β-Sitosterol: esterification by intestinal acylcoenzyme A:cholesterol acyltransferase (ACAT) and its effect on cholesterol esterification

F. Jeffrey Field and Satya N. Mathur

Department of Internal Medicine, University of Iowa School of Medicine, Iowa City, IA 52242

Abstract Rabbits were fed either 10% coconut oil, 10% coconut oil and 1% β-sitosterol, 10% coconut oil and 1% cholesterol, or 10% coconut oil and 1% β-sitosterol plus 1% cholesterol for 4 weeks. Microsomal membranes from intestines of animals fed the 1% β-sitosterol diet had 48% less cholesterol and were enriched twofold in β-sitosterol compared to membranes from animals fed the coconut oil diet alone. Acylcoenzyme A:cholesterol acyltransferase (ACAT) activity in jejunum and ileum was decreased significantly in animals fed the plant sterol alone. In membranes from animals fed 1% β-sitosterol and 1% cholesterol, β-sitosterol content increased 50% whereas cholesterol was modestly decreased compared to their controls fed only cholesterol. Intestinal ACAT was unchanged in the animals fed both sterols when compared to their controls. β-Sitosterol esterification was determined by incubating intestinal microsomal membranes with either [^{14}C]β-sitosterol–albumin emulsion or [^{14}C]β-sitosterol:dipalmitoyl phosphatidylcholine (DPPC) liposomes to radiolabel the endogenous sterol pool. Oleoyl-CoA was then added. The CoA-dependent esterification rate of β-sitosterol was very slow compared to that of cholesterol using both techniques. An increased amount of endogenous microsomal β-sitosterol, which occurs in animals fed 1% β-sitosterol, did not interfere with the stimulation of ACAT activity secondary to cholesterol enrichment of the membranes. Enriching microsomal membranes three- to fivefold with β-sitosterol did not affect ACAT activity. Freshly isolated intestinal cells were incubated for 1 hour with [^{3}H]oleic acid and β-sitosterol:DPPC or 25-hydroxycholesterol:DPPC. Incorporation of oleic acid into cholesteryl esters did not change in the presence of β-sitosterol but increased fourfold after the addition of 25-hydroxycholesterol. We conclude that the CoA-dependent esterification rate of cholesterol is at least 60 times greater than that of β-sitosterol. Membrane β-sitosterol does not interfere with nor compete with cholesterol esterification. Inadequate esterification of this plant sterol may play a role in the poor absorption of β-sitosterol by the gut.—**Field, F. J., and S. N. Mathur.** β-Sitosterol: esterification by intestinal acylcoenzyme A:cholesterol acyltransferase (ACAT) and its effect on cholesterol esterification. *J. Lipid Res.* 1983. **24:** 409–417.

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Phytosterols have interested investigators of cholesterol absorption for many years. The mammalian gut handles phytosterols very differently from cholesterol, despite their structural resemblances. β-Sitosterol, the main plant sterol in the diet, has been studied most extensively. Only 5% or less of ingested β-sitosterol is absorbed in man (1–5), about one-tenth the amount of cholesterol absorption (4, 6–10). In other animals, the percent of ingested sitosterol that is absorbed varies (11–13), but the substantial difference between the proportion of ingested cholesterol that is absorbed compared to that of sitosterol still remains. The intestinal mucosa can thus discriminate between sterols that differ only by an ethyl group at the C-24 position. The reasons for this are not clear.

Swell et al. (11), postulated that β-sitosterol was not esterified in the intestinal wall of lymph-fistula rats. Most of the sitosterol found within the intestinal wall in these experiments was in the unesterified form. Very small amounts were detected in the lymph. Sylven and Borgström (12), also working with lymph-fistula rats, saw no evidence for competition between dietary cholesterol and sitosterol during absorption. In contrast to the findings of Swell et al. (11), no accumulation of sitosterol was observed within the intestinal wall. Therefore, Sylven and Borgström suggested that lack of esterification of β-sitosterol was not the reason for differences observed in cholesterol and sitosterol absorption. Kuksis and Huang (14), however, found that virtually all of the plant sterols in chyle of thoracic duct-fistula dogs were not esterified. This was in contrast to cholesterol which was predominantly esterified in chyle. This suggests that phytosterols are not esterified in the gut and yet can be transferred into the lymph. Acetone-dried powder preparations of cholesterol esterase from rat intestine have been shown to esterify plant sterols (15). Bhattacharyya and Connor (16), after describing two sisters with β-sitosterolemia, suggested that esterification of the plant sterol may well be the limiting factor in its absorption. Salen, Ahrens, and Grundy (5) reached the same conclusion while studying β-sitosterol metabolism in man.

Abbreviations: ACAT, acylcoenzyme A:cholesterol acyltransferase; DPPC, dipalmitoyl phosphatidylcholine; BSA, bovine serum albumin; GLC, gas–liquid chromatography.

Thus, the question of the intestinal esterification of β-sitosterol and its place in absorption is far from settled.

The ingestion of β-sitosterol lowers serum cholesterol levels in hypercholesterolemic humans (17–20), but no one knows how it does so. Lees et al. (19), pointed out three ways in which sitosterol ingestion could affect cholesterol absorption. One of these was by interference with cholesterol incorporation into chylomicrons by interfering with cholesterol esterification. The other two ways occur within the intestinal lumen by decreasing cholesterol solubility within the micelle, or competing with cholesterol for uptake by the absorptive cell.

Evidence has been accumulating to suggest that acyl-coenzyme A:cholesterol acyltransferase (ACAT) is the enzyme responsible for catalyzing cholesterol esterification in the enterocyte (21–24). The ability of this enzyme to catalyze the esterification of β-sitosterol, and the degree to which β-sitosterol competes with and/or interferes with cholesterol esterification are matters of importance in understanding the metabolism of cholesterol in the gut. For this reason, we looked at the effects of dietary β-sitosterol on intestinal microsomal ACAT activity. Studies were designed in vitro to see if β-sitosterol competes with or interferes with cholesterol esterification by ACAT.

MATERIALS AND METHODS

[1-^{14}C]Oleoyl coenzyme A, [4-^{14}C]cholesterol, [9,10-^{3}H]oleic acid, and [1,2-^{3}H(N)]cholesterol were purchased from New England Nuclear. β-[4-^{14}C]Sitosterol came from Amersham. Oleoyl coenzyme A, β-sitosterol, dipalmitoyl phosphatidylcholine, oleic acid, and cholesterol were obtained from Sigma, and 25-hydroxycholesterol came from Steraloid. All other reagents were reagent grade.

Animals and diets

Male New Zealand white rabbits, weighing 0.7 kg ± 0.2 kg, were housed in a windowless room that was illuminated from 0700 to 1900 hr. Two groups of five animals were each fed one of two diets: Purina rabbit chow with 10% coconut oil and 1% β-sitosterol, or rabbit chow with 10% coconut oil, 1% cholesterol, and 1% β-sitosterol. The control diets for these diets (rabbit chow with 10% coconut oil, and rabbit chow with 10% coconut oil and 1% cholesterol) have been described previously (23). Animals were maintained on these diets for 4 weeks with free access to water. At weekly intervals, the animals were weighed and bled for lipoprotein cholesterol analyses. The animals in each group gained weight at similar rates over a 4-week feeding period.

Preparation of microsomes

All rabbits were killed between 0800 and 1000 hr by cervical dislocation. In each animal, the entire small intestine was removed. For the dietary experiments, the small intestine was divided into six equally-long segments from the pylorus to the terminal ileum. Only the jejunum and proximal ileum (segment 3) were used in the experiments in which ACAT activity was determined using radiolabeled sterol as substrate or in the preparation of microsomes for liposome sterol enrichment. The intestinal segments were washed thoroughly with ice-cold saline and opened along the mesenteric border. The intestinal mucosa was gently scraped from the muscularis mucosae and homogenized in a buffered sucrose solution (0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4, 0.03 M EDTA, pH 7.4) in a glass Dounce homogenizer with ten passes with a loose-fitting pestle. The whole homogenate was spun for 20 min at 10,000 *g* at 4°C. The resulting supernatant was then centrifuged at 105,000 *g* for 1 hr at 4°C. The resulting microsomal pellet was washed once and resuspended in cold buffered sucrose solution.

Preparation of the [^{14}C]cholesterol- or [^{14}C]β-sitosterol-BSA emulsion

The method used was that of Stokke and Norum (25). Twenty μCi of either [^{14}C]cholesterol (60 mCi/mmol) or [^{14}C]β-sitosterol (58 mCi/mmol) was dried under nitrogen. Acetone, 0.5 ml, was then added to dissolve the dried sterol. This solution was slowly added with continuous stirring to 5 ml of an albumin solution (250 mg of fatty acid-poor bovine serum albumin in 5 ml of buffered sucrose solution). This mixture was placed under a stream of nitrogen until the odor of acetone was no longer detectable. The [^{14}C]cholesterol- or [^{14}C]β-sitosterol-BSA emulsion was kept at 4°C for no longer than 2 weeks.

ACAT assay

Radiolabeled sterol as substrate. The method was that of Haugen and Norum (24). The total volume of each assay was 0.5 ml consisting of 0.05 to 0.1 mg of microsomal protein in the buffered sucrose solution. Approximately 75,000 dpm of the BSA emulsion, containing either [^{14}C]cholesterol or [^{14}C]β-sitosterol, was added to the microsomal membranes and incubated at 37°C for 2 hr. After 2 hr, the reaction was started with 21 nmol of oleoyl-CoA. The reaction was stopped at 5 min with 7 ml of chloroform–methanol 2:1 (v/v). Approximately 8,000–10,000 cpm of [^{3}H]cholesteryl oleate was added as an internal standard to calculate recoveries. One ml of 0.04 N HCl was added. The samples

were vortexed and allowed to stand at 4°C overnight to separate the phases completely. The aqueous phase was then removed and the chloroform phase was dried under N_2. The residue was dissolved in 0.125 ml of chloroform and spotted on thin-layer chromatography plates layered with Silica Gel 60 H (E. Merck). The chromatograms were developed with a solvent system containing hexane–ethyl acetate 9:1 (v/v). Lipids were visualized by exposure of the chromatograms to I_2 vapor, and the area corresponding to cholesteryl ester or β-sitosteryl ester was scraped directly into liquid scintillation vials containing 10 ml of a Liquifluor (New England Nuclear)–toluene scintillation solution. β-Sitosteryl oleate was prepared by the pyridine method (26). This sterol ester migrated with cholesteryl oleate in the TLC system. Radioactivity was measured in a Beckman model LS8100-liquid scintillation counter. Quenching was monitored by an external standard. Efficiency for ^{14}C was 75% and did not vary between assays. Recoveries ranged from 75 to 90%. Spillover of ^{3}H counts into the ^{14}C channel were calculated for each assay and subtracted from the total ^{14}C counts. ACAT activity was expressed as pmol of the sterol ester formed min^{-1} mg^{-1} of microsomal protein. Specific activity of the substrate differed slightly between assays depending upon the amount of sterol present in the microsomal membranes. The specific activity of cholesterol and β-sitosterol was approximately 14 dpm/pmol and 100 dpm/pmol, respectively.

In the experiment in which liposomes were used to deliver [^{14}C]β-sitosterol to the microsomes instead of the BSA emulsion, the method is fully described under 'Enrichment of microsomal membranes with cholesterol or β-sitosterol'. [^{14}C]β-Sitosterol was incorporated into the phospholipid liposome and incubated with intestinal microsomal membranes. The membranes were recovered after removing the liposomes, and ACAT was determined as outlined in the next section. Oleoyl-CoA, 8.6 nmol, was used as substrate in place of radiolabeled oleoyl-CoA.

Radiolabeled oleoyl-CoA as substrate. The total volume of each sample was 0.2 ml consisting of 0.05–0.100 mg of microsomal protein, 0.5 mg of fatty acid-poor bovine serum albumin, 8.6 nmol of [^{14}C]oleoyl-CoA with specific activity 12,364 dpm/nmol. Buffered sucrose solution was added to adjust the final volume to 0.2 ml. The assay mixture was incubated at 37°C for 5 min before the addition of the substrate [^{14}C]oleoyl-CoA to start the reaction. At 2 min the reaction was stopped by the addition of 5 ml of chloroform–methanol 2:1 (v/v). [^{3}H]Cholesteryl oleate was added as an internal standard and the samples were processed as stated above.

Time-dependence, protein-dependence, and optimal substrate concentrations were determined for each assay technique before this study.

Preparation of liposomes

A chloroform solution containing dipalmitoyl phosphatidylcholine (DPPC), cholesterol–dipalmitoyl phosphatidylcholine 2:1 (mol/mol) or β-sitosterol–dipalmitoyl phosphatidylcholine 2:1 (mol/mol) was evaporated under N_2 to dryness. A buffered sucrose solution containing 0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4, was added to give 1.5 mM of phospholipid. This mixture was dispersed for 2 min with a Vortex mixer and sonicated under N_2 at 10 watts output for 15 min at 50°C using a Bronson sonifier. The liposome solution was then centrifuged at 12,000 *g* for 30 min to remove any metal particles released by the sonifier.

Enrichment of microsomal membranes with cholesterol or β-sitosterol

Cholesterol-DPPC, β-sitosterol-DPPC, or DPPC alone was incubated with 3.0–5.0 mg of intestinal microsomal membranes at 37°C from 1 to 4 hr in a Dubnoff metabolic shaker. Fusion of intact liposomes with microsomes was minimized by keeping a 1:4 ratio of DPPC:microsomal phospholipid, inclusion of 1 mM EDTA, and 5 mg/ml bovine serum albumin in the incubation mixture. Phospholipid content in the microsomal membranes did not change over the 4-hr incubation, evidence that no net transfer of phospholipids took place. After the designated time of incubation, the membranes were layered over a 20% sucrose solution and centrifuged at 40,000 rpm for 45 min. The supernatant was removed by aspiration and the microsomal pellet was resuspended in Tris buffer and again layered on top of a 20% sucrose solution. After centrifugation at 40,000 rpm for 45 min, the recovered microsomal pellet was resuspended in Tris buffer. This preparation was used to determine sterol content, phospholipid content, and ACAT activity in the membranes.

Chemical analysis

Lipids were extracted from the microsomes using chloroform–methanol 2:1 (v/v). Free cholesterol and β-sitosterol were determined by GLC with cholestane as an internal standard to determine recoveries. The sterols were not derivatized prior to GLC analysis. Good separation was obtained on the column, which is described below. Cholesterol and β-sitosterol (Applied Science) were used as standards. GLC was done on a Hewlett Packard 5840 A Gas Chromatograph with a 3-ft glass column containing 3% OV-1 on 80/100 Supelcoport. Microsomal phospholipid content was estimated

according to the procedure of Raheja et al. (27) using dipalmitoyl phosphatidylcholine as the standard. Protein was determined by the method of Lowry et al. (28) with bovine serum albumin as the standard.

Isolated cell preparation

Approximately 25 cm of mid-gut from animals fed normal rabbit chow was used for cell isolation with a modification of the method described by Weiser (29). The intestinal segment was flushed thoroughly with ice-cold saline. The lumen was then filled with Buffer A (8 mM KH_2PO_4, pH 7.3, containing 1.5 mM KCl, 96 mM NaCl, 27 mM Na citrate) and the segment was clamped at both ends and placed in a beaker filled with normal saline at 37°C. The beaker was oscillated in a Dubnoff metabolic shaker for 20 min after which Buffer A was discarded. The segment was then filled with Buffer B (phosphate-buffered 0.9% NaCl containing 1.5 mM EDTA, 0.5 mM dithiothreitol at pH 7.3) at 37°C. The mucosal cells were collected by draining the intestinal luminal contents into separate plastic centrifuge tubes at 6-min intervals. Cells from exchanges 4 and 5 were used for the experiment. The cells were diluted with Krebs buffer without Ca^{2+} and recovered by centrifugation at 2,000 rpm for 5 min. The cells were washed twice with the Krebs buffer before use.

Measurement of cholesterol esterification in isolated enterocytes

[^{3}H]Oleic acid, purified by TLC, was mixed with 500 nmol of unlabeled oleic acid and 500 nmol of KOH. This mixture was dried completely and then 3 ml of Krebs buffer without calcium, containing 8.5 mg of fatty acid-poor bovine serum albumin, was added with constant stirring.

Isolated intestinal cells were incubated in oxygenated Krebs buffer (without Ca^{2+}) and 50 μM K^+[^{3}H]oleate-BSA solution (sp act 70,000 dpm/nmol). Buffer, DPPC, β-sitosterol-DPPC, or 25-OH cholesterol-DPPC was added, and the incubation was continued for 1 hr. The cell suspensions were collected, diluted with buffer, and kept on ice. The cells were washed twice with buffer and the cell pellet was extracted twice with 2 ml of chloroform–methanol 1:1 (v/v). After centrifugation, 2 ml of chloroform and 1.3 ml of 0.04N HCl were added to the lipid extract. An aliquot of the chloroform phase was taken for determination of β-sitosterol content and the remainder was dried. The residue was dissolved in 125 μl of chloroform and applied to TLC plates coated with silica gel 60 H. The plates were eluted with hexane–diethyl ether–methanol–acetic acid 85:20:1:1 (v/v). The lipids were visualized with iodine vapor and scraped into counting vials containing 10 ml of toluene-Liquifluor (New England Nuclear). In this system, cholesteryl esters migrated with an R_f value of 0.87. R_f values for triglycerides and fatty acid methyl esters were equal at 0.73. The phospholipids remained at the origin.

RESULTS

Microsomal lipid modification and ACAT activity

Table 1 shows the effect of dietary β-sitosterol on intestinal microsomal cholesterol and β-sitosterol content. The values obtained from animals fed coconut oil or coconut oil plus cholesterol are shown for comparison (23). In animals fed a 1% β-sitosterol diet, microsomal membranes contained two times the amount of this sterol and 48% less cholesterol than membranes prepared from animals fed the coconut oil diet alone, (8.4 vs. 4.2 μg β-sitosterol/mg protein and 8.4 vs. 16.2 μg cholesterol/mg protein). In membranes from animals fed 1% β-sitosterol and 1% cholesterol, β-sitosterol content increased 50% (6.6 vs. 4.4 μg/mg protein), whereas cholesterol was modestly decreased (26 μg vs. 31 μg/mg protein) compared to their controls.

The fact that β-sitosterol was incorporated into the microsomal membrane indicates that a substantial amount of this plant sterol enters the mucosa. In animals fed cholesterol and β-sitosterol in equal amounts, both sterols were incorporated into the membranes. Both were present in smaller amounts, however, than if they were given alone without the other. Microsomal fatty acid composition in animals fed coconut oil and β-sitosterol was no different than that in animals fed the diet containing coconut oil alone.

The effects of dietary β-sitosterol on intestinal microsomal ACAT activity as measured with [^{14}C]oleoyl CoA are also shown in Table 1. β-Sitosterol ingestion had a profound effect on microsomal ACAT activity in every segment of small bowel. In those animals fed β-sitosterol without added cholesterol, ACAT activity was significantly decreased in segments two through five. In contrast, in animals fed cholesterol and β-sitosterol in equal amounts, this inhibitory effect on ACAT was abolished. There were no significant changes observed in any segment if compared to microsomes prepared from cholesterol-fed animals, although a trend to higher values was observed.

Microsomes prepared from animals fed coconut oil plus β-sitosterol contained less cholesterol and more β-sitosterol than those from controls. The recognized substrate for ACAT is endogenous microsomal free cholesterol. The observed decrease in enzyme activity may be explained simply by substrate depletion. One cannot exclude, however, that ACAT activity may have been affected directly or indirectly by the increased

TABLE 1. Effect of dietary β-sitosterol and cholesterol on intestinal microsomal ACAT activity and sterol content

Diet	N[a]	Cholesterol[b] / β-Sitosterol	Cholesterol[c]	ACAT activity[d] Intestinal Segments[e] 1	2	3	4	5	6
Coconut oil	5	4.1 ± 1.0	16.2 ± 1.1	62 ± 13	252 ± 61	269 ± 61	305 ± 64	266 ± 64	109 ± 22
Coconut oil + β-sitosterol	5	1.0 ± 0.2	8.4 ± 0.51	37 ± 4	100 ± 19[f]	122 ± 15[f]	119 ± 14	117 ± 29[f]	52 ± 17
Coconut oil + cholesterol	5	7.2 ± 1.2	31.0 ± 1.7	131 ± 22[f]	366 ± 74	488 ± 92	538 ± 79	387 ± 30	118 ± 34
Coconut oil + β-sitosterol + cholesterol	5	3.9 ± 0.8	25.0 ± 1.7	195 ± 17[g]	589 ± 88[h]	578 ± 96[f]	604 ± 57[i]	531 ± 79[f]	145 ± 24

[a] Number of animals.
[b] Sterol ratio, mean ± SE, from segment 3.
[c] μg/mg Microsomal protein, mean ± SE, from segment 3.
[d] Values represent mean ± SE (pmol/mg per min).
[e] Segments 1 and 6 are duodenum and terminal ileum, respectively. Segments 2–5 are equal segments distal to the duodenum.
[f] $P < 0.05$ vs. coconut oil.
[g] $P < 0.001$ vs. coconut oil.
[h] $P < 0.02$ vs. coconut oil.
[i] $P < 0.01$ vs. coconut oil.

amount of phytosterol that accumulated in the membrane. In animals fed both cholesterol and β-sitosterol, intestinal microsomal ACAT was unchanged from microsomes prepared from animals fed cholesterol alone. In this group, however, only a modest increase in β-sitosterol and a modest decrease in cholesterol content was observed in the microsomal membranes. Therefore, the manner in which exogenous β-sitosterol decreases intestinal ACAT activity remains in question. The in vitro studies that follow investigate the esterification of β-sitosterol by intestinal ACAT and the possible competition between the two similar sterols for esterification.

ACAT activity

A modification of the technique of Haugen and Norum (24), was used to label the endogenous cholesterol and β-sitosterol pools within the microsomal membranes (see Methods). No difficulty was encountered in demonstrating cholesterol esterification by ACAT using this method. **Table 2** shows values obtained for ACAT activity in microsomes prepared from five rabbits fed either chow plus coconut oil or chow and coconut oil plus 1% β-sitosterol. The activities obtained for the rate of cholesterol esterification were similar to those obtained using radiolabeled oleoyl-CoA as substrate (23). Difficulty was encountered, however, in demonstrating β-sitosterol esterification with this technique. The average production rate of β-sitosteryl oleate was 5.3 and 1.6 pmol mg^{-1} min^{-1} in microsomes prepared from rabbits fed coconut oil and coconut oil plus 1% β-sitosterol, respectively. However, the range of activities varied from no β-sitosteryl oleate production to 16.8 pmol mg^{-1} min^{-1}. The reaction rate of β-sitosteryl oleate synthesis was linear with time and protein resembling the parameters of cholesteryl oleate production (data not shown).

Another technique was used to label the endogenous β-sitosterol pool. β-Sitosterol that was solubilized in liposomes was exchanged with endogenous membrane β-sitosterol in two preparations of microsomes from rabbits fed normal rabbit chow. [^{14}C]β-Sitosterol 3.8 nmol (sp act 58 mCi/mmol) and 1.9 nmol of dipalmitoyl phosphatidylcholine in liposomes were incubated with each mg of microsomal protein for 4 hr at 37°C. After removal of liposomes, 2.3 nmol of [^{14}C]β-sitosterol was found to be incorporated per mg of microsomal protein. By this procedure, 6% of the microsomal sterol was replaced by the [^{14}C]β-sitosterol. Using unlabeled oleoyl-CoA as substrate, the microsomal preparations yielded 1.3 and 1.1 pmol of [^{14}C]β-sitosteryl oleate mg protein^{-1} min^{-1}. These same microsomal preparations formed

TABLE 2. Cholesteryl oleate and β-sitosteryl oleate production in rabbit intestinal microsomes

Diet	Cholesteryl Oleate	β-Sitosteryl Oleate
	pmol/mg per min	
Chow + coconut oil (5)	330 ± 41	5.3 (0.0–16.8)[a]
Chow + coconut oil + 1% β-sitosterol (5)	94 ± 12[b]	1.6 (0.7–3.7)[a]

[a] Range for the five microsomal preparations.
[b] Significantly different than chow and coconut oil group at $P < 0.001$.

Intestinal microsomal membranes 0.1 mg protein, prepared from animals fed chow and coconut oil, or chow, coconut oil, and 1% β-sitosterol were incubated for 2 hr at 37°C with [^{14}C]cholesterol or [^{14}C]β-sitosterol-BSA emulsion. See Methods. The reaction was started with unlabeled oleoyl-CoA, 43 μM, and stopped after 5 min with 5 ml of chloroform–methanol 2:1 (v/v). Cholesteryl oleate and β-sitosteryl oleate production were measured after thin-layer chromatography separation of lipid classes as described in Methods and were expressed as the mean ± SE.

488 and 324 pmol cholesteryl oleate mg protein^{-1} min^{-1} when radiolabeled oleoyl-CoA was used as substrate.

Enrichment of microsomes with cholesterol

Microsomes prepared from the intestine of animals fed β-sitosterol are enriched two fold with this sterol. Membrane cholesterol is decreased. To test if the dietary plant sterol that has been incorporated into the membrane effects the amount of ACAT enzyme present or the action of ACAT to catalyze cholesterol esterification, intestinal microsomal membranes prepared from five rabbits fed coconut oil or four animals fed coconut oil plus β-sitosterol were enriched with cholesterol. Cholesterol-DPPC liposomes were prepared and incubated with the microsomes as described in Methods. ACAT activity was determined using [^{14}C]oleoyl-CoA as substrate. The results are shown in **Table 3.** As can be seen by the cholesterol to β-sitosterol ratio at 1, 2, and 4 hr of incubation with DPPC and cholesterol, cholesterol enrichment of microsomal membranes was time-dependent. In microsomes prepared from animals fed the coconut oil diet, ACAT activity was stimulated 4.5-fold over control incubations containing DPPC alone at 4 hr. ACAT activity was stimulated 5.8-fold over control incubations in microsomes prepared from animals fed 1% β-sitosterol. This difference was not significant. Actual ACAT activities were equal in the two microsomal preparations after cholesterol loading of the microsomes. The data suggest therefore, the membranes from animals fed β-sitosterol probably have similar amounts of enzyme compared to membranes prepared from animals fed coconut oil alone. It also suggests that a modest increase in membrane β-sitosterol does not interfere with cholesterol esterification as catalyzed by ACAT. DPPC alone decreased ACAT activity 22–44% as compared to microsomes that were incubated with buffer alone. This was not associated with a decrease in membrane cholesterol as measured by GLC. Microsomes that were labeled with [^{14}C]cholesterol and then incubated with DPPC liposomes or buffer for 4 hr had the same amount of label associated with them after thoroughly washing the membranes. Thus, we feel that DPPC is a direct inhibitor of ACAT activity. It was also observed that ACAT activity progressively increased over the 4 hr of incubation with buffer alone (data not shown). In removing the liposomes through a 20% sucrose gradient, approximately 30–40% of the microsomal protein is recovered. Thus, this step in actuality partially purifies the enzyme and hence increases the specific activity.

One reason proposed to explain why β-sitosterol interferes with cholesterol absorption in the human intestine is that it competes with cholesterol for esterification in the enterocyte (19). The data presented so far neither reject nor support this idea. Large doses of β-sitosterol are usually required to inhibit cholesterol absorption and lower serum cholesterol levels in man (17). Therefore, large amounts of β-sitosterol may need to be incorporated into intestinal microsomal membranes to compete with active sites on the enzyme. β-Sitosterol, because it is a poor substrate for ACAT, could decrease ACAT activity by attaching to the available sites. To test this idea, intestinal microsomes from rabbits fed normal chow were enriched with β-sitosterol. The re-

TABLE 3. Effect of cholesterol enrichment of intestinal microsomal membranes on microsomal ACAT activity

Diet	1 hr		2 hr		4 hr	
	Cholesterol[a] / β-Sitosterol	Fold Increase of ACAT Activity over Control	Cholesterol / β-Sitosterol	Fold Increase of ACAT Activity over Control	Cholesterol / β-Sitosterol	Fold Increase of ACAT Activity over Control
Chow + coconut oil (5)						
Control incubation	4.0 ± 0.7		3.5 ± 0.5		3.5 ± 0.6	
DPPC + cholesterol	5.8 ± 1.0	3.7 ± 1.1	6.9 ± 0.9	3.9 ± 1.2	9.5 ± 1.7	4.5 ± 1.1 (1131 ± 105)[b]
Chow + coconut oil + 1% β-sitosterol (4)						
Control incubation	0.61 ± 0.1		0.63 ± 0.07		0.57 ± 0.1	
DPPC + cholesterol	1.5 ± 0.4	5.1 ± 1.2	1.9 ± 0.7	6.1 ± 2.0	2.3 ± 0.8	5.8 ± 1.6 (1287 ± 174)[b]

[a] Sterol ratio in jejunal and proximal ileal microsomal membranes, mean ± SE.
[b] ACAT activity (pmol/mg per min), mean ± SE.
Cholesterol-DPPC-liposomes were incubated for 1–4 hr at 37°C with 3.0–5.0 mg of intestinal microsomal membranes prepared from animals fed chow and coconut oil, or chow, coconut oil, and 1% β-sitosterol. Initial ACAT activities of the microsomal preparations are given in Table 2 under cholesteryl oleate production. Control incubations contained equal amounts of DPPC but without cholesterol. After the liposomes were removed and the membranes were washed, ACAT activity was measured with radiolabeled oleoyl-CoA, 43 μM, as the substrate. See Methods. Membrane cholesterol and β-sitosterol were measured by GLC.

TABLE 4. Effect of β-sitosterol enrichment of microsomal membranes on microsomal ACAT activity

	1 hr			2 hr			4 hr		
	β-Sitosterol/Cholesterol[a]	Cholesterol[b]	ACAT[c]	β-Sitosterol/Cholesterol	Cholesterol	ACAT	β-Sitosterol/Cholesterol	Cholesterol	ACAT
Control (4)[d]	0.39 ± 0.09	19 ± 2	384 ± 24	0.41 ± 0.07	18 ± 1	473 ± 73	0.44 ± 0.08	21 ± 3	496 ± 88
DPPC + β-sitosterol (4)	1.22 ± 0.36	19 ± 1	367 ± 18	1.53 ± 0.40	20 ± 2	394 ± 71	2.10 ± 0.51	19 ± 2	478 ± 56

[a] Sterol ratio in microsomal membrane, mean ± SE.
[b] μg/mg Microsomal protein, mean ± SE.
[c] ACAT activity, pmol cholesteryl oleate formed/mg per min, mean ± SE.
[d] Number of animals.
Intestinal microsomal membranes were incubated with DPPC-liposomes alone (control) or liposomes containing β-sitosterol for 1, 2, and 4 hr. The experiment was performed as described in Table 3.

sults of four separate experiments are shown in **Table 4.** As noted before with cholesterol enrichment, enrichment of membranes with β-sitosterol is time-dependent over 4 hr (microsomal sterol ratio in incubations with DPPC and β-sitosterol). In control incubations containing DPPC alone or in incubations with DPPC and β-sitosterol, membrane cholesterol content remain unchanged. Despite an increase of β-sitosterol fivefold over control incubations (2.10 vs. 0.44), there was no effect on ACAT activity at the end of 4 hr. This suggests that β-sitosterol does not compete with cholesterol for the enzyme.

To ensure that the effects observed on ACAT activity after enrichment of microsomal membranes with β-sitosterol reflect what is occurring in the cell, isolated intestinal cells were used to determine changes in cholesterol esterification in the presence or absence of β-sitosterol. Isolated intestinal cells were incubated for 1 hr in oxygenated Krebs Medium that contained K^+[^{3}H]oleate attached to albumin (see Methods). The experimental dishes contained buffer alone, DPPC, β-sitosterol-DPPC liposomes, or 25-hydroxycholesterol-DPPC liposomes. The results of two separate experiments are shown in **Table 5.** In 1 hr, enterocytes incubated with β-sitosterol and DPPC contained twice as much β-sitosterol as cholesterol. Despite this increase in plant sterol content, the incorporation of oleic acid into phospholipid, triglyceride, or cholesteryl ester did not change. In contrast, 25-hydroxycholesterol, a sterol known to stimulate cholesterol esterification by ACAT in isolated hepatocytes (30), hepatic microsomes (26), and intestinal explant cultures (22), stimulated oleic acid incorporation into cholesteryl ester fivefold. This increase in oleic acid incorporation into cholesteryl ester was not associated with changes in the β-sitosterol:cholesterol ratio in the cells. This suggests that, despite a significant amount of β-sitosterol entering intact isolated intestinal cells, the phytosterol has no effect on cholesterol esterification.

DISCUSSION

This study clearly shows that the phytosterol, β-sitosterol, is a poor substrate for intestinal ACAT. This

TABLE 5. Effect of β-sitosterol and 25-hydroxycholesterol on the rate of oleic acid incorporation into phospholipids, triglycerides, and cholesteryl esters in isolated intestinal cells

	β-Sitosterol/Cholesterol[a]	Phospholipid	Triglyceride	Cholesteryl Ester
		nmol of [^{3}H]oleic acid incorporated/mg protein per hr		
Experiment #1				
Buffer	0.64	3.29	10.37	0.413
DPPC	0.75	3.56	10.40	0.386
DPPC + β-sitosterol	1.30	3.18	11.31	0.392
Experiment #2				
Buffer	0.29	2.45	5.75	0.217
DPPC	0.30	2.48	5.93	0.225
DPPC + β-sitosterol	0.78	2.36	5.95	0.259
DPPC + 25-hydroxycholesterol		2.26	5.64	1.052

[a] Sterol ratio in isolated cells.
Freshly isolated intestinal cells (approximately 3 mg protein per dish) were incubated for 1 hr with [^{3}H]oleic acid (50 μM, sp act 70 dpm/pmol). To the dishes, buffer, DPPC, β-sitosterol-DPPC, or 25-hydroxycholesterol-DPPC were added. Incorporation of [^{3}H]oleic acid into each lipid class was determined as described in Methods.

finding substantiates the recent observations by Tavani, Nes, and Billheimer (31). They found that the esterification rate of β-sitosterol as catalyzed by ACAT was 5% or less as compared with the esterification rate of cholesterol. We found that the rate of esterification for cholesterol was at least 60-fold greater than that for the phytosterol. This observation would explain why Swell et al. (11) found that most of the β-sitosterol within the intestinal wall was in the unesterified form. Only small amounts of the sterol were detected in the lymph. The incorporation of β-sitosterol into microsomal membranes prepared from animals fed 1% β-sitosterol does suggest that a substantial amount of this plant sterol enters the enterocyte from the lumen. It implies that sterol esterification may play no role or only a small role in the uptake of sterols from the lumen into the cell. It is still in question whether esterification plays a major part, however, in sterol transfer into the lymph.

We have shown that feeding β-sitosterol decreases ACAT activity in the gut. There are three possible reasons to explain this. During the 4 weeks of feeding β-sitosterol to the animals, the amount of ACAT protein in the membrane could decrease. We think this is unlikely, however, as ACAT activity in microsomes prepared from animals fed β-sitosterol for 1 month increased equally in response to membrane cholesterol enrichment as compared to ACAT activity measured in membranes prepared from animals fed a control diet (Table 3). The presence of increased amounts of β-sitosterol in the membrane could interfere with cholesterol esterification (19). We have excluded this possibility as well. Isolated intestinal cells that were enriched twofold with β-sitosterol have the same rate of cholesteryl oleate formation as controls (Table 5). Intestinal microsomal β-sitosterol content was increased fivefold over control microsomes (Table 4). Despite this obvious abnormal physiological state of the membrane, the rate of cholesterol esterification was not affected. The last possibility, which seems the most feasible to us, is that large dietary consumption of β-sitosterol decreases microsomal cholesterol content (Table 1). Since cholesterol and β-sitosterol are equally soluble within micelles (32), displacement of cholesterol from the micelle by the abundant luminal β-sitosterol, could decrease the absorption of cholesterol (19). This would explain the lipid changes which we observed within the membranes. The decrease in available cholesterol, as substrate for ACAT, would lead to a decrease in the activity of the enzyme (33–35) within the gut. In contrast, in the in vitro experiments, microsomal cholesterol content was not changed when the membranes were enriched with β-sitosterol. ACAT activity, therefore, was not affected under these conditions.

The pathway for sterol absorption in intestine involves many steps from the partitioning of the sterols in the oil-micellar phase to the synthesis of lipoproteins. Previous investigators have suggested that differences in esterification of plant sterols might explain the differences observed between the absorption of β-sitosterol and cholesterol (11, 14–16). Our results support this view. We have shown that a substantial amount of β-sitosterol becomes incorporated into intestinal microsomal membranes when animals ingest this sterol. Despite the fact that a substantial amount of β-sitosterol enters the absorptive cell, the esterification of this sterol as catalyzed by ACAT proceeds very slowly as compared to cholesterol esterification. This agrees with the observations by Kuksis and Huang (14) that plant sterols in chyle are not esterified, whereas most of the cholesterol in chyle is esterified. More information will be necessary, however, to determine if esterification of sterols in the gut is, indeed, rate-limiting for absorption.

Lees et al. (19), while studying the serum cholesterol-lowering effects of β-sitosterol in man, suggested that the phytosterol might interfere with incorporation of cholesterol into chylomicrons, possibly by interfering with its esterification. Our studies demonstrate that β-sitosterol does not interfere with nor competes with the esterification of cholesterol via ACAT.

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