Compared with Acyl-CoA:Cholesterol O-Acyltransferase (ACAT) 1 and Lecithin:Cholesterol Acyltransferase, ACAT2 Displays the Greatest Capacity to Differentiate Cholesterol from Sitosterol*

Ryan E. Temels, Abraham K. Gebre, John S. Parks, and Lawrence L. Rudels

From the Department of Pathology, Section on Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157

The capacity of acyl-CoA:cholesterol O-acyltransferase (ACAT) 2 to differentiate cholesterol from the plant sterol, sitosterol, was compared with that of the sterol esterifying enzymes, ACAT1 and lecithin:cholesterol acyltransferase (LCAT). Cholesterol-loaded microsomes from transfected cells containing either ACAT1 or ACAT2 exhibited significantly more ACAT activity than their sitosterol-loaded counterparts. In sitosterol-loaded microsomes, both ACAT1 and ACAT2 were able to esterify sitosterol albeit with lower efficiencies than cholesterol. The mass ratios of cholesterol ester to sitosterol ester formed by ACAT1 and ACAT2 were 1.6 and 7.2, respectively. Compared with ACAT1, ACAT2 selectively esterified cholesterol even when sitosterol was loaded into the microsomes. To further characterize the difference in sterol specificity, ACAT1 and ACAT2 were compared in intact cells loaded with either cholesterol or sitosterol. Despite a lower level of ACAT activity, the ACAT1-expressing cells esterified 4-fold more sitosterol than the ACAT2 cells. The data showed that compared with ACAT1, ACAT2 displayed significantly greater selectivity for cholesterol compared with sitosterol. The plasma cholesterol esterification enzyme lecithin:cholesterol acyltransferase was also compared. With recombinant high density lipoprotein particles, the esterification rate of cholesterol by LCAT was only 15% greater than the ACAT2 cells. The data showed that compared with ACAT1, ACAT2 displayed significantly greater selectivity for cholesterol compared with sitosterol. The plasma cholesterol esterification enzyme lecithin:cholesterol acyltransferase was also compared. With recombinant high density lipoprotein particles, the esterification rate of cholesterol by LCAT was only 15% greater than sitosterol. Thus, LCAT was able to efficiently esterify both cholesterol and sitosterol. In contrast, ACAT2 demonstrated a strong preference for cholesterol rather than sitosterol. This sterol selectivity by ACAT2 may reflect a role in the sorting of dietary sterols during their absorption by the intestine in vivo.

On an average day, humans will consume 300–500 mg of dietary cholesterol and through the action of the small intestine will absorb 40–50% of this sterol (1). In addition to cholesterol, 250–500 mg of plant and shellfish-derived sterols can be ingested daily and, depending upon their chemical structure, will be differentially absorbed by the intestine (2–5). The most abundant of the dietary non-cholesterol sterols is sitosterol, which comes from plant and vegetable products. Even though sitosterol only differs from cholesterol by the presence of an ethyl group at the C-24 position of the side chain, the intestine will absorb only 5% of the sitosterol consumed on a daily basis (2, 3).

The factor(s) determining the differential absorption of cholesterol and sitosterol in intestinal enterocytes is not identified. The ATP binding cassette transporters, ABCG5 and ABCG8, are one set of proteins that are essential in intestinal sterol sorting and absorption. Loss of function mutations in both alleles encoding either ABCG5 or ABCG8 results in sitosterolemia, a disorder in which sitosterol and all other dietary sterols are hyperabsorbed (6, 7). ABCG5 and ABCG8 are believed to form a heterodimer that localizes to the apical plasma membrane of the enterocyte (8) to return plant and shellfish sterols back to the lumen of the intestine.

Acyl-CoA:cholesterol O-acyltransferase 2 (ACAT2) may be another protein that facilitates differential absorption of dietary sterols. ACAT2 is highly expressed in enterocytes where it functions to esterify cholesterol for packaging, as cholesteryl esters, into the neutral lipid core of nascent chylomicrons (9, 10). ACAT2 may also aid in sorting dietary sterols in the enterocytes by discriminating among them as substrate candidates. Several in vivo studies (11–15) have reported that a substantial amount of dietary free sitosterol can be internalized by enterocytes but that the vast majority of the sitosterol is not esterified and absorbed. In addition, in vitro studies using whole cells or isolated microsomes have indicated that sitosterol is poorly esterified by ACAT (14, 16, 17). Therefore, because of its limited ability to esterify sitosterol, ACAT2 could not only limit the incorporation of sitosterol into chylomicrons but may also allow ABCG5 and ABCG8 to return the remaining free sitosterol back to the lumen of the intestine.

However, in many of the previous studies, the expression of ABCG5 and ABCG8 in the experimental models should have limited, although to an unknown extent, the access of sitosterol to ACAT2. Moreover, several of the in vitro systems likely contained not only ACAT2 but also ACAT1. Therefore, in the current study, the ability of ACAT2 to esterify sitosterol was tested in AC29 cells expressing ACAT2 exclusively but not ABCG5 and ABCG8. AC29 cells expressing only ACAT1 were compared to ascertain whether ACAT1 and ACAT2 differ in their specificity toward sitosterol. Finally, it has been reported

* This work was supported in part by National Institutes of Health Grants HL 24736, HL 49373, and HL 54176. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by National Institutes of Health Training Grant HL 07115. To whom correspondence may be addressed. Dept. of Pathology, Wake Forest University School of Medicine, Hanes Bldg., 8th Floor, Winston-Salem, NC 27157. Tel.: 336-716-2822; Fax: 336-716-6279; E-mail: rtmele@wfubmc.edu.

§ To whom correspondence may be addressed: Dept. of Pathology, Wake Forest University School of Medicine, Hanes Bldg., 8th Floor, Winston-Salem, NC 27157. Tel.: 336-716-2821; Fax: 336-716-6279; E-mail: lruedel@wfubmc.edu.

1 The abbreviations used are: ACAT, acyl-CoA:cholesterol O-acyltransferase; LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; CD, cyclodextrin; FC, free cholesterol; CE, cholesteryl ester; ANOVA, analysis of variance; POPC, 1-stearoyl-2-oleoyl-phosphatidylcholine, 2-oleoyl-1-palmitoyl.
that 60–70% of the sitosterol in the plasma of patients with sitosterolemia is esterified (18, 19). The capacity of a third cholesteryl esterifying enzyme, lecithin:cholesterol acyltransferase or LCAT, to esterify sitosterol was determined using apoA-I-POPC recombinant HDL particles containing sitosterol or cholesterol. This enzyme is present in lymph and could contribute sitosterol esters to lipoproteins during post-absorptive transport.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents used for culturing the ACAT1 and ACAT2 cells were purchased from the listed vendors: Ham’s F-12 medium with 2 mM l-glutamine, 100× minimum Eagle’s medium vitamins, 100× penicillin/streptomycin (Mediatech), heat denatured fetal bovine serum (Atlanta Biologicals), and 50 mg/ml geneticin (Invitrogen). [1-14C]Oleic acid (52.00 mCi/mmol) and unlabeled oleic acid were from PerkinElmer Life Sciences and Sigma, respectively.

**Isolation of Microsomes from ACAT1 and ACAT 2 Cells—**AC29 cells stably transfected with African green monkey ACAT1 or ACAT2 (9) were seeded into 150-mm dishes (Phoenix Research Products) containing Medium A (Ham’s F-12 with 2 mM l-glutamine, 10% heat denatured fetal bovine serum, 1% minimum Eagle’s medium vitamins, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml geneticin) and maintained in a 37 °C humidified 95% air, 5% CO2 incubator. Upon reaching confluency, the cells were washed with balanced salt solution, scraped from the plates, and pelleted by centrifugation at 1,500 x g for 5 min at room temperature. The cells were resuspended in ACAT homogenization buffer (0.25 M sucrose, 1 mM EDTA, 0.1 M K2HPO4,pH 7.4) supplemented with protease inhibitor mixture. The cell lysate was centrifuged at 16,000 x g for 30 min at 4 °C and the supernatant was transferred to a 15 × 51-mm polycarbonate tube (Beckman) and was centrifuged at 38,000 rpm for 60 min at 4 °C in a SW55Ti rotor. After removing the supernatant, the microsomal pellet was resuspended in ACAT reaction buffer (0.1 M K2HPO4, pH 7.4) supplemented with protease inhibitor mixture. The microsomal suspension was stored at −80 °C until used.

**Solubilization of Cholesterol or Sitosterol in a Hydroxypropyl-β-Cyclodextrin Solution—**A 308 mM hydroxypropyl-β-cyclodextrin (CD) (Aldrich) solution was created by dissolving CD in distilled H2O. Into a 16 × 100-mm glass tube containing 5 ml of CD solution was placed either 8 mg of free cholesterol (NU Chek) or 10 mg sitosterol (ICN). To facilitate the solubilization of the sterol, the solutions were incubated at 60 °C and periodically vortexed. In addition, the sterol-containing solution was sonicated. After cooling to room temperature, the solutions were passed through a syringe filter with a 0.45-μm HT Tuffryn membrane (Gelman) and stored at 4 °C in 16 × 100-mm glass tubes. Using the Cholesterol/High Density Lipoprotein (Roche Diagnostic GmbH), the sterol concentrations of the cholesterol and sitosterol solutions were determined to be 4.9 and 4.6 nmol/μl, respectively. The molar ratios of CD to sterol for the cholesterol and sitosterol solutions were ~63 and 67, respectively.

**ACAT Activity in Microsomes Loaded with Cholesterol or Sitosterol—**[7-n]-1HCholesterol (Amersham Biosciences) in toluene or [22,23-3H]sitosterol (PerkinElmer Life Sciences) in ethanol was dried in a 16 × 100-mm glass tube under N2 gas. The radiolabeled sterol was redissolved in ethanol and subsequently mixed with ACAT reaction buffer containing fatty acid-free human serum albumin (Miles). This solution was placed into a 16 × 100-mm glass tube containing either 100 μg of ACAT1 or 50 μg of ACAT2 microsomes. Cholesterol-CD, sitosterol-CD, or an equimolar mix of cholesterol and sitosterol (1:1 molar ratio) was added to the tube such that the final concentration of the exogenous sterol was 0.5 nmol/μg microsomal protein. The final volumes for the ACAT1 and ACAT2 reactions were 300 and 150 μl, respectively, whereas the final concentration of human serum albumin was 3.3 mg/ml, ethanol was 1.3% (v/v), and [3H]sterol was 1400–3000 dpm/μl. The tube containing the ACAT reaction mixture was incubated for 30 min in a shaking water bath set at 37 °C. After adding 30 nmol of [1-14C]oleoyl CoA (7885 dpm/nmol) (Amersham Biosciences), the tube was vortexed and placed back into the water bath for 5 min. To terminate the ACAT reaction, 6 ml of CHCl3/CH3OH (2:1) was added to the tube. Following incubation at room temperature for 1 h, 1.2 ml of 0.88% KCl was added to the tube, and the phases were allowed to separate overnight at 4 °C. After centrifugation at 1,500 x g for 5 min at 4 °C, the upper, aqueous phase was aspirated from the tube, and 3 ml of the lower, organic phase was removed and dried at 60 °C under N2 gas. The lipid was resuspended in CHCl3 containing 1 mg/ml free cholesterol and 1 mg/ml cholesterol oleate, was applied to a silica Gel 60 thin layer chromatography plate (EM Science), and was separated using 70:30:1 hexane/ethyl ether/acetic acid as the running solvent. The separated lipids were visualized using L2 vapor, and the FC and CE bands were scraped from the plate into scintillation vials. To the vials was added 4 ml of Bio-Safe II scintillation fluid (Research Products International). The free sterol band was subjected to scintillation counting to determine [3H]sterol content, and the CE band was counted for both [3H]sterol and [3H]oleate.

**Determination of the Specific Activity of the [3H]Cholesterol and [3H]Sitosterol in the microsomes, ACAT1 and ACAT2 microsomes were sterol-loaded as described above. After the incubation at 37 °C, the microsomes were transferred to a 15 × 51-mm polycarbonate tube and were centrifuged at 100,000 rpm in a TLA 100.3 rotor for 10 min at 20 °C. After removing the supernatant, the pellet was washed three times with 0.083 M EDTA, 0.74 M NaCl, pH 7.4, suspended in 1% SDS, 0.1 M NaOH. The protein concentration of the sample was determined by modified Lowry assay, and the [3H]sterol content was determined by scintillation counting. In addition, a Bligh-Dyer extraction was carried out on the microsomal lysates in the presence of 10 μg of 5 α-cholestan as an internal standard. Analysis of the extracted free sterol was conducted by injecting the sample on a ZS50 (0.53-mm inner diameter × 15 m × 1 μm) gas-liquid chromatography column (Phenomenex) at 250 °C and installed in a Hewlett-Packard 5890 gas chromatograph equipped with a Hewlett-Packard 7673A automatic injector using on-column injection and a flame ionization detector.

**Esterification by ACAT1 or ACAT2 Cells of Cholesterol or Sitosterol Delivered by Cyclodextrin—**On day 0, 0.75 × 106 ACAT2 cells were seeded into a 6-well cluster (Costar) containing Medium A. On day 1, [3H]cholesterol (10–20 μCi) and [3H]sitosterol (25–50 μCi) were dried in glass vials to which were then added cholesterol-CD and sitosterol-CD, respectively. On day 2, the cholesterol-CD and sitosterol-CD was added to pre-heated medium A (37 °C) such that the final concentration of the CD was 2 mM, and the exogenous sterol was ~30 nmol/ml. After allowing the medium plus sterol-CD to incubate at 37 °C for 1 h, the medium was passed through a sterile syringe filter with a 0.22-μm polyvinylidene difluoride Durapore membrane (Millipore). The specific activity of the [3H]sterol in the filtered medium was calculated by determining [3H]sterol concentration via scintillation spectroscopy and the total sterol concentration by gas-liquid chromatography for total sterol mass. The difference between free and total sterol was multiplied by 1.67 to calculate the esterified mass.

**Creation of POPC:apoA-I Recombinant HDL Particles Containing Cholesterol or Sitosterol—**POPC: cholesterol (or sitosterol):human apoA-I recombinant HDL particles with a 80:5:1 molar ratio were created as described previously (20). A slightly modified protocol was used to make recombinant HDL with an 80:15:1 molar ratio. Briefly, 12 ml Pyrex tube was placed 13 mg of POPC (Sigma), 1.24 mg of cholesterol or 1.33 mg of sitosterol (all lipids were dissolved in CHCl3, at 1 mg/ml). In addition, 65 μCi of [3H]cholesterol or 100 μCi of [3H]sitosterol was added to the appropriate tube. After evaporating the CHCl3 under N2 at 60 °C, the tube was covered with paraffin, and its contents were lyophilized for 2 h. To the tube was added 118 μl of cholesterol (212 mg/ml) of 600 μl of disc buffer (10 mM NaCl, 0.27 mM EDTA, pH 7.4), which created a 5:1 molar ratio of sodium cholate:POPC. After vortexing the tube, it was flushed with argon and placed in a 37 °C shaking water bath for 20 min. To the tube was added 2.9 ml of human apoA-I (2.084 mg/ml) and 400 μl of 10% disc buffer. The tube was vortexed, flushed with argon, and placed in a 37 °C water bath for 30 min. The recombinant HDL particles were dialyzed six times against 1 liter of argon purged disc buffer. The protein concentration of the HDL was determined using modified Lowry assay, and the sterol concentration was determined using the Cholesterol-HPLC reagent.
Esterification of Sitosterol by ACAT2

To determine the diameters of the recombinant HDL, a volume of HDL containing 5 μg of apoA-I was diluted with 5× loading buffer (0.1% bromphenol blue, 40% sucrose). The HDL particles along with high molecular weight calibration proteins (Amersham Biosciences) were separated on a 4–30% non-denaturing gradient gel. After a pre-run at 125 V for 30 min, the samples were loaded onto the gel and run at 70 V for 20 min and then at 150 V for 20 h. The gel was washed for 5 min with water, and the apoA-I on the HDL was visualized using GelCode blue stain reagent (Pierce).

**Assay of LCAT Esterification of Cholesterol or Sitosterol**—Using an established protocol, an exogenous LCAT assay was conducted using purified His-tagged human LCAT and the POPC:cholesterol (or sitosterol):human apoA-I recombinant HDL (21, 22). In one assay utilizing only the 80:5:1 recombinant particles, 3 nmol of HDL sterol was present in each reaction. In a second assay, employing both the 80:5:1 and 80:15:1 recombinant HDL, each reaction contained 17.5 μg of HDL apoA-I and the following amount of sterol: 80:5:1 HDL, 3.4 nmol of cholesterol, and 2.9 nmol of sitosterol; 80:15:1 HDL, 9.5 nmol of cholesterol, and 8.6 nmol of sitosterol.

**Statistical Evaluations**—Using the StatView statistical package (version 5.0; SAS Institute Incorporate), statistically significant differences were determined by either unpaired t test or ANOVA with post hoc analysis by Fisher’s protected least significant difference test. Statistical significance was considered at p ≤ 0.05. Statistically significant differences among the analyzed values are denoted by different letters.

**RESULTS**

**Esterification of Sitosterol by ACAT1 or ACAT2 in Microsomes**—To determine whether ACAT1 and ACAT2 differ in their ability to esterify sitosterol, AC29 cells stably expressing African green monkey ACAT1 or ACAT2 were employed (9). These cell lines have no endogenous ACAT protein expression or activity (23, 24), and their use allows ACAT1 and ACAT2 to be studied separately.

For the initial experiments, microsomes isolated from the ACAT1 and ACAT2 cells were incubated with cholesterol or sitosterol solubilized in hydroxypropyl-β-cyclodextrin compared with buffer alone. The sterol concentrations in the microsomes were determined by ANOVA with post hoc analysis and are indicated by different superscript letters (p < 0.03).

**TABLE I**

| Microsome type | Incubation conditions | mmol cholesterol/ mg protein | mmol sitosterol/ mg protein | mmol total sterol/mg protein |
|----------------|-----------------------|-----------------------------|-----------------------------|-----------------------------|
| ACAT1 Buffer only | 201 (10)        | 201                         | 201                         | 201                         |
| ACAT1 Cholesterol-CD | 452 (20)    | 452                         | 452                         | 452                         |
| ACAT1 Sitosterol-CD | 169 (4)       | 169                         | 169                         | 169                         |
| ACAT2 Buffer only | 168 (4)        | 168                         | 168                         | 168                         |
| ACAT2 Cholesterol-CD | 443 (16)    | 443                         | 443                         | 443                         |
| ACAT2 Sitosterol-CD | 131 (3)       | 131                         | 131                         | 131                         |

**Notes:**
- ACAT1 and ACAT2 were expressed in microsomes isolated from ACAT1 or ACAT2 transfected cells.
- ACAT1 and ACAT2 expressed in microsomes isolated from ACAT1 or ACAT2 transfected cells.
- The ACAT activity in the microsomes was measured by incorporation of [14C]oleoyl CoA into [14C]steroyl ester (1). For ACAT1 microsomes, pre-incubation with cholesterol-cyclodextrin increased the activity decreases (5-50) 0.18 versus 0.87 nmol/min/mg protein. The microsomes pre-incubated with sitosterol-cyclodextrin showed a doubling of ACAT1 activity even though the cholesterol concentration was decreased from 201 to 169 nmol/mg protein. For ACAT2 microsomes, pre-incubation with cholesterol-cyclodextrin increased the activity almost 39-fold (0.68 to 26.4 nmol/min/mg protein). Following enrichment of the membrane with sitosterol, the ACAT2 activity was increased by over 6-fold (0.68 to 4.65 nmol/min/mg protein), despite the fact that the microsomal cholesterol concentration was decreased from 168 to 131 nmol/mg protein. Compared with the ACAT1 microsomes with comparable sterol concentrations, the ACAT2 microsomes consistently displayed significantly higher activity; 4-fold with buffer only (0.18 versus 0.68 nmol/min/mg protein), 30-fold with cholesterol-cyclodextrin (0.87 versus 26.4 nmol/min/mg protein), and 12-fold with sitosterol-cyclodextrin (0.40 versus 4.65 nmol/min/mg protein). Higher ACAT2 activity in transfected cell lines has been a consistent observation.

For comparison, sitosterol and cholesterol loaded microsomes were then incubated with tracer amounts of [3H]cholesterol or [3H]sitosterol. The inclusion of the radiolabeled sterols allowed direct assessment of the esterification efficiency of both sterols. For ACAT1 microsomes, the results were similar to those seen with the [14C]oleoyl CoA substrate (Fig. 2A). In the buffer control microsomes, the rate of [3H]cholesteryl ester formation was 2.6-fold less than the rate in microsomes loaded with cholesterol (0.49 versus 1.28 nmol/min/mg protein). After loading the microsomes with sitosterol, the esterification rates of cholesterol and sitosterol were 0.29 and 0.18 nmol/min/mg protein, so that the total sterol esterification rate of 0.47 nmol/min/mg protein was essentially equivalent to that in the non-loaded state.

For the ACAT2 microsomes, the activities were again much higher than was seen with the ACAT1 microsomes, a difference similar to what was observed when [14C]oleoyl CoA was used as substrate (Fig. 2B). The rate of [3H]cholesterol esterification was some 18-fold higher in cholesterol-loaded microsomes compared with the buffer-treated control (36.5 versus 1.97 nmol/min/mg protein). When the ACAT2 microsomes were loaded with sitosterol, the total esterification rate was 5.38 nmol/min/mg protein, with only 0.66 nmol/min/mg protein of [3H]sitosteryl ester formation included in this number whereas the remainder, 4.72 nmol/min/mg protein, was [3H]cholesteryl ester formation.

The data showed that when loaded into microsomes, sitosterol was esterified by both ACAT1 and ACAT2, albeit at a reduced efficiency compared with cholesterol (Fig. 2). In addition, the two ACATs in sitosterol-loaded microsomes displayed differing sterol substrate specificities. Although the rate of [3H]sitosteryl ester formation by ACAT2 microsomes was higher overall than for ACAT1 microsomes (0.66 versus 0.18 nmol/min/mg), the ratio of cholesterol to sitosterol products must be the benchmark for interpretation because of the much higher ACAT2 activities achieved in transfected cell lines. The ratio of cholesteryl ester to sitosteryl ester formed by ACAT1 and ACAT2 was 1.6 and 7.2, respectively (Fig. 2). In other words, the efficiency of ACAT1 for cholesterol esterification was modestly greater than for sitosterol esterification. In contrast, ACAT2 showed a much higher efficiency for esterification of cholesterol compared with sitosterol. The results demonstrate that ACAT1 and ACAT2 have distinctly different preferences for individual sterol substrates.

**Esterification of Sitosterol by ACAT1 and ACAT2 Expressed in Intact Cells**—After finding that ACAT1 and ACAT2 in microsomes varied in their capacity to esterify sitosterol, the sterol specificity of the enzymes was studied in intact cells. To facilitate sterol loading of the cells, serum containing tissue culture medium (complete medium) was supplemented with
cholesterol-cyclodextrin or sitosterol-cyclodextrin and trace amounts of [3H]cholesterol or [3H]sitosterol. The medium was placed onto the ACAT1 and ACAT2 cells for 24 h, and during the last 2 h of the incubation, the cells were pulsed with [14C]oleate conjugated to albumin. As determined by [14C]oleate incorporation into [14C] steryl ester (Fig. 3), the ACAT activities of ACAT1 cells exposed to complete medium plus or minus sterol-cyclodextrin were similar (±5.5 nmol/h/mg protein). ACAT2 cells incubated with sitosterol-cyclodextrin had the highest activity (12 nmol/h/mg protein) whereas ACAT2 cells exposed to complete medium or cholesterol-cyclodextrin had significantly lower activities (10 and 8.8 nmol/h/mg protein). These results showed that ACAT2 cells consistently had more ACAT activity than ACAT1 cells and that incubation of cells with sitosterol-cyclodextrin significantly increased ACAT2 activity.

At the same time, the sterol mass of the ACAT1 and ACAT2 cells was determined (Table II). ACAT1 cells treated with complete medium or sitosterol-cyclodextrin had similar free cholesterol and cholesteryl ester contents (73.0 versus 75.1 nmol FC/mg protein and 280 versus 275 nmol CE/mg protein) whereas the cells exposed to cholesterol-cyclodextrin showed a significant increase in free cholesterol and cholesteryl ester mass (89.5 nmol FC/mg protein and 325 nmol CE/mg protein). ACAT2 cells exposed to complete medium or sitosterol-cyclodextrin had less free cholesterol (62.8 versus 60.6 nmol FC/mg protein) and more cholesteryl ester (325 versus 317 nmol CE/mg protein) than ACAT1 cells, and although these values were significantly increased in ACAT2 cells treated with cholesterol-cyclodextrin (67.1 nmol FC/mg protein and 405 nmol CE/mg protein), they were still significantly lower and higher, respectively, than in ACAT1 cells.
Incubation of cells with sitosterol-cyclodextrin left the free cholesterol and cholesteryl ester concentrations unchanged from those in cells incubated in complete medium. The differences in the free and esterified sitosterol content of the ACAT1 and ACAT2 cells were contrary to the free cholesterol and cholesteryl ester mass of these cells. ACAT2 cells had significantly more free sitosterol (29 versus 22.5 nmol/mg protein) and significantly less cholesteryl ester (4.3 versus 21.4 nmol/mg protein) than ACAT1 cells. Loading either cell line with sitosterol did not appear to alter the handling of cholesterol, rather the sitosterol appeared to be processed in a unique fashion.

In addition to measuring the mass of steryl esters in the ACAT1 and ACAT2 cells, the amounts of cholesteryl ester and sitosteryl ester formed by the cells were calculated from the isotopic labeling with [3H]cholesterol and [3H]sitosterol (Fig. 4). Based upon the specific activity of the [3H]cholesterol in the cholesteryl-cyclodextrin containing medium, the ACAT1 cells internalized 187 nmol of total cholesterol/mg cell protein and converted 74% (139 nmol/mg cell protein) of it to cholesteryl ester (Fig. 4A). For the ACAT2 cells, 193 nmol of medium-derived cholesteryl/mg cell protein was present in the cells with 85% (164 nmol/mg cell protein) of it in an esterified form. Although the calculated amounts of sterol were not the same as for the sterol mass data (Table II), the trend for relative amounts of [3H]cholesterol and [3H]cholesteryl ester in the ACAT1 and ACAT2 cells was the same as for the mass data and consistent with the observation that the ACAT2 cells had higher ACAT activity.

Under conditions where the cells were exposed to medium supplemented with sitosterol-cyclodextrin, the ACAT1 cells accumulated significantly less free sitosterol than the ACAT2 cells (19.9 versus 24.4 nmol/mg cell protein) (Fig. 4B). In contrast, the ACAT1 cells esterified 43% of the total sitosterol compared with only 15% for the ACAT2 cells (15.1 versus 4.3 nmol/mg cell protein). Again, the same trend occurred in the mass data of Table II. Moreover, when the ratio of cellular steryl ester content to ACAT activity (Table III) was considered, the esterification efficiency of sitosterol was 9- to 12-fold higher for ACAT1 compared with ACAT2. From this outcome, which is similar to that seen in the assays with microsomes (Fig. 2), it could be concluded that compared with ACAT1, ACAT2 within intact cells more readily distinguishes sitosterol from cholesterol and produces significantly less esterified sitosterol product.

**Esterification of Sitosterol by LCAT**—Using recombinant HDL particles that had either an 80:15:1 or 80:5:1 molar ratio of POPC:cholesterol (or sitosterol):human apoA-I, the sterol specificity of LCAT was tested. Analysis of the particles by non-denaturing gel electrophoresis showed that the cholesterol and sitosterol containing HDL with the same constituent molar ratios had similar diameters and particle subpopulations (Fig. 5A). The rate of cholesterol esterification by LCAT was similar when either the 80:15:1 or 80:5:1 recombinant HDL were used as substrate (420 versus 402 nmol/min/mg LCAT) (Fig. 5B). Likewise LCAT esterified the sitosterol on both the 80:15:1 or 80:5:1 HDL at nearly identical rates (316 versus 336 nmol/min/mg LCAT). Comparison of the rates of esterification of the two sterols indicated that LCAT esterifies cholesterol 15% more efficiently than sitosterol.

**DISCUSSION**

The purpose of this study was to compare the efficiency with which the three known cholesterol esterification enzymes, namely ACAT1, ACAT2, and LCAT, esterify sitosterol versus cholesterol. As expected, all three enzymes esterified cholesterol with a greater efficiency than sitosterol; however, their selectivity for the two sterols varied considerably. In microsomes loaded with equal concentrations of sitosterol, ACAT2 esterified 7.2-fold more endogenous cholesterol than sitosterol whereas ACAT1 esterified only 1.6-fold more cholesterol than sitosterol. When whole cells were incubated with sitosterol-containing medium, 43% of the internalized sitosterol was esterified by ACAT1 whereas only 15% of it was esterified by ACAT2. In comparison to the cellular sterol esterifying enzymes, the capacity of LCAT to esterify sitosterol on apoA-I-POPC recombinant HDL particles was 85% of that shown for cholesterol. From these results it can be concluded that compared with ACAT1, ACAT2 can more readily distinguish and limit the esterification of sitosterol. On the other hand, the sterol substrate specificity of LCAT appears to be limited allowing both cholesterol and sitosterol on recombinant HDL to be esterified at similar rates.

The novel findings of this study were that although both ACAT1 and ACAT2 were able to esterify sitosterol, ACAT2 was much less efficient at esterifying this plant sterol. Using more complex biological systems, many previous studies have examined the esterification of sitosterol by ACATs. By feeding animals diets enriched in sitosterol, several *in vivo* studies have shown that the small intestinal mucosal cell, which predominantly expresses ACAT2 (9), could accumulate a significant amount of free sitosterol but that very little of this plant sterol was esterified and subsequently absorbed (11–15). In addition, microsomes isolated from rat liver and rabbit intestine, which likely contain both ACAT1 and ACAT2, esterified sitosterol 20- to 60-fold less efficiently than cholesterol (14, 16). Although consistent with the data from the current study concerning ACAT2, the limited cellular esterification of sitosterol observed in the earlier studies cannot be definitively ascribed to either ACAT2 or ACAT1 expression.

Handling of sitosterol and cholesterol was examined in microsomal preparations isolated from both ACAT1 and ACAT2 cells. The sitosterol to cholesterol ratio in microsomes from ACAT1 cells was 0.58, which was higher than the sitosterol to cholesterol ratio in microsomes from ACAT2 cells, which was 0.43 (Table I). This different ratio was because of the fact that there was less cholesterol in the microsomes from the ACAT2 cells, whereas equivalent amounts of sitosterol were present in
The differences for free sitosterol and sitosteryl ester were determined by unpaired t test for 24 h in complete medium plus [3H]cholesterol-cyclodextrin (110 nmol cholesterol/ml) ([3H]sitosterol in the medium. Each column represents mean ± S.E.) of six samples from two experiments. Values for free cholesterol and cholesteryl ester were analyzed by ANOVA with post hoc analysis, and different superscript letters indicate statistically significant differences (p < 0.05). Statistically significant differences for free sitosterol and sitostery ester were determined by unpaired t test and are indicated by different superscript letters (p < 0.001).

Table II
Sterol content of ACAT1 or ACAT2 cells exposed to complete medium plus or minus cholesterol or sitosterol suspended in cyclodextrin

The values represent the mean (±S.E.) of six samples from two experiments. Values for free cholesterol and cholesteryl ester were analyzed by ANOVA with post hoc analysis, and different superscript letters indicate statistically significant differences (p < 0.05). Statistically significant differences for free sitosterol and sitostery ester were determined by unpaired t test and are indicated by different superscript letters (p < 0.001).

Table III
Esterification efficiency of cellular ACAT1 and ACAT2 for sitosterol

The values represent the mean (±S.E.) of six samples from two experiments. *, ACAT activity based upon [14C]oleate incorporation into [14C]cholesterol oleate. #, sitosteryl ester content of the cells measured by gas-liquid chromatography. *, sitosteryl ester content of the cells based upon conversion of [3H]sitosterol to [3H]sitosteryl ester.

ACAT2 activity. At the same time, the concentration of free sitosterol was higher, and the concentration of sitostery ester was lower in the sitosterol-loaded ACAT2 versus ACAT1 cells, suggesting free sitosterol was not efficiently converted into sitosteryl ester by ACAT2. The small decrease in oleate incorporation into steryl esters when cholesterol in cyclodextrin was incubated with the ACAT2 cells (Fig. 3) may reflect the fact that significantly more CE has already been accumulated in cholesterol-loaded ACAT2 cells, and the equilibrium may have shifted such that additional CE formation may have been down-regulated during the 22–24-h period of the incubation with labeled oleate. This situation did not pertain when sitosterol in cyclodextrin was incubated with cells, because CE accumulation was limited.

Significantly greater ACAT activity and cholesterol esterification were observed in microsomes and whole cells containing ACAT2 compared with ACAT1. However, under conditions of sitosterol loading, ACAT2 esterified sitosterol much less efficiently than ACAT1. From these results, it can be suggested that ACAT2, the main ACAT enzyme in enterocytes, would not readily esterify sitosterol thus limiting its incorporation into chylomicrons and its subsequent absorption into the body. However, any absorbed sitosterol that is internalized by peripheral tissues expressing ACAT1, such as macrophage and adrenal, may be esterified without difficulty. Although it is recognized that tissue culture cells may not always reflect the physiologic systems of the whole body, the consistencies between the data here with the observations in the literature suggest that our findings are relevant.

Besides the difference in steryl specificity displayed by ACAT1 and ACAT2, a consistent finding from this study was that cellular expression of ACAT2, compared with that of both. The difference in free cholesterol concentrations of ACAT1 and ACAT2 microsomes suggests that the higher endogenous amounts of ACAT activity in the ACAT2 cells are sufficient to convert more of the cholesterol into the esterified form thereby reducing the concentration of free cholesterol in the membrane. In contrast, ACAT2 could not effectively reduce the free sitosterol concentration.

In addition to analysis of microsomes from ACAT1 and ACAT2 cell lines, studies using intact cells were performed. The data in Fig. 3 show that ACAT2 cells have a higher total ACAT activity than ACAT1 cells when measured by oleate incorporation into steryl esters at the end of a 24-h incubation with or without sterol-cyclodextrin preparations. When the data in Table II are taken into account, the indications are that for each of the three conditions of these experiments, ACAT2 cells have lower free cholesterol and higher CE concentrations than ACAT1 cells, presumably reflecting a new equilibrium established during the 24-h incubation because of higher ACAT2 activity. At the same time, the concentration of free sitosterol was higher, and the concentration of sitostery ester was lower in the sitosterol-loaded ACAT2 versus ACAT1 cells, suggesting free sitosterol was not efficiently converted into sitosteryl ester by ACAT2. The small decrease in oleate incorporation into steryl esters when cholesterol in cyclodextrin was incubated with the ACAT2 cells (Fig. 3) may reflect the fact that significantly more CE has already been accumulated in cholesterol-loaded ACAT2 cells, and the equilibrium may have shifted such that additional CE formation may have been down-regulated during the 22–24-h period of the incubation with labeled oleate. This situation did not pertain when sitosterol in cyclodextrin was incubated with cells, because CE accumulation was limited.

Significantly greater ACAT activity and cholesterol esterification were observed in microsomes and whole cells containing ACAT2 compared with ACAT1. However, under conditions of sitosterol loading, ACAT2 esterified sitosterol much less efficiently than ACAT1. From these results, it can be suggested that ACAT2, the main ACAT enzyme in enterocytes, would not readily esterify sitosterol thus limiting its incorporation into chylomicrons and its subsequent absorption into the body. However, any absorbed sitosterol that is internalized by peripheral tissues expressing ACAT1, such as macrophage and adrenal, may be esterified without difficulty. Although it is recognized that tissue culture cells may not always reflect the physiologic systems of the whole body, the consistencies between the data here with the observations in the literature suggest that our findings are relevant.

Besides the difference in steryl specificity displayed by ACAT1 and ACAT2, a consistent finding from this study was that cellular expression of ACAT2, compared with that of ACAT2 activity. At the same time, the concentration of free sitosterol was higher, and the concentration of sitostery ester was lower in the sitosterol-loaded ACAT2 versus ACAT1 cells, suggesting free sitosterol was not efficiently converted into sitosteryl ester by ACAT2. The small decrease in oleate incorporation into steryl esters when cholesterol in cyclodextrin was incubated with the ACAT2 cells (Fig. 3) may reflect the fact that significantly more CE has already been accumulated in cholesterol-loaded ACAT2 cells, and the equilibrium may have shifted such that additional CE formation may have been down-regulated during the 22–24-h period of the incubation with labeled oleate. This situation did not pertain when sitosterol in cyclodextrin was incubated with cells, because CE accumulation was limited.

Significantly greater ACAT activity and cholesterol esterification were observed in microsomes and whole cells containing ACAT2 compared with ACAT1. However, under conditions of sitosterol loading, ACAT2 esterified sitosterol much less efficiently than ACAT1. From these results, it can be suggested that ACAT2, the main ACAT enzyme in enterocytes, would not readily esterify sitosterol thus limiting its incorporation into chylomicrons and its subsequent absorption into the body. However, any absorbed sitosterol that is internalized by peripheral tissues expressing ACAT1, such as macrophage and adrenal, may be esterified without difficulty. Although it is recognized that tissue culture cells may not always reflect the physiologic systems of the whole body, the consistencies between the data here with the observations in the literature suggest that our findings are relevant.

Besides the difference in steryl specificity displayed by ACAT1 and ACAT2, a consistent finding from this study was that cellular expression of ACAT2, compared with that of
ACAT1, led to significantly greater levels of cholesterol esterification. The esterification rate of cholesterol by the ACAT2-containing microsomes compared with the ACAT1-containing microsomes was 30-fold greater under cholesterol-loaded conditions (see Figs. 1 and 2). In whole cells, the steady-state mass of cholesterol ester was 15% greater (Table II), and the rate of sterol esterification from oleate incorporation into sterol esters was 2-fold higher in cells expressing ACAT2 compared with ACAT1. The difference in ACAT activity in the ACAT1- and ACAT2-expressing cells may be because of several factors. One possibility is that on a per molecule basis ACAT2 is more efficient at esterifying cholesterol than ACAT1. However, because both enzymes have very similar amino acid sequences, and no method is currently available to directly compare purified ACAT1 and ACAT2, the specific activity of the two enzymes could not be determined. A more likely explanation for the difference in cellular ACAT activity is the more rapid turnover of ACAT1 versus ACAT2 protein. Metabolic labeling of ACAT1 and ACAT2 cells with [35S]Met/Cys followed by immunoprecipitation of the two enzymes showed that the half-lives of ACAT1 and ACAT2 protein were ~30 min and 6 h, respectively (data not shown). This difference in protein stability likely results in fewer ACAT1 molecules and lower activity in ACAT1- compared with ACAT2-expressing cells. Further studies will be needed to determine the exact basis for the difference in ACAT1 and ACAT2 stability.

As expected from the results of previous studies (14, 25–27), significant increases in ACAT1 and ACAT2 activity were observed after loading microsomes with cholesterol (see Figs. 1 and 2). This stimulatory effect of cholesterol may be attributed not only to an increase in substrate availability but also to the ability of cholesterol to act as an allosteric activator of ACAT (28). Unexpectedly, the current results support the possibility that sitosterol may also activate ACAT2 through an allosteric mechanism. Even though sitosterol loading resulted in a 20% decrease in microsomal cholesterol content (Table I), 2.4-fold more cholesteryl ester was produced in the sitosterol-loaded versus buffer-treated ACAT2 microsomes (Fig. 2B). This result indicates that sitosterol may activate ACAT2 thereby allowing the endogenous cholesterol pool to be esterified more efficiently.

Similar to ACAT1 and ACAT2, LCAT was found to preferentially esterify cholesterol compared with sitosterol when the sterols were incorporated into POPC:apoA-I recombinant HDL particles. However, the esterification rate of the two sterols by LCAT differed by only 15%. These results are in agreement with a previous study (29) showing that in the presence of apoA-I, LCAT esterified sitosterol that was incorporated into phospholipid vesicles at a rate that was 80% of that observed for cholesterol. In contrast, another study (30) determined that the esterification rate of sitosterol associated with all lipoprotein species in whole plasma was only 40% of that seen for cholesterol. The inconsistency of these findings is likely because of the varying capacity of LCAT to esterify sitosterol on different classes of lipoproteins. It has been reported that in patients with sitosterolemia, 14–65% of the sitosterol and 70–74% of the cholesterol was esterified in LDL. In contrast, ~80% of the not only the cholesterol but also the sitosterol was esterified in the HDL isolated from these individuals (18, 19). Taking all of the findings into consideration, it seems appropriate to conclude that the ability of LCAT to distinguish sitosterol from cholesterol is limited and should not be confused with the more limited activity of LCAT on apoB-containing lipoproteins.

In summary, the current study has shown that ACAT1, ACAT2, and LCAT can all esterify sitosterol. However, ACAT2 displayed the greatest capacity to distinguish cholesterol from sitosterol and limit sitosterol esterification. The sterol specificity of ACAT2 may reflect an in vivo role in limiting the absorption of sitosterol and other plant and shellfish sterols. Future studies are needed to elucidate the physiological significance of these findings.
REFERENCES
1. Wilson, M. D., and Rudel, L. L. (1994) J. Lipid Res. 35, 943–955
2. Heinemann, T., Axmann, G., and von Bergmann, K. (1995) Eur. J. Clin.
   Invest. 23, 827–831
3. Salen, G., Ahrens, E. H., Jr., and Grundy, S. M. (1970) J. Clin. Invest.
   49, 952–967
4. Conner, W. E., and Lin, D. S. (1981) Gastroenterology 81, 276–284
5. Gregg, R. E., Connor, W. E., Lin, D. S., and Brewer, H. B., Jr. (1986)
   J. Clin. Invest. 77, 1864–1872
6. Berge, K. E., Tian, H., Graf, G. A., Yu, L., Grishin, N. V., Schultz, J., Kwiterov-
   ich, P., Shan, B., Barnes, R., and Hobbs, H. H. (2000) Science 290,
   1771–1775
7. Lee, M. H., Lu, K., Hazard, S., Yu, H. W., Shulenin, S., Hidaka, H., Kojima, H.,
   Alkilnets, R., Sakuma, N., Pegoara, R., Srivastava, A. K., Salen, G., Dean,
   M., and Patel, S. B. (2001) Nat. Genet. 27, 79–83
8. Graf, G. A., Li, W.-P., Gerard, R. D., Gelissen, I., White, A., Cohen, J. C., and
   Hobbs, H. H. (2002) J. Clin. Invest. 110, 659–669
9. Lee, R. G., Willingham, M. C., Davis, M. A., Skinner, K. A., and Rudel, L. L.
   (2000) J. Lipid Res. 41, 1991–2001
10. Buhman, K. R., Accad, M., Nowak, S., Choi, R. S., Wang, J. S., Hamilton, R. L.,
     Turley, S., and Farese, R. V., Jr. (2000) Nat. Med. 6, 1341–1347
11. Swell, L., Trout, E. C., Jr., Field, H., Jr., and Treadwell, C. R. (1959) Proc. Soc.
     Exp. Biol. Med. 104–142
12. Bhattacharyya, A. K., and Lopez, L. A. (1979) Biochim. Biophys. Acta. 574,
     146–153
13. Bhattacharyya, A. K. (1981) Am. J. Physiol. 240, G50-G55
14. Field, F. J., and Mathur, S. N. (1983) J. Lipid Res. 24, 409–417
15. Ikeda, I., Tanaka, K., Sugano, M., Vahouny, G. V., and Gallo, L. L. (1988) J.
     Lipid Res. 29, 1583–1591
16. Tavani, D. M., Nes, W. R., and Bilheimer, J. T. (1982) J. Lipid Res. 23,
     774–781
17. Field, F. J., Born, E., and Mathur, S. N. (1997) J. Lipid Res. 38, 348–360
18. Salen, G., Kwiterovich, P. O., Jr., Sheler, S., Tint, G. S., Horak, I., Shore, V.,
     Dayal, R., and Horak R. (1986) J. Lipid Res. 26, 203–209
19. Miettinen, T. A. (1980) Eur. J. Clin. Invest. 10, 27–35
20. Matsu, C. E., and Jonas, A. (1982) J. Biol. Chem. 257, 4535–4540
21. Parks, J. S., Gebre, A. K., and Parbee, J. W. (1999) in Lipase and Phospho-
     lipase Protocols (Dolett, M., and Reue, K., eds) pp. 123–131, Humana
     Press, Totawa, NJ
22. Chisholm, J. W., Gebre, A. K., and Parks, J. S. (1999) J. Lipid Res. 40,
     1512–1519
23. Cadigan, K. M., Heider, J. G., and Chang, T.-Y. (1988) J. Biol. Chem. 263,
     274–282
24. Chang, C. C. Y., Chen, J., Thomas, M. A., Cheng, D., Del Priore, V. A., Newton,
     R. S., Pape, M. E., and Chang, T. Y. (1995) J. Biol. Chem. 270, 29532–29540
25. Goldstein, J. L., Dana, S. E., and Brown, M. S. (1974) Proc. Natl. Acad. Sci.
     U. S. A. 71, 4298–4292
26. Dosolittle, M. G., and Chang, T. Y. (1982) Biochim. Biophys. Acta. 713,
     529–537
27. Field, E. J., Albright, E., and Mathur, S. N. (1987) J. Lipid Res. 28, 1057–1066
28. Chang, C. C. Y., Lee, C.-Y. C., Chang, E. T., Cruz, J. C., Levesque, M. C., and
     Chang, T.-Y. (1998) J. Biol. Chem. 273, 35132–35141
29. Piran, U., and Nishida, T. (1979) Lipids 14, 478–482
30. Nordby, G., and Norum, K. R. (1975) Scand. J. Clin. Lab. Invest. 35, 677–682