# Effect of micellar β-sitosterol on cholesterol metabolism in CaCo-2 cells

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**Abstract** CaCo-2 cells were used to address the effect of the plant sterol, β-sitosterol, on cholesterol trafficking, cholesterol metabolism, and apoB secretion. Compared to cells incubated with micelles (5 mM taurocholate and 250 μM oleic acid) containing cholesterol, which caused an increase in the influx of plasma membrane cholesterol to the endoplasmic reticulum and increased the secretion of cholesteryl esters derived from the plasma membrane, β-sitosterol did not alter cholesterol trafficking or cholesteryl ester secretion. Including β-sitosterol in the micelle together with cholesterol attenuated the influx of plasma membrane cholesterol and prevented the secretion of cholesteryl esters derived from the plasma membrane. Stigmasterol and campesterol had effects similar to β-sitosterol, although campesterol did promote a modest influx of plasma membrane cholesterol. Including β-sitosterol in the micelle with cholesterol decreased the uptake of cholesterol. Compared to cholesterol, 60% less β-sitosterol was taken up by CaCo-2 cells. No observable esterification of β-sitosterol was appreciated and the transport of the plant sterol to the basolateral medium was negligible. Cholesterol synthesis and HMG-CoA reductase activities were decreased in cells incubated with β-sitosterol. This was associated with a decrease in reductase mass and mRNA levels. Cholesteryl ester synthesis and ACAT activities were unaltered by β-sitosterol. Both stigmasterol and campesterol decreased reductase activity, but only campesterol increased ACAT activity. β-sitosterol did not affect the secretion of apoB mass. The results suggest that β-sitosterol does not promote cholesterol trafficking from the plasma membrane to the endoplasmic reticulum. β-sitosterol interferes with the uptake of micellar cholesterol causing less plasma membrane cholesterol to influx and less cholesteryl ester to be secreted. Despite its lack of effect on cholesterol trafficking, β-sitosterol decreases cholesterol synthesis at the level of HMG-CoA reductase gene expression.—**Field, F. J., E. Born, and S. N. Mathur.** Effect of micellar β-sitosterol on cholesterol metabolism in CaCo-2 cells. *J. Lipid Res.* 1997. **38:** 348–360.

**Supplementary key words** apoB • HMG-CoA reductase • ACAT • cholesterol trafficking • cholesterol absorption

The human small intestine can differentiate between luminal cholesterol and the plant sterol β-sitosterol. Despite differing from cholesterol by only an ethyl group on carbon number 24, the absorption of β-sitosterol is approximately 1/10 that of cholesterol (1). Although a number of mechanisms have been addressed in an attempt to explain this observation, it remains unresolved (2–7). In individuals suffering from the rare disorder β-sitosterolemia, the intestinal absorption and plasma levels of plant sterols are markedly increased. This disorder is also characterized by accelerated atherosclerosis resulting in cutaneous xanthomas, coronary heart disease, and death at a young age (8, 9, review). For reasons that are not clear, cholesterol metabolism in these patients is also abnormal. Absorption of cholesterol from the small intestine is not altered, but results from balance and isotopic turnover studies have suggested that total body cholesterol synthesis in individuals with β-sitosterolemia is decreased (10, 11). In support of these studies, the activity of HMG-CoA reductase in liver and circulating monocytes has been found to be significantly decreased in these patients, whereas LDL receptor activity is increased (12, 13). It has been suggested that there is an inherent defect in the HMG-CoA reductase gene to explain these observations.

The intestine is the organ that regulates the entry of β-sitosterol into the body and yet there is no information regarding the effect of the plant sterol on lipoprotein secretion, cholesterol metabolism, or cholesterol trafficking in this tissue. We have recently postulated that during fat absorption, cholesterol derived predominately from the plasma membrane is used for lipoprotein assembly and secretion and that luminal micellar cholesterol displaces plasma membrane cholesterol causing the sterol to influx to the endoplasmic reticulum for esterification (14). These observations and results from previous studies in individuals with β-sitosterolemia have raised questions concerning whether β-sitosterol might also regulate lipoprotein assembly,

Abbreviations: ACAT, acylcoenzyme A:cholesterol acyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TC, taurocholate; OA, oleic acid

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cholesterol metabolism, and/or cholesterol trafficking in intestinal cells. The present study was done in the human intestinal cell line, CaCo-2, to specifically address these questions.

The results suggest that β-sitosterol does inhibit cholesterol biosynthesis in cultured intestinal cells and does so by decreasing HMG-CoA reductase mRNA levels and mass. Although β-sitosterol itself is taken up by CaCo-2 cells, the plant sterol does not displace cholesterol from the plasma membrane. Inclusion of β-sitosterol in a bile salt micelle together with cholesterol decreases the influx of plasma membrane cholesterol to the endoplasmic reticulum resulting in a decrease in the esterification of cholesterol and the secretion of cholesteryl esters. β-Sitosterol, therefore, inhibits cholesterol synthesis in CaCo-2 cells without causing an influx of cholesterol from the plasma membrane to the endoplasmic reticulum.

## MATERIALS AND METHODS

[7-$^{3}$H]cholesterol, [$^{14}$C]cholesterol, [5-$^{3}$H]mevalonolactone, 3-hydroxy-3-methyl[3-$^{14}$C]glutaryl coenzyme A, [cholesterol-1,2,6,7-$^{3}$H]cholesteryl linoleate, [oleoyl-1-$^{14}$C]oleoyl CoA, $^{3}H_2O$, and [9,10-$^{3}$H]oleic acid were purchased from New England Nuclear (Boston, MA). [$^{14}$C]β-sitosterol was a generous gift from Dr. Lawrence Rudel, Bowman Gray School of Medicine. β-Sitosterol was purchased from Supelco, Inc. (Bellefonte, PA) and Sigma Chemical Co. (St. Louis, MO). β-Sitosterol was 97% pure as estimated by gas–liquid chromatography. Similar results were obtained with the two preparations. Cholesterol, sodium taurocholate, oleic acid, campesterol, stigmasterol, oleoyl CoA, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and nucleotide adenine diphosphate were purchased from Sigma Chemical Co. (St. Louis, MO). HMG-CoA was from Pharmacia (Piscataway, NJ). ApoB monoclonal antibody (clone No. 1607) (immunoglobulin G26 fraction purified by column chromatography), and apoB sheep immunopurified polyclonal antibody conjugated to horseradish peroxidase were from Biodesign International (Kennebunkport, ME). TMB Microwell Peroxidase Substrate System was from Kirkegaard and Perry Labs Inc. (Gaithersburg, MD). Ninety-six-well Nunc-Immuno plates were from VWR Scientific (Batavia, IL). The murine monoclonal antibody A9, with specificity for human HMG-CoA reductase, was obtained from American Type Culture Collection (Rockville, MD). Anti-mouse Ig, horseradish peroxidase-linked whole antibody (from sheep) was obtained from Amersham Life Science, Inc. (Arlington Heights, IL). SuperSignal™ Substrate was from Pierce (Rockford, IL). A cDNA probe of human HMG-CoA reductase, pH RED 102, was obtained from ATCC, Rockville, Maryland.

### Cell culture

CaCo-2 cells were grown in T-75 flasks as described previously (15). They were subcultured on polycarbonate micropore membranes inserted in Transwells (Costar, Cambridge, MA). Medium was changed every 2 days and the cells were used for experiments after 14 days.

### Esterification of plasma membrane cholesterol

Plasma membrane cholesterol was labeled by incubating cells for 90 min a 4°C with 3 μCi [$^{3}$H]cholesterol in 0.4 ml of M199 (Medium #199, Earle's base, Gibco, Grand Island, NY) containing 1% delipidated fetal calf serum. The radiolabeled cholesterol in ethanol was added to this medium and the final concentration of ethanol was less than 1%. The solution containing the labeled cholesterol was added to the top well (apical side) only. Cells were washed twice with cold M199 to remove unincorporated labeled cholesterol. They were then incubated for 5 h at 37°C in 1 ml of the different micellar solutions (5 mM sodium taurocholate and 250 μM oleic acid) to be tested. After the treatment incubation, basal medium was collected and the lipids were extracted with chloroform–methanol 1:1 (v/v). The cells were washed with cold phosphate-buffered saline (PBS) and the lipids were extracted twice directly from the cells on the filter by adding 1 ml hexanes–isopropanol–water 3:2:0.1 (v/v/v). Unlabeled cholesterol and cholesteryl oleate were added as carriers. The medium and cell lipids were separated by thin-layer chromatography using solvent system containing hexanes–diethyl ether–methanol–acetic acid 85:15:1:1 (v/v). The free cholesterol and cholesteryl esters were localized by authentic standards, scraped from the plate, and counted.

### Cholesterol and β-sitosterol uptake and esterification

Cells were incubated at 37°C for specified times in micellar solutions containing 5 mM sodium taurocholate and 250 μM oleic acid, 100 μM [$^{3}$H]cholesterol with or without 100 μM unlabeled β-sitosterol. At the specified times, the apical medium was removed and discarded. The cells were washed with cold PBS and the lipids were extracted from the cells and basolateral media as described above. Carrier lipids were added and the solvent was dried under a stream of nitrogen. Cholesterol and cholesteryl esters were separated by thin-layer chromatography and measured.

To investigate β-sitosterol uptake, cells were incubated with micelles containing 100 μM [$^{14}$C]β-sitosterol. The cells and basolateral media were analyzed as described above.

### Enzyme analyses

The activity of HMG-CoA reductase was estimated as previously described (16, 17) and acylcoenzyme A:cholesterol acyltransferase (ACAT) activity was estimated as described (18).

### Estimation of cholesterol synthesis

Cholesteryl ester synthesis was estimated, as described previously (19) by the incorporation of labeled oleic acid for 4 h after an overnight incubation with the micellar solutions with and without cholesterol, and with and without β-sitosterol. The Silica Gel G thin-layer plates were developed with hexanes–diethyl ether–methanol–acetic acid 85:15:1:1 (v/v). The incorporation of labeled water into cholesterol was used to estimate the rate of cholesterol synthesis. Cells were incubated overnight with the micellar solutions described above. The next morning, approximately 20 mCi of [$^3$H]water, (0.4 cpm/pmol), was added to each filter of cells and allowed to incubate for 6 h at 37°C. The cells were washed extensively with cold PBS and the lipids extracted from the cells on the filter as described above using hexanes–isopropanol–water. The solvent was dried under a stream of nitrogen. The lipids were saponified with 1 ml alkaline methanol. The non-saponifiable fraction was extracted twice with hexanes. The hexane layer was dried under nitrogen and lipids were separated by thin-layer chromatography. The plates were eluted in hexanes–diethyl ether–methanol–acetic acid 85:15:1:1 (v/v) and when dried, stained by iodine vapors. The band corresponding to cholesterol was scraped and counted for radioactivity.

### Estimation of HMG-CoA reductase mass by Western blot

Cells were incubated overnight with the micellar solutions with/without 200 μM cholesterol and with/without 200 μM β-sitosterol. The cells were washed well with warm M199, harvested with 1 mL M199, and recovered by centrifugation at 10 *g* for 10 min. The cells were lysed in 30 μL of radioimmunoprecipitation assay buffer, pH 7.4 (10 mM sodium phosphate, 5 mM ethylenediaminetetraacetic acid, 5 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid, 100 mM NaCl, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) sodium dodecyl sulfate, 0.5% (wt/vol) sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 105 μM leupeptin, and 1 mM dithiothreitol) and kept on ice for 10 min. After vortexing, the samples were diluted with 5 mM Tris/HCl, pH 7.4, 50% (vol/vol) glycerol and 0.5% (vol/vol) Triton X-100. They were again vortexed and allowed to sit on ice for another 10 min. The cell suspension was centrifuged at 850 *g* for 5 min to remove cell debris and nuclear material. The supernatant was transferred to a new tube and diluted with an equal volume of 2× Laemmli sample buffer. Proteins were resolved using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (5% stacking and 8% separating). Proteins were transferred to a polyvinylidene difluoride membrane overnight at 15 V. The next morning the voltage was increased to 100 for 30 min to assure complete transfer. The membrane was blocked with 5% milk in Tris-buffered saline (10 mM Tris, 0.9% (wt/vol) NaCl, pH 7.4), containing 0.1% (vol/vol) Triton X-100, for 1 h at 37°C with shaking. The membrane was treated with 1 μg/ml anti-HMG-CoA reductase monoclonal antibody A9 suspended in 1% milk in the TBS solution mentioned above, for 2 h at room temperature with shaking. The excess antibody at each step was removed by washing the membrane three times with a TBS/0.2% (vol/vol) Triton X-100 solution. The membrane was then treated with the anti-mouse antibody–horseradish peroxidase for 1 h at room temperature with shaking. After incubating with the secondary antibody, the membrane was washed three times with the TBS/0.2% Triton X-100 and then washed finally three times with TBS only. The membrane was kept wet with TBS until the color was developed using the Pierce SuperSignal™ Substrate chemiluminescence kit. The amount of HMG-CoA reductase protein on the blot was quantitated using HP ScanJet cx scanner equipped with a transparency adapter and Sigma gel software (Jandel Scientific Software, San Rafael, CA).

### HMG-CoA reductase mRNA estimation

Total cellular RNA was extracted from CaCo-2 cells by the method described by Chomczynski and Sacchi (20) using guanidium thiocyanate. Northern blots were prepared using 1.1% agarose–formaldehyde gels as described by Sambrook, Fritsch, and Maniatus (21). RNA was transferred to membrane filters (Nytran, Midwest Scientific, Valley Park, MO) by capillary transfer and dried for 4 h at 80°C in a vacuum oven. The radiolabeled probe was prepared by labeling 100 ng of the cDNA for HMG-CoA reductase with [α-$^{32}$P]-dCTP using a random priming DNA kit (Pharmacia, Piscataway, NJ). Hybridization was performed for 18 h at 68°C in 0.25 M $Na_2HPO_4$ (pH 7.2), 1 mM EDTA, 10% SDS, and 0.5% blocking reagent (Schleicher & Schuell, Keene, NH). The blot was washed for 20 min each of 3 times at 60°C, with 20 mM $Na_2HPO_4$(pH 7.2), 1 mM EDTA and 1% SDS. It was then exposed to KODAK XAR-5 film at −70°C for up to 5 days. The mRNA on the blot was quantitated using HP ScanJet cx scanner equipped with a transparency adapter and Sigma gel software (Jandel Scientific Software, San Rafael, CA). RNA isolated from

proper controls and treatment groups were applied on the same gel. RNA loaded on the gel was normalized using the density of 28S and 18S RNA stained with ethidium bromide.

### ApoB mass and protein estimation

The estimation of apoB mass in cells and that secreted basolaterally was determined as we have described (22). Protein was determined according to the method of Lowry, et al. (23).

For statistical analysis of the data, Bonferroni's method using SIGMASTAT software (Jandel Scientific Software, San Rafael, CA) was used to compare 5 mM taurocholate + 250 μM oleic acid control group with other treatment groups at $P < 0.05$.

## RESULTS

### Plasma membrane cholesterol influx and secretion of plasma membrane-derived cholesteryl esters

To address whether β-sitosterol causes the influx of plasma membrane cholesterol to the endoplasmic reticulum, cells were labeled with cholesterol for 90 min at 4°C to label the plasma membrane pool. After the labeling period, cells were incubated for 5 h at 37°C with micelles containing 5 mM taurocholate and 250 μM oleic acid with or without cholesterol and/or β-sitosterol. The amount of labeled plasma membrane cholesterol that was esterified was used to estimate the influx of plasma membrane cholesterol to the endoplasmic reticulum (site of ACAT). The results are shown in **Fig. 1.** When cholesterol was included in the micelle, significantly more plasma membrane cholesterol influxed to the endoplasmic reticulum and more plasma membrane-derived cholesteryl esters were secreted into the basolateral medium. In contrast, β-sitosterol did not alter the amount of plasma membrane cholesterol esterified. Compared to control cells, significantly less labeled cholesteryl esters were secreted. When β-sitosterol was included in micelles containing cholesterol, less plasma membrane cholesterol influxed into the cell compared to that observed in cells incubated with micelles containing cholesterol alone. Moreover, the inclusion of the plant sterol in the micelle together with cholesterol also prevented the increase in secretion of plasma membrane-derived cholesteryl esters.

**Figures 2A and 2B** show the effect of including β-sitosterol in micelles containing cholesterol on the esterification of plasma membrane cholesterol and their secretion into the basolateral medium over time and at increasing concentrations of the plant sterol. In Fig. 2A, cholesterol-containing micelles caused a significant increase in the influx of plasma membrane-derived cholesterol to the endoplasmic reticulum and also increased the secretion of plasma membrane-derived cholesteryl esters. The inclusion of the plant sterol, however, markedly decreased the influx of plasma membrane-derived cholesterol and completely prevented the increase in secretion of plasma membrane-derived cholesteryl esters. The effect of β-sitosterol was directly related to its concentration within the cholesterol micelle (Fig. 2B). These data suggest that micellar β-sitosterol does not displace cholesterol from the plasma membrane. The results indicate, however, that the addition of the plant sterol to the cholesterol-containing micelle interferes with the effects of micellar cholesterol on cholesterol trafficking and the secretion of plasma membrane-derived cholesteryl esters.

To address whether this effect of β-sitosterol was unique to this plant sterol, other plant sterols were similarly tested for their ability to promote an influx of plasma membrane-derived cholesterol. After the labeling period of 4°C, cells were incubated at 37°C with micelles containing taurocholate and oleic acid with or without cholesterol, campesterol, or stigmasterol. After 5 h, the amount of labeled cholesteryl esters was estimated. **Table 1** shows these results. Again, cholesterol caused a significant increase in the movement of plasma membrane cholesterol to the endoplasmic reticulum. Campesterol also promoted the influx of plasma membrane cholesterol to the ER, but campesterol was not as potent as cholesterol in doing so. In contrast, similar to β-sitosterol, stigmasterol did not displace cholesterol from the plasma membrane. The inclusion of either plant sterol in the micelle together with cholesterol decreased the ability of cholesterol to promote the influx of plasma membrane cholesterol. Moreover, unlike cholesterol, which increased the secretion of plasma membrane-derived cholesteryl esters, neither plant sterol caused an increase in cholesteryl ester secretion. Both campesterol and stigmasterol prevented the increase in secretion of cholesteryl esters caused by micellar cholesterol.

### Effect of β-sitosterol on the uptake of micellar cholesterol

As β-sitosterol decreased the displacement of plasma membrane cholesterol by micellar cholesterol, it was postulated that the plant sterol likely interfered with the uptake of cholesterol from the micelle. To address this, control cells were incubated with micelles containing 5 mM taurocholate, 250 μM oleic acid, and 100 μM labeled cholesterol. Another set of cells was incubated with the cholesterol micelles containing 100 μM β-sitosterol. **Figure 3** shows these data. Very reproducibly, early in the

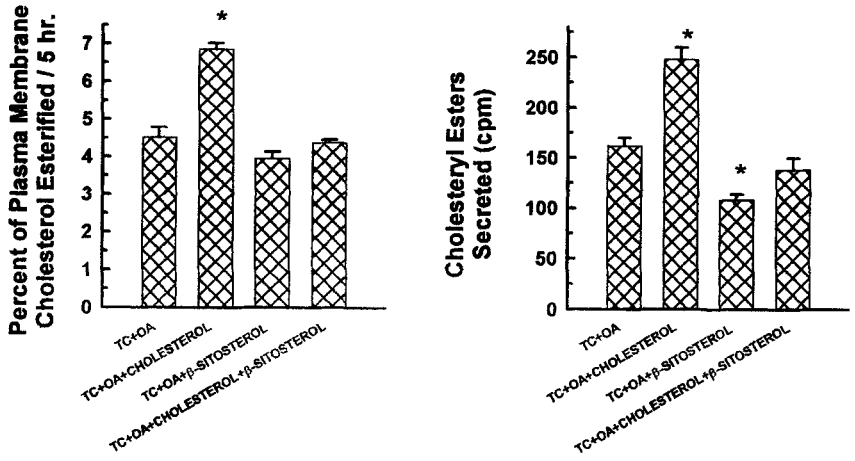


**Fig. 1.** Effect of micellar β-sitosterol and/or cholesterol on the esterification of plasma membrane cholesterol and its basolateral secretion. Plasma membrane cholesterol was labeled at 4°C for 90 min. The cells were then incubated for 5 h with 5 mM taurocholate (TC) + 250 μM oleic acid (OA) with or without 200 μM of cholesterol or β-sitosterol. The percent of plasma membrane cholesterol esterified and the secretion of labeled cholesteryl esters were estimated as described in Methods. Each value represents the mean ± SE of 4 filters. * $P < 0.05$ vs. 5 mM taurocholate + 250 μM oleic acid.

incubation, cells incubated with micelles containing β-sitosterol contained more labeled cholesterol. By 2 h and thereafter, however, the inclusion of β-sitosterol decreased the amount of labeled cholesterol associated with the cells. By 4 h, cells incubated with cholesterol micelles alone had accumulated 60% more cholesterol compared to cells incubated with micelles containing both cholesterol and β-sitosterol. In cells incubated with β-sitosterol, maximal uptake of cholesterol occurred at 30 min and essentially plateaued thereafter. In contrast, cells incubated with cholesterol without the plant sterol accumulated cholesterol incrementally over time. The reasons for this particular observation remain unclear. It appears, however, that the inclusion of β-sitosterol within the micelle does decrease the uptake of micellar cholesterol by CaCo-2 cells.

### Uptake of micellar β-sitosterol

To address the specific question of whether β-sitosterol was taken up by CaCo-2 cells, cells were incubated with micelles containing 5 mM taurocholate, 250 μM oleic acid, and 100 μM of either labeled cholesterol or β-sitosterol (**Fig. 4**). Compared to cells incubated with micelles containing cholesterol, cells incubated with micelles containing β-sitosterol contained approximately 2-fold less cell-associated labeled sterol. This suggests that uptake of the plant sterol, as estimated by cell-associated radiolabeled sterol, was significantly less than that of cholesterol. The amount of unesterified sterol transported and secreted into the basolateral medium, however, was quite different. Whereas labeled cholesterol was easily detected in the basolateral well and increased with time, the amount of β-sitosterol secreted was negligible. Moreover, in contrast to the esterification of micellar-derived cholesterol and its basolateral secretion, micellar-derived β-sitosterol was not esterified within cells and no β-sitosterol ester was recovered in the basolateral medium.

### Effect of β-sitosterol on cholesterol metabolism

As β-sitosterol did not displace cholesterol within the plasma membrane, it seemed unlikely that the plant sterol would alter cholesterol metabolism within intestinal cells. However, because individuals with β-sitosterolemia appear to have lower rates of cholesterol synthesis when compared to normal controls (11), we felt it was important to determine whether β-sitosterol would alter rates of cholesterol synthesis and/or esterification in CaCo-2 cells. Cells were incubated with micelles containing 5 mM taurocholate and 250 μM oleic acid with or without cholesterol and/or β-sitosterol. The activities of HMG-CoA reductase and ACAT were estimated in cell homogenates following the incubations. **Figure 5** shows these results. As expected, compared to control cells, in cells incubated with micelles containing cholesterol, HMG-CoA reductase activity was significantly decreased and ACAT activity was significantly increased. Unexpectedly, β-sitosterol also caused a significant decrease in reductase activity similar to what was observed with cholesterol. Unlike cholesterol, however, β-sitosterol did not alter ACAT activity. Including the plant sterol in the micelle together with cholesterol, decreased the

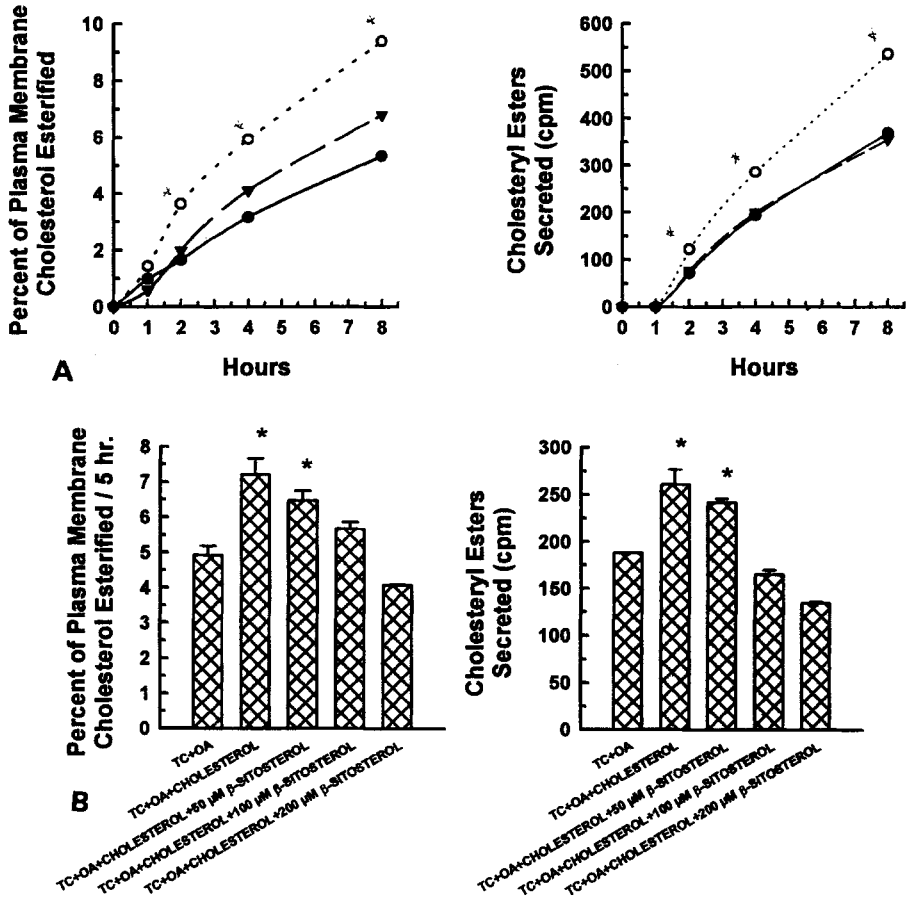


**Fig. 2.** Effect of micellar β-sitosterol and cholesterol on the esterification of plasma membrane cholesterol and its basolateral secretion. Plasma membrane cholesterol was labeled at 4°C for 90 min. (A) The cells were then incubated with the 5 mM taurocholate + 250 μM oleic acid, ●; 5 mM taurocholate + 250 μM oleic acid + 200 μM cholesterol, ○; or 5 mM taurocholate + 250 μM oleic acid + 200 μM cholesterol + 200 μM β-sitosterol, ▼; values are mean of duplicate dishes at each time point. (B) Cells were incubated with micelles containing cholesterol or cholesterol and increasing concentrations of β-sitosterol; values are mean ± SE of 4 filters. * $P < 0.05$ vs. 5 mM taurocholate + 250 μM oleic acid.

effect of micellar cholesterol on ACAT activity and caused a modest, but significant, additional inhibitory effect on reductase activity.

To further support these observations, cells were again incubated with one of the four micellar preparations described above and the rates of cholesterol synthesis and cholesterol esterification were estimated by labeled water incorporation into cholesterol and labeled oleic acid incorporation into cholesteryl esters, respectively **(Fig. 6).** Interestingly, micellar β-sitosterol was as effective as cholesterol in inhibiting the rate of cholesterol synthesis. Adding cholesterol and β-sitosterol together in the micelle did not further inhibit cholesterol synthetic rates. In contrast to the effect of micellar cholesterol on cholesterol esterification, the plant sterol had no effect on oleic acid incorporation into cholesteryl esters. As expected, however, the inclusion of β-sitosterol in micelles containing cholesterol markedly diminished the stimulatory effect of cholesterol on cholesteryl ester synthesis.

As β-sitosterol did not cause an influx of cholesterol from the plasma membrane to the endoplasmic reticulum, we considered whether this pathway of cholesterol trafficking was required for the regulation of ACAT activity by plant sterols. As shown in Table 1, in contrast to β-sitosterol, campesterol did cause an increase in the influx of plasma membrane cholesterol. Similar to β-sitosterol, stigmasterol did not. ACAT and HMG-CoA reductase activities were therefore estimated in cells incubated with micelles containing either campesterol or stigmasterol **(Table 2).** Indeed, ACAT activity was increased in cells incubated with campesterol, whereas it

TABLE 1. Effect of cholesterol and/or various plant sterols on the esterification of plasma membrane cholesterol and its basolateral secretion

| | Fold-Increase | Cholesteryl Esters Secreted |
|---|---|---|
| | | *cpm* |
| 5 mM taurocholate + 250 μM oleic acid | 1.0 | 196 ± 18 |
| 5 mM taurocholate + 250 μM oleic acid + 250 μM cholesterol | 2.4[a] | 296 ± 5[a] |
| 5 mM taurocholate + 250 μM oleic acid + 250 μM campesterol | 1.7[a] | 213 ± 7 |
| 5 mM taurocholate + 250 μM oleic acid + 250 μM stigmasterol | 1.2 | 200 ± 17 |
| 5 mM taurocholate + 250 μM oleic acid + 250 μM cholesterol + 250 μM campesterol | 1.9[a] | 213 ± 7 |
| 5 mM taurocholate + 250 μM oleic acid + 250 μM cholesterol + 250 μM stigmasterol | 1.8[a] | 215 ± 8 |

Plasma membrane cholesterol was labeled at 4°C for 90 min, followed by a 5-h incubation with micelles alone or micelles containing 250 μM of sterol. The percent of plasma membrane cholesterol esterified and the basolateral secretion of labeled cholesteryl esters were estimated as described in Methods. Each value is mean ± SE of 4 filters.

[a] $P < 0.05$ vs. 5 mM taurocholate + 250 μM oleic acid.

was unaltered in cells incubated with stigmasterol. Reductase activity was decreased in cells incubated with either of the plant sterols, however, campesterol was more potent than stigmasterol. The data suggest that cholesterol influx from the plasma membrane to the endoplasmic reticulum is important in the regulation of intestinal ACAT. Cholesterol influx does not appear to be required for the regulation of HMG-CoA reductase activity by plant sterols.

To determine whether β-sitosterol inhibited HMG-CoA reductase activity by decreasing the mass of enzyme protein, cells were incubated with micelles containing cholesterol or β-sitosterol. After the incubation, the amount of reductase protein was estimated within cells by immunoblot analysis. The results are illustrated in **Fig. 7.** HMG-CoA reductase mass was significantly decreased in cells incubated with micelles containing either cholesterol or β-sitosterol. Compared to the plant sterol, cholesterol seemed to be more potent in decreasing the amount of reductase protein. Moreover, adding β-sitosterol to micelles containing cholesterol did not have a significant additive effect in decreasing reductase protein mass.

To further investigate mechanisms for the inhibition of HMG-CoA reductase activity by the two sterols, steady-state mRNA levels for reductase were estimated by Northern analysis after the incubation of cells with micelles containing either cholesterol or β-sitosterol (**Fig. 8**). Incubating cells with micelles containing 200 μM cholesterol significantly decreased mRNA levels. β-Sitosterol also decreased message levels but the two sterols together did not have an additive effect. During a large lipid flux, therefore, both β-sitosterol and cholesterol regulate reductase activity at the level of its protein and message.

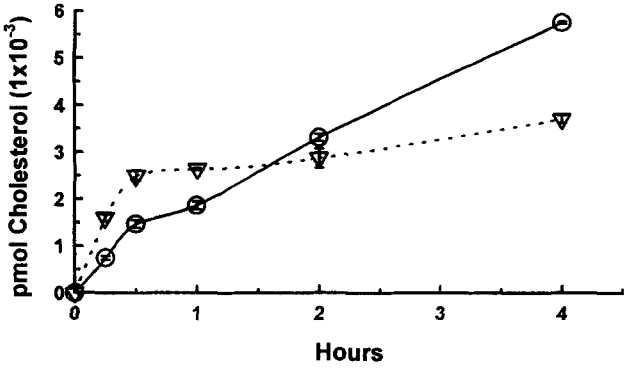


**Fig. 3.** Effect of β-sitosterol on the uptake of micellar cholesterol. Cells were incubated at 37°C with 5 mM taurocholate + 250 μM oleic acid + 100 μM [$^3$H]cholesterol (specific activity, 48 cpm/pmol), ○; or 5 mM taurocholate + 250 μM oleic acid + 100 μM [$^3$H]cholesterol + 100 μM β-sitosterol, ▽. The uptake of labeled cholesterol was estimated. Each value is mean ± SE of 3 filters. Data from one of the two experiments with similar results are shown.

### Effect of β-sitosterol on apoB secretion

As β-sitosterol decreased the secretion of cholesteryl esters that originated from cholesterol of the plasma membrane, we addressed whether β-sitosterol might interfere with the number of lipoprotein particles being secreted. To estimate lipoprotein secretion, apoB mass was determined within cells and the basolateral medium after an incubation of cells with micelles containing either 200 μM cholesterol or β-sitosterol. Neither cholesterol nor β-sitosterol altered cellular apoB or the amount of apoB secreted, suggesting that micellar sterols do not regulate the number of lipoprotein particles secreted by CaCo-2 cells (data not shown).

## DISCUSSION

In a recent study performed in CaCo-2 cells, we have shown that during lipid flux, micellar cholesterol is not used directly for the assembly of a lipoprotein particle; rather, micellar cholesterol displaces cholesterol from the plasma membrane causing it to influx to the endoplasmic reticulum (14). It is this cholesterol, originating from the plasma membrane, that is used primarily for triacylglycerol-rich lipoprotein assembly and secretion. In the present study, we again confirmed that adding cholesterol to cells in a micellar solution causes an in-

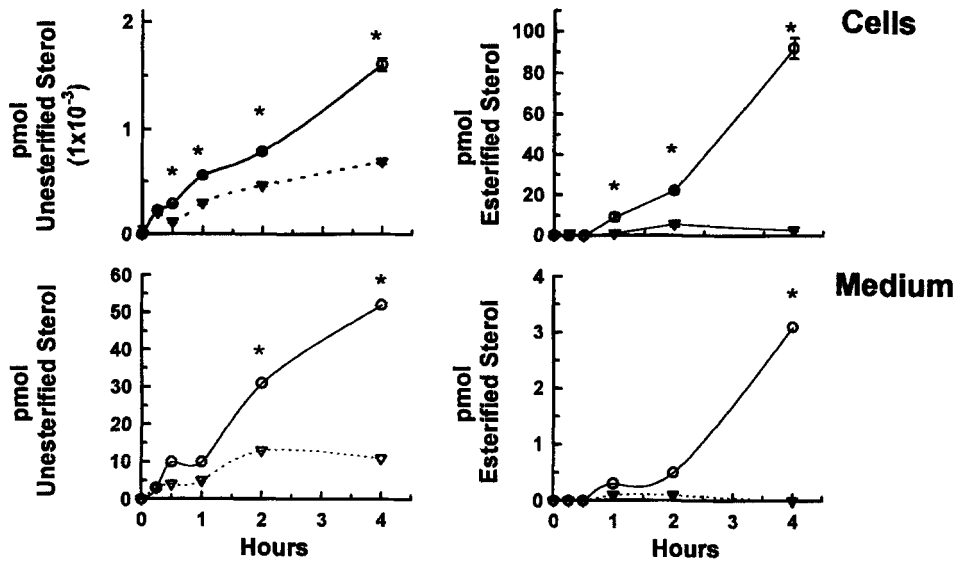


**Fig. 4.** Uptake, esterification and secretion of micellar cholesterol or β-sitosterol. Cells were incubated at 37°C with 5 mM taurocholate + 250 μM oleic acid + 100 μM [$^{14}$C]cholesterol (specific activity, 85 cpm/pmol), ○; or 5 mM taurocholate + 250 μM oleic acid + 100 μM [$^{14}$C]β-sitosterol (specific activity, 85 cpm/pmol), ▽. The radioactivity in cellular and basolaterally secreted esterified and unesterified sterol was estimated as described in Methods. Each value is mean ± SE of 3 filters. * $P < 0.05$, [$^{14}$C]cholesterol vs. [$^{14}$C]β-sitosterol.

crease in the movement of plasma membrane cholesterol to the endoplasmic reticulum resulting in an increase in the secretion of cholesteryl esters derived from plasma membrane cholesterol. In contrast, β-sitosterol, which differs from cholesterol only by the addition of an ethyl group on the side chain of carbon 24, neither alters the influx of plasma membrane cholesterol nor the secretion of cholesteryl esters. One could argue that the plant sterol is "neutral," i.e., it is not taken up by intestinal cells and therefore does not interact with cholesterol of the plasma membrane. Indeed, there is evidence to suggest that the intestinal cell does discriminate between cholesterol and β-sitosterol at the level of the apical absorptive surface (3). Other evidence, how-

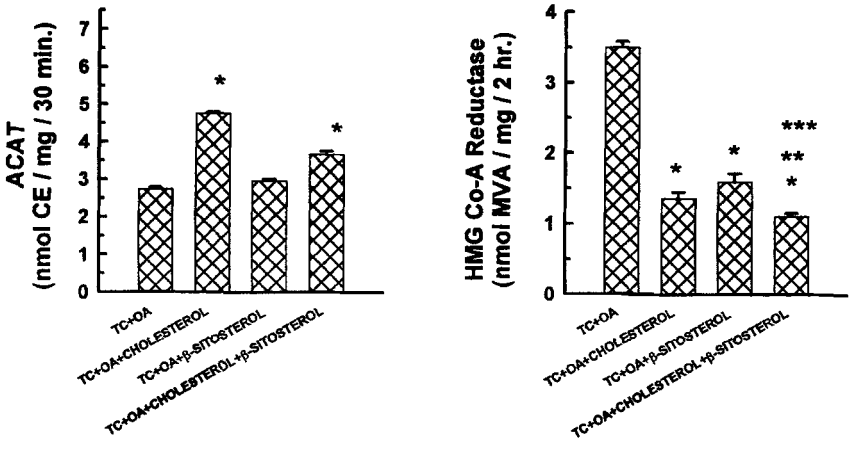


**Fig. 5.** Effect of micellar cholesterol and/or β-sitosterol on ACAT and HMG-CoA reductase activities. Cells were incubated at 37°C for 18 h with 5 mM taurocholate + 250 μM oleic acid, 5 mM taurocholate + 250 μM *oleic acid + 200 μM cholesterol, 5 mM taurocholate + 250 μM oleic acid + 200 μM β-sitosterol, or 5 mM* taurocholate + 250 μM oleic acid + 200 μM cholesterol + 200 μM β-sitosterol. The enzyme activity in the total membrane preparations was assayed as described in Methods. Each assay was performed in triplicate. The values are mean ± SE of 3 experimental preparations. *$P < 0.05$ vs. 5 mM taurocholate + 250 μM oleic acid. **$P < 0.05$ vs. 5 mM taurocholate + 250 μM oleic acid + 200 μM cholesterol. ***$P < 0.05$ vs. 5 mM taurocholate + 250 μM oleic acid + 200 μM β-sitosterol.

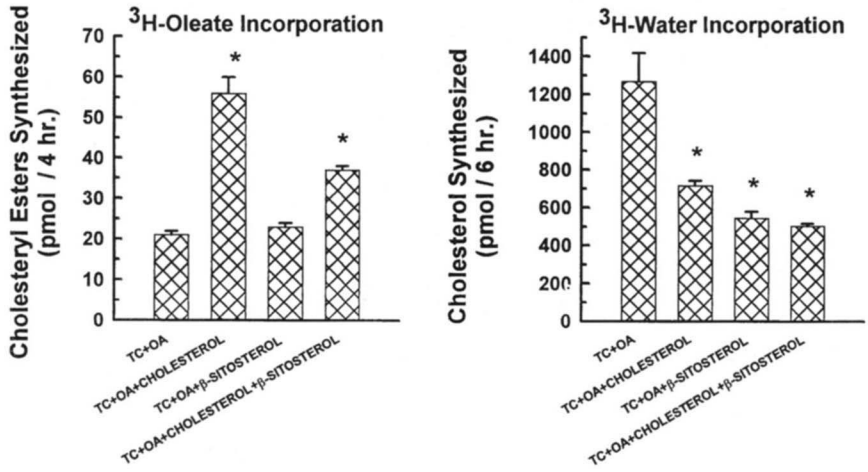


**Fig. 6.** Effect of micellar cholesterol and/or β-sitosterol on the synthesis of cholesteryl esters or cholesterol. Cells were incubated at 37°C for 18 h with 5 mM taurocholate + 250 μM oleic acid, 5 mM taurocholate + 250 μM oleic acid + 200 μM cholesterol, 5 mM taurocholate + 250 μM oleic acid + 200 μM β-sitosterol, or 5 mM taurocholate + 250 μM oleic acid + 200 μM cholesterol + 200 μM β-sitosterol. During the last 4 h of incubation, radiolabeled oleate was added to measure cholesteryl [$^3$H]oleate synthesis. In another experiment [$^3$H]water was added during the last 6 h of the incubation to measure cholesterol synthesis. Values are mean ± SE of 6 filters. *$P < 0.05$ vs. 5 mM taurocholate + 250 μM oleic acid control.

ever, would argue that β-sitosterol is taken up by intestinal cells but the discrimination between the two sterols occurs intracellularly, perhaps at the level of sterol esterification (4, 5). CaCo-2 cells do take up β-sitosterol from a micellar solution albeit at a reduced amount compared to the uptake of cholesterol. Moreover, as we will discuss later, β-sitosterol is not only taken up by the cell, but the plant sterol also regulates cholesterol metabolism. Thus, in CaCo-2 cells, β-sitosterol does interact with the apical absorptive membrane, but unlike micellar cholesterol, it does not promote the influx of plasma membrane cholesterol.

TABLE 2. Effect of campesterol or stigmasterol on ACAT or HMG-CoA reductase activities

| | ACAT | Reductase |
|---|---|---|
| | *pmol/mg/30 min* | *pmol/mg/2 h* |
| 5 mM taurocholate + 250 μM oleic acid | 1555 ± 82 | 4413 ± 209 |
| 5 mM taurocholate + 250 μM oleic acid + 200 μM campesterol | 1948 ± 9[a] | 1988 ± 118[a] |
| 5 mM taurocholate + 250 μM oleic acid + 200 μM stigmasterol | 1384 ± 47 | 3501 ± 160[a] |

CaCo-2 cells were incubated with 5 mM taurocholate + 250 μM oleic acid with or without addition of 200 μM campesterol or stigmasterol for 18 h. The enzyme activity in the total membrane preparations was assayed as described in Methods. Each assay was performed in triplicate. The values are the mean ± SEM of 4 preparations.

[a] $P < 0.05$ vs. 5 mM taurocholate + 250 μM oleic acid.

When similar amounts of β-sitosterol are included in a micelle along with cholesterol, the movement of cholesterol from the plasma membrane inward and the secretion of plasma membrane-derived cholesteryl esters are markedly decreased. This can best be explained by a displacement of cholesterol from the micellar solution by β-sitosterol. Because absorption of cholesterol by the gut is dependent upon its solubilization in bile salt micelles, a decrease in the concentration of choles-

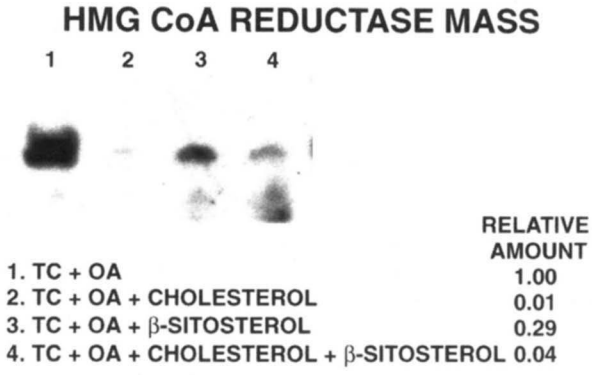


**Fig. 7.** Effect of micellar cholesterol and/or β-sitosterol on HMG-CoA reductase mass. Cells were incubated at 37°C for 18 h with (1) 5 mM taurocholate + 250 μM oleic acid, (2) 5 mM taurocholate + 250 μM oleic acid + 200 μM cholesterol, (3) 5 mM taurocholate + 250 μM oleic acid + 200 μM β-sitosterol, or (4) 5 mM taurocholate + 250 μM oleic acid + 200 μM cholesterol + 200 μM β-sitosterol. The reductase mass in the cell homogenates was estimated by Western blot as described in Methods. A representative blot from 4 replicates is shown.

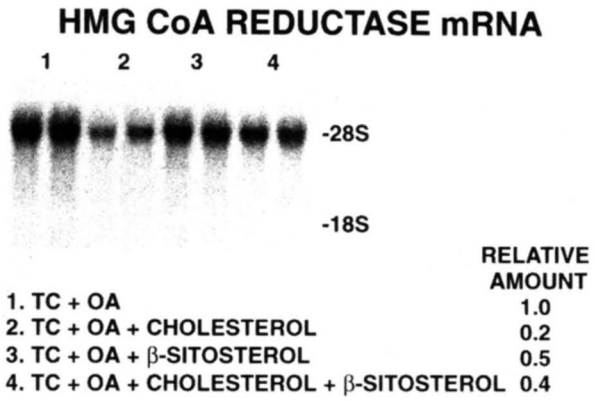


**Fig. 8.** Effect of micellar cholesterol and/or β-sitosterol on HMG-CoA reductase mRNA. Cells were incubated at 37°C for 18 h with (1) 5 mM taurocholate + 250 μM oleic acid, (2) 5 mM taurocholate + 250 μM oleic acid + 200 μM cholesterol, (3) 5 mM taurocholate + 250 μM oleic acid + 200 μM β-sitosterol, or (4) 5 mM taurocholate + 250 μM oleic acid + 200 μM cholesterol + 200 μM β-sitosterol. The reductase mRNA in the cell homogenates was estimated by Northern blot as described in Methods. A representative blot from 4 replicates is shown.

terol within micelles would lead to a decrease in the uptake of cholesterol by the absorptive cell (24,25). Results in animal and human studies have suggested that this is the likely mechanism to explain why excess ingestion of β-sitosterol leads to a decrease in cholesterol absorption (7, 26). If, however, cholesterol uptake from the intestinal lumen is mediated by a specific transport protein as suggested by Thurnhofer and Hauser (27), it is possible that β-sitosterol competes with cholesterol for this protein. The present data cannot prove or refute either hypothesis. What is clear, however, is that in CaCo-2 cells, β-sitosterol does interfere with the amount of cholesterol taken up by the cell. The experiment that was performed to support this conclusion requires further explanation (Fig. 3). In this experiment, at early time points, more labeled cholesterol was associated with cells incubated with micelles containing both cholesterol and β-sitosterol than cells incubated with cholesterol alone, suggesting that β-sitosterol caused an increase in the uptake of labeled cholesterol. In a micellar solution containing labeled cholesterol and equal amounts of unlabeled β-sitosterol, more cholesterol would be in the aqueous phase and accessible for exchange with cholesterol of the plasma membrane. Because exchange occurs rapidly depending upon the concentration of the donor particle (28), this would explain why the amount of labeled cholesterol associated with cells appeared to plateau early in the incubation and increased only slowly thereafter. In contrast, in cells incubated with micelles containing only cholesterol, the amount of labeled cholesterol associated with cells due to exchange would be less, thus resulting in the observed time-dependent uptake of labeled cholesterol. Nonetheless, because plasma membrane cholesterol is used for normal lipoprotein assembly during lipid flux (14), the inclusion of β-sitosterol in micelles containing cholesterol will result in a decrease in the uptake of micellar cholesterol; less plasma membrane cholesterol will move to the endoplasmic reticulum and less unesterified and esterified cholesterol will be secreted in a lipoprotein particle.

It is well established that plant sterols are poorly absorbed by the gut (1). Although we show here that CaCo-2 cells take up β-sitosterol at their apical membrane, it must be recognized that uptake of a sterol by an intestinal cell cannot be equated with absorption. Uptake is obviously necessary for absorption of β-sitosterol to occur, but absorption also requires that the plant sterol be incorporated normally into a lipoprotein particle and then be secreted within this particle into mesenteric lymphatics. It has been estimated that the absorption of β-sitosterol by the small intestine is approximately one-tenth that of cholesterol (1). In the present study, the uptake of β-sitosterol by CaCo-2 cells was one-half that of cholesterol. Moreover, in line with what we and others have observed regarding the lack of intracellular esterification of β-sitosterol (4, 5, 29), essentially no esterified β-sitosterol was observed within cells and no β-sitosterol esters were recovered in the basolateral media. In addition, the transport of unesterified β-sitosterol was negligible to that of unesterified cholesterol suggesting that although CaCo-2 cells do take up β-sitosterol, they lack a mechanism to effectively transport the plant sterol into the basolateral medium.

The effect of micellar β-sitosterol on cholesterol metabolism was somewhat unexpected. We initially postulated that the influx of plasma membrane cholesterol to the endoplasmic reticulum and the ensuing expansion of "regulatory cholesterol pools" would be required for regulating both ACAT and HMG-CoA reductase activities. For the regulation of ACAT activity, this turned out to be true. In CaCo-2 cells, similar to what has been observed in other cell types, cholesterol of the plasma membrane is the major substrate for ACAT (30–34). Normal cholesterol trafficking from the plasma membrane to the endoplasmic reticulum appears to be the important pathway for supplying substrate for ACAT. Although more data will be required to establish other possible mechanisms for ACAT regulation, most data to date would suggest that ACAT is regulated by substrate supply (35, 36). Thus, a molecule that increases the delivery of plasma membrane cholesterol to the endoplasmic reticulum will result in an increase in ACAT activity. The present results support that postulate. As we have shown previously and further confirmed in the present study, ACAT activity, as estimated

by direct measurement of activity and oleic acid incorporation into cellular cholesteryl esters, was increased in CaCo-2 cells incubated with micelles containing cholesterol (37). This was associated with a marked increase in the movement of plasma membrane cholesterol to the endoplasmic reticulum. In contrast, in cells incubated with micelles containing β-sitosterol or stigmasterol, which do not cause cholesterol influx, ACAT activity was not altered. Campesterol, however, a plant sterol that does cause an increase in the influx of plasma membrane cholesterol, increased the activity of ACAT. As one might expect from these results, the inclusion of β-sitosterol within a micelle containing cholesterol will cause less cholesterol to be taken up, cause less cholesterol to influx from the plasma membrane to the endoplasmic reticulum, and cause an attenuation of ACAT activity compared to cells incubated with cholesterol alone. The results, therefore, are very consistent with what is already known about the regulation of cellular ACAT activity.

In cells incubated with micelles containing oleic acid and cholesterol, cholesterol synthesis and HMG-CoA reductase activity were significantly decreased. This makes good sense and agrees with results from others demonstrating a decrease in intestinal cholesterol synthesis after the ingestion of cholesterol (38). Unexpectedly, however, cholesterol synthesis was also decreased in cells incubated with micelles containing β-sitosterol. As micellar β-sitosterol did not promote the influx of plasma membrane cholesterol and, therefore, would not be expected to expand intracellular pools of cholesterol, one cannot invoke a change in cholesterol trafficking to explain this effect. In addition, reductase activity was also decreased in cells incubated with micelles containing stigmasterol, another related plant sterol that did not alter cholesterol influx. A decrease in reductase activity in cells incubated with micellar β-sitosterol was associated with a decrease in reductase mass and mRNA levels. In studies done in livers and isolated mononuclear cells from individuals with β-sitosterolemia, HMG-CoA reductase activities were decreased and LDL binding was increased, compared to controls (12, 13). Additionally, hepatic mRNA levels of reductase and enzyme mass were decreased as well. Although it is unclear why there is a decrease in the expression of HMG-CoA reductase in individuals with β-sitosterolemia, it has been postulated that there is an inherent defect in the gene for the enzyme (12). This presumption is based on previous data showing that β-sitosterol, added in ethanol, does not regulate HMG-CoA reductase activity in cultured fibroblasts (39) or in livers of rats infused intravenously with Intralipid containing β-sitosterol (40). If these observations can be applied to intestine and to our present results in CaCo-2 cells (that β-sitosterol does not have a direct effect on reductase activity), then other mechanisms must be entertained for our observations. It would be unlikely that β-sitosterol is metabolized to a more polar sterol within the cell. In data not shown, analysis by gas–liquid chromatography of cells and medium prior to and after incubation with β-sitosterol revealed no qualitative or quantitative changes to the sterol. Moreover, if this were a potential mechanism, ACAT activity would be increased and more cholesterol would move from the plasma membrane to the endoplasmic reticulum for esterification (unpublished observations in CaCo-2 cells). This did not occur. Although the present results suggest that β-sitosterol does not cause the influx of plasma membrane cholesterol, it remains possible that the plant sterol alters the flux of cholesterol in another intracellular pool that regulates reductase but not ACAT activity.

When sterols accumulate within cells, membrane proteins that bind to the sterol-regulatory element of the HMG-CoA reductase gene escape proteolysis and hence do not bind to the promoter. This leads to suppression of gene transcription for reductase (41). It is possible that high concentrations of β-sitosterol within the cell could also interfere with proteolysis of a regulatory binding protein or proteins thus causing a decrease in HMG-CoA reductase expression. In the previous studies cited, it would be unlikely that sufficient plant sterol was taken up to cause regulation of reductase activity (39, 40).

In previous studies from our laboratory, we have demonstrated that unlike fatty acids, phosphatidylcholine, or lysophosphatidylcholine, which cause an increase in apoB secretion, cholesterol does not induce lipoprotein secretion (19, 42). Likewise, in the present study, β-sitosterol had no effect on the secretion of apoB by CaCo-2 cells. In individuals with β-sitosterolemia, there are no recognized nutritional deficiencies and lipid absorption by the small intestine appears to be normal (8, 9). Moreover, treating hypercholesterolemic patients with gram quantities of β-sitosterol does not alter plasma triacylglycerol levels and has no observed nutritional side effects (26). We would conclude, therefore, that like cholesterol, plant sterols do not alter the number of lipoprotein particles secreted by the intestine. The data would suggest, however, that in individuals ingesting large amounts of β-sitosterol as a lipid lowering agent, lipoprotein particles secreted by the intestine will be deficient in cholesteryl esters.

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