Inhibition of cholesterol absorption in rats by plant sterols

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Abstract The extent and site(s) of inhibition of cholesterol absorption by plant sterols, sitosterol and fucosterol, were studied in rats. The intragastric administration of a single emulsified lipid meal containing 25 mg [^{3}H]cholesterol and 25 mg of either sitosterol or fucosterol inhibited the lymphatic absorption of cholesterol by 57% and 41%, respectively, in 24 hr. Less than 2% of each plant sterol was absorbed in the 24-hr period. In contrast, neither plant sterol (50 μM) inhibited cholesterol absorption when co-administered with equimolar amounts of cholesterol in phospholipid-bile salt micelles nor was either absorbed from the micellar solution. A series of in vitro studies was conducted to identify the site(s) of plant sterol inhibition of cholesterol absorption and to account for the difference in inhibitory effectiveness of sitosterol and fucosterol. A comparison of the micellar solubility of each sterol alone and in equimolar binary mixtures (to 2.0 mM) revealed that the solubility of individual sterols decreased in the following order: cholesterol, fucosterol, sitosterol, and that in binary mixtures cholesterol solubility was decreased by sitosterol and, to a lesser extent, by fucosterol relative to its solubility alone. A comparison between micellar-solubilized cholesterol and either sitosterol or fucosterol for binding to isolated brush border membranes, intestinal mucin, or for esterification by either cholesterol esterase or acyl coenzyme A:cholesterol acyltransferase revealed moderate to no competition. The data suggest that plant sterols displace cholesterol from bile salt (taurocholate) micelles and that sitosterol is more effective than fucosterol in this capacity.—**Ikeda, I., K. Tanaka, M. Sugano, G. V. Vahouny, and L. L. Gallo.** Inhibition of cholesterol absorption in rats by plant sterols. *J. Lipid Res.* 1988. **29:** 1573–1582.

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Plant sterols are effective inhibitors of cholesterol absorption, i.e., they display hypocholesterolemic properties (1–3). From a structural standpoint, this inhibition is related most clearly to substitutions in position 24 (1–6) on the sterol side chain as demonstrated with sitosterol (24-ethyl cholesterol), stigmasterol (δ^{22},24-ethyl cholesterol), sitostanol (5-dihydro, 24-ethyl cholesterol), and campesterol (24-methyl cholesterol). Fucosterol (24-ethylidene cholesterol), although untested as an inhibitor of dietary cholesterol absorption, by contrast, does not inhibit the lymphatic absorption of endogenous cholesterol (5).

It has been suggested that these structural analogs function (1–3) by competing with cholesterol at steps essential for absorption including: micellar solubilization, uptake by the brush border membrane, intracellular esterification, and/or incorporation into chylomicrons. With respect to lumenal events, cholesterol and sitosterol, as well as their 7-dehydro derivatives (7), are reported to bind to isolated rat brush border membranes (7), to the brush border membranes of either rat jejunal loops in situ (8) or isolated rat jejunal villus cells (7), and to brush border isolated from rat proximal small intestine 2 hr after the in vivo administration of each sterol in a lipid meal (9). In each case, less sitosterol associates with this membrane. Nonetheless, if sitosterol were present in high doses, it might inhibit cholesterol binding. Another major lumenal event, micellar solubilization of sterols, represents a likely site for sterol competition. Related to this, a recent study (10) suggests that sitosterol has a greater affinity than cholesterol for taurocholate micelles and would displace micellar cholesterol with a favorable free energy change.

With respect to intracellular steps, the amount of plant sterol taken up by intestinal cells may be insufficient to account for significant inhibition of cholesterol processing, i.e., esterification or incorporation into chylomicrons. In this regard Swell et al. (11) reported that sitosterol was taken

Abbreviations: C, cholesterol; S, sitosterol; F, fucosterol; BSA, bovine serum albumin; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); ACAT, acyl coenzyme A:cholesterol acyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; MO, monoolein; OA, oleic acid; BS, bile salt; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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up by the intestinal wall, but Borgström (12) did not observe any accumulation of administered sitosterol and our recent observations support the latter finding (9). Moreover, it has been reported that microsomal sitosterol does not compete with cholesterol for ACAT-catalyzed esterification (13).

The present studies represent a systematic approach to examining the relative inhibitory effect of sitosterol and fucosterol (plant sterols with ethyl and ethylidene substitutions at carbon 24, respectively) on the lymphatic absorption of exogenous cholesterol and to accounting for this inhibition with in vitro studies that examine *1*) the micellar solubility of each plant sterol and the effect of each on the micellar solubility of cholesterol, and *2*) the competition between plant sterols and cholesterol for binding to brush border membranes, for binding to mucin, and for esterification by mucosal cholesterol esterase and ACAT.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA) fraction V (fatty acid-poor), ATP, coenzyme A, monoolein, oleic acid (> 99% purity), fucosterol (>95% purity), and egg yolk phospholipids (Type IX-E) were obtained from Sigma Chemical Co., St. Louis, MO. Cholesterol (>99% purity) was purchased from Serdary Research Laboratories, Ontario, Canada. Sitosterol (from ICN Pharmaceuticals, Cleveland, OH) was recrystallized from ethyl acetate-methanol to >98% purity. Sodium taurocholate (>96% purity) was from Calbiochem, LaJolla, CA. [1-^{14}C]Oleic acid, [4-^{14}C]cholesterol and [1α,2α-^{3}H]cholesterol and [4-^{14}C]sitosterol were supplied by Amersham, Arlington Heights, IL. All other chemicals were reagent grade and purchased from Fisher Scientific, Silver Spring, MD.

Animals

Adult male Wistar rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 200-240 g were housed under a normal (12 hr) light cycle and allowed laboratory chow (Ralston-Purina Co., St. Louis, MO) and water ad libitum prior to use.

Surgical procedures

Animals were anesthetized with sodium pentobarbital (50 mg/kg body weight) and subjected to cannulation of the left thoracic lymphatic cephalad to the cisterna chyli as previously described (14). A second indwelling catheter was placed either in the stomach or in the duodenum for later administration of either an emulsified or a micellar lipid meal, respectively. In some studies, where indicated, bile was drained by an indwelling bile duct catheter without interruption of pancreatic juice flow. After surgery, animals were placed in restraining cages in a warm recovery room. All animals post-surgery were allowed free access to drinking water containing 5% glucose and 0.9% NaCl; those rats that would later receive the emulsified lipid meals were infused intragastrically with this same solution at a rate of 3.0 ml/hr. Animals with bile diversion received, instead, a continuous infusion of "artificial" bile (preparation described below). This was infused intraduodenally at a rate of 3.4 ml/hr from the time of surgery to killing with a brief interruption for intraduodenal administration of the radiolabeled micellar sterol(s). Animals with lymph flow rates of >2.0 ml/hr were given the lipid meals as described in the individual studies between 9 and 10 the morning after surgery.

Preparation of lipid meals and "artificial" bile for in vivo sterol absorption studies

Lipid emulsions for intragastric administration were prepared as described earlier (5) and contained per 3 ml of physiologic saline: 50 mg BSA, 292 mg oleic acid, 279 mg sodium taurocholate, and 25 mg of [^{3}H]cholesterol either alone or plus 25 mg of sitosterol or fucosterol. These emulsions were prepared immediately before use and rehomogenized before administration.

Sterol-containing micellar solutions were prepared for intraduodenal infusion and contained 6.6 mM sodium taurocholate, 0.6 mM egg yolk phospholipids, and 50 μM [^{3}H]cholesterol either alone or plus 50 μM of [4-^{14}C]sitosterol or fucosterol in 15 mM sodium phosphate buffer, pH 7.4, containing 62 mM NaCl and 2.5% glucose. The solutions were sonicated, filtered through ACRODISC (0.2 μm, Gelman Sciences, Inc., Ann Arbor, MI), and kept at 37°C. These preparations were used as described in the individual studies.

"Artificial" bile for intraduodenal infusion into bile-fistula rats was prepared as a micellar solution containing 6.6 mM sodium taurocholate and 0.6 mM egg yolk phospholipids with no sterol, with 150 μM cholesterol, or with 150 μM each of cholesterol and sitosterol in 15 mM sodium phosphate buffer, pH 7.4, containing 62 mM NaCl and 2.5% glucose. The procedure for preparation was the same as that described above for the micellar sterol meals.

Preparation of micellar solutions for in vitro studies

A series of micellar solutions were prepared for the in vitro studies. These varied in the number and type of component, as well as in the concentration of sterol. Each contained 6.6 mM sodium taurocholate and 25 μM to 2.0 mM sterol (cholesterol, sitosterol, or fucosterol, alone or in binary combinations) and where indicated in the individual experiments, 0.6 mM egg yolk phospholipids (67.7 mol % PC and 22.7 mol % PE based on phosphorus content), 1 mM oleic acid, and 0.5 mM monoolein. These were prepared in Hank's balanced salts solution containing 15 mM HEPES, pH 7.4, when used in brush border binding studies (to preserve the membrane) or in 15 mM sodium phosphate buffer, pH 7.4, containing 132 mM NaCl when used

in the mucin binding and micellar sterol solubility studies. All were sonicated, filtered, and gassed as described above.

Preparation and administration of the semi-purified diet

A semi-purified diet was prepared (Dyets, Inc., Bethlehem, PA) to contain 20% casein (vitamin-free), 10% safflower oil (plant sterol-free), 4% mineral mixture (Rogers-Harper), 1% vitamin mixture (Harper), 4% cellulose, 0.2% choline chloride, and 60.8% sucrose. Sterols were added at a level of 0.25% at the expense of sucrose. Rats were meal-fed 10 g of the diet over a period of 1 hr for 7 days. On the final day of the study, 5 μCi of [^{3}H]cholesterol was added to each diet.

Collection of lymph

Lymph was collected at 3, 6, 9, and 24 hr in iced heparinized tubes containing DTNB (1 mM), an inhibitor of lecithin:cholesterol acyltransferase.

Determination of micellar solubility of sterols in vitro and in vivo

In the in vitro studies, 2 ml of each micellar solution containing sterols singly or in binary combinations (prepared as described above with a specific composition as described in the individual experiments) at 37°C was monitored immediately for turbidity by reading absorbance at 430 nm (Beckman, Acta V spectrophotometer). The micellar solutions were maintained at 37°C overnight and observed for precipitates. In a separate study, selected micellar solutions (3 ml), as defined in the individual experiments, were centrifuged at forces of 10,000 *g* up to 100,000 *g* (Beckman L8, 50 Ti rotor). The concentration of each sterol in the clear supernatant was determined either by radioactivity or mass measurement.

In the in vivo studies, rats were killed 2 hr after consumption of the final sterol-containing meal. Lumenal content (2 ml average volume) was collected from each animal, heated for 15 min at 70°C to inactivate lipolytic enzymes, and centrifuged at 100,000 *g* for 60 min. [^{3}H]Cholesterol in the clear micellar solutions was determined by radioactivity measurement.

Preparation of brush border membranes

Brush border membranes were prepared from mucosa scraped from the proximal half of the small intestine as described by others (15). The membrane pellet was resuspended in 5.0 ml of 50 mM mannitol 2 mM HEPES, pH 7.1, the preparation buffer. After assay for alkaline phosphatase (16), sucrase (17), and protein (18), the membranes were pelleted by centrifugation for 30 min at 27,000 *g* and resuspended in Hank's balanced salts solution containing 15 mM HEPES, pH 7.4, 5 mM EGTA, and 4% BSA (fatty acid-free) to give a final protein concentration of 2 mg/ml. The suspension was gassed with O_2–CO_2 95:5 as suggested (7). The brush border membranes were consistently enriched 20 times in sucrase activity and 15 times in alkaline phosphatase activity relative to the whole homogenate. Recovery of ACAT (19), succinic dehydrogenase (20), and DNA (21), markers for microsomes, mitochondria, and nuclei, respectively, in the brush border was less than 0.5% of levels in the whole homogenate.

Preparation of mucin

Surface material was aspirated under line vacuum from the proximal half of everted small intestine that had been flushed with 100 ml of iced 0.9% NaCl. Crude mucins were suspended in iced 0.154 M sodium phosphate buffer, pH 7.2, and homogenized for 30 sec in a Potter-Elvehjem tube with a motor-driven Teflon pestle. Mucins were isolated from the suspension by centrifugation at 4°C for 30 min at 6000 *g* (22) and were diluted with the same buffer to a final protein concentration of 10 mg/ml. Mucin enrichment (threefold) in the supernatant was assessed by radioimmunoassay (22).

Transfer of sterols from micellar solutions to brush border membranes and to mucin

In the brush border experiments, 4 ml of the micellar solution (prepared as described above with a specific composition as described in individual experiments) was incubated with 1 ml of brush border membrane suspension (2 mg protein) at 37°C (7). At 30 min (representing initial rate of binding), 1–2-ml samples were withdrawn and released into 5 ml iced 0.9% saline containing 7 mM sodium taurocholate, which decreases nonspecific sterol adsorption. This was centrifuged for 30 min at 27,000 *g* at 5°C. The resulting pellet was washed once in 10 ml of the same solution and recollected by centrifugation. The brush border membrane pellet was suspended in 1.0 ml distilled water, sonicated, and subjected to radioactivity and protein determinations (18). The data were corrected for zero-time binding ($<$ 10 nmol/mg protein).

In the mucin experiments, 3 ml of the micellar solution (composition as described in individual experiments) was incubated with 1.0 ml of mucin suspension (10 mg protein) at 37°C. At 15 min (representing initial rate of binding), 1-ml samples were withdrawn and released into 5.0 ml of iced 0.3 M sucrose containing 7 mM sodium taurocholate, mixed, and centrifuged at 5°C for 15 min at 100,000 *g*. The mucin pellet was washed once with the same solution and again centrifuged. Pellet-associated sterol was determined by radioactivity counting and pellet protein was assayed (18). The data were corrected for zero-time binding ($<$ 20 nmol/mg protein).

Esterification enzyme assays

Cholesterol esterase activity was assayed as previously described (23) in cytosol (2.0 mg protein) prepared from a

20% homogenate of mucosa scraped from the proximal small intestine. The conversion of sterol into steryl ester was measured in the presence of either 0.5 μmol [^{3}H]cholesterol or [^{14}C]sitosterol alone or in the presence of 0.5 μmol of both sterols with first one and then the other radiolabeled.

ACAT activity was assayed as previously described (19) (but scaled up tenfold) in microsomes (1.5 mg protein) prepared from a 10% homogenate of mucosa scraped from the proximal small intestine and preincubated for 30 min at 37°C with either 84 nmol [^{3}H]cholesterol or [4-^{14}C]sitosterol, or mixtures containing 84 nmol of both sterols with first one and then the other radiolabeled. The sterols were added in 5 μl dioxane-propylene glycol 2:1 (v/v) (19). Under the preincubation conditions described, each sterol alone and in equimolar concentrations (over a range of 10–175 nmol total sterol added) bound to the same extent and linearly to microsomal protein (results not shown). For example, when 175 nmol of cholesterol, sitosterol, or 87.5 nmol of each was preincubated with microsomes, 154 ± 8 nmol of total sterol was bound.

In both enzyme assays, aliquots were removed from the digest with time and added to Folch solvent (24) containing [^{3}H]- or [^{14}C]cholesteryl oleate (20,000 dpm) as an internal standard to correct for procedural losses. Free and esterified sterol were separated by thin-layer chromatography as previously described (19).

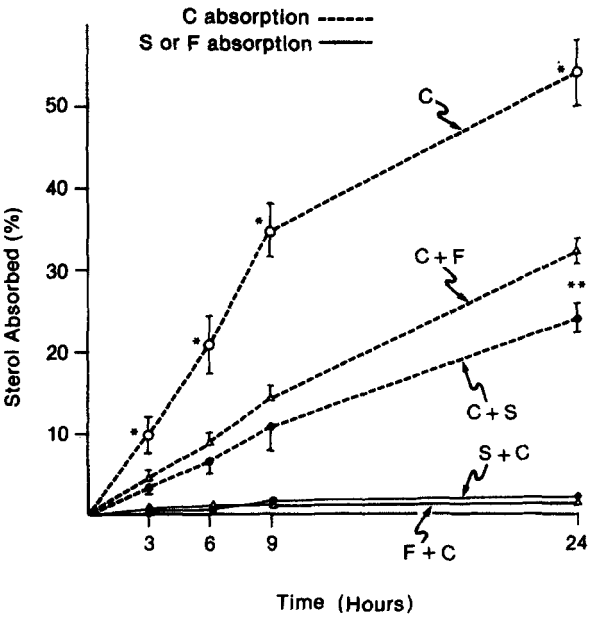

Fig. 1. Inhibition of exogenous cholesterol absorption by sitosterol and fucosterol. Lymph fistula rats were administered intragastrically an emulsion containing 25 mg [^{3}H]cholesterol (C) alone or plus 25 mg of either sitosterol (S) or fucosterol (F). Lymph was collected with time for 24 hr and analyzed for sterol radioactivity (C) by liquid scintillation counting or sterol mass (S and F) by GLC. Each data point is the mean ± SE for n = 6; *C group significantly different from C + S and C + F groups, $P < 0.05$; **C + S group significantly different from C + F group, $P < 0.05$.

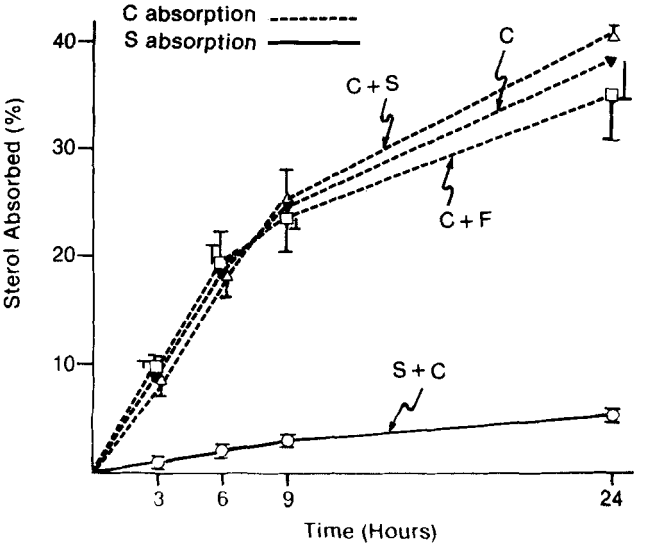

Fig. 2. Effect of micellar sitosterol and fucosterol on micellar cholesterol absorption. Lymph and bile duct-cannulated rats were intraduodenally infused for 24 hr with "artificial" bile (3.4 ml/hr). Then 2 ml of micellar sterol(s), in the concentrations given below, was infused. Lymph was collected with time for 24 hr and analyzed for sterol radioactivity by liquid scintillation counting. Each data point is the mean for n = 4–6; C, 50 μM [^{3}H]cholesterol; C + S, 50 μM [^{3}H]cholesterol + 50 μM [^{14}C]sitosterol; C + F, 50 μM [^{3}H]cholesterol + 50 μM fucosterol.

Sterol analyses

Sterols were estimated by liquid scintillation counting of radioactivity (19) or by GLC determination of mass (5).

RESULTS

Inhibition of exogenous cholesterol absorption by sitosterol and fucosterol

The lymphatic absorption of cholesterol alone and in the presence of plant sterols was followed for 24 hr after administration in an intragastric meal. As shown in **Fig. 1**, the absorption of exogenous cholesterol was significantly ($P < 0.05$) decreased by each plant sterol at all time points. Sitosterol inhibited cholesterol absorption by 57% at 24 hr compared to 41% inhibition with fucosterol. This difference was significant ($P < 0.05$). The absorption of sitosterol and fucosterol from the binary mixtures was less than 2% in 24 hr. The average lymph flow was constant at each time in the three groups with a total volume (ml) in 24 hr of 185 ± 13, 163 ± 17, and 151 ± 19, respectively (data not shown).

Micellar cholesterol absorption in the presence of micellar sitosterol or fucosterol

In bile fistula rats, receiving "artificial" bile (no sterols), the lymphatic absorption of cholesterol alone and in the presence of plant sterols was followed for 24 hr after intraduodenal administration of the micellar-solubilized sterols.

The results shown in **Fig. 2** indicate that neither micellar sitosterol nor fucosterol inhibited the lymphatic absorption of micellar cholesterol. In addition, although in micellar solution, sitosterol was poorly absorbed from the binary mixture with about 5% of the labeled dose appearing in the lymph in 24 hr.

In a second study, the "artificial" bile infusate contained 150 μM cholesterol (~5 mg sterol delivered overnight) or 150 μM of both cholesterol and sitosterol (~10 mg sterols delivered) to increase the sterol challenge. As in the first study, micellar sitosterol did not inhibit the absorption of micellar cholesterol (results not shown).

Micellar solubility of cholesterol, sitosterol, and fucosterol alone and in binary combinations in vitro

A set of "solubility" curves for individual and binary (equimolar) mixtures of sterols solubilized in micelles containing 6.6 mM sodium taurocholate, 0.6 mM egg yolk phospholipids, 1 mM oleic acid, and 0.5 mM monoolein is shown in **Fig. 3**. Sterol "solubility" was assessed qualitatively by absorbance measurement at a wavelength of 430 nm. The initial preparations of individual sterols were clear with a baseline absorbance of 0.1. However, as the concentration of each sterol was increased, turbidity appeared, although at different concentrations for each, and the sterols precipitated upon standing. Specifically, the micellar solutions containing either cholesterol, fucosterol, or sitosterol were clear at sterol concentrations up to 0.8 mM, 0.6 mM, and 0.27 mM, respectively. This suggests that cholesterol is more soluble than fucosterol which is more soluble than sitosterol. In binary mixtures prepared with cholesterol and sitosterol (1:1), a precipitate appeared at a total sterol concentration of 0.4 mM and absorbance rose sharply after this. By contrast, equimolar combinations of fucosterol and either cholesterol or sitosterol up to 2 mM total sterol displayed no turbidity even after standing overnight.

Since absorbance measurements are indicators of micellar size and provide only a "qualitative" estimate of sterol solubility, the micellar solubility of cholesterol and how it is affected by plant sterols was assessed quantitatively by the standard ultracentrifugation method. Micellar solutions prepared with either 1 mM or 2 mM [^{3}H]cholesterol alone or with 1 mM [^{3}H]cholesterol plus either 1 mM [^{14}C]sitosterol or 1 mM fucosterol were incubated at 37°C overnight. After centrifugation the micellar concentration of each sterol was measured in the clear supernatants (micellar phase). As shown in **Table 1**, about 95% of the original cholesterol was in micellar solution after centrifugation at 100,000 *g*. Although some degree of turbidity was visualized, no precipitate was collected at either concentration. With the binary mixtures of cholesterol and either sitosterol or fucosterol, 1 mM each, the respective 100,000 *g* supernatants contained 0.33 mM cholesterol and 0.22 mM

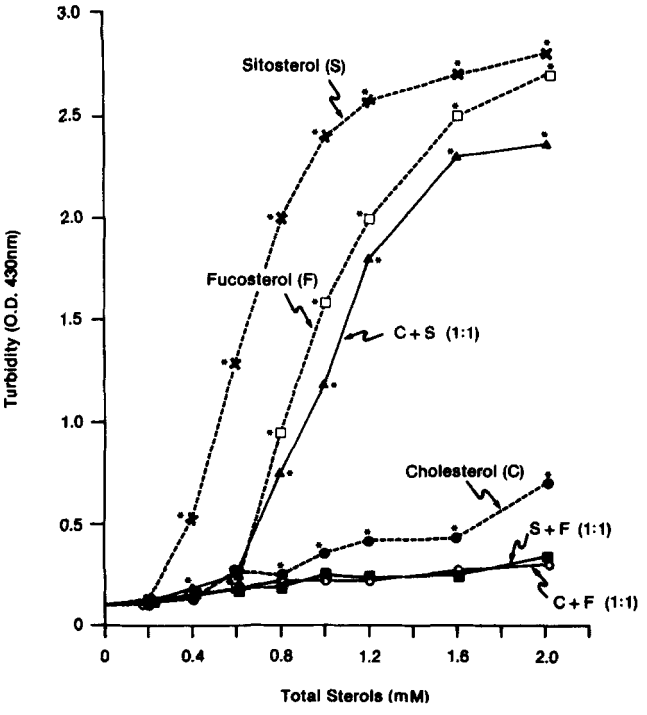

Fig. 3. Micellar solubility of sterols alone and in binary mixtures. Cholesterol (C), sitosterol (S), and fucosterol (F) alone and C + S, C + F, and S + F in equimolar binary mixtures containing 0.2 to 2.0 mM total sterol were sonicated at 37°C in 2.0 ml of 15 mM sodium phosphate buffer, pH 7.4, containing 6.6 mM sodium taurocholate, 0.6 mM egg yolk phospholipids, 1 mM oleic acid, 0.5 mM monoolein, and 132 mM sodium chloride. Turbidity was measured spectrophotometrically at 430 nm. Suspending solution (no sterols) absorbance = 0.101. The results are the mean for n = 2. Variation in absorbance ≤1.0%. *Turbidity or precipitates were observed.

sitosterol for a total sterol concentration of 0.55 mM, and 0.49 mM cholesterol and 0.35 mM fucosterol for a total sterol concentration of 0.84 mM. These represent 66% and 50% less micellar cholesterol, respectively, than that in bile salt micelles prepared with 1 mM cholesterol alone. However, with the cholesterol–fucosterol mixtures, the amount of each sterol in the clear supernatant was dependent upon the centrifugal force, with 1.7 mM total sterol determined in the 10,000 *g* supernatant and half this amount in the 100,000 *g* supernatant. This anomalous behavior of the cholesterol–fucosterol mixture was not observed with cholesterol alone and to a very limited extent with the cholesterol–sitosterol mixture. The above results were unchanged relatively (data not shown) when the sterols were solubilized in bile salt micelles containing either phospholipids or monoolein and oleic acid.

Micellar solubility of cholesterol alone and in binary combination with sitosterol and fucosterol in vivo

As shown in **Table 2**, the percentage of [^{3}H]cholesterol in the micellar phase prepared from the lumenal content collected from the small intestine was reduced 33% by

TABLE 1. Micellar solubility of cholesterol alone and in binary mixtures with plant sterols

Group	Sterol	mM Sterol in Supernatant at 10,000 *g*	25,000 *g*	50,000 *g*	100,000 *g*
Cholesterol (1 mM)	C	0.99	0.98	0.96	0.94
Cholesterol (2 mM)	C	1.9	1.8	1.8	1.8
Cholesterol (1 mM) +	C	0.42	0.40	0.37	0.33
Sitosterol (1 mM)	S	0.29	0.27	0.25	0.22
Sum		0.71	0.67	0.62	0.55
Cholesterol (1 mM) +	C	0.93	0.82	0.67	0.49
Fucosterol (1 mM)	F	0.75	0.64	0.49	0.35
Sum		1.68	1.46	1.16	0.84

[^{3}H]Cholesterol alone (1 and 2 mM) or in binary mixtures containing [^{3}H]cholesterol (1 mM) plus either [^{14}C]sitosterol (1 mM) or fucosterol (1 mM) were sonicated for 2 min at 37°C in 12.0 ml of 15 mM sodium phosphate buffer, pH 7.4, containing 6.6 mM sodium taurocholate, 0.6 mM egg yolk phospholipids, 1 mM oleic acid, 0.5 mM monoolein, and 132 mM sodium chloride. The solutions were divided into 3-ml aliquots, incubated at 37°C overnight, and centrifuged at the indicated force for 60 min at 37°C. The supernatants were collected, and cholesterol (C) and sitosterol (S) concentrations were determined by liquid scintillation counting and fucosterol (F) concentration was determined by gas-liquid chromatography. The results are the mean for n = 2. Variation in mM sterol values ≤ 2%.

sitosterol and 25% by fucosterol. On the basis of the cholesterol content per ml of micellar phase, the concentration of cholesterol alone was estimated at 1.0 mM. This was reduced to 0.67 mM and 0.75 mM by sitosterol and fucosterol, respectively.

Concentration dependency of micellar sterol binding to brush border membranes

Brush border membranes incubated with increasing (25–325 μM) concentrations of either [^{3}H]cholesterol or [^{14}C]sitosterol in micellar solution bound both sterols. As shown in **Fig. 4**, two- to threefold more cholesterol than sitosterol bound at each concentration. Binding did not reach a maximum at the highest level of cholesterol, although at cholesterol concentrations greater than 100 μM, binding was no longer linear. In contrast, binding reached a maximum as 200 μM sitosterol was approached.

Competition of micellar sitosterol and fucosterol with micellar cholesterol for binding to brush border membranes

The brush border binding of cholesterol and sitosterol from micellar binary mixture was independent at low total sterol concentrations (≤ 75 μM, 1C:2S) as shown in **Fig. 5**, panel A. In contrast, in a second study (Fig. 5, panel B) that included a fucosterol group and where the total sterol concentration in the micellar binary mixtures was increased (200 μM, 1C:1S or 1F), the brush border binding of cholesterol was inhibited an average of 38% and 35% by sitosterol and fucosterol, respectively.

Competition of micellar sitosterol and fucosterol with micellar cholesterol for binding to mucin

The mucin binding of cholesterol and plant sterols from micellar binary mixtures containing cholesterol (60 μM) and either sitosterol (60 or 120 μM) or fucosterol (60 μM) was independent as shown in **Fig. 6**.

Competition of sitosterol with cholesterol for esterification

Cholesterol esterase: Cytosolic cholesterol esterase in the presence of saturating levels of sterol (0.5 μmol) esterified [^{3}H]cholesterol at more than twice the rate of [4-^{14}C]sitos-

TABLE 2. Effect of sitosterol and fucosterol on the micellar solubility of cholesterol in lumenal content

Group	Micellar Cholesterol: Percentage of Dose Fed	μg/2 ml
Cholesterol	3.22 ± 0.29	805 ± 73
Cholesterol + sitosterol	2.13 ± 0.46	532 ± 115
Cholesterol + fucosterol	2.41 ± 0.18	602 ± 45

Rats that were meal-fed a semi-purified diet (10 g) containing 0.25% cholesterol (C) alone or plus 0.25% sitosterol (C + S) or fucosterol (C + F) for 7 days were killed 2 hr after they consumed the final meal to which 5 μCi of [^{3}H]cholesterol was added. Lumenal content was collected (2–3 ml), heated to inactive lipolytic enzymes, and centrifuged at 100,000 *g* for 60 min. Micellar [^{3}H]cholesterol in the clear supernatant was determined by liquid scintillation counting. Results are the mean ± SE, n = 5.

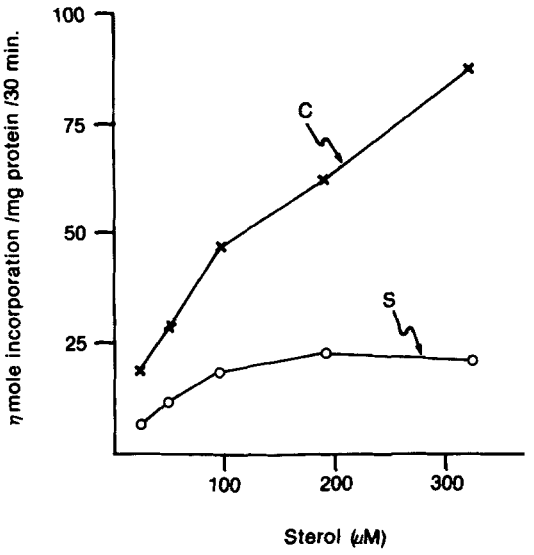

Fig. 4. Concentration dependency of sterol binding to brush border membranes. Brush border membranes (2.0 mg protein) were incubated in a volume of 5.0 ml at 37°C for 30 min with increasing concentrations (25 to 325 μM) of [^{3}H]cholesterol or [^{14}C]sitosterol in micellar solution containing 6.6 mM sodium taurocholate and 0.6 mM egg yolk phospholipids. Sterol bound was determined by liquid scintilation counting. Sterol binding at zero time (< 10 nmol/mg protein) was subtracted from that at 30 min. The results represent one study.

terol (25 vs. 10 nmol ester formed/per mg protein). Further, the rate of [^{3}H]cholesterol esterification was not altered by the addition of an equimolar amount (0.5 μmol) of sitosterol (results not shown).

ACAT. Microsomal ACAT catalyzed [^{14}C]cholesterol (84 nmol) esterification at a rate of 560 pmol/min per mg protein in the absence or presence of an equimolar amount of sitosterol. [^{14}C]Sitosterol (84 nmol) was not esterified in the absence or presence of added exogenous cholesterol. These results (not shown) are in agreement with those reported by others (13).

DISCUSSION

The present study supports numerous previous reports that plant sterols inhibit cholesterol absorption (1–3) and extends these studies to include fucosterol and to assess the mechanism of the inhibitory effects. The anticipated inhibition of absorption occurred only when sitosterol or fucosterol was given with cholesterol (25 mg each sterol) as an intragastric emulsion (Fig. 1). Comparatively, sitosterol was a significantly more effective inhibitor of exogenous cholesterol absorption than fucosterol (57 vs. 41%). However, neither plant sterol (50 μM) inhibited cholesterol absorption when co-administered with cholesterol (50 μM) in micellar solution. This was true whether the micellar sterol meal was given to bile-fistula animals receiving an overnight continuous infusion of sterol-free (Fig. 2) or sterol-loaded "artificial" bile (results not shown).

Differences in feeding protocols per se, i.e., intragastric emulsions of sterols to animals with normal bile flow versus intraduodenal micellar solutions of sterols to bile-fistula animals, were unlikely to have accounted for the difference in effect on cholesterol absorption. Predictably, emulsified sterol (25 mg cholesterol + 25 mg sitosterol or fucosterol) would be solubilized physiologically in the small intestine

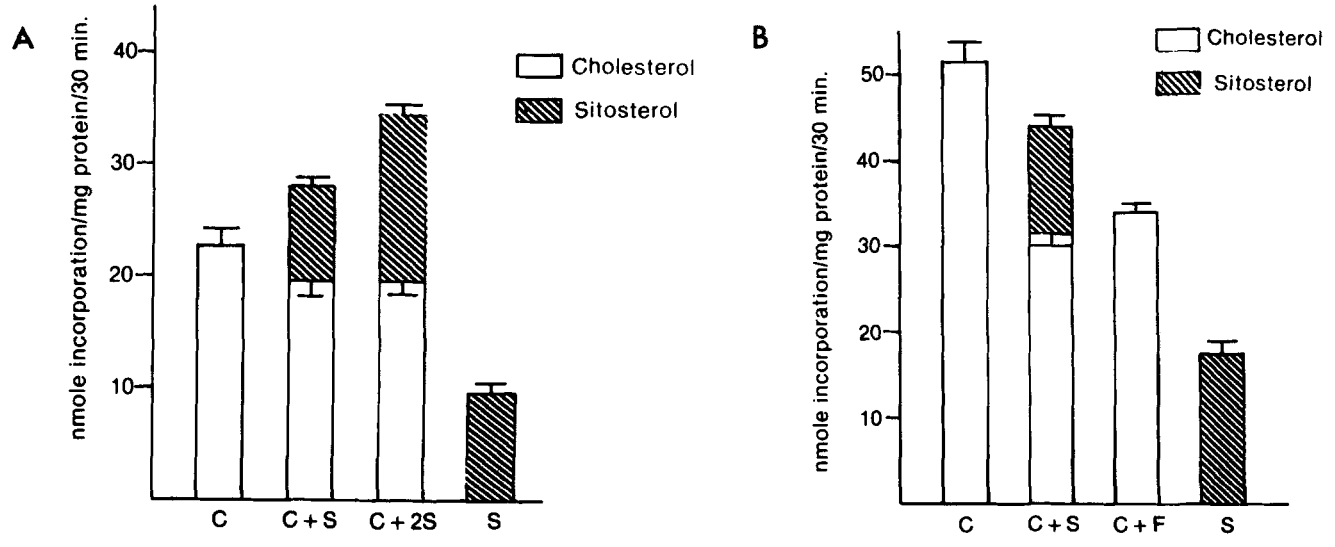

Fig. 5. Competition of micellar sitosterol and fucosterol with micellar cholesterol for binding to brush border membranes. Brush border membranes (2.0 mg protein) were incubated in a volume of 5.0 ml for 30 min at 37°C with micellar solutions containing 6.6 mM sodium taurocholate, 0.6 mM egg yolk phospholipids, and sterols singly or in binary mixtures as indicated. Panel A: C, 25 μM [^{3}H]cholesterol; C + S, 25 μM [^{3}H]cholesterol + 25 μM [^{14}C]sitosterol; C + 2S, 25 μM [^{3}H]cholesterol + 50 μM [^{14}C]sitosterol; and S, 25 μM sitosterol. Panel B: C, 100 μM [^{3}H]cholesterol; C + S, 100μM [^{3}H]cholesterol + 100 μM [^{14}C]sitosterol; C + F, 100 μM [^{3}H]cholesterol + 100 μM fucosterol; and S, 100 μM [^{14}C]sitosterol. Sterols bound were determined by liquid scintillation counting. Results are the mean ± SE for n = 3 in both studies.

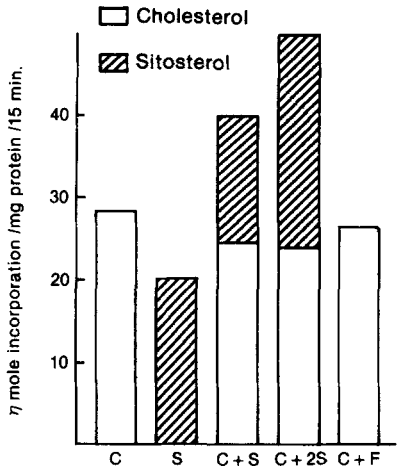

Fig. 6. Competition of micellar sitosterol and fucosterol with micellar cholesterol for binding to mucin. Mucin (10 mg protein) was incubated in a volume of 4.0 ml for 15 min at 37°C with micellar solutions containing 6.6 mM sodium taurocholate, 0.6 mM egg yolk phospholipids, and sterols singly or in mixtures as indicated: C, 60 μM [^{3}H]cholesterol; C + S, 60 μM [^{3}H]cholesterol + 60 μM [^{14}C]sitosterol; C + 2S, 60 μM [^{3}H]cholesterol + 120 μM [^{14}C]sitosterol; C + F, 60 μM [^{3}H]cholesterol + 60 μM fucosterol; and S, 60 μM [^{14}]sitosterol. Sterols bound were determined by liquid scintillation counting. Results are corrected for sterol binding at zero time (<20 nmol/mg protein). Results are the mean for n = 2. Variation in nmol incorporation/mg protein ≤ 5.

at a concentration of approximately 1 mM total sterol in micelles formed from the biliary phospholipids and bile salts. Dilution with endogenous cholesterol (well less than 1.0 mg contributed during absorption since bile contains about 0.15 mg/ml per hr in a 200-g rat) would be insignificant. By comparison, the small intestine of bile-fistula rats which had continuously received "artificial" bile supplemented with micellar sterols (about 5 mg cholesterol + 5 mg sitosterol or fucosterol delivered overnight) was presented with 0.3 mM total sterol. Thus, the major difference between the two feeding protocols was that, in the former, sterol selection for micellar solubilization was by physiological choice and in the latter it was not (both sterols were in micellar solution prior to infusion). As the result of this selection process, the data suggest that plant sterols were selected preferentially and limited the micellar solubilization of cholesterol. This is borne out by the in vivo study in which the effect of sitosterol on the lumenal micellar concentration of cholesterol was assessed (Table 2). Mechanistically, the simplest explanation of the limitation would be sterol dilution, but this does not explain the significant difference in inhibitory effectiveness between sitosterol and fucosterol.

In vitro studies (Fig. 3, Table 1) were designed to further characterize the in vivo inhibitory effects of plant sterols on cholesterol absorption. For the individual sterols, the turbidity data pointed to the formation of larger, less stable micelles with sitosterol followed by fucosterol and cholesterol, in that order. Equimolar binary mixtures of sterols ranging in total sterol concentration from 0.2–2 mM behaved anomalously by these same measurements. Specifically in the cholesterol + sitosterol combination, turbidity appeared and sterol precipitated at a lower total sterol concentration than with cholesterol alone (0.4 mM total sterol vs. 0.8 mM cholesterol). However, equimolar binary combinations of sitosterol + fucosterol and cholesterol + fucosterol remained clear at total sterol concentrations of 2 mM. Curiously, fucosterol, which differs from sitosterol by one double bond in the side chain, appeared much less effective than sitosterol in restricting the micellar solubility of cholesterol. When the quantitative effect of these two plant sterols on the micellar solubility of cholesterol was assessed by the conventional ultracentrifugation approach (Table 1), the 100,000 *g* supernatants from micellar solutions prepared with 1.0 mM each of cholesterol and sitosterol contained 0.33 mM cholesterol while those prepared with 1 mM each of cholesterol and fucosterol contained 0.49 mM cholesterol. It was noted in this study, as in the spectrophotometric study, that the cholesterol + fucosterol combination gave rise to clear solutions, but the micellar concentration of each sterol decreased sharply with increased centrifugal force. We have not attempted to characterize this effect, which may be explained by the generation of either unstable micelles or, possibly, a mixed population of micelles of variable density. The in vitro effects of sitosterol and fucosterol on the micellar content of cholesterol hold up in every particular in vivo (Table 2). The magnitude of the in vivo effect with each plant sterol was less, but normal bile contains chenodeoxycholate in which cholesterol can be solubilized and predictably (10) not displaced by plant sterols. Overall, since solubilization of cholesterol in bile salt micelles is a requirement for its efficient absorption (25, 26), the displacement of cholesterol from micelles by plant sterols in general and the greater displacement with sitosterol relative to fucosterol measured in vitro and in vivo are consistent with the observed inhibitory effects on cholesterol absorption in vivo. Moreover, these data are consistent with a recent report (10) in which the importance of the side chain substitution at carbon 24 to sterol solubilization in taurocholate micelles in vitro was demonstrated. Sitosterol, which is less hydrophilic than cholesterol, has a lower capacity but higher affinity for binding to cholic acid micelles and was predicted to displace cholesterol with a favorable free energy change. Our recent in vitro studies (9) revealed that the rate of sitosterol movement from the micellar phase to triolein was 3.5-fold less than cholesterol. This is consistent with the suggested differences in their micellar affinity. By the same reasoning, it is predictable that fucosterol, to a lesser extent, would displace cholesterol from the micelle. Related to this, in feeding studies, sitosterol is reported to produce a greater hypocholesterolemic effect than fucosterol when each is included at 1.0% in the diet of rats (27).

Although displacement of cholesterol from micelles may represent the major mechanism of plant sterol-mediated inhibition of cholesterol absorption, other mechanisms have been suggested. These include plant sterol inhibition of cholesterol binding to brush border membranes and/or cell surface mucins and inhibition of intracellular cholesterol esterification.

With respect to the cell surface, early studies (28) suggested the presence of specific binding sites for cholesterol. If such sites exist, micellar sitosterol did not compete with micellar cholesterol for these in rat jejunal loops in situ (8). In addition, during absorption no accumulation of sitosterol occurs in and/or on the intestine (e.g., refs. 9, 12). Consistent with this, the present in vitro study confirmed independence of binding of the two sterols to isolated brush border at low micellar sterol concentrations (Fig. 5A, 25 and 50 μM) but at higher levels of cholesterol (100 μM) in combination with either sitosterol or fucosterol (100 μM), less (−38%) cholesterol bound (Fig. 5B). At the higher total sterol concentration, this result is expected since sterol binding to the brush border is approaching saturation (Fig. 4).

To the extent that these in vitro and the in vivo results are comparable, cholesterol absorption was not inhibited when intestine was exposed to a continuous overnight challenge with 150 μM each of cholesterol and sitosterol, a concentration of total sterol that was 100 μM greater than that which produced competition in vitro. This may be explained by the greater accessibility of sterols to isolated brush border which has a minimal unstirred water and mucin layer and to differences in a closed in vitro versus an open in vivo system. However, under physiological conditions, the results suggest that the monomolecular concentration of sitosterol attained at the cell surface was below that required for competition for binding.

Mucins which blanket the cell surface bind cholesterol (29) and may represent a barrier through which cholesterol must pass in the manner suggested for the unstirred water layer (30). If so, plant sterols might be competitive for binding. When sterols, singly and in mixtures, were incubated with mucin, a small sterol binding differential of less than 2:1 was observed in favor of cholesterol, and sterol binding from binary mixtures was independent at low sterol concentrations (60 μM cholesterol + 60 or 120 μM sitosterol or fucosterol). However, as mentioned above, concentrations of plant sterols attained in the aqueous phase under physiological conditions are likely below that required for binding competition with cholesterol.

Intracellular esterification of exogenous cholesterol is substantial (70–90%) and considered essential to efficient absorption (25). The in vitro effect of sitosterol on cholesterol esterification by the two cholesterol esterification enzymes in the mucosa, cholesterol esterase and ACAT, reveals that sitosterol is not competitive with cholesterol for either enzyme. These ACAT data are consistent with those reported in a more critical study (13). In the esterase assay, sitosterol, as the sole substrate, was esterified at about half the rate of cholesterol as reported earlier (11) but failed to compete with cholesterol when present at equimolar concentrations. Since the esterifying enzymes either do not or poorly use sitosterol as a substrate and because sitosterol uptake into the cell is extremely limited, these enzymes cannot account for plant sterol inhibition of cholesterol absorption.

The basis for the inhibitory action of plant sterols on cholesterol absorption has been assessed. Plant sterol inhibition of cholesterol absorption appears unrelated to competition with cholesterol for binding to the cell surface membrane or its mucin coat. Neither are the plant sterols competitive inhibitors of the cholesterol esterifying enzymes. Rather, the data demonstrate that plant sterols restrict the solubility of cholesterol in micelles prepared with taurocholate, the principal bile salt in rat and human bile (31). Sitosterol relative to fucosterol restricts the micellar solubility of cholesterol more severely, which accounts for its greater inhibitory effect on cholesterol absorption.

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