Unesterified plant sterols and stanols lower LDL-cholesterol concentrations equivalently in hypercholesterolemic persons[1–3]

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ABSTRACT

Background: Plant sterols, in various forms, have been shown to reduce total and LDL-cholesterol concentrations. Particularly controversial at present is the effect of the degree of hydrogenation of the plant sterols on cholesterol-lowering efficacy and the responsible mechanisms.

Objective: Our goal was to examine the effect of supplementation with unesterified plant sterols and stanols on plasma lipid and phytosterol concentrations and cholesterol absorption, synthesis, and turnover.

Design: Fifteen otherwise healthy hypercholesterolemic subjects consumed each of 4 dietary treatments in a randomized crossover design. Unesterified sterols and stanols were blended into the butter component of the diet at a dosage of 1.8 g/d. The diets contained plant sterols (NS), plant stanols (SS), a 50:50 mixture of sterols and stanols (NSS), or cornstarch (control).

Results: Plasma total cholesterol concentrations were 7.8%, 11.9%, and 13.1% lower ($P < 0.01$) in the NS, SS, and NSS groups, respectively, than in the control group. LDL-cholesterol concentrations were 11.3%, 13.4%, and 16.0% lower ($P < 0.03$) in the NS, SS, and NSS groups, respectively, than in the control group. Plasma triacylglycerols and HDL-cholesterol concentrations did not differ significantly across diets. Cholesterol absorption efficiency was 56.0%, 34.4%, and 48.9% lower ($P < 0.001$) in the NS, SS, and NSS groups, respectively, than in the control group. The fractional synthesis rate was higher by 45.5% ($P < 0.003$) in the NSS group than in the control group. Plasma campesterol and sitosterol concentrations were higher ($P < 0.01$) in the NS group and sitosterol concentrations were lower ($P < 0.01$) in the SS group than in the control group.

Conclusion: These data indicate that, in their free unesterified form, sterols and stanols lower plasma LDL cholesterol equivalently in hypercholesterolemic persons by suppressing cholesterol absorption. *Am J Clin Nutr* 2002;76:1272–8.

INTRODUCTION

Plant sterols and stanols, structural analogues of cholesterol, have been shown to substantially reduce total and LDL-cholesterol concentrations under a variety of study conditions. Several researchers have claimed that consumption of stanol-containing mixtures is more effective in reducing circulating cholesterol concentrations than is consumption of sterols (1–8). Recently, however, the paradigm has shifted to the position that sterol and stanol esters are comparable plasma cholesterol modulators. It was observed that circulating total and LDL-cholesterol concentrations were equally reduced by 8–13% with both sitosterol-ester and sitostanol-ester margarines at dosages of 1.5–3.3 g/d (9). Similar lowering of total and LDL-cholesterol concentrations was reported with ingestion of sitosterol-esters and sitostanol-esters (10).

This controversy raises another important question: whether unesterified (free) plant sterol and stanol mixtures possess the same cholesterol-lowering efficacy regardless of their degree of hydrogenation, or whether esterification and solubilization of plant sterol mixtures are responsible for their equal effectiveness. Comparison of free sitosterol and sitostanol in pastil form given to children with severe hypercholesterolemia showed that hydrogenation improved the LDL-cholesterol lowering by increasing fecal neutral sterol output to a greater degree than that in the sitosterol-supplemented group (11).

The plasma cholesterol-lowering efficacy of phytosterols varies according to the composition and dose of the phytosterol mix and the vehicle in which they are given. It has been suggested that high intakes of saturated fat and cholesterol may improve the efficacy of phytosterols (12) and phytosterol esters (3). To date, the relative effectiveness of these materials in a dietary context in which saturated fat and cholesterol intakes are at the higher end of the normal physiologic range has not been assessed, nor have the mechanisms of action been fully explored. Therefore, the objective of this study was to examine the effect of supplementation with unesterified sitosterols and sitostanols on plasma lipid and phytosterol concentrations and on cholesterol absorption and synthesis in subjects consuming precisely defined diets.

SUBJECTS AND METHODS

Subjects

Ten male and 6 female otherwise healthy, free-living volunteers with primary familial hyperlipidemia were recruited. The subjects were aged between 35 and 58 y. Female subjects were either post-

TABLE 1
Baseline characteristics of the subjects[1]

Variables	Men ($n = 9$)	Women ($n = 6$)	All ($n = 15$)
Age (y)	46.4 ± 2.7	49.8 ± 2.6	47.8 ± 1.9
Weight (kg)	92.2 ± 3.5	82.4 ± 7.3	88.3 ± 3.6
BMI (kg/m^2)	30.5 ± 1.5	31.3 ± 2.5	30.8 ± 1.3
Lipids (mmol/L)			
Total cholesterol	6.48 ± 0.43	6.57 ± 0.28	6.52 ± 0.27
LDL cholesterol	4.31 ± 0.44	4.29 ± 0.28	4.30 ± 0.26
HDL cholesterol	0.93 ± 0.04	1.42 ± 0.08	1.16 ± 0.08
Triacylglycerols	2.53 ± 0.36	1.86 ± 0.26	2.26 ± 0.25

[1] $\bar{x} \pm$ SEM.

menopausal or had undergone a hysterectomy. Subjects were screened for total circulating cholesterol and triacylglycerol concentrations. Inclusion criteria included a plasma total cholesterol concentration in the range of 5.2–9.0 mmol/L and a triacylglycerol concentration <3.5 mmol/L. Before acceptance, subjects were required to provide a medical history and to undergo a complete physical examination. Fasting blood and urine samples were collected for serum biochemistry, hematology, and urinalysis. Subjects were screened for chronic illness, including hepatic, renal, thyroid, and cardiac dysfunction, before admission in the study. Subjects were required to refrain from using drug therapy for hypercholesterolemia during and for ≥8 wk before the start of the study. Before study commencement, the subjects received a thorough explanation of the study protocol and were given the opportunity to discuss any queries with either the primary investigator, the physician, or the study coordinator before signing the consent form. The experimental protocol was approved by the Human Ethical Review Committee of the Faculty of Agriculture and Environmental Sciences for the School of Dietetics and Human Nutrition at McGill University. The baseline characteristics of the study subjects are presented in **Table 1**.

Experimental design and diets

The study was a randomized, crossover, double-blind clinical trial. Subjects consumed each of 4 dietary treatments. Each dietary treatment phase consisted of 21 feeding days and was separated by a 4-wk washout period during which the subjects consumed their habitual diets. To reduce the error term associated with diet sequencing, subjects were randomly assigned to 1 of 4 predetermined Latin squares, each of which possessed 4 sequenced phases and 4 subjects. In this manner, we ensured that the crossover design was balanced.

Diets were designed on the basis of the recommended nutrient intakes for Canadians to provide 3000 kcal per 70-kg individual daily. The Mifflin equation was used to estimate each subject's basal energy requirement (13), which was then multiplied by an activity factor of 1.7 to compensate for the additional energy needs of mildly to moderately active healthy adults. If subjects gained or lost weight during the first week of each treatment phase, energy adjustments were made to meet individual requirements and ensure that baseline body weights were maintained.

The diets comprised solid foods, typical of those consumed in North America, and were provided as 3 meals daily in a 3-d rotating menu. The nutrient content of the basal diet was calculated by using FOOD PROCESSOR (Food Processor, Salem, OR), a computerized dietary analysis system with a Canadian database. Dietary carbohydrate, fat, and protein made up 50%, 35%, and 15% of ingested energy, respectively, with 70% of the fat provided as butter.

The diets contained plant sterols (NS), plant stanols (SS), a 50:50 mixture of sterols and stanols (NSS), or cornstarch (control).

The NS treatment consisted of purified phytosterols derived from soybeans and contained sitosterol (43%), campesterol (26%), stigmasterol (17%), and other identified phytosterols (14%). For the SS treatment, the same soybean phytosterols were hydrogenated to produce a composition of sitostanol (66%) and campestanol (33%). Equal parts of phytosterols and phytostanols were mixed together to create the NSS treatment. The control product was cornstarch, which strongly resembled the white, powdery phytosterol-containing mixtures. The phytosterol and phytostanol mixtures and the cornstarch control were blended into the butter component of the diet at a dosage of 1.8 g/d; the butter was warmed to 37 °C and administered equally across the 3 daily meals. To achieve double blinding, the containers of plant sterols, stanols, and the cornstarch control were coded so that neither the researcher giving the test mixture nor the subject receiving it knew its true identity.

Diets were prepared in the metabolic kitchen of the Mary Emily Clinical Nutrition Research Unit of McGill University. Subjects consumed a minimum of 2 of the 3 daily meals at the unit under supervision. All subjects were required to consume breakfast at the unit and 1 of the other 2 meals was available for take out.

Protocol

At the start (day 1) and end (day 22) of each dietary phase, fasting blood samples were taken for measurement of circulating lipid concentrations. Ninety-six hours before the end of each phase, subjects provided a baseline blood sample before receiving an intravenous injection of 15 mg [25,26,26,26,27,27,27-D$_7$]cholesterol and a 75-mg oral dose of [3,4-^{13}C]cholesterol for cholesterol absorption determination. The ratio of ingested [3,4-^{13}C]cholesterol to injected [25,26,26,26,27,27,27-D$_7$]cholesterol enrichment in serum cholesterol after 24, 48, and 72 h was taken as an indicator of the cholesterol fractional absorption rate. The [D$_7$]cholesterol isotope was prepared for injection by first dissolving it in ethanol at a concentration of 5 mg/mL under sterile conditions at the Royal Victoria Hospital pharmacy. The isotope-ethanol mixture was then added drop-wise to an intravenous fat emulsion (Baxter Corp, Toronto), for a total injectable volume of 9 mL. Cholesterol synthesis was also measured at the end of each diet period by using the deuterium incorporation approach. Seventy-two hours after dosing with [^{13}C]cholesterol and [D$_7$]cholesterol, subjects were dosed with 0.7 g D$_2$O/kg estimated body water (99.8 atom percent excess; CDN Isotopes, Montreal). Body water was estimated to be 60% for calculation of the dose. Deuterium oxide was given immediately after a fasting blood sample was collected at ≈0800 on day 21 of each diet phase.

Analyses

Plasma lipid concentrations

Blood samples were centrifuged for 15 min at 520 × g and 4 °C to separate plasma from red blood cells (RBCs) and were stored at −80 °C until analyzed. Plasma total cholesterol, HDL-cholesterol, and triacylglycerol concentrations were analyzed in quadruplicate with standardized reagents in a VP Autoanalyser (Abbott Laboratories, North Chicago, IL). The analyzer was calibrated before each run as per the standardization protocol of the Canadian Reference Laboratory. The Friedewald equation was used to calculate LDL-cholesterol concentrations (14).

Cholesterol absorption

Free cholesterol extracted from RBCs was used to determine [^{13}C]cholesterol and [D$_7$]cholesterol enrichments. Lipid was

extracted from the RBCs in duplicate by using a modified Folch extraction procedure (15). Thin-layer chromatography (20 × 20 cm, 250 μ; Scientific Adsorbents Inc, Atlanta) was used to separate free cholesterol from cholesteryl ester. The free cholesterol band was then scraped from the silica gel plate and saponified with 0.5 mol methanolic KOH/L to eliminate any fatty acid contaminants. Free cholesterol extracts were dried under nitrogen and transferred into 18-cm sealed combustion tubes (Vycor; Corning Glass Works, Corning, NY). Cupric oxide (0.6 g) and a 2-cm piece of silver wire were added and the tubes were sealed under a vacuum for ≥ 5 min at < 20 mTorr. Dual-tracer-labeled cholesterol samples were then combusted to deuterium-enriched water and ^{13}C-enriched carbon dioxide over 4 h at 520 °C. The generated carbon dioxide was transferred under vacuum into Vycor tubes for measurement of ^{13}C enrichment, and water was vacuum-distilled into sealed tubes containing 0.06 g Zn (Biogeochemical Laboratories, Indiana University, Bloomington, IN) for deuterium enrichment analysis. Tubes containing the water and zinc were then reduced to deuterium-labeled hydrogen gas at 520 °C for 30 min.

Nuclear magnetic resonance was used to verify that the isotopic enrichments of the tracers, [3,4-^{13}C]cholesterol and [D$_7$]cholesterol (CDN Isotopes, Pointe Claire, Canada), were > 99 atom percent excess. The ^{13}C enrichments of free cholesterol were measured by differential isotope ratio mass spectrometry (IRMS) with an automated dual-inlet system (SIRA 12; Isomass, Cheshire, United Kingdom). Enrichments were then expressed relative to PeeDee Belemnite (PDB) limestone, which is used as the international reference standard for expressing carbon stable isotopic ratios, from the National Bureau of Standards (NBS). The linearity and gain of response of the SIRA IRMS instrument were assessed by using a carbon dioxide reference tank and NBS standards of known isotopic enrichment. The deuterium enrichments of free cholesterol were measured by differential IRMS with the use of a manually operated dual-inlet system with electrical H^{3+} compensation (VG Isomass 903D). For deuterium, enrichments were expressed relative to standard mean ocean water (SMOW) and a series of standards of known enrichment from the NBS, which were analyzed concurrently on each day of measurement to correct for any variations in linearity of gain of response of the IRMS.

The average ^{13}C and deuterium enrichments of 48- and 72-h RBC free cholesterol relative to baseline ($t = 0$) samples were used to calculate the cholesterol absorption coefficient (CAC) by using the ratio of orally ingested [^{13}C]cholesterol to intravenously administered [D$_7$]cholesterol as described by Bosner et al (16):

$$\text{CAC (pool/pool)} = (\Delta^{13}C/\Delta D_7) \times$$
$$(\text{15-mg intravenous dose}$$
$$[D_7]\text{cholesterol/75-mg oral dose}$$
$$[^{13}C]\text{cholesterol}) \times$$
$$[(7/46)/(2/27)] \times (0.0112/0.000155) \qquad (1)$$

where Δ (‰) for ^{13}C and D$_7$ is the difference between the average of the enriched samples at 48 or 72 h and the baseline abundance (at $t = 0$) in parts per thousand relative to the PDB and SMOW standards, respectively. The factors 7/46 and 2/27 reflect the ratios of labeled to unlabelled hydrogen and carbon atoms in the cholesterol tracers, respectively. The constants 0.0112 and 0.000155 represent factors converting the part per thousand units to equivalent atom percent excess for the PDB and SMOW scales, respectively.

Cholesterol biosynthesis

Cholesterol biosynthesis was determined as the rate of incorporation of deuterium from body water into RBC membrane free cholesterol over the period between 72 and 96 h at the end of each feeding period. Deuterated water equilibrates quickly between intracellular and extracellular water pools and permits direct determination of cholesterol formation rates (17). Deuterium enrichment was measured in both RBC free cholesterol and plasma water. To determine plasma cholesterol deuterium enrichment, total RBC lipids were extracted and isolated by using the same procedure described above.

To measure the deuterium enrichment of plasma water, additional plasma samples were diluted 7-fold with water to reduce deuterium enrichment to within the normal analytic range. Baseline samples were not diluted. Triplicate samples were then vacuum-distilled into zinc-containing (0.06 g) Vycor tubes. Cholesterol and plasma water samples were then reduced to hydrogen gas at 520 °C for 30 min and were analyzed by differential IRMS, as previously described above.

The cholesterol fractional synthesis rate (FSR) was taken to represent the RBC free cholesterol deuterium enrichment values relative to the corresponding plasma water sample enrichment after correction for the free cholesterol pool. The FSR represents that fraction of the cholesterol pool that is synthesized in 24 h and was calculated with the following formula (18):

$$\text{FSR (pools/d)} = (\Delta_{\text{cholesterol}}/\Delta_{\text{plasma}}) \times 0.478 \qquad (2)$$

where Δ (‰) for deuterium cholesterol is the difference between enriched free cholesterol and plasma water at 96 and 72 h in parts per thousand relative to a SMOW standard. The factor 0.478 reflects the ratio of labeled H atoms replaced by deuterium (22/46) during in vivo biosynthesis (18).

Cholesterol turnover measured by [D7]cholesterol decay

Plasma turnover of unesterified cholesterol represents the rate of flux of incoming sterol from synthesis and the diet relative to that being esterified, eliminated, or transferred into other pools. Turnover rates for RBC free cholesterol were determined from the decay rate of [D$_7$]cholesterol. Exponential curves were fitted to 24-, 48-, and 72-h RBC [D$_7$]cholesterol enrichments after subtraction of baseline deuterium abundance at 0 h.

Plasma phytosterol concentrations

Plasma phytosterol concentrations were determined in duplicate by gas-liquid chromatography from the nonsaponifiable material of plasma lipid as reported previously (19). Briefly, 1.0-mL plasma samples were saponified with 0.5 mol methanolic KOH/L for 1 h at 100 °C, and the nonsaponifiable materials were extracted with petroleum ether. Samples with 250 μg 5-α-cholestane added as an internal standard were injected into a gas-liquid chromatograph equipped with a flame ionization detector (HP 5890 Series II; Hewlett Packard, Palo Alto, CA) and a 30-m capillary column (SAC-5; Supelco, Bellefonte, PA). Detector and injector temperatures were 310 and 300 °C, respectively. Duplicate samples were run isothermically at 285 °C. Phytosterol peaks were identified by comparison with authenticated standards (Supelco).

Statistics

All data are expressed as means ± SEMs. Lipoprotein cholesterol, triacylglycerol, and phytosterol concentrations; absolute values at the beginning and end of each dietary period; and percentage

TABLE 2
Plasma lipid concentrations on days 0 and 21 of each dietary period[1]

Lipid	NS	SS	NSS	Control
Total cholesterol				
Day 0 (mmol/L)	5.97 ± 0.26	6.23 ± 0.26	6.40 ± 0.31	6.14 ± 0.33
Day 21 (mmol/L)	5.54 ± 0.22[b,2]	5.57 ± 0.22[b,3]	5.59 ± 0.26[b,4]	6.15 ± 0.25[a]
Change (%)	−6.3 ± 2.9[b]	−10.4 ± 2.3[b]	−11.6 ± 3.2[b]	1.5 ± 3.2[a]
Change relative to control (%)	−7.8	−11.9	−13.1	
LDL cholesterol				
Day 0 (mmol/L)	4.0 ± 0.20	4.11 ± 0.18	4.18 ± 0.23	4.06 ± 0.26
Day 21 (mmol/L)	3.6 ± 0.17[b,2]	3.59 ± 0.18[b,2]	3.55 ± 0.17[b,4]	4.01 ± 0.20[a]
Change (%)	−9.1 ± 2.9[b]	−11.2 ± 3.0[b]	−13.8 ± 3.2[b]	2.2 ± 5.4[a]
Change relative to control (%)	−11.3	−13.4	−16.0	
Triacylglycerol				
Day 0 (mmol/L)	1.91 ± 0.22	2.10 ± 0.24	2.21 ± 0.35	1.94 ± 0.20
Day 21 (mmol/L)	1.85 ± 0.23	1.90 ± 0.20	1.99 ± 0.32	2.15 ± 0.33
Change (%)	2.2 ± 9.1	−5.4 ± 6.7	−3.1 ± 8.0	7.0 ± 6.3
Change relative to control (%)	−4.8	−12.4	−10.1	
HDL cholesterol				
Day 0 (mmol/L)	1.14 ± 0.08	1.17 ± 0.07	1.20 ± 0.09	1.19 ± 0.07
Day 21 (mmol/L)	1.10 ± 0.07	1.14 ± 0.09	1.14 ± 0.09[2]	1.16 ± 0.08
Change (%)	−2.0 ± 2.9	−2.6 ± 3.7	−5.6 ± 2.5	−2.3 ± 2.1
Change relative to control (%)	0.3	−0.3	−3.3	
LDL:HDL				
Day 0	3.71 ± 0.29	3.69 ± 0.26	3.81 ± 0.39	3.62 ± 0.34
Day 21	3.40 ± 0.25	3.48 ± 0.30	3.61 ± 0.42	3.73 ± 0.29
Change (%)	−7.9 ± 3.2	−6.4 ± 4.1	−6.7 ± 6.4	4.0 ± 6.0
Change relative to control (%)	−11.9	−10.4	−10.7	

[1] $\bar{x}$ ± SEM; n = 15. Percentage change is based on individual data. Percentage change relative to control is based on the mean of day 21 values. NS, plant sterols; SS, plant stanols; NSS, a 50:50 mixture of sterols and stanols. Means within a row with different superscript letters are significantly different, $P < 0.05$.
[2–4] Significantly different from day 0: [2] $P < 0.05$, [3] $P < 0.001$, [4] $P < 0.01$.

changes relative to baseline were compared by using a crossover analysis of variance (ANOVA) design for determination of the diet effects. Although a 4-wk washout period separated each dietary period, a carryover term was used in the model to reduce any error associated with dietary sequence (20). When treatment effects were identified as significant, a Tukey test was used for identification of significant effects between diets at particular time points. Student's paired t tests were used to compare baseline values with final time points within each diet. Cholesterol absorption, synthesis, and turnover values were compared at the end of each treatment phase by using a crossover ANOVA. When treatment effects were identified, a Tukey test was used for identification of significant effects of each diet treatment. Pearson's correlation coefficient analysis was used to test for relations between variables. A level of statistical significance at $P < 0.05$ was used in all analyses. The data were analyzed by using the PROC general linear model procedure in SAS (version 6.12; SAS Institute Inc, Cary, NC).

RESULTS

Sixteen subjects were enrolled in the study. One male subject dropped out at the end of the first feeding cycle because of difficulties with daily transportation to the unit. Therefore, complete data for 9 men and 6 women were collected and analyzed as per the study protocol. All individuals tolerated the diet without any reported adverse events. Subjects reported no abnormal or atypical smells, tastes, colors, or mouth-feel effects when consuming any of the 4 mixtures and thus were unable to distinguish between dietary treatments. There were no significant mean group weight changes across any of the 3 treatment phases. Blood and urine samples at the beginning and end of each phase

for all 15 subjects were sent to LDS Diagnostic Laboratories (Pointe Claire, Canada), where complete blood counts, biochemistry analyses (sequential multiple analysis level C), and urinalyses were carried out. Results from all 4 phases of the feeding trial remained within normal ranges throughout the study period, and the results of regular physical exams showed no suggestion of any clinical irregularities.

Circulating lipids in response to treatment

Concentrations of plasma lipids at the beginning and end of each treatment phase are shown in **Table 2**. Total cholesterol concentrations and the changes in cholesterol concentrations varied greatly from one subject to another across all phases of the feeding trial. Plasma total cholesterol concentrations were 7.8%, 11.9%, and 13.1% lower at the end of the dietary period ($P < 0.01$) in the NS, SS, and NSS groups, respectively, than in the control group. LDL-cholesterol concentrations were 11.3%, 13.4%, and 16.0% lower ($P < 0.03$) in the NS, SS, and NSS groups, respectively, than in the control group. Plasma triacylglycerol and HDL-cholesterol concentrations did not differ significantly across diets. Over the study period, however, HDL cholesterol was lower on day 21 of the NSS treatment period than on day 0 ($P < 0.05$).

Cholesterol absorption in response to treatment

Cholesterol absorption at the end of each feeding phase was taken as an average of the 48- and 72-h measurements. The mean cholesterol absorption coefficient was lower ($P < 0.001$) after ingestion of the NS, SS, and NSS diets than after the control diet (**Table 3**). Therefore, relative to the control period, absorption was 56.0%, 34.4%, and 48.9% lower after the NS, SS, and NSS dietary periods, respectively. Absorption values for the NS and SS dietary

TABLE 3
Cholesterol absorption coefficients and fractional synthesis rates for each dietary period[1]

Phytosterol	NS	SS	NSS	Control
Absorption				
Efficiency (pool/pool)	0.200 ± 0.03^c	0.298 ± 0.04^b	$0.232 \pm 0.04^{b,c}$	0.454 ± 0.04^a
Change relative to control (%)	−56.0	−34.4	−48.9	
Synthesis				
Rate (pool/d)	$0.055 \pm 0.008^{a,b}$	$0.059 \pm 0.01^{a,b}$	0.064 ± 0.007^a	0.044 ± 0.007^b
Change relative to control (%)	25.0	34.1	45.5	

[1] $\overline{x} \pm$ SEM; $n = 15$. Percentage change relative to control is based on the mean of day 21 values. NS, plant sterols; SS, plant stanols; NSS, a 50:50 mixture of sterols and stanols. Means within a row with different superscript letters are significantly different, $P < 0.05$.

periods were significantly ($P < 0.05$) different from each other; however, the value for the NSS period was not significantly different from that for either the NS or the SS period.

Cholesterol biosynthesis in response to treatment

The FSR in the control group was measured to be 0.044 ± 0.007 pool/d. Ingestion of the NSS diet resulted in a 45.5% higher FSR (0.064 ± 0.007 pool/d; $P < 0.003$) than did consumption of the control diet. The FSR also tended to be higher after consumption of both the NS and SS diets by 25.0% (0.055 ± 0.008 pool/d) and 34.1% (0.059 ± 0.01 pool/d), respectively (NS). No significant difference in synthesis was observed between groups supplemented with phytosterols or phytostanols.

Cholesterol turnover in response to treatment

Rates of cholesterol turnover were calculated from $[D_7]$cholesterol enrichment values obtained during the 24–72 h period following injection of the isotope at the end of each treatment phase. Turnover rates of free cholesterol, extracted from RBCs, were 0.381 ± 0.05, 0.346 ± 0.06, 0.324 ± 0.04, and 0.364 ± 0.04 pools/d, for the NS, SS, NSS, and control diets, respectively. No significant differences were found between any of the diets.

Plasma plant sterol concentrations in response to treatment

Plasma plant sterol concentrations and ratios relative to total cholesterol are presented in **Table 4**. Plasma campesterol and sitosterol concentrations were not significantly different between groups at the beginning of each feeding phase. There were, however, differences between groups at the end of phytosterol supplementation. Plasma campesterol and sitosterol concentrations were higher ($P < 0.01$) in the NS group than in the control and SS groups. Mean plasma campesterol and sitosterol concentrations were 99.3% and 38.6% higher ($P < 0.0001$), respectively, after the NS diet than after the control diet. Sitosterol concentrations were lower ($P < 0.01$) by 23.6%

TABLE 4
Plasma plant sterol concentrations on days 0 and 21 of each dietary period[1]

Phytosterol	NS	SS	NSS	Control
Campesterol				
Day 0 (μmol/L)	14.1 ± 1.2	17.3 ± 3.0	13.8 ± 1.5	15.6 ± 2.1
Day 21 (μmol/L)	$24.3 \pm 2.3^{a,2}$	$8.5 \pm 0.8^{c,2}$	$17.7 \pm 2.1^{a,b}$	$12.7 \pm 2.4^{b,c}$
Change (%)	84.2^a	-43.1^c	$39.2^{a,b}$	$-10.9^{b,c}$
Change relative to control (%)	99.3	−27.9	54.6	
β-Sitosterol				
Day 0 (μmol/L)	8.6 ± 1.2	9.2 ± 1.0	7.2 ± 0.7	8.4 ± 1.1
Day 21 (μmol/L)	9.6 ± 1.0^a	$4.8 \pm 0.5^{c,2}$	$7.4 \pm 0.9^{a,b}$	7.0 ± 0.9^b
Change (%)	26.3^a	-35.9^c	$10.0^{a,b}$	-12.3^b
Change relative to control (%)	38.6	−23.6	22.3	
β-Sitosterol:campesterol				
Day 0 (mmol/mmol)	0.61 ± 0.05	0.57 ± 0.07	0.55 ± 0.04	0.56 ± 0.04
Day 21 (mmol/mmol)	0.40 ± 0.03^c	$0.57 \pm 0.04^{a,b}$	$0.43 \pm 0.03^{c,b}$	0.64 ± 0.03^a
Change (%)	-26.8 ± 8.2^c	$14.5 \pm 12.1^{a,b}$	$-18.9 \pm 5.1^{c,b}$	22.2 ± 14.4^a
Change relative to control (%)	−37.5	−10.9	−32.8	
Campesterol:total cholesterol				
Day 0 (mmol/mol)	2.45 ± 0.23	2.76 ± 0.37	2.29 ± 0.31	2.57 ± 0.30
Day 21 (mmol/mol)	4.41 ± 0.39^a	1.54 ± 0.15^c	$3.29 \pm 0.46^{a,b}$	$2.12 \pm 0.45^{b,c}$
Change (%)	95.8 ± 17.0^a	-35.6 ± 7.5^b	61.9 ± 18.1^a	-11.7 ± 16.3^b
Change relative to control (%)	108	−27.3	55.2	
β-Sitosterol:total cholesterol				
Day 0 (mmol/mol)	1.44 ± 0.17	1.47 ± 0.20	1.16 ± 0.13	1.40 ± 0.17
Day 21 (mmol/mol)	1.72 ± 0.18^a	0.89 ± 0.11^b	1.32 ± 0.14^b	1.14 ± 0.15^b
Change (%)	-24.8 ± 6.3^a	-64.2 ± 4.6^c	$-32 \pm 7.5^{a,b}$	$-53.4 \pm 4.6^{b,c}$
Change relative to control (%)	50.9	−21.9	15.8	

[1] $\overline{x} \pm$ SEM; $n = 15$. Percentage change is based on individual data. Percentage change relative to control is based on the mean of day 21 values. NS, plant sterols; SS, plant stanols; NSS, a 50:50 mixture of sterols and stanols. Means within a row with different superscript letters are significantly different, $P < 0.05$.
[2] Significantly different from day 0, $P < 0.01$.

after the SS diet than after the control. Campesterol concentrations were also lower with SS feeding, but not significantly so. The NSS diet produced few changes in circulating plant sterol concentrations.

Associations between plasma lipid concentrations and kinetic measurements

Across all subjects, both plasma total ($r = 0.42$, $P < 0.001$) and LDL cholesterol ($r = 0.35$, $P < 0.006$) concentrations varied directly with the cholesterol absorption coefficient. Similarly, β-sitosterol ($r = 0.40$, $P < 0.002$) concentrations varied directly with circulating LDL-cholesterol concentrations. The FSR varied inversely with LDL cholesterol ($r = -0.29$, $P < 0.03$) concentrations, further supporting the compensatory relation between cholesterol lowering and increased synthesis rates. The ratio of sitosterol to campesterol correlated directly with plasma LDL cholesterol ($r = 0.49$, $P < 0.0001$) and inversely with the FSR ($r = -0.42$, $P < 0.0008$). Notably, neither campesterol nor the ratio of campesterol to cholesterol correlated with the cholesterol absorption coefficient.

DISCUSSION

The major novel finding of the present study is the demonstration that unsaturated, saturated, and an equal mixture of unsaturated and saturated phytosterols, in their unesterified form, significantly and equally reduce both plasma total and LDL-cholesterol concentrations. The degree of cholesterol lowering observed was entirely due to the action of the plant sterols and stanols and not to the basal diet, because plasma total and LDL-cholesterol concentrations marginally increased with the control diet. This reduction in circulating cholesterol concentrations was achieved through inhibition of intestinal cholesterol absorption as evidenced by lower absorption coefficients; however, these reductions were accompanied by a partial compensatory desuppression of cholesterol synthesis, which may be an indication that other mechanisms are also at work.

Despite the relatively high content of saturated fat and cholesterol in the basal diet, sterols and stanols were efficacious in lowering circulating total and LDL-cholesterol concentrations. It has been postulated that elevated intakes of dietary fat and cholesterol (400–450 mg/d) may increase the effectiveness of phytosterols in the intestinal lumen. Unesterified plant sterols blended in butter and supplemented in doses of 0.74 g for 4 wk were shown to decrease total and LDL-cholesterol concentrations by 10% and 15%, respectively, despite a phytosterol dosage of < 1 g/d (12). These authors attributed their results to the high cholesterol intake obtained from butter. Several researchers, however, achieved similar degrees of cholesterol suppression when the total fat and cholesterol contents of the diet were much lower (7, 21, 22). These and other studies showed the efficacy of plant sterols and stanols when blended into a fat source such as margarine, butter, mayonnaise, or vegetable oils before supplementation (23). Conversely, when provided as a powder-filled capsule as part of a low-fat diet, plant stanols failed to exert any lipid-modulating effect (24), suggesting that the amount of fat and cholesterol in the diet are not strong modulators of the effectiveness of plant sterols. It is more likely that the effectiveness of plant sterols and stanols depends more on the vehicle in which they are matrixed and added to the diet than on the composition of the diet.

Although structurally similar to cholesterol, plant stanols are believed to be negligibly absorbed by the intestine (1). Therefore, they do not enter the cell and displace cholesterol at the level of the micelle, interrupting absorption (25). Several methods exist to directly measure cholesterol absorption; however, many require fecal collections, radiolabeled cholesterol administration, or both (26, 27). The current study is one of few to use the dual-stable-isotope method. A coefficient of absorption for cholesterol is derived through a time-step comparison of the proportion of an orally administered, labeled bolus of tracer cholesterol appearing in blood relative to the appearance of a bolus labeled with a second tracer administered intravenously (16, 28). The pattern of decay of the intravenous tracer permits correction of the oral tracer response in plasma for loss into routes of excretion or deeper metabolic pools. Previously, selected ion monitoring mass spectrometry was used to measure isotope enrichments (16), whereas this study used more sensitive IRMS to improve precision with lower isotopic dosages. The cholesterol absorption coefficient was determined by calculating the average of the 48 and 72 h time points by using the approach described by Bosner et al (16), who showed that the plasma ratio of oral and intravenous tracers becomes constant between 48 and 72 h after dosing, allowing for accurate assessment of intestinal cholesterol absorption.

The relative effect of unesterified sterols and stanols compared with a control diet on cholesterol absorption has not been previously studied, particularly in the context of a rigidly controlled dietary paradigm. The cholesterol absorption efficiency of 45.4% in the control group is comparable to values reported elsewhere (16, 29, 30). Similarly, the 34–56% lower cholesterol absorption rates after sterol and stanol supplementation agree with data for sterol and stanol esters reported elsewhere for humans (6, 10, 29, 30). The present data indicate that the decrease in circulating cholesterol concentrations in subjects supplemented with unesterified phytosterols or phytostanols is due to this inhibition in the cholesterol absorption efficiency.

Cholesterol absorption varied directly with both total and LDL-cholesterol concentrations, suggesting that circulating cholesterol concentrations are dependent on the uptake of cholesterol in the intestine and that plant sterols and stanols effectively inhibit cholesterol absorption. The group with the lowest absorption coefficient was not, however, the group with the greatest degree of cholesterol lowering. The sterol diet lowered cholesterol absorption by 56% and raised synthesis by 25%; however, a smaller effect on cholesterol lowering was seen than in the other groups. The stanol diet decreased the absorption coefficient by 34.4% and raised synthesis by the same amount (34.1%), and cholesterol concentrations fell more dramatically. Interestingly, the 50:50 mix of sterols and stanols decreased cholesterol absorption by 48.9% and increased synthesis almost 50%, yet lowered cholesterol concentrations to the greatest degree.

Free sitosterol has been shown to more effectively lower cholesterol absorption than does sitosterol ester (29). However, most previous reports concluded that sitostanol more effectively inhibits cholesterol absorption than does sitosterol (1, 6, 7) or results in equal reductions in cholesterol absorption efficiency (10, 30). Although our results are in contrast with those previously reported, the present study enforced a strict dietary regimen, ensuring that all subjects consumed identical foods, in equal proportions, while maintaining a steady weight. This regimen minimized several dietary confounders, making the comparisons between groups more accurate.

Consumption of plant sterols and stanols significantly induces changes in circulating plant sterol and cholesterol concentrations, indicating mutually competitive inhibition between all sterol forms

(25). On this basis, plasma plant sterol concentrations have been used as indicators of compliance. Absolute values and percentage changes in campesterol and sitosterol concentrations were similar to those previously reported after phytosterol feeding (8, 10, 23), signaling that the subjects were in fact consuming the treatment.

Plasma plant sterol concentrations have also been used as an indirect measure of cholesterol absorption. Specifically, serum campesterol concentrations and the ratio of campesterol to cholesterol have been shown to correlate positively with intestinal cholesterol absorption. This association would be expected to reflect cholesterol absorption under static dietary conditions. However, different plant sterols are variably absorbed and metabolized; therefore, it is unclear whether the use of campesterol is appropriate for measuring cholesterol absorption under conditions in which plant sterol and stanol intakes are changing. Supplementation with stanols inhibits cholesterol absorption and has consistently produced decreases in sterol concentrations (7, 10, 23). During sitosterol feeding, however, sitosterol and campesterol concentrations have been shown to increase (10) or remain unchanged (11) despite a clear inhibition in the cholesterol absorption efficiency, making this correlation inapplicable as a method of estimating cholesterol absorption in any situation in which phytosterol intakes would be expected to change. In the present study, plasma sterol concentrations decreased with stanols and increased with sterols, whereas concentrations remained similar to those with the control with a 50:50 mix of sterols and stanols. Furthermore, the cholesterol absorption coefficient was not associated with either campesterol concentration or the ratio of campesterol to cholesterol, as would be predicted from investigating stanols alone.

In summary, the results of the present study showed that in free form, sterol and stanol feeding results in eqivalent reductions in total and LDL-cholesterol concentrations. Cholesterol absorption was reduced in response to sterol and stanol feeding and varied directly with reductions in LDL-cholesterol concentration. Cholesterol synthesis was increased, however, but not to an extent that prevented cholesterol lowering. Both unesterified plant sterols and stanols favorably lower LDL cholesterol, independent of their degree of hydrogenation, in hypercholesterolemic individuals. ♥

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