects of

individual plant sterols on plasma and biliary levels of cholesterol and plant sterols could not be determined. Although hepatic cholesterol levels were also reduced in the Wistar rats, the difference was not statistically significant. Plasma cholesterol levels increased after SCH 58235 feeding to WKY rats whereas biliary cholesterol levels showed a tendency to increase (P = NS) (Table 3). The effect of SCH 58235 feeding was apparent in both the untreated WKY rats and WKY rats that were also fed plant sterols (Tables 2 and 3). The increased plasma and biliary cholesterol levels after SCH 58235 feeding suggested increased hepatic synthesis [as shown by increased biliary levels of cholesterol precursor (Table 3)] resulting from reduced intestinal absorption (Table 8), as has been shown in both rodents and human beings [22,23,26]. Recently, in patients with normal plasma cholesterol levels, it was shown that after a 2-week treatment with 10 mg/d ezetimibe, cholesterol absorption was reduced by 54% whereas cholesterol synthesis increased 89% leading to a 15% decrease in plasma cholesterol levels [26]. On the other hand, we have shown in a more recent study that ezetimibe caused progressive reduction in plasma plant sterol levels in patients with the lipid storage diseases, sitosterolemia (with mutation in ABCG5 or ABCG8 and hyperabsorption of plant sterols), during 8 weeks of feeding, whereas cholesterol levels declined for the first 2 weeks and then started to increase. That this increase in plasma levels was due to increased hepatic synthesis was evident from increased biliary lathosterol/cholesterol levels in these patients [41].

The activity of the rate-limiting enzyme, HMG-CoA reductase, was similar in the Wistar and the WKY rats at baseline even though plasma cholesterol was higher in WKY rats because the suppression of cholesterol absorption by plant sterols and 7α-hydroxylation of the hepatic cholesterol would both affect hepatic cholesterol influx that would determine the activity of HMG-CoA reductase. After feeding 1% plant sterol-enriched diet, HMG-CoA reductase activity was increased 1.57- and 1.27-fold in the Wistar and WKY rats, respectively, apparently due to the reduced cholesterol levels in the hepatocytes resulting from competitive inhibition of intestinal cholesterol absorption by plant sterols (Table 8). In human sitosterolemia, the activities of HMG-CoA reductase and the other enzymes involved in cholesterol biosynthesis pathway are all greatly diminished as compared to healthy controls [42]. As shown in in vitro studies in human fibroblasts, and in rats infused intravenously with sitosterol, the increased levels of plant sterols in the hepatocytes do not affect HMG-CoA reductase activity [42-44]. Both cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase activities were lower in the WKY rats because of the competitive inhibition of enzymes by sitosterol [43,45,46]. The activities of these enzymes increased after plant sterol feeding in both rat species due to increased cholesterol synthesis caused by up-regulated HMG-CoA reductase activity. This cholesterol would displace plant sterol from the microsomal cholesterol 7α-hydroxylase and mitochondrial cholesterol 27-hydroxylase, thereby increasing their activities.

Absorption of sterols first involves their micelle formation with bile acids in the small intestine, uptake to the brush border membrane, intracellular esterification, and incorporation into chylomicrons [47]. Cholesterol is preferentially absorbed because the small intestine is able to differentiate between luminal cholesterol and plant sterols that differ from cholesterol in having an extra methyl or ethyl group in the side chain [48,49]. It has been shown that approximately 5% of ingested sitosterol is absorbed by this mechanism as compared to 50% absorption of cholesterol [1]. Another mechanism to control the body pool of plant sterols is rapid hepatic removal [31,50-52]. This occurs even though plant sterols are not biotransformed by hepatic enzymes [53] and their plasma pool is always very low. However, it has been shown in numerous studies that plant sterols interfere with cholesterol absorption when given in large amounts [54]. The precise mechanism by which plant sterols inhibit intestinal cholesterol absorption is not fully understood, but it is generally believed that plant sterols compete with cholesterol for solubilization in mixed micelles, a prerequisite to sterol absorption [47]. The increased plasma concentration of plant sterols in rats after ingestion is a direct consequence of their micellar incorporation in the small intestine. The WKY rats seem special in that they accumulate plant sterols so that their plasma concentrations increased almost 3-fold as compared to the Wistar rats, and their increased plasma levels continued after large amounts of plant sterols were fed to these rats. Ikeda et al [55] have shown that feeding 0.5% plant sterol to stroke-prone hypertensive (SHR) rats does not affect plasma cholesterol levels, but we find that feeding 1% plant sterols in diet lowers plasma cholesterol levels by 21% WKY rats (P = NS) (Table 2). This suggests that when present in sufficient amounts, the plant sterols partially displace cholesterol from the intestinal mixed micelles so that less cholesterol is available for absorption. The somewhat reduced biliary secretion of plant sterols in the WKY rats, suggesting their reduced hepatic clearance as compared to the Wistar rats, and the somewhat increased absorption of plant sterols in the WKY rats may result in their retention in the plasma. Thus, the mutation in Abcg5 in WKY rats is associated with enhanced absorption and reduced hepatic secretion of plant sterols [19,20].

A detailed analysis of the fecal sterols showed the presence of the 5β-H derivatives and the corresponding 3-oxo-5β-H-stanol derivatives of cholesterol and dietary campesterol and sitosterol. Small amounts of the 3-oxo derivatives of campesterol and sitosterol were present, but the corresponding compounds from brassicasterol and stigmasterol were not detected. The side-chain double bond was found to be resistant to bacterial saturation or degradation. The fecal outputs of cholesterol and its bacterial metabolites increased from their pretreatment levels in all animals after they were fed the plant sterol-rich diet in accordance with interference with cholesterol

absorption in the small intestine. It may be noted that even though such large amounts of the 5β-H derivatives of plant sterols are excreted (8%-21% of total plant sterols), their colonic absorption is nil. The 5β-H sterols have not been reported in the plasma or other tissues of animals, supporting micellar solubilization as a prerequisite for intestinal sterol absorption, whereas the bile acids, being soluble in ionized form, are passively absorbed from the colon.

The small intestine can differentiate between luminal cholesterol and plant sterols that differ from cholesterol by having an extra methyl or ethyl group in the side chain with the result that plant sterols are absorbed at a rate of 1/5 to 1/10 of that of cholesterol [1]. The absorbability of plant sterols has been shown to depend on the structure of the side chain and campesterol, with an extra methyl group in the side chain, is absorbed better than the sitosterol, which has an ethyl group in the side chain, and both compounds suppress intestinal cholesterol absorption. It has been suggested that the absorptive discrimination against the plant sterols is the result of greater reverse sterol transport by ABCG5/ABCG8 located in the enterocytes. The presence of the double bond in the side chain in addition to the extra methyl or ethyl group makes these compounds even more difficult to pass. Our results show that in the rat, both brassicasterol and stigmasterol are poorly absorbed; the 2 compounds were found in quantities below our GLC detection limit in the plasma from Wistar rats, and brassicasterol was barely detected whereas less than 0.5% stigmasterol was found in the WKY rats that were fed the plant sterol-rich diet that contained as much as 22% of these Δ^{22}-plant sterols. These findings are in agreement with earlier studies in the rabbit where it was shown that stigmasterol was not absorbed [56] whereas the lymphatic absorption of stigmasterol has been shown to be similar to that of sitosterol when administered intragastrically to rat [57]. It can be speculated that these side-chain unsaturated compounds may work even better than their parent sterols to compete with cholesterol absorption, whereas their plasma and biliary concentrations would be low.

Thus, we have shown that WKY rats are hypercholesterolemic and their cholesterol levels may be lowered when large amounts of plant sterols are fed to these rats. SCH 58235 feeding suppresses intestinal cholesterol absorption, as also shown in human beings, but contrary to the finding in human beings, SCH 58235 increases both biliary and plasma cholesterol levels in WKY rats. This may be the result of slightly increased biliary secretion of cholesterol and increased hepatic synthesis. Because plant sterols are not endogenously synthesized, their reduced intestinal absorption caused by SCH 58235 results in reduced plasma and biliary levels. Plant sterol feeding increases their plasma levels to a certain extent only. Further plant sterol feeding does not increase their plasma levels, as the intestinal absorptive sites are already saturated. However, the extra plant sterols present in the intestinal lumen compete with cholesterol for absorption and thereby suppress its absorption.

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References

[1] Salen G, Arhens EH, Grundy SM. Metabolism of β-sitosterol in man. J Clin Invest 1970;49:952-67.
[2] Mellies MJ, Ishikawa TT, Glueck CJ, et al. Phytosterols in aortic tissue in adults and infants. J Lab Clin Med 1976;88:914-21.
[3] Koivisto PV, Miettenen TA. Plasma and biliary cholestanol related to steroid metabolism in familial hypercholesterolemia patients with and without ileal exclusion. Clin Chim Acta 1988;174:197-205.
[4] Miettenen TA. Diurnal variation of cholesterol precursors squalene and methyl sterols in human plasma lipoproteins. J Lipid Res 1982;23:466-73.
[5] Becker M, Staab D, von Bergmann K. Treatment of severe familial hypercholesterolemia in childhood with sitosterol and sitostanol. J Pediatr 1993;122:292-6.
[6] Kuksis A, Myher JJ, Marai L. Usefulness of gas chromatographic profiles of plasma total lipids in diagnosis of phytosterolemia. J Chromatogr 1986;381:1-12.
[7] Miettenen TA, Tilvis RS, Kesaniemi YA. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. Am J Epidemiol 1990;131:20-31.
[8] Lee M-H, Lu K, Hazard S, et al. Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. Nat Gen 2001;27:79-83.
[9] Berge KE, Tian H, Graf GA, et al. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. Science 2000;290:1771-5.
[10] Lu K, Lee M-H, Hazard S, et al. Two genes that match to the STSL locus cause sitosterolemia: genomic structure and spectrum of mutations involving sterolin-1 and steroin-2 encoded by ABCG5 and ABCG8, respectively. Am J Hum Genet 2001;69:278-90.
[11] Salen G, Tint GS, Shefer S, et al. Increased sitosterol absorption is offset by rapid elimination to prevent accumulation in heterozygotes with sitosterolemia. Arterioscler Thromb 1992;12:563-8.
[12] Aringer L. 7-Hydroxylation of 3-oxygenated C27-, C28-, and C29-steroids in rat liver 18,000 g supernate. J Lipid Res 1978;19:933-44.
[13] Boyd GS, Brown MJG, Hattersley NG, et al. Biochim Biophys Acta 1974;337:132-5.
[14] Arthur JR, Blair HAF, Boyd GS, et al. Biochem Soc Trans 1975;3:963-4.
[15] Slota T, Kozlov NA, Ammon HV. Comparison of cholesterol and β-sitosterol: effects on jejunal fluid secretion induced by oleate, and absorption from mixed micellar solutions. Gut 1983;24:653-8.
[16] Lees AM, Mok HYI, McCluskey MA, et al. Plant sterols as cholesterol-lowering agents: clinical trial in patients with hypercholesterolemia and studies of sterol balance. Atherosclerosis 1977;28:325-38.
[17] Grundy SM, Arhens EH, Davignon J. The interaction of cholesterol absorption and cholesterol synthesis in man. J Lipid Res 1969;10:304-15.
[18] Heinemann T, Aztmann G, von Bergmann K. Comparison of intestinal absorption of cholesterol with different plant sterols in man. Eur J Clin Invest 1993;23:827-31.
[19] Scoggan KA, Gruber H, Lariviere K. A missense mutation in the rat Abcg5 gene causes phytosterolemia in stroke-prone spontaneously hypertensive (SHRSP), spontaneously hypertensive (SHR), and normotensive (WKY inbred) rats. J Lipid Res 2003;44:911-6.

[20] Yu H, Pandit B, Klett E, et al. The rat STSL locus: characterization, chromosomal assignment, and genetic variations in sitosterolemic hypertensive rats. BMC Cardiovasc Disord 2003;3:4.
[21] Kramer W, Glombik H, Petry S, et al. Identification of binding proteins for cholesterol absorption inhibitors as components of the intestinal cholesterol transporter. FEBS Lett 2000;487:293-7.
[22] van Heek M, Farley C, Compton DS, et al. Ezetimibe selectively inhibits intestinal cholesterol absorption in rodents in the presence and absence of exocrine pancreatic function. Br J Pharmacol 2001; 134:409-17.
[23] van Heek M, France CF, Compton DS, et al. In vivo metabolism-based discovery of a potent cholesterol absorption inhibitor, SCH58235, in the rat and rhesus monkey through the identification of the active metabolites of SCH48461. J Pharmacol Exp Ther 1997; 283:157-63.
[24] Davis HR, Compton DS, Hoos L, et al. Ezetimibe (SCH58235) localizes to the brush border of small intestinal enterocyte and inhibits enterocyte cholesterol uptake and absorption. Eur Heart J 2000; 21(Suppl):636.
[25] Bays H. Ezetimibe. Expert Opin Investig Drugs 2002;11:1587-604.
[26] Sudhop T, Lutjohann D, Kodal A, et al. Inhibition of intestinal cholesterol absorption by ezetimibe in humans. Circulation 2002;106: 1943-8.
[27] Knopp RH, Dujovne CA, Le Beaut A, et al. Evaluation of the efficacy, safety, and tolerability of ezetimibe in primary hypercholesterolaemia: a pooled analysis from two controlled phase III clinical studies. Int J Clin Pract 2003;57:363-8.
[28] Melani L, Mills R, Hassman D, et al. Efficacy and safety of ezetimibe coadministered with pravastatin in patients with primary hypercholesterolemia: a prospective, randomized, double-blind trial. Eur Heart J 2003;24:717-28.
[29] Ballantyne CM, Houri J, Notarbartolo A, et al. Effect of ezetimibe coadministered with atorvastatin in 628 patients with primary hypercholesterolemia: a prospective, randomized, double-blind trial. Circulation 2003;107:2409-15.
[30] Kerzner B, Corbelli J, Sharp S, et al. Efficacy and safety of ezetimibe coadministered with lovastatin in primary hypercholesterolemia. Am J Cardiol 2003;91(4):418-24.
[31] Salen G, Shore V, Tint GS, et al. Increased sitosterol absorption, decreased removal, and expanded body pools compensate for reduced cholesterol synthesis in sitosterolemia with xanthomatosis. J Lipid Res 1989;30:1319-30.
[32] Batta AK, Salen G, Batta P, et al. Simultaneous quantitation of fatty acids, sterols and bile acids in human stool by capillary gas-liquid chromatography. J Chromatogr B 2002;775:153-61.
[33] Shefer S, Salen G, Batta AK. Methods of assay. In: Fears R, Sabine JR, editors. Cholesterol 7α-hydroxylase (7α-monooxygenase). Boca Raton (FL): CRC Press; 1986. p. 43-9.
[34] Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.
[35] Nguyen LB, Shefer S, Salen G, et al. A molecular defect in hepatic cholesterol biosynthesis in sitosterolemia with xanthomatosis. J Clin Invest 1990;86:923-31.
[36] Shefer S, Cheng FW, Hauser S, et al. Regulation of bile acid synthesis. Measurement of cholesterol 7α-hydroxylase activity in rat liver microsomal preparations in the absence of endogenous cholesterol. J Lipid Res 1981;22:532-6.
[37] Shefer S, Kren BT, Salen G, et al. Regulation of bile acid synthesis by deoxycholic acid in the rat: different effects on cholesterol 7α-hydroxylase and sterol 27-hydroxylase. Hepatology 1995;22:1215-21.
[38] Lutjohann D, Meese CO, Crouse JR, et al. Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in humans. J Lipid Res 1993;34:1039-46.
[39] McNamara DJ, Proia A, Miettinen TA. Thin-layer and gas-liquid chromatographic identification of neutral steroids in human and rat feces. J Lipid Res 1981;22:474-84.
[40] Weststrate JA, Ayesh R, Bauer-Plank C, et al. Safety evaluation of phytosterol esters: Part 4. Faecal concentrations of bile acids and neutral sterols in healthy normolipidaemic volunteers consuming a controlled diet either with or without a phytosterol ester-enriched margarine. Food Chem Toxicol 1999;37:1063-71.
[41] Salen G, von Bergmann K, Kwiterovitch P, et al. Ezetimibe is an effective treatment for homozygous sitosterolemia. Circulation 2002; 106(Suppl. II):II-185.
[42] Honda A, Salen G, Nguyen LB, et al. 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.
[43] Shefer S, Salen G, Bullock J, et al. The effect of increased hepatic sitosterol on the regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and cholesterol 7α-hydroxylase in the rat and sitosterolemic homozygotes. Hepatology 1994;20:213-9.
[44] Boberg KM, Akerlund JE, Bjorkhem I. Effect of sitosterol on the rate-limiting enzymes in cholesterol biosynthesis and degradation. Lipids 1989;23:9-12.
[45] Aringer L, Eneroth P. Studies on the formation of C_7-oxygenated cholesterol and β-sitosterol metabolites in cell-free preparations of rat liver. J Lipid Res 1973;14:563-72.
[46] Nguyen LB, Shefer S, Salen G, et al. Competitive inhibition of hepatic sterol 27-hydroxylase by sitosterol: decreased activity in sitosterolemia. Proc Assoc Am Physicians 1998;110:32-9.
[47] Liscum L, Underwood KW. Intracellular cholesterol transport and compartmentation. J Biol Chem 1995;270:15443-6.
[48] Igel M, Giesa U, Lütjohann D, et al. Comparison of the intestinal uptake of cholesterol, plant sterols, and stanols in mice. J Lipid Res 2003;44:533-8.
[49] Ikeda I, Tanaka K, Sugano M, et al. Discrimination between cholesterol and sitosterol for absorption in rats. J Lipid Res 1988; 29:1583-91.
[50] McNamara DJ, Kolb R, Parker TS, et al. Heterogeneity of cholesterol homeostasis in man. J Clin Invest 1987;79:729-39.
[51] Kesaniemi YA, Ehnholm C, Miettinen TA. Intestinal cholesterol absorption efficiency in man is related to apoprotein E phenotype. J Clin Invest 1987;80:578-81.
[52] Bhattacharyya AK, Connor WE, Lin DS, et al. Sluggish sitosterol turnover and hepatic failure to excrete sitosterol into bile cause expansion of body pool of sitosterol inpatients with sitosterolemia and xanthomatosis. Arterioscler Thromb 1991;11:1287-94.
[53] Bogert KM, Einarsson K, Bjorkhem I. Apparent lack of conversion of sitosterol into C_{24}-bile acids in humans. J Lipid Res 1990;31:1083-8.
[54] Heinemann T, Kullak-Ublick GA, Pietruck B, et al. Mechanism of action of plant sterols on inhibition of cholesterol absorption: comparison of sitosterol and sitostanol. Eur J Clin Pharmacol 1991; 40(Suppl. 1):S59-S63.
[55] Ikeda I, Nakagiri H, Sugano M, et al. Mechanism of phytosterolemia in stroke-prone spontaneously hypertensive and WKY rats. Metabolism 2001;50:1361-8.
[56] Schonheimer R, Behring H, Hummel RH. Uber die spezifitat der resorption von sterin, abhanging von ihren konstitution. Hoppe-Seylers Z Physiol Chem 1930;192:117-24.
[57] Vahouny GV, Connor WE, Subramaniam S, et al. Comparative lymphatic absorption of sitosterol, stigmasterol, and fucosterol and differential inhibition of cholesterol absorption. Am J Clin Nutr 1983; 37:805-9.